

Discovery and characterisation of yolk-sac derived Endothelial-Macrophage progenitor cells in postnatal murine tissues

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Abstract

New blood vessel formation is central to many pathophysiological processes, such as atherosclerosis and cancer, and is closely intertwined with inflammation. Macrophages accumulate at sites of neovascularisation and support endothelial cells that line new blood vessels. It follows that a better understanding of how the intricate relationships between macrophages and endothelial cells come about, may open up new therapeutic opportunities to target these major disease processes. One important clue may lie in the ontogenies of both cell lineages, which increasingly appear to overlap, at least during early prenatal life. Converging lines of evidence indicate that macrophages and endothelial cells share a common ancestor, c-Kit⁺CSF1R⁺ erythromyeloid progenitors (EMP), which arise in extra-embryonic yolk sac (YS), as shown by murine lineage mapping studies. These recent observations align well with historical descriptions of bipotent haemangioblasts in YS and haemogenic endothelium in both the YS and aorta-gonad-mesonephros (AGM) during embryogenesis. Current expert consensus contends that YS-derived macrophages and endothelial cells are seeded in tissues prenatally and are maintained after birth by local self-renewal in their mature, differentiated state. However, an alternative, largely neglected explanation is that the YS also releases EMP-like progenitors to different tissues that persist postnatally to sustain local myeloid and endothelial populations under steady-state and mediates their expansion in disease.

Using complementary experimental approaches, this thesis has uncovered for the first time the existence of clonogenic progenitor cells in postnatal murine tissues (aorta, heart, lung, skin, skeletal muscle), that express CD45, Sca-1, c-Kit, CX₃CR1 and CSF1R and derive embryonically from early-wave YS EMPs. These tissue-resident progenitors have finite capacity for self-renewal, decreasing in prevalence with postnatal ageing (e.g. ~100 per 10⁵ cells in neonatal aorta, ~55 at 3 weeks, ~15 at 12 weeks, ~5 at 52 weeks, p<0.01). Analysis of *Flt3*^{Cre} x *Rosa*^{mT/mG} mice demonstrated that these progenitors arise from a Flt3^{-ve} source, indicating they are not derived or maintained from definitive haematopoiesis as occurs in postnatal bone marrow. Rather, embryonic profiling and tamoxifen-induced lineage mapping revealed

that they arise in extra-embryonic yolk sac at E8.5 and are seeded in intra-embryonic tissues around E10.5, where they persist until after birth. Using *in vitro* and *ex vivo* differentiation studies, we have determined that these YS-derived CD45^{+/Lo}Sca-1⁺c-Kit⁺CX₃CR1⁺CSF1R⁺ progenitors are bipotent for both macrophages and endothelial cells, also possessing striking vasculogenic capacity. This was also confirmed *in vivo* by adoptive transfer into different murine models of disease or physiological conditions, including atherosclerosis, skin wound injury, melanoma and peripheral ischaemia, where progenitors achieved robust engraftment, proliferative expansion and generation of intact, perfused neovessels.

We propose that these previously unidentified Endothelial-Macrophage (EndoMac) progenitor cells provide a local ancestral source for inflammatory and vasculogenic responses in postnatal tissues, and contribute to the apparent self-maintenance of YS-derived tissue-resident macrophages and endothelium after birth.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint award of this degree.

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.....

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Publications

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Abbreviations

acLDL	Acetylated LDL
ADAM	A Disintegrin and Metalloprotease
AGM	Aorta-Gonad-Mesonephro
AMPK	Amp-Activated Protein Kinase
AngII	Angiotensin II
ATAC	Assay for Transposase-Accessible Chromatin
BL-CFC	Blast Colony Forming Cells
BM	Bone Marrow
BrdU	Bromodeoxyuridine
CFU-G	Colony Forming Unit Granulocyte
CFU-GEMM	Colony Forming Unit Granulocyte, Erythrocyte, Monocyte, Megakaryocyte
CFU-GM	Colony Forming Unit Granulocyte, Monocyte
CFU-M	Colony Forming Unit Macrophage
CLP	Common Lymphoid Progenitors
cMOP	Common Monocyte Progenitor
CMP	Common Myeloid Progenitors
Cre	Cre Recombinase
CSF1/MCSF	Macrophage Colony-Signalling Factor 1
DC	Dendritic Cell
DNA	Deoxyribonucleic Acid
E	Embryonic Day
EC	Endothelial Cell
ECFC	Endothelial Colony-Forming Cells
ECM	Extracellular Matrix
eEPC	Embryonic Endothelial Progenitor Cells
EMP	Erythromyeloid Progenitors
EndoMac progenitor	Endothelial-Macrophage Progenitor
EPC	Endothelial Progenitor Cells
ER	Recombinase
EVP	Endovascular Progenitors
FGF	Fibroblast Growth Factor
Gata2	GATA-binding Factor 2
GDF3	Growth Differentiation Factor-3
GFP	Green Fluorescent Protein
GMP	Granulocyte and Macrophage Progenitors
h	Hour(s)
HAEC	Human Aortic Endothelial Cells
HCD	High Cholesterol Diet
HGF	Hepatocyte Growth Factor
HSC	Haematopoietic Stem Cells
HUVEC	Human Umbilical Vein Endothelial Cells
IGF	Insulin-like Growth Factor
IL	Interleukin
iPSC	Induced Pluripotent Stem Cells
JNK	c-Jun N-terminal Kinase
LC	Langerhans Cells
LDL	Low Density Lipoprotein
LIN	Lineage Markers
MAC	Myeloid Angiogenic Cells
MAPK	Mitogen-Activated Protein Kinases
M-CSF	Macrophage Colony Stimulating Factor

MDP	Macrophage/Dendritic Cell Progenitors
MIC	Muscle Interstitial Cells
min	Minute(s)
MPO	Myeloperoxidase
MSC	Mesenchymal Stem Cells
Mϕ	Macrophages
N/A	Not Available/Not Applicable
NF-κB	Nuclear Factor-Kappa- β
NK	Natural Killer Cells
Notch	Notch homolog 1, translocation-associated
oxLDL	Oxidised LDL
PAMPs	Pathogen-Associated Molecular Patterns
PCR	Polymerase Chain Reaction
PDGF	Platelet Derived Growth Factor
Prog	Progenitor
RANTES	Regulated on Activation, Normal T cell Expressed and Secreted
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
scRNA-seq	Single cell RNA sequencing
SDEC	Somite Derived Endothelial Cells
SMA	Smooth Muscle Actin
SMC	Smooth Muscle Cell
STAT3	Signal Transducer and Activator of Transcription 3
TAM	Tamoxifen
TAMϕ	Tumour-Associated Macrophages
tdTom	tdTomato Fluorescent Protein
TGF- β	Transforming Growth Factor B
TH1	T-helper Type 1
TNF	Tumour Necrosis Factor
TSP-1	Thrombospondin-1
UV	Ultraviolet Radiation
VEGF	Vascular Endothelial Growth Factor
VLA-4	Very Late Antigen-4
VWPCs	Vascular Wall-Resident Progenitor/Stem Cells
w	Week(s)
WT	Wild Type
YS	Yolk Sac

Protein abbreviations

ACE	Angiotensin Converting Enzyme, CD143
ATR1	Angiotensin II Receptor 1
ATR2	Angiotensin II Receptor 2
Bry	Brachyury
CBFβ	Core Binding Factor β
CCL5	Chemokine (C-C motif) Ligand 5
CCR2	C-C Chemokine Receptor Type 2
CD105	Endoglin
CD106	Vascular Cell Adhesion Molecule-1 (VCAM1)
CD11b	Integrin Alpha M
CD11c	Integrin Alpha X
CD133	Prominin-1
CD14	Cluster of Differentiation 14
CD146	Cluster of Differentiation 146
CD16	Fc γ RIII
CD16/32	Fc receptor 16/32
CD206	Mannose Receptor
CD207	Langerin
CD24	Sialoglycoprotein
CD29	Very late antigen-4 (VLA-4)
CD31	Platelet Endothelial Cell Adhesion Molecule (Pecam1)
CD34	Cluster of Differentiation 34
CD36	Cluster of Differentiation-36
CD4	Cluster of Differentiation 4
CD41	Integrin Alpha-IIb
CD44	Cluster of Differentiation 44
CD45	Protein Tyrosine Phosphatase, Receptor Type, C
CD5	Cluster of Differentiation 5
CD64	Cluster of Differentiation 64
CD68	Cluster of Differentiation 68
CD73	Ecto-5'-nucleotidase
CD8	Cluster of Differentiation 8
CD86	Cluster of Differentiation 86
CD90	Thy-1
CD93 (AA4.1)	Cluster of Differentiation 93
CDH5	VE-Cadherin/CD144
c-Kit	Proto-oncogene c-KIT
c-Myc	Myc Proto-Oncogene Protein
CSF1R	Macrophage Colony-Signalling Factor 1 Receptor
CX₃CR1	CX3C Chemokine Receptor 1
CXCL1	Chemokine (C-X-C motif) Ligand 1
F4/80	EGF-like Module-containing Mucin-like Hormone Receptor-like 1
Flt3	Fms Related Receptor Tyrosine Kinase 3
Gr-1	GPI-linked Myeloid Differentiation Marker
HLA-DR	Human Leukocyte Antigen – DR isotype
ICAM-1	Intercellular Adhesion Molecule 1
IFN-γ	Interferon- γ
LDLR	Low-Density Lipoprotein Receptor
Ly6C	Lymphocyte Antigen 6 C
LYVE-1	Lymphatic Vessel Endothelial Hyaluronan Receptor 1
MerTK	Proto-oncogene Tyrosine-Protein Kinase MER

MHCII	Class II Major Histocompatibility Complex
Nanog	Homeobox Protein NANOG
Oct4	Octamer-binding Transcription Factor 4
PDGFRα	Platelet Derived Growth Factor Receptor α ; CD140a
PDGFRβ	Platelet Derived Growth Factor Receptor β ; CD140b
PI3	Phosphoinositide 3
PKA	Protein Kinase A
PKC	Protein Kinase C
PPAR	Peroxisome Proliferator-Activated Receptor
PPT	Palmitoyl-Protein Thioesterases
RUNX1	Runt-related Transcription Factor 1
Sca-1	Stem Cell Antigen 1; also known as Ly6a
SM22	Smooth Muscle Protein 22
Sox2	Sex determining region Y
SR-A1	Scavenger Receptor Type A1
SRB1	Scavenger Receptor B1
Tal1	Erythroid Differentiation Factor
Ter119	Glycophorin A
TIE2	Angiopoietin-1 Receptor
TLR	Toll-Like Receptor
TLR2	Toll-Like Receptor 2
TREM2	Triggering Receptor Expressed on Myeloid Cells 2
VEGFR2	Vascular Endothelial Growth Factor Receptor 2; Foetal Liver Kinase 1 (Flk1)
vWF	von Willebrand Factor

Gene abbreviations

<i>Abca1</i>	ATP Binding Cassette Subfamily A Member 1
<i>Abcg1</i>	ATP Binding Cassette Subfamily G Member 1
<i>Adgre1</i>	Adhesion G Protein-Coupled Receptor E1; F4/80
<i>Agtr1</i>	Angiotensin II Receptor Type 1
<i>Agtr2</i>	Angiotensin II Receptor Type 2
<i>Arg1</i>	Arginase 1
<i>C4b</i>	Complement Component 4B
<i>Calcr</i>	Calcitonin Receptor
<i>Cbr2</i>	Carbonyl Reductase [NADPH] 2
<i>Ccl12</i>	Chemokine (C-C motif) Ligand 12
<i>Ccna1</i>	Cyclin A1
<i>Ccna2</i>	Cyclin A2
<i>Ccnb1</i>	Cyclin B1
<i>Ccne1</i>	Cyclin E1
<i>Ccr2</i>	C-C Motif Chemokine Receptor 2
<i>Cd163</i>	Cluster of Differentiation 163
<i>Cd209f</i>	C-type Lectin Domain Family 4 Member f
<i>Cd248</i>	Endosialin
<i>Cd36</i>	Cluster of Differentiation 36
<i>Cd52</i>	CAMPATH-1
<i>Cd74</i>	Cluster of Differentiation 74
<i>Cd84</i>	Cluster of Differentiation 84
<i>Cd9</i>	Cluster of Differentiation 9
<i>Cdki</i>	Cyclin-Dependent Kinase Inhibitor
<i>Cebpb</i>	CCAAT Enhancer Binding Protein Beta
<i>Ctsb</i>	Cathepsin B
<i>Ctsd</i>	Cathepsin D
<i>Ctsz</i>	Cathepsin Z
<i>Cxcl1</i>	CXCL1 Gene C-X-C Motif Chemokine Ligand 1
<i>Cxcl10</i>	C-X-C Motif Chemokine Ligand 10
<i>Cxcl2</i>	C-X-C Motif Chemokine Ligand 2
<i>Egr1</i>	Early Growth Response 1
<i>Fcgrt</i>	Neonatal Fc Receptor
<i>Fcna</i>	Ficolin 1
<i>Flk1</i>	Foetal Liver Kinase 1; Vascular Endothelial Growth Factor Receptor 2
<i>Folr2</i>	Folate Receptor Beta
<i>Fosb</i>	Protein fosB,
<i>Gas6</i>	Growth Arrest Specific 6,
<i>Gata4</i>	GATA Binding Protein 4
<i>Gfap</i>	Glial Fibrillary Acidic Protein
<i>Hes1</i>	Hairy and Enhancer of Split-1
<i>Hvcn1</i>	Voltage-gated Hydrogen Channel 1
<i>Ier</i>	Immediate Early Gene
<i>Ifitm3</i>	Interferon Induced Transmembrane Protein 3
<i>Il1β</i>	Interleukin 1 Beta
<i>Irf7</i>	Interferon Regulatory Factor 7
<i>Irf8</i>	Interferon Regulatory Factor 8
<i>Isl1</i>	Insulin Gene Enhancer Protein 1
<i>Isyna1</i>	Inositol-3-Phosphate Synthase 1
<i>Itga9</i>	Integrin Alpha-9/Beta-1
<i>Itgam</i>	Integrin Subunit Alpha M

<i>Itgb2</i>	Integrin Beta 2
<i>Klf4</i>	Kruppel-Like Factor 4
<i>Klf7</i>	Kruppel-Like Factor 7
<i>Lgals3</i>	Galectin-3
<i>Lyz2</i>	Lysozyme 2
<i>Mertk</i>	Proto-oncogene Tyrosine-Protein Kinase MER
<i>Mnda</i>	Myeloid Cell Nuclear Differentiation Antigen
<i>Mrc1</i>	Mannose Receptor C-type 1
<i>Mrf</i>	Myogenic Regulatory Factor
<i>Myb</i>	MYB Proto-Oncogene
<i>Myc</i>	MYC Proto-Oncogene
<i>Myf5</i>	Myogenic Factor 5
<i>Myod</i>	Myoblast Determination Protein 1
<i>Nfatc1</i>	Nuclear Factor of Activated T-cells, Cytoplasmic 1
<i>Nfkb1q</i>	NF-Kappa-B Inhibitor Alpha
<i>Nkx2-5</i>	Homeobox Protein Nkx-2.5
<i>Nlrp3</i>	NLR Family Pyrin Domain Containing 3
<i>Nr4a1</i>	Nuclear Receptor Subfamily 4 Group A Member 1
<i>Pax7</i>	Paired Box Protein Pax-7
<i>Pdgfc</i>	Platelet-derived Growth Factor C
<i>Pf4</i>	CXC Chemokine 4
<i>Psmd11</i>	Proteasome 26S Subunit, Non-ATPase 11
<i>Rab14</i>	Ras-related Protein Rab-14
<i>Rpl24</i>	Ribosomal Protein L24
<i>Rpl27</i>	Ribosomal Protein L27
<i>Sepp1</i>	Selenoprotein P
<i>Sox18</i>	Transcription Factor SOX-18
<i>Spi1</i>	Transcription Factor PU.1
<i>Spp1</i>	Secreted Phosphoprotein 1
<i>Stat3</i>	Signal Transducer and Activator of Transcription 3
<i>Swi5</i>	SWI5 Homologous Recombination Repair Protein
<i>Tek</i>	Angiopoietin-1 Receptor (TIE2)
<i>Timd4</i>	T-cell Immunoglobulin and Mucin Domain Containing 4
<i>Tlr2</i>	Toll-like Receptor 2
<i>Tlr5</i>	Toll-like Receptor 5
<i>Tnf</i>	Tumour Necrosis Factor
<i>Trem2</i>	Triggering Receptor Expressed on Myeloid Cells 2
<i>Tslp</i>	Thymic Stromal Lymphopoietin
<i>Txnip</i>	Thioredoxin-Interacting Protein
<i>Zfp612</i>	Zinc Finger Protein 612

Chapter 1

Introduction

1.1 Complementary roles of endothelial and myeloid cells in neovascularisation

The formation of neovessels by angiogenesis and/or vasculogenesis is central to many pathophysiological processes and is closely intertwined with inflammation (3). Endothelial cells line blood vessels, forming a monolayer that provides an interface between blood and the surrounding tissue/organ, called the endothelium. In healthy blood vessels and tissues, the endothelium helps to maintain vascular tone and permeability through vasodilation, anti-inflammatory, anti-thrombotic and anti-proliferative actions (3). It also helps to mediate tissue repair and regeneration after injury or ischaemic insult. However, in some disease states, dysregulated endothelial responses may exacerbate injury and contribute to angiogenesis-related pathological conditions, such as in atherosclerosis or cancer.

Angiogenesis is the process by which new blood vessels develop through outgrowths from the endothelium of existing blood vessels (4, 5). Adult endothelial cells migrate and proliferate to create new sprouts from pre-existing vessels when exposed to proangiogenic signals. In contrast, vasculogenesis occurs via contributions from undifferentiated ancestral cells, such as endothelial progenitor cells (EPCs), rather than established endothelium (6). A multistage process occurs that first involves collective endothelial cell migration, which is coordinated by cellular and molecular interactions in the local vascular microenvironment. Through these interactions, endothelial cells receive and integrate proangiogenic cues which coordinate the outward migration of an endothelial “tip” cell, with the expansion of adjacent “stalk” cells that maintain the growth of the nascent vessel branch (7, 8).

These cellular interactions among endothelial cells are known to be critical for successful neovascular remodelling. Macrophages are among several different cell types that support endothelial cells during

neovascularisation. Macrophages are a type of inflammatory haematopoietic cell that forms a key part of the body's innate immune system. They play numerous essential roles during normal tissue development in embryogenesis and in maintaining healthy tissue homeostasis postnatally. During tissue injury and inflammation, macrophages engulf pathogens, foreign material and dying cells, while also secreting growth factors, cytokines, chemokines, and proteolytic enzymes which help recruit and activate other inflammatory and non-inflammatory cells to the region. Fundamentally, their properties enable them to help fight infection, limit tissue injury and promote healthy tissue remodelling (4).

Among their other effects, macrophages are integrally involved during angiogenesis and vasculogenesis. Although known to undergo phenotypic changes in response to their surrounding microenvironment, it is important to note that they are not believed to have *bona fide* endothelial differentiation capacity (4, 9-11). Their contributions to neovascularisation are therefore widely regarded as being supportive or paracrine (12). Macrophages proliferate and assemble around endothelial-lined neovessels, extending their coverage from the base of filopodia to the tip of endothelial cells, and their neighbouring vessel sprouts which promote endothelial anastomosis into perfused vessel loops (7). Microvessel remodelling in adult tissue also requires the vessel wall to undergo a coordinated phase of disassembly, involving endothelial cell plasticity in response to angiogenic cues. Macrophages partly mediate this by producing pro-angiogenic cytokines and growth factors (e.g. vascular endothelial growth factor, VEGF). They also act downstream of VEGF as vascular fusion cells to support endothelial tip cell anastomosis and vascular branching (13). Macrophages also facilitate angiogenesis by secreting proteases that enable the release of proangiogenic growth factors embedded in the perivascular matrix (4). Furthermore, macrophages produce semaphorins that help with “vascular guidance” by modulating endothelial cell migration and survival (14). In this way, macrophages are able to support directional vascular growth and sprouting (4, 15).

Endothelial cells in turn can influence macrophage plasticity to mediate inflammation and angiogenesis. Adult endothelial cells express macrophage colony-signalling factor 1 (CSF1, M-CSF), which promotes selective growth and differentiation of macrophages from haematopoietic progenitors (10). In addition, endothelial cells provide a niche for the differentiation and functional polarisation of anti-inflammatory, pro-angiogenic macrophages, traditionally referred to as M2-like macrophages (10, 16). Therefore, throughout the formation, maturation and remodelling of neovessels, macrophages and endothelial cells are able to influence each other's function through both direct structural interactions and the secretion of different molecules. Despite decades of research, the continued pursuit of a better understanding of how these interactions arise is crucial in order to effectively target neovascularisation in different pathophysiological conditions.

Interim Conclusion

A better understanding of how these intimate relationships between macrophages and endothelial cells are so well-orchestrated is needed in order to understand and target neovascularisation in aforementioned physiological and pathological conditions.

1.2 The emergence of the haematopoietic system during embryogenesis

1.2.1 Early stages of embryogenesis

After fertilisation of an egg, the initial growth of a multicellular organism begins when the zygote cell undergoes rapid cell division through cleavage to create an initial cell cluster called a blastula. The type of cleavage that occurs within the developing embryo is dependent on the volume of “yolk” that forms inside the egg itself (**Figure 1-1**). In placental mammals, such as humans and mice, the eggs have a very small volume of yolk, and thus undergo a type of cleavage called “holoblastic cleavage” (17). Other animal species, such as birds and reptiles, have a larger yolk and undergo “meroblastic cleavage” which is used to nourish the developing embryo (18, 19).

In the next stage of its initial development, the mammalian blastula forms a blastocyst. In the blastocyst stage, cells begin to arrange themselves into two distinct layers: an outer layer and an inner layer, termed trophoblast and embryoblast, respectively (17, 20) (**Figure 1-1**). At this stage of embryogenesis, the embryoblast consists of embryonic stem cells which will differentiate into the many different cell types that are needed by the organism to function, forming the embryo. This occurs in mice at embryonic day 3.25 (E3.25) and in humans at day 6 (20).

The next stage of embryonic development is the basic conception of the body plan, occurring in mice at E6.5 and 16 days of gestation in humans. The process is known as “gastrulation”, where the cells within the blastocyst structure rearrange themselves by folding in on itself and forming three distinct layers of cells called germ layers (17, 20). These are the ectoderm (outer layer), mesoderm (middle layer), and endoderm (inner layer). Each layer differentiates into a different organ system within the body: 1) the ectoderm initiates the formation of the nervous system and layers of skin; 2) the mesoderm

activates the formation of muscle cells and connective tissue; and 3) the endoderm starts the formation of columnar cells found inside the digestive system, as well as many other internal organs.

Soon after gastrulation, proliferating progenitors from the lateral and posterior mesoderm migrate towards the extra-embryonic yolk sac (YS). These mesodermal cells aggregate to form small clusters called “blood islands” (21). Central cells in the blood islands will accumulate primitive haematopoietic precursors, while outer cells give rise to endothelial precursors (angioblasts), which flatten and form the endothelium (22). These blood islands develop alongside the endoderm (**Figure 1-1**). In mouse development, this is observed to occur during E7-7.5 (20). These haematopoietic and endothelial lineages form the first differentiated cells in the mammalian embryo. Therefore, the relationship between blood cells and endothelial cells commences very early during the initial stages of embryonic development, through the formation of mesoderm-derived blood islands. Once the three germ layers have become fully established and start to develop separately, the body begins to form and the embryo becomes a foetus.

Interim Conclusion

Haematopoietic and endothelial lineages form the first differentiated cells in the mammalian embryo as they develop in parallel to create a functional circulatory system.

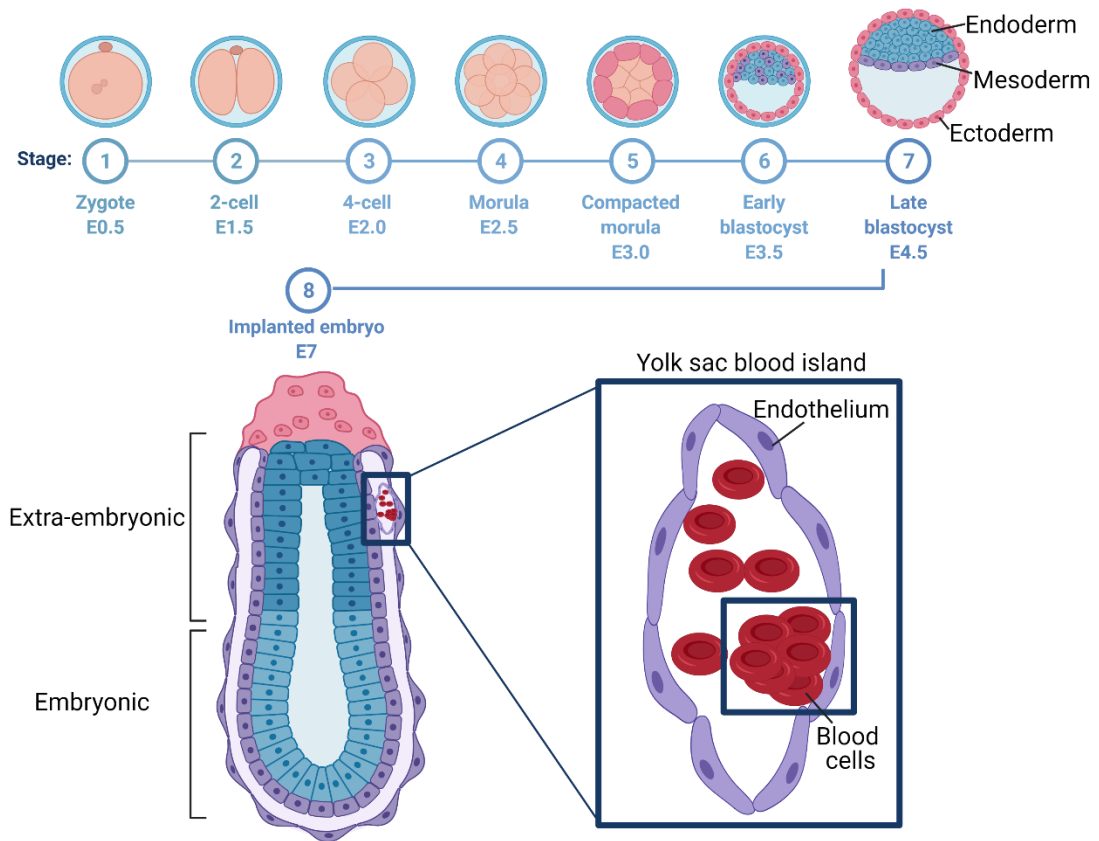


Figure 1-1. Development of yolk sac haematopoiesis.

A schematic of early stages of embryonic development. In the E7.5 mouse embryo, blood islands start to develop alongside the endoderm. Here, there is segregation of the haematopoietic erythroblasts (blood cells) and endothelial lineages.

1.2.2 Haematopoiesis in embryonic development

During murine embryogenesis, there are three major waves of haematopoietic potential (**Figure 1-2**). These waves are classified under two broad terms: primitive and definitive haematopoiesis. The differences between cells arising from primitive and definitive haematopoiesis are summarised in **Table 1-1**. Primitive haematopoiesis represents the first wave of blood cells that emerges in the YS starting around E7 in mice and gestational day 18 in humans (23, 24). Primitive haematopoiesis is marked by the short-lived emergence of primitive erythroid cells, as well as primitive macrophages, which are established in parallel with endothelial cells. Overlapping with primitive haematopoiesis, is the second wave of haematopoiesis, classified as transient-definitive haematopoiesis, which emerges in the YS from erythromyeloid progenitors (EMPs) between E7.5-E8.5. The EMPs then enter the embryo proper and seed the foetal liver around E10.5 (25). Within the foetal liver EMPs rapidly differentiate, giving rise to the first circulating definitive erythrocytes. These two waves of haematopoiesis also contain megakaryocyte and myeloid potential. The third wave of haematopoiesis subsequently occurs through the emergence of definitive haematopoietic stem cells (HSCs) appearing around E10–E11 in the aorta-gonad-mesonephros (AGM) region. Thereafter, HSCs relocate to the foetal liver and eventually migrate to the bone marrow (BM) around the time of birth (**Figure 1-2**).

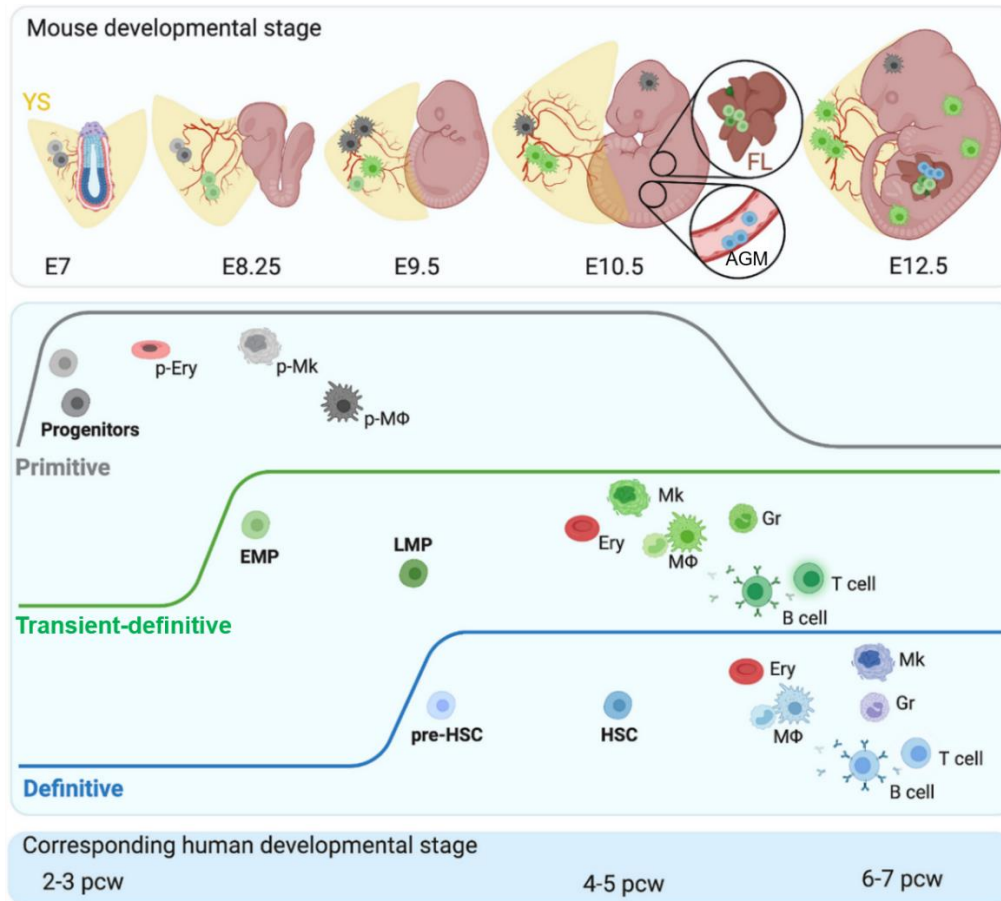


Figure 1-2. Three waves of haematopoiesis in prenatal development.

During murine development there are three successive waves of haematopoiesis, represented in the top panel: primitive (grey cells), transient-definitive (green cells), and definitive haematopoiesis (blue cells). Primitive haematopoiesis emerges in the YS, along with transient-definitive haematopoiesis (EMP-wave) while haematopoietic stem cells (HSCs) arise in the aorta-gonad-mesonephros (AGM). At E10.5 EMPs and HSCs begin to co-exist in the foetal liver (FL).

Middle panel: The primitive wave gives rise to erythrocytes (p-Ery), megakaryocytes (p-Mk), and macrophages (p-MΦ). The transient-definitive wave produces erythromyeloid progenitors (EMPs) and lympho-myeloid progenitors (LMPs). The definitive wave generates pre-HSCs, which differentiate into HSCs. In the foetal liver, both EMP and definitive wave progenitors generate erythrocytes (Ery), megakaryocytes (Mk), granulocytes (Gr), T cells and B cells as well as monocyte-derived macrophages (MΦ).

Bottom panel: The corresponding haematopoietic developmental stages in humans. pcw, Post-conception week *Image from Canu et al. (25)*

	Primitive lineage	Transient-definitive early EMP lineage	Transient-definitive late EMP lineage	Definitive lineage (HSC)	References
Erythrocytes	Nucleated when released in blood, before enucleation. Diameter 12 µm. Embryonic haemoglobins (βH1, ε, ζ)		Enucleation before release into circulation Diameter 10 µm. Foetal haemoglobins (βH1 and β1-adult)	Enucleation before releasing into circulation. Diameter 8 µm. Adult haemoglobin (β-major)	Craig <i>et al.</i> (26), Wong <i>et al.</i> (27), McGrath <i>et al.</i> (28), Kingsley <i>et al.</i> (29), McGrath <i>et al.</i> (30)
Megakaryocytes	Average size 9 µm. Polyploidy < 4N	No information	No information	Average size 20 µm Polyploidy > 16N	Tober <i>et al.</i> (31), Xu <i>et al.</i> (32)
Macrophages	Maternal-derived macrophages: Not produced by the YS. Initially described as primitive macrophages bypassing the monocytic stage. YS-derived macrophages: CD45 ⁺ Mac1 ⁺ CSF1R ⁺ CX ₃ CR1 ⁺ F4/80 ⁺ Flt3 ⁻ . No monocyte intermediate stage. Proliferative capacity.	YS EMP-derived macrophages: CD45 ⁺ Mac1 ⁺ CSF1R ⁺ CX ₃ CR1 ⁺ F4/80 ^{Hi} LYVE-1 ⁺ Flt3 ⁻ No monocyte intermediate stage Proliferative capacity.	Foetal liver EMP-derived macrophages: CD45 ⁺ Mac1 ⁺ CSF1R ⁺ CX ₃ CR1 ⁺ F4/80 ^{Hi} Flt3 ^{+/-} Monocyte intermediate stage Proliferative capacity	BM-derived macrophages: CD45 ⁺ Mac1 ⁺ , CSF1R ⁺ CX ₃ CR1 ⁺ F4/80 ^{Lo} Flt3 ⁺ . Derive from a Common Myeloid Progenitor, through a monocytic stage. No proliferative capacity	Palis <i>et al.</i> (24), Bertrand <i>et al.</i> (33), Ovchinnikov <i>et al.</i> (34)

Table 1-1. Cells arising from primitive and definitive haematopoiesis.

CD45, Protein tyrosine phosphatase, receptor type, C; CSF1R, colony stimulating factor 1 receptor; CX₃CR1, CX3C chemokine receptor 1; EMPs, Erythromyeloid progenitor; F4/80, EGF-like module-containing mucin-like hormone receptor-like 1; Mac1, Consisting of CD11b (integrin αM) and CD18 (integrin β2); N, Haploid; YS, Yolk Sac. *Table adapted from Wittamer et al. (35)*

1.2.2.1 Primitive erythrocytes

Blood islands in the YS are comprised of developing primitive erythroblasts surrounded by endothelial cells. Primitive erythrocytes, identified as large nucleated erythroid cells, are the predominant haematopoietic population early in development. These primitive erythroid cells are functionally and morphologically distinct from later erythroid progenitors. They are large, nucleated, express embryonic globins, and are exclusively detected in the extra-embryonic YS during a narrow time window, between E7.25–8.75, before they disappear (26, 27). Hence, primitive erythropoiesis is transient and appears to be restricted to the YS stage of haematopoiesis. The close association of primitive erythroblasts with the developing endothelial cells in the YS strongly suggests that haematopoiesis is also initiated by a common bipotent progenitor, with two possible ancestral candidates proposed, namely: the haemangioblast or the haemogenic endothelium.

1.2.2.2 Haemangioblasts

During embryonic development, haematopoietic and endothelial cells are first thought to arise from a common mesodermal precursor, the haemangioblast, which first appears in murine YS at ~E7.5 (**Figure 1-3**). The notion of the embryonic haemangioblast first arose during the early 1900s by His *et al.* This study observed a very close association between the first blood cells and vascular progenitor cells in blood islands within the YS of chick embryos which they inferred was due to a common bipotent ancestral progenitor (22, 36).

A century later, the existence of haemangioblasts still remains somewhat controversial. Studies have identified specific human embryonic stem cells (ESC) lines that are able to form blast colony forming cells (BL-CFC) and can be induced to differentiate into both haematopoietic and endothelial cells at the single-cell level (37-40). These BL-CFCs were first identified as expressing the mesoderm-specific marker, mesodermal gene *T* (*Brachyury*) and Foetal Liver Kinase 1 (*Flk1*) (41). In pluripotent model

systems, their differentiation and regulation have been linked to various genes and signalling pathways involving Runt-related transcription factor [*Runx-1* (AML1)] (42), T-cell acute lymphocytic leukaemia protein 1 [*Scl* (Tal1)] (43), *Flk1* (37, 38, 40, 44, 45), *brachyury* (41) and angiotensin converting enzyme (*Ace*, CD143) (46). Fate mapping experiments in Zebrafish gastrula have shown the existence of an analogous clonal population with both haematopoietic and endothelial biopotency, localised within the ventral-lateral mesoderm during Zebrafish embryogenesis (39, 47). Moreover, blast colonies from E7-7.5 mouse embryos have also been shown to give rise to adherent and non-adherent cells when exposed to endothelial and haematopoietic growth media conditions (37). The non-adherent population expressed the haematopoietic markers β H1 and β -major globin, as well as erythroid transcription factor (*Gata1*), *Csf1r* and MYB Proto-Oncogene (*Myb*), indicating the presence of primitive erythroid, macrophage and immature haematopoietic cells, respectively. The adherent cells expressed genes attributed to vascular cells, including *Flk1*, *Tie2* and platelet endothelial cell adhesion molecule (*Pecam1* or *Cd31*), as well as smooth muscle actin (*Acta2*), smooth muscle protein 22 kDa (*Sm22*) and calponin (*Cnn1*), signifying endothelial and smooth muscle cell (SMC) development, respectively (37).

Another study has shown that haemangioblasts are also generated from human induced pluripotent stem cells (iPSCs) undergoing haemopoietic-to-endothelial induction *in vitro* (48). iPSCs exposed in culture to VEGF and CSF-1 first formed *Flk1*⁺ haemangioblast-like cells, which could then differentiate into mature macrophages that were positive for CD45, CD11b, F4/80, fractalkine receptor (CX₃CR1) and negative for the stem cell marker, CD41 (Integrin alpha-IIb). In order to achieve this differentiation pathway, the authors used an agonist to activate the *Wnt* pathway, which is known to regulate the development of the primitive streak, formation of haemangioblasts and primitive haematopoiesis during normal embryogenesis.

Over recent decades, there has also been indirect evidence that the haemangioblast may persist into postnatal life, particularly in humans. One such line of support comes from the observation that some

patients with chronic myelogenous leukaemia possess the same BCR/ABL rearrangement fusion gene in both their BM-derived haematopoietic cells and endothelial cells, suggesting that the pathogenic translocation occurred in a common precursor for both blood and endothelial lineages (49, 50). Another study reported that human CD34⁺VEGFR2 (Flk1)⁺ BM progenitor cells generate blastic colonies, which can be differentiated into endothelial and haematopoietic cells (51). Notably, this population was found to be exceedingly rare in older individuals, indicating that they may “burn out” as a result of ageing.

Despite the evidence from pluripotent differentiation models, definitive confirmation of the *in vivo* existence of a bipotent haemangioblast ancestor is still lacking. Although it has been interpreted from single cell labelling and tracking of gastrula-stage zebrafish embryos that individual mesodermal cells can give rise to both blood and endothelial cells, the majority of these two lineages were found to be independently fated (52). Murine lineage-tracing data have also suggested that the cellular composition of individual YS blood islands may be derived from distinct mesodermal progenitors (53) and that the VEGFR2⁺ mesodermal subpopulation is not exclusively specific to endothelial and haematopoietic lineages, also giving rise to muscle cell progeny (54). Therefore, the paradigm of a common haemangioblast progenitor for both early vasculogenesis and primitive haematopoiesis remains controversial and unproven *in vivo*.

Despite these revelations, there has still not been any conclusive or direct *in vivo* proof that such a bipotent haemangioblast or haemangioblast-like progenitor cell actually exists in higher vertebrates postnatally. *Ex vivo* experiments using mouse embryos that show BL-CFCs can give rise to both haematopoietic and endothelial cells have led to speculation that the haemangioblast is a state of competence, whereby experimental manipulations may uncover a dormant competency state, as distinct from an inherent bipotent progenitor state that is active *in vivo* (55). However, until now the assays used for *in vivo* studies have arguably lacked the sensitivity needed to confidently refute the true existence of haemangioblasts. Emerging techniques, such as single cell sequencing (scRNA-seq), may help to

definitively define whether haemangioblasts do exist, and potentially whether they persist beyond embryonic development to be also present in postnatal mammalian tissues. Other sophisticated experimental approaches, including lineage-mapped multi-colour mice (Confetti or the Rainbow Cre) (56), may in turn help to assess the clonal contribution of haemangioblasts to tissue development both pre- and postnatally.

1.2.2.3 Haemogenic endothelium

As stated above, it has long been documented that blood and endothelium are two closely related lineages that emerge in close proximity during embryonic development (22). Although some have speculated that haemangioblasts give rise to the first haematopoietic cells, a contrary theory is that they instead arise from haemogenic endothelium (57). Haemogenic endothelium is a phenotypically differentiated endothelial cell that has the potential to also become a blood cell. This is thought to occur through a process of endothelial-to-haematopoietic transition which involves asymmetric cell division, rather than via a single cell giving rise to two daughter cells (58). The discovery of haemogenic endothelium was first made through both mice and zebrafish lineage-tracing studies, involving the use of a CDH5-Cre system, and time-lapsed imaging to demonstrate that CDH5⁺ endothelial cells generate definitive HSCs in the AGM at ~E10.5 (59, 60). Subsequent murine studies have shown that haemogenic endothelium in extra-embryonic YS also gives rise to EMPs at ~E8.25 (61), T and B cell progenitors at ~E9.5 (62, 63), and AGM HSCs at ~E10.5 (64). Hence, it is now believed that haemogenic endothelium gives rise to both the YS EMP and AGM HSC definitive waves of haematopoiesis (**Figure 1-3**). Moreover, several groups have successfully differentiated endothelial precursors into HSCs by using an endothelial “niche” population (65-72), further supporting the notion of haemogenic endothelium.

In contrast to the two waves of definitive haematopoiesis described above, the cellular origin of primitive haematopoiesis during early embryogenesis is still disputed (42, 73). It remains unclear

whether primitive haematopoiesis emerges directly from mesoderm, from haemogenic endothelium or from the haemangioblast. There is strong evidence showing that primitive haematopoiesis arises from precursors expressing endothelial markers, such as TIE2, CDH5 and CD31 (38, 74-77). Interestingly, these precursors are not located within the wall of blood vessels but rather within a mass of endothelial-expressing cells (74, 78). Consequently, Lancrin *et al.* have presented a theory that the haemangioblast itself may generate haematopoietic cells through a haemogenic endothelium intermediate (74). This followed a study in which they were able to differentiate mouse haemangioblasts/BL-CFCs into haematopoietic cells through a two-step process *in vitro*. First, the haemangioblasts transformed into a tightly adherent cell layer that expressed endothelial cell markers, the transitory haemogenic endothelium stage, then after 36-48h of culture became non-adherent cells, resembling a change into haematopoietic colonies.

Although it is uncertain what role haemogenic endothelium plays in primitive haematopoiesis, its importance to the emergence of definitive haematopoiesis (both the EMP and HSC waves) is much better established. In addition, it regulates the transition of progenitor/stem cells to mature haematopoietic cells through various signalling pathways, namely: *Runx*; Notch homolog 1, translocation-associated (*Notch*); and Kit Proto-Oncogene, Receptor Tyrosine Kinase (*Kit*). Although RUNX1 is dispensable for primitive haematopoiesis, loss of RUNX1 is detrimental for the generation of both EMPs and HSCs (79, 80). Furthermore, RUNX1 has been found to regulate the loss of endothelial properties during blood generation (74, 81, 82), indicating a crucial role in the endothelial-to-haematopoietic transition of haemogenic endothelium. Meanwhile, *Notch* signalling indirectly regulates the activation of *Runx1* (through the upregulation of *Gata2*). *Notch* is also an important regulator of endothelial-to-haematopoietic transition that gives rise to definitive haematopoiesis, but not primitive haematopoiesis (65, 83-86). Haemogenic endothelium is also induced by retinoic acid signalling, which upregulates expression of c-Kit (87). A recent study has determined that c-Kit, expressed on both EMPs and HSCs from haemogenic endothelium, is essential for definitive erythropoiesis in the foetal liver (88). However, it is not required for haemogenic endothelial

contributions to angiogenesis in the brain (88). Hence, c-Kit may be a mediator for switching from developmental angiogenesis to haematopoiesis. Importantly, the abovementioned studies present a clear distinction between the signalling pathways involved in primitive and definitive haematopoiesis. This has prompted recent speculation that haemogenic endothelium may only give rise to definitive haematopoietic cells *in utero*, while primitive haematopoietic cells may originate from another mesoderm precursor, possibly the enigmatic haemangioblast.

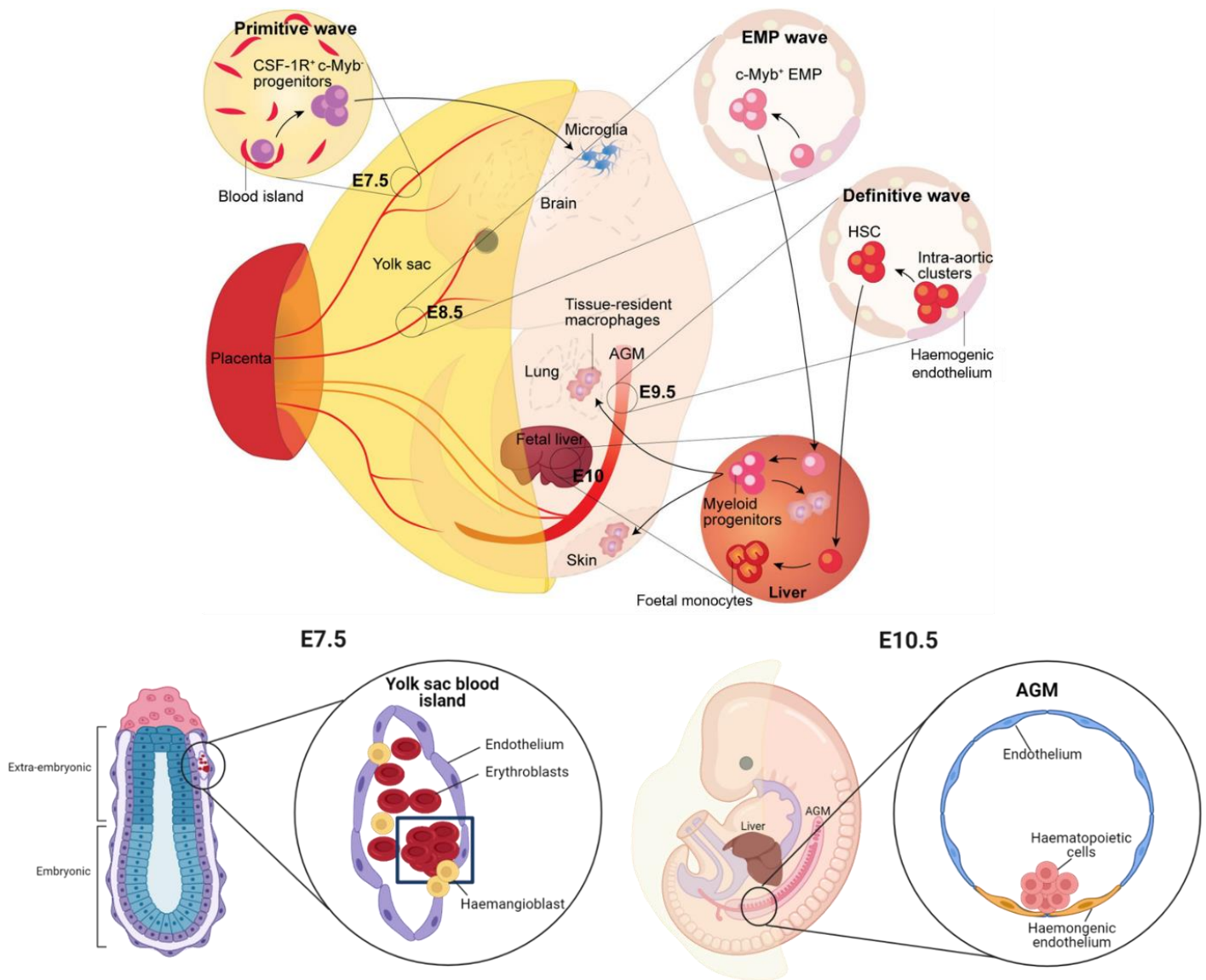


Figure 1-3. Origins of the three waves of haematopoiesis in the developing murine embryo: haemangioblasts and haemogenic endothelium.

Top image: during murine development, there are three successive waves of haematopoiesis. The first wave, called primitive haematopoiesis, begins at E7.5 in the blood islands of the extra-embryonic YS. The second wave arises from the haemogenic endothelium of YS around E8.5 and generates c-Myb⁺ erythromyeloid progenitor (EMPs). The third wave arises from the haemogenic endothelium in the aorta-gonad-mesonephros (AGM) region, which gives rise to foetal haematopoietic stem cells (HSC), occurring between E10-E11. Note that both EMPs from YS and HSCs from AGM subsequently colonise the foetal liver. CSF1R, Colony stimulating factor 1 receptor. Bottom image: Haemangioblasts (yellow) arise in the extra-embryonic at E7.5 embryo. Haemangioblasts give rise to circulating primitive

erythroblasts (red) and to endothelial cells (purple). In the E10.5 embryo, AGM has clusters of haematopoietic cells (red) in close association with the ventral endothelium (blue). Intra-arterial blood cells are thought to arise from haemogenic endothelium (orange). *Top image from Wu et al. (89).*

1.2.2.4 The emergence of the first macrophages: Primitive macrophages

In mice, macrophages can first be detected as early as E7. During embryonic development, macrophages can be separated into two classes, primitive and definitive, generated from the three different waves. Unlike macrophages arising from the HSC- definitive haematopoiesis wave, primitive macrophages are generated directly from progenitors bypassing the monocyte intermediate (90, 91). As for macrophages generated from the transient-definitive EMP wave, studies are contradictory. Studies have indicated that macrophages arising from early EMPs bypass the monocyte intermediate (92-94), while those macrophages arising from late EMPs, follow the classical phase of myelopoiesis (2, 94) (**Figure 1-3**).

Studies in mice have found that the first wave of primitive macrophages are from maternal origin (24, 90, 91, 95). These macrophages have a limited lifespan, such that they do not seed the developing embryo but rather the extra-embryonic YS only (33, 96). A second wave of primitive macrophages arises during the onset of primitive haematopoiesis (YS E7.5) independently from maternal macrophages. Lineage-tracing studies in Zebrafish development show that these primitive macrophages do not originate from the embryonic blood island that gives rise to erythroblasts and endothelial cells (97). Rather, they come from a mesoderm progenitor which gives rise to macrophages that then migrate straight to the YS where they give rise to vasculogenic cells, which develop into blood islands in Zebrafish. This particular line of evidence suggests a common mesodermal origin (perhaps via the haemangioblast) for primitive macrophages and vasculogenic cells at this early stage of development (97).

In contrast, murine lineage-tracing studies have provided compelling evidence that primitive macrophages instead derive directly from YS progenitors *in vivo*. Using inducible *Runx1* promoter-driven GFP reporter mice, Ginhoux *et al.* initially demonstrated that primitive macrophages seeded in the brain (microglia) originate from E7.5 YS CD45⁻c-Kit⁺ progenitors (98). These c-Kit⁺ progenitors were also found to exclusively give rise to haemopoietic macrophage colonies when cultured in

haemopoietic media *in vitro* (96). Subsequent work has used tamoxifen inducible *Csf1r*-Cre mice to identify that the source of primitive macrophages is predominantly YS progenitors that express CSF1R at E8.5 (99). Moreover, a large proportion of tissue-resident macrophages in embryonic and postnatal liver, brain, skin epidermis and lung have been shown to originate from a *Tie2* expressing cell in the YS before E10.5 (93). Macrophages generated from these YS progenitors appear to have a high proliferative potential, which was thought to propagate their expansion and self-maintenance in different tissues during foetal development (91, 100).

Although it has been recognised that macrophages arise during primitive haematopoiesis, the underlying mechanisms that regulate their development remains largely unknown. Nonetheless, it has been established that primitive myelopoiesis in YS occurs independently of c-Myb, although it is required for the subsequent EMP and HSC definitive waves which seed foetal liver (101). At E16.5, *Myb*^{-/-} mouse embryos, therefore, exhibit depletion of most myeloid cell populations, except macrophages derived from YS such as liver Kupffer cells, epidermal Langerhans cells and brain microglia (101).

The most dominant YS-derived primitive macrophages to persist after birth into adulthood, are brain microglia, which are highly proliferative (89). Therefore, to study the developmental mechanisms of primitive macrophages, groups have mainly focused on the regulation of microglia. Transcription factors PU.1/*Spi1* and Interferon regulatory factor-8 (*Irf8*) are both essential for microglia development (99). Kierdorf *et al.* observed that primitive c-Kit⁺ YS progenitors at E8 developed into mature CD45⁺c-Kit⁻CX₃CR1⁺ precursor cells with downregulation of CD31 and associated upregulation of F4/80 and CSF1R, the receptor for M-CSF (99). These proliferating CD45⁺c-Kit⁻CX₃CR1⁺ precursor cells were seen to infiltrate the developing brain and subsequently develop into microglia.

Microglia development is also dependant on transforming growth factor β (TGF- β) signalling. *In vitro* culture of adult microglia without the presence of TGF- β 1, results in their failure to express microglia signature genes. Moreover, adult *Tgfb1*^{-/-} mice do not maintain microglia (102). Interestingly, compared to their wild type (WT) littermates, *Tgfb1*^{-/-} embryos had no deficiency in their content of primitive macrophages in the YS at E10.5. Depletion of brain microglia only occurred from E14.5 onwards when the blood-brain barrier begins to form. Based on these observations, the authors concluded that TGF- β 1 does not regulate the ancestral progenitors of microglia in the YS but rather their maturation in brain after E14.5. Another study employed mice expressing a *Vav1*-driven inducible Cre to delete *Tgfb2* (gene encoding Transforming Growth Factor, β Receptor II) in haematopoietic cells (103). In this model, microglia were significantly reduced in the brain at E11.5. More importantly, this study also revealed two distinct brain macrophage subpopulations arising from the primitive macrophage pool: 1) mannose receptor (CD206) positive microglia and 2) CD206 negative border-associated macrophages. Through different fate-mapping models, it was revealed that both populations arise from E8.5 YS primitive precursors and not from definitive haematopoiesis. Although it remains unclear whether CD206⁻ macrophages differentiate into CD206⁺ microglia, it is currently believed that microglia development occurs without an intermediate transitional precursor in brain. Therefore, the authors concluded that embryonic CD206⁻ border-associated macrophages and CD206⁺ microglia are distinct populations that arise in parallel from primitive YS precursors.

Interim Conclusion

The early cells resulting from primitive haematopoiesis sustain the developing embryo's survival until the definitive haematopoietic system is established. Although the first sign of primitive haematopoiesis occurs in YS, the actual origin of each primitive haematopoietic cell is still unknown. However, it seems certain that primitive haematopoiesis begins when early progenitor cells form blood islands around E7.5. Those cells found in the centre of these islands give rise to primitive erythroid and myeloid cells and the cells lining the blood islands become endothelial cells. Blood islands then merge to form a vascular network throughout the YS, which ultimately helps to create blood flow.

1.2.3 Second wave of haematopoiesis: The EMP-wave

The second wave of haematopoiesis arises from EMPs in the YS and begins the transition from primitive to definitive haematopoiesis. From E7.5, early EMPs start to arise in the YS which contributes to primitive haematopoiesis. Definitive haematopoiesis starts with late EMPs arising in the YS around E8.5-9.5 (**Figure 1-3**). EMPs are highly proliferative, expanding predominantly in the YS, and migrating to the liver between E10.5-11. Overlapping with EMP production, is the wave of HSCs that emerge intra-embryonically from the AGM region and other haemogenic sites (placenta, vitelline and umbilical arteries) between E10 and E12 (104-107).

Early EMPs differentiate into YS macrophages, which do not involve a monocyte intermediate but rather sequential differentiation into CX₃CR1⁺ pre-macrophages (~E9.5) and mature F4/80^{Hi} macrophages (~E10). On the other hand, late EMPs differentiate into a monocyte intermediate. Interestingly, EMPs only start to differentiate into different lineages at ~E10; thus, any macrophages present before this time are thought to represent only progeny of primitive YS myelopoiesis (either from EMP pre-macrophages or primitive precursors). At ~E10.5, EMPs enter the circulation and start to seed the developing foetal liver. Once there, they produce the first enucleated erythrocytes and give rise to bipotent granulocyte/macrophage progenitors and mast cells (**Table 1-1**). From E12.5 onwards, these EMP-derived macrophages gradually replace those produced during the primitive wave, and hence, are thought to comprise the majority of tissue-resident macrophages found at birth, with the exception of the brain (2, 92, 93, 108). EMPs are thought to be short-term haematopoietic progenitors, as transplantation of E10.5 EMPs into either normal or immunocompromised adult mice produces erythroid cells but very few myeloid cells or platelets (30). Due to this lack of long-term haematopoietic potential in adult recipients, the EMP wave of haematopoiesis is also called the “transient definitive wave” (109).

Although the origin of YS EMPs is still debated, they are thought to derive from haemogenic endothelium (42, 110-113). As stated above, studies using timed-induction of CDH5 lineage-tracing have shown little contribution from E9.5 YS CDH5⁺ cells to primitive haematopoietic cells but rather robust labelling of definitive haematopoietic cells (59). EMPs have been shown to be efficient at expanding and differentiating into macrophages and megakaryocytes within their foetal liver niche (30, 114, 115). Within the foetal liver environment, they rapidly produce megakaryocyte-erythroid progenitors, mast cell progenitors and granulocyte-monocyte progenitors. Up until ~E16, the majority of foetal liver myeloid cells are of EMP origin (114). By comparison, definitive HSCs lag behind in this process and only begin to contribute to myeloid progenitors, megakaryocyte-erythroid progenitors, mast cell progenitors and granulocyte-monocyte progenitor intermediates in foetal liver around late gestation (after E16) (114).

Due to temporal and spatial overlap of EMP-mediated haematopoiesis with both primitive and HSC-derived myelopoiesis, it has been necessary to try to distinguish these three processes by identifying unique cell surface markers. In mouse E9.5 YS, EMPs are c-Kit⁺CD41⁺ and share other markers with haemogenic endothelial cells and developing AGM HSCs, including CD31, CD45, and Integrin beta-3 (CD61) (89, 116-118). However, only EMPs express Fcγ receptor (CD16/32) (30, 119, 120). In addition, they lack stem cell antigen-1 (Sca-1), which conversely is expressed on approximately 50% of AGM HSCs at that time (E9.5-10.5) (30, 119, 121). Furthermore, although CD93 (AA4.1) is highly expressed by foetal liver HSCs, it is present on only ~15% of E9.5 EMPs (30, 89). Notably, the majority of CD93⁺ cells in E9.5 YS have been found to be c-Kit⁺CDH5⁺ endothelial cells (30).

Using a combination of lineage mapping and adoptive transfer irradiation studies in mice, Hoeffel *et al.* demonstrated that YS-derived c-Kit⁺CD41⁺CD16/32⁺CD93⁻ EMPs in foetal liver give rise to resident macrophages in several different adult tissues via monocytic intermediates (2). Other groups have also used lineage-tracing to show that macrophages residing in postnatal aorta, skeletal muscle, lung, heart

brain and liver originate from CSF1R⁺ EMPs in E8.5 YS and/or CX₃CR1⁺ precursors in E9.5 YS. However, it appears that neither CSF1R nor CX₃CR1 is indispensable for the generation of EMPs (98, 122). Currently, it is not precisely known when or where CSF1R⁺ EMPs acquire CX₃CR1 expression. However, intravital live imaging microscopy has revealed that CX₃CR1⁺ progenitor cells start to leave the YS by accessing the bloodstream at E9. By E12.5, all CX₃CR1⁺ cells are relocated in foetal liver, as well as other tissues in the developing embryo, including AGM and the brain (1). Notably, in *Cx3cr1*-deficient mice there is delayed colonisation of resident macrophages in the embryonic head, caudal region and limbs, indicating the dependence of tissue colonisation on CX₃CR1 (123).

Complicating matters somewhat is the fact that both CX₃CR1 and CSF1R have also been linked to primitive haematopoietic precursors and primitive macrophages in YS and therefore cannot be reliably used to track only EMP-derived cells. One distinguishing receptor between these two haematopoietic waves could be Toll-like receptor 2 (TLR2), which has been found to be essential for the development and function of E8.5 YS c-Kit⁺ EMPs, but was dispensable for primitive erythropoiesis (124). However, this study did not report the effect of TLR2 deficiency on other primitive haematopoietic cells, such as macrophages, meaning that TLR2 has not yet been definitively proven to discriminate between primitive and EMP-derived haematopoiesis (124).

YS EMPs and definitive AGM HSCs appear to share even greater phenotypic overlap. Foetal livers from *Myb*^{-/-} mice lack all c-Kit⁺ populations, indicating the importance of c-Myb for regulating both EMPs and HSCs (101) as distinct from primitive haematopoiesis which is c-Myb independent. Indeed, late EMPs are c-Myb⁺ both in E9.5 YS and foetal liver at later gestational ages, while early EMPs are c-Myb⁻ (2). RUNX1 also regulates both the EMP and HSC waves of definitive haematopoiesis, and *Runx1*^{-/-} mice are embryonically lethal at ~E12.5 due to deficiency of foetal liver haematopoiesis. Moreover, *Runx1*^{-/-} mouse ESCs can neither form haematopoietic colonies *in vitro*, nor generate myeloid cells after blastocyst injection into WT recipients (125). The binding partner of RUNX1, core

binding factor β (CBF β), is also indispensable in EMP development. In *Cbfb*^{-/-} mice, forced expression of CBF β driven by the *Tie2* promoter rescues the number of EMPs in E12.5 foetal liver (126). As discussed previously, RUNX1 and CBF β are also specifically involved in the generation of EMPs and HSCs via endothelial-to-haematopoietic transition (100). However, there may be some differences in the regulatory effect of CBF β between EMPs and HSCs, as restoration of CBF β expression in *Tie2*⁺ cells, which encodes *Tie2*, was able to rescue EMP formation, but not HSC formation (126). On the other hand, another study showed that expression of CBF β in Sca-1⁺ cells was sufficient for HSC but not EMP formation, adding further complexity to haemogenic endothelial giving rise to EMPs or HSCs. These studies further supports the idea that YS haemogenic endothelial cells may be different from their intraembryonic counterparts and are dependent on their developmental timing, their phenotype, and the surrounding microenvironment (127).

There has been conjecture around the extent to which early versus late EMPs gives rise to different tissue macrophages. Whereas the study by Hoeffel *et al.* concluded that c-Myb⁺ EMPs in liver were an important source of resident macrophages in skin, lung, liver, kidney and gut before birth (2), work by Schulz *et al.* has argued that primitive YS c-Myb⁻ EMPs seed resident macrophages in skin, liver and brain (101, 128). Under homeostatic conditions, activation of *Csf1r* or *Runx1* promoter-driven reporters at E8.5-9.5 leads to labelling of E10.5 EMPs and, hence, tissue resident macrophages (98, 129, 130). However, when *Csf1r* and *Runx1* reporters are activated at E13.5 many different myeloid cells are labelled including foetal macrophages, foetal liver monocytes, granulocytes and adult foetal-derived macrophages. Since no phenotypic marker is known to distinguish macrophages derived from distinct haematopoietic sources (primitive-wave, EMP-wave and HSC-wave), these lineage-tracing studies describing the origin of tissue-resident macrophages are highly dependent on the time window of the labelling and tracing studies, relative to the onset of the distinct haematopoietic waves. As a result, the temporal overlap of the different waves of haematopoiesis during embryonic development confounds the interpretation of these studies (89).

1.2.3.1 Other EMP progeny

Recently, Plein *et al.* used inducible *Csf1r*-Cre and *Kit*-Cre mice to demonstrate that E8.5 early EMPs also give rise to endothelial cells lining blood vessels in the YS, as well as embryonic and postnatal liver, brain, heart and lung. Unlike the classic differentiation of angioblasts to endothelium, these EMPs are believed to return to their initial endothelial fate and combine within existing vessels (131, 132). However, a subsequent study that predominantly used immunofluorescence staining and confocal microscopy of different tissue sections could not find any evidence of EMP-derived vascular endothelial cells in the above-mentioned organs, postnatally (133).

There is now emerging evidence from inducible *Cx3cr1*-iCre lineage-tracing and scRNA-seq data that early EMPs also give rise to osteoclasts in neonatal bones (134). In particular, this study provided evidence that EMP-derived macrophages fuse with local HSC-derived macrophages in bone to form multinucleated osteoclasts, which contribute to postnatal bone remodelling under both homeostatic and inflammatory conditions (134).

On the basis of timed lineage-tracing with tamoxifen inducible *Csfr1* and/or *Runx1*-iCre mice, other groups have determined that c-Myb⁺ late EMPs in foetal liver are the predominant source of circulating erythrocytes (135) and skin mast cells (136, 137) *in utero*, while YS early EMPs also produce a distinct subtype of natural killer (NK) cells which have a potent degranulation response (138). While liver EMPs have been shown to contribute to megakaryocyte and erythrocytes (139).

Interim Conclusion

From E7.5, Myb-independent macrophage progenitors, in both multipotent EMP and pre-macrophage stages, expand in YS and travel via the bloodstream to embryonic tissues where they complete their maturation (1). This migration peaks around E10.5 and is mostly complete by E12.5. From ~E8.5, late Myb⁺ EMPs also differentiate into YS macrophages and traffic to liver, where they expand and differentiate into lineage-specific haematopoietic progenitors and foetal blood cells, including monocytes (2).

1.2.4 Third haematopoiesis wave: HSC-wave

The third wave of haematopoiesis, the HSC wave, begins in the ventral floor of the dorsal embryonic aorta (AGM) at around E10.5 in mice (17) and 27 days of gestation in humans (140) (**Figure 1-4**). The discovery that HSCs are first derived from the AGM was first shown in chick-quail embryo chimaeras (141, 142). In humans, HSCs express both haematopoietic and endothelial markers that include: CD34, CDH5, c-Kit, CD90, CD45, and Endoglin (CD105) (143). In the AGM region, intra-aortic haematopoietic clusters are located around the ventral wall and cover the endothelial lining. Due to the nature of these clusters, it is hypothesised that HSCs, like EMPs, are generated from haemogenic endothelium (**Figure 1-3; Figure 1-4**), which undergoes RUNX1-dependent endothelial-to-haematopoietic transition. This is also supported by the fact that HSCs are able to be generated from AGM CDH5⁺ progenitors in coculture with OP9 mesenchymal stromal cells or endothelial cells *in vitro* (68, 144-147).

From the AGM, HSCs are released into the circulation and migrate into the foetal liver between E11.5-E12.5 in mice where they join YS-derived EMPs. Between E11.5 and E16.5, HSCs in the foetal liver undergo dramatic proliferative expansion, which is an important point of difference from the inherently quiescent nature of postnatal BM HSCs (148, 149). As indicated previously, HSCs only start to contribute to myeloid progenitors, megakaryocyte-erythroid progenitors, mast cell progenitors and granulocyte-monocyte progenitors in foetal liver after E16.5. Around this stage HSCs also begin to migrate to spleen (E16.5-17.5) and later BM (E17.5), which becomes the main haematopoietic site after birth. Once HSCs begin to seed in the BM, their numbers begin to drop off in foetal liver and spleen. They can, however, still be detected in the spleen until a few weeks after birth. Their lineage commitment is considered to be mostly limited to erythropoiesis in adult mice under steady state conditions (150).

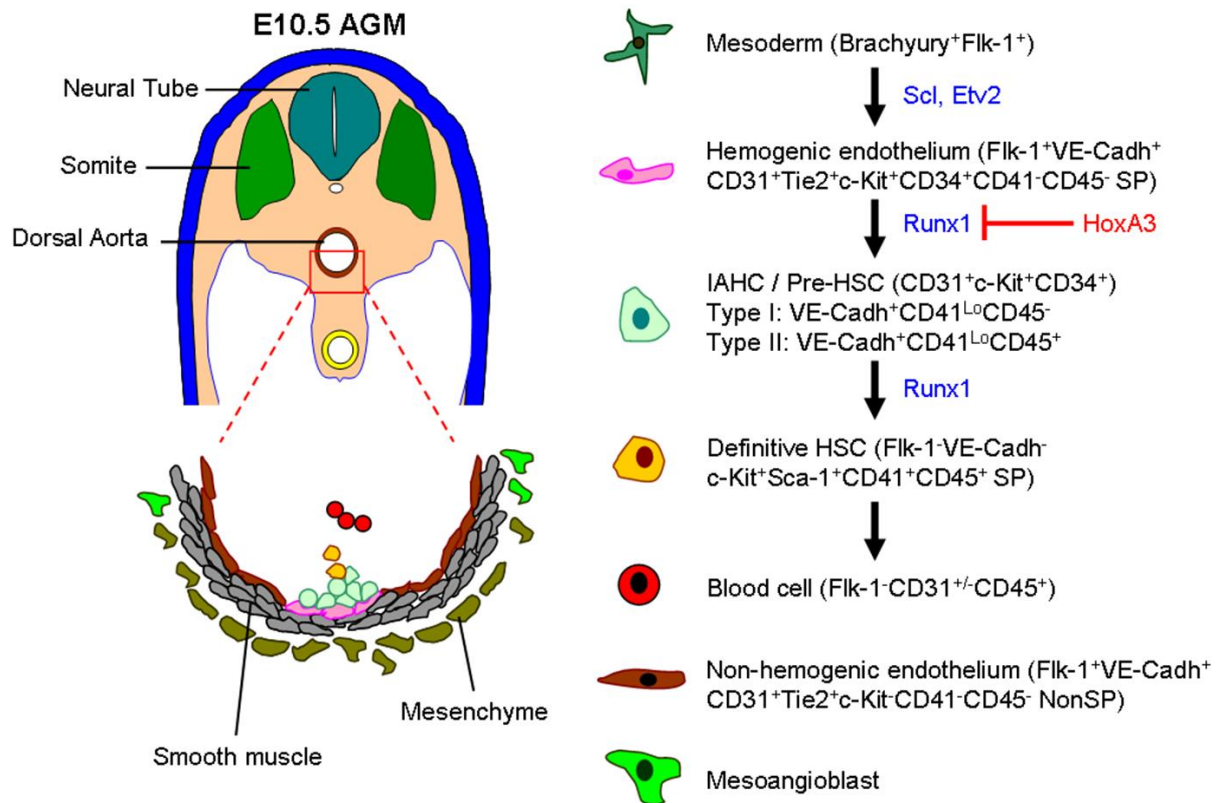


Figure 1-4. Vascular origins of definitive haematopoiesis during embryogenesis.

In embryonic development definitive haematopoiesis starts at E10.5–12.5 in mice with the emergence of HSCs from haemogenic endothelium in the ventral floor of the dorsal aorta in the para-aortic splanchnopleure and subsequent aorta-gonad-mesonephros (AGM) region. Intermediary pre-HSCs begin to aggregate to form intra-aortic haematopoietic clusters (IAHCs). Mesoangioblasts, which lack haematopoietic potential, reside in the roof and lateral walls of the dorsal embryonic aorta and may possibly originate from extra-embryonic endothelium. Image from Psaltis *et al.* (73)

1.2.5 Ontogeny of macrophages in postnatal tissues

1.2.5.1 Postnatal macrophage origins: Traditional BM-haematopoiesis

Throughout the postnatal lifespan of mammals, monocytes and macrophages are continuously generated via the hierarchical process of definitive haematopoiesis occurring in BM (114). This begins with CD34⁺ long-term (LT)-HSCs that differentiate into CD34⁺ short-term (ST)-HSCs, which then give rise to multipotent progenitors (MPPs) and subsequently into different lineage-committed progenitor cells (151). These comprise of common lymphoid progenitors (CLPs) for the differentiation of lymphocytes, such as T, B, and NK cells, and common myeloid progenitors (CMPs) for the differentiation of innate myeloid cells (152). CMPs further produce granulocyte and macrophage progenitors (GMPs), which are characterised as being LIN⁻c-Kit⁺Flt3⁺CSF1R^{Lo}FcγR^{Hi} and thereafter GMPs give rise to LIN⁻Sca-1⁻c-Kit^{+/Int}Flt3⁺CSF1R⁺CX₃CR1⁺ macrophage/dendritic cell progenitors (MDPs). These, in turn, differentiate into LIN⁻c-Kit⁺Flt3⁻CSF1R⁺Ly6C⁺CD11b⁻ common monocyte progenitors (cMoPs) that are specifically committed to become monocytes (152, 153).

The heterogeneity of human monocytes was first identified in the late 1980s, when they were classified into two main subpopulations: CD14⁺CD16⁻ and CD14^{Lo}CD16⁺ (154). In mice, monocytes can be identified by their expression of CSF1R and CD11b. Over the past decade, the use of *Cx3cr1*^{GFP} mice has further facilitated the distinction of murine monocyte subsets between BM and blood with other peripheral tissues (155, 156). Mouse monocytes are commonly classified into two main subtypes: Ly6C^{Hi} classical monocytes, which typically are also CCR2⁺/CX₃CR1^{Lo} and Ly6C^{Lo} nonclassical monocytes, which are also CCR2⁻/CX₃CR1^{Hi} (157, 158). Comparatively, murine Ly6C^{Hi} monocytes and Ly6C^{Lo} monocytes correspond to the CD14⁺CD16⁻ classical monocytes and CD14^{Lo}CD16⁺ nonclassical monocytes in humans, respectively, while CD14⁺CD16⁺ cells are referred to as intermediate monocytes (159). Despite similar transcriptional profiles and cell surface marker expression, there are differences between human and mouse monocytes. On one hand, Ly6C^{Lo} monocytes express major

histocompatibility complex (MHCII), while it is absent on mouse Ly6C^{Hi} monocytes. On the other hand, human monocytes generally express MHCII (160).

The development of circulating monocytes is controlled by a variety of factors, including stress, diet, sleep, environmental temperature, and caloric intake. Produced in the BM, Ly6C^{Hi} classical monocytes migrate to the blood vasculature where they have the potential to extravasate to peripheral tissues or differentiate into Ly6C^{Lo} monocytes in blood (161). Fate mapping approaches, adoptive monocyte transfer, and BrdU pulse-chase studies have shown that Ly6C^{Hi} blood monocytes have a half-life of ~1 day before differentiating into Ly6C^{Lo} which has a lifespan of ~2.2 days (162). Similar to murine monocytes, human classical monocytes differentiate to the nonclassical monocytes with a lifespan of 7 days via CD14⁺CD16⁺ intermediate monocyte populations (163).

It was long thought that BM-derived circulating monocytes were the only source of all tissue-resident macrophages in adult mammals. However, as mentioned above, this paradigm has been challenged over the past decade. Numerous studies have shown embryonically-derived macrophage subpopulations in different tissues that have originated either from primitive YS precursors or EMPs (89, 92, 160, 164). Although monocytes certainly also generate adult tissue macrophages, they seem to make a minimal contribution to replacing embryonically-seeded, YS-derived macrophages in healthy brain, liver and skin epidermis (93). In other healthy tissues, a growing body of evidence demonstrates that YS- and HSC-derived macrophages coexist to maintain normal homeostasis. As shown for dermis (165), heart (166, 167), aorta (168, 169), lung (170, 171) and skeletal muscle (172), the different macrophage origins typically correspond to different macrophage subtypes, characterised by different biological functions, transcriptomic and phenotypic signatures (173).

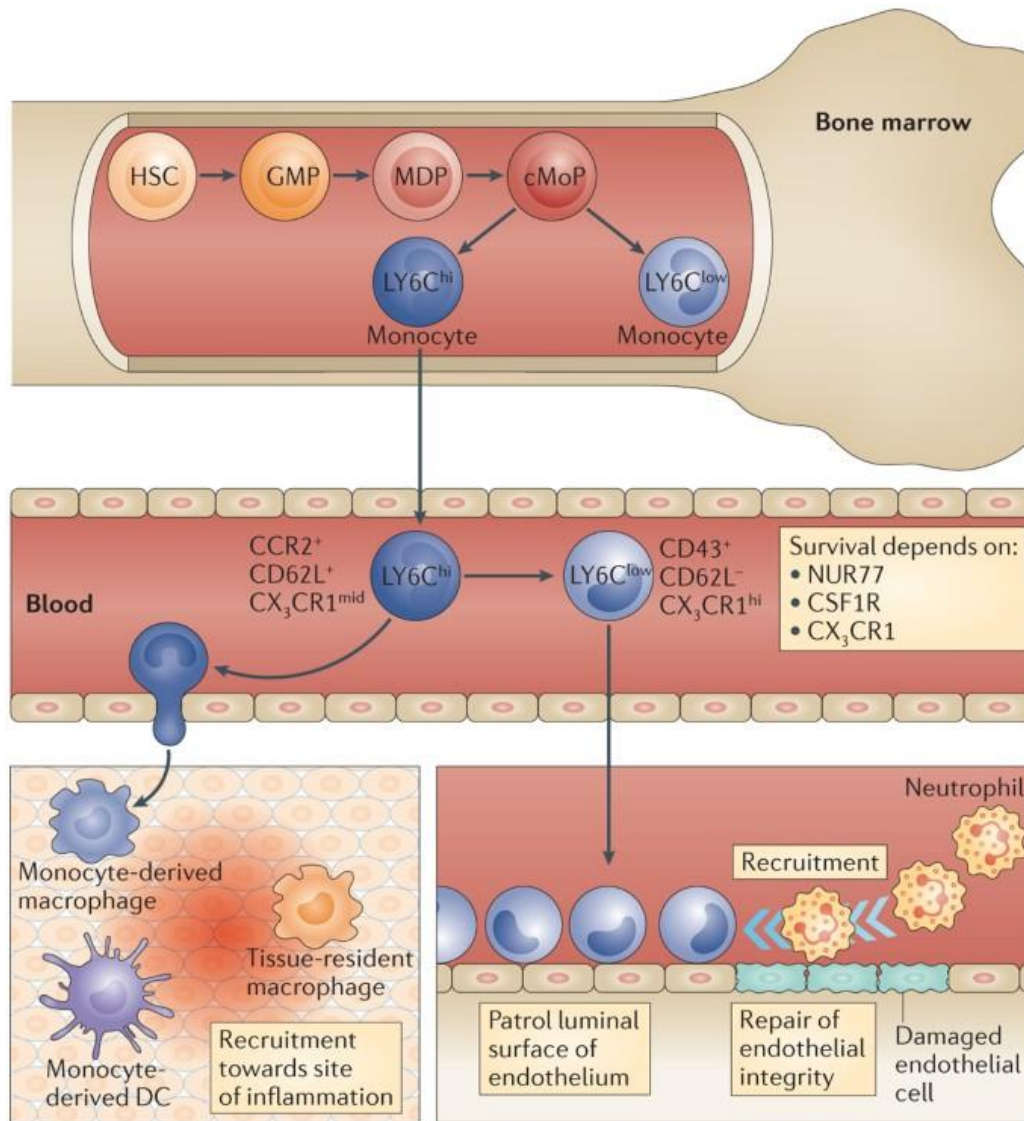


Figure 1-5. Postnatal monocyte development from definitive haematopoiesis.

Monocytes are generated in the BM from HSCs via MDP and cMoP intermediates. In steady state, circulating Ly6C^{hi} monocytes differentiate into Ly6C^{Lo} monocytes. Circulating Ly6C^{Lo} monocytes patrol the endothelial surface and coordinate its repair by recruiting neutrophils. While circulating Ly6C^{hi} monocytes are recruited to sites of inflammation or injury where they give rise to monocyte-derived dendritic cells (DCs) and macrophages. cMoP, Common monocyte progenitor; CCR2, CC-chemokine receptor 2; CSF1R, Colony-stimulating factor 1 receptor; CX₃CR1, CX3C-chemokine receptor 1; DC, Dendritic cell; GMP, Granulocyte-macrophage progenitor; HSC, Haematopoietic stem cell; MDP, Macrophage and dendritic cell progenitor. *Image from Ginhoux et al. (174)*

1.2.5.2 Postnatal macrophage origins: tissue-resident macrophages

There is now compelling evidence from murine studies that many populations of tissue resident macrophages are derived embryonically from early YS origins (89, 92, 160, 164). This includes the microglia in brain (175) and macrophages in the arterial adventitia (168, 169), heart (175), skin (epidermis and dermis) (176), lung (171), liver (175) and skeletal muscle (172) (**Table 1-2**). It has become increasingly apparent that the ontogeny of different YS-derived tissue-resident macrophages is complex. Until now it remains unclear whether they derive specifically from early EMPs, late EMPs, primitive macrophages or even a YS macrophage-specific precursor.

A critical and consistent finding throughout the existing literature is that YS-derived, postnatal tissue macrophages are able to be maintained independently of circulating monocyte and BM haematopoiesis. This has led experts in the field to conclude that they self-renew in their functional and differentiated state (177). However, direct evidence that mature macrophages can achieve self-renewal seems to be primarily based on observations from genetically manipulated (*Mac^{f/-}*) cells (178), but definitive *in vivo* proof for its occurrence in wildtype mice is lacking. Most mature cell types rely on the self-renewal of tissue-specific stem cells and/or the proliferation and differentiation of transient amplifying progenitor cells for their homeostatic turnover and recovery after tissue injury (177). This has led to speculation that local YS-derived progenitors in postnatal tissues may exist that are responsible for the apparent self-maintenance of tissue-resident macrophages after birth (177, 179). As such, there are currently opposing views as to whether self-maintenance of YS-derived tissue macrophages occurs due to *bona fide* self-renewal or via an ancestral progenitor intermediate, with both arguments mostly based on under-developed evidence. On the one hand, a study by Hashimoto *et al.* concluded that local macrophage expansion in brain, skin, lung and spleen does not require a progenitor population. This is because during recovery from cytotoxic depletion, macrophages were observed to have the same probability of entering the cell cycle, irrespective of whether they had divided previously (164). Ghigo *et al.* (180) used lineage-mapped multi-colour mice to show that division of epidermal Langerhans cells occurs from local, clonal proliferative units composed of dividing parent cells and their terminally differentiated daughter cells. This suggested the existence of a clonogenic macrophage precursor that

gives rise to Langerhans cells. Similarly, using the same multi-colour lineage-mapping approach, another study revealed that in response to neuronal damage there was local clonal expansion leading to clusters of daughter cells (microglia) in the brain (181). Moreover, it also showed that most individual adult microglia do not display extended longevity. Whilst this seems to suggest that microglia do not uniformly maintain self-renewal, the authors still argued that this is likely explained by individual CX₃CR1⁺ microglia (rather than microglial precursors) replicating and dividing in a clonal fashion. In contrast, Elmore *et al.* claimed to have identified a Nestin⁺ progenitor cell in the brain capable of generating microglia (182). More recently, another study found that after induced depletion of microglia in mice, a CX₃CR1⁺ myeloid cell was able to replace microglia with no interference from BM-derived cells (183). These cells displayed upregulation of cell cycle genes (*Pak3*, *Swi5*, *Psmc11*, *Stat3*), developmental genes (*Gfap*, *Rab14*, *Zfp612*) and myeloid genes (*Ccl12*, *Cd52*, *Lyz2*, *Itgb2*, and *Cd84*) and could potentially be a dedicated microglia precursor.

Interim Conclusion

In most tissues self-renewal is a characteristic of undifferentiated stem or progenitor cells and despite expert assertions. However, with the exception of a few studies (164, 177), there is limited proof that mature macrophages actually achieve this *in vivo*.

Tissue	Macrophage	Origin	Reference
Bone Marrow	Stromal macrophages	BM-derived	Udagawa <i>et al.</i> (184)
	Osteoclasts	Early EMP-derived BM-derived	Yahara <i>et al.</i> (134) Udagawa <i>et al.</i> (184)
Spleen	Red pulp	Early EMP-derived Foetal liver HSC-derived	Kurotaki <i>et al.</i> (185), Sheng <i>et al.</i> (175), Gonzalez <i>et al.</i> (186)
	Marginal zone, metalophids	BM-derived	Gonzalez <i>et al.</i> (186), Gordon <i>et al.</i> (176), Silva <i>et al.</i> (187)
	Outer marginal zone, White pulp	BM-derived	Gonzalez <i>et al.</i> (186), Gordon <i>et al.</i> (176), Silva <i>et al.</i> (187)
Lymph nodes	Subcapsular macrophages	Early EMP-derived	Louie <i>et al.</i> (188)
	Medulla	BM-derived	Louie <i>et al.</i> (188)
Gut	Lamina propria macrophages	BM-derived	Bain <i>et al.</i> (189), Viola <i>et al.</i> (190)
	Submucosal macrophages	Early EMP-derived	Bain <i>et al.</i> (189), Viola <i>et al.</i> (190)
Peritoneal cavity	Large resident macrophages	Early EMP-derived	Cassado <i>et al.</i> (191)
	Small resident macrophages	BM-derived	Cassado <i>et al.</i> (191)
Liver	Kupffer cells	Early EMP-derived Late EMP-derived Foetal liver HSC-derived	Sheng <i>et al.</i> (175), Gordon <i>et al.</i> (176)
Lung	Alveolar macrophages	Late EMP-derived Foetal liver HSC-derived Early EMP-derived BM-derived	Evren <i>et al.</i> (170), Gordon <i>et al.</i> (176), Guilliams <i>et al.</i> (171)
	Bronchial macrophages and DCs	Late EMP-derived BM-derived	Evren <i>et al.</i> (170), Gordon <i>et al.</i> (176)
Heart	CCR2 ⁻ resident macrophages	Unknown but presumably early EMP-derived	Epelman <i>et al.</i> (192), Wong <i>et al.</i> (167), Sansonettil <i>et al.</i> (193)
	CCR2 ⁺ resident macrophages	BM-derived	Epelman <i>et al.</i> (166), Wong <i>et al.</i> (167), Sansonettil <i>et al.</i> (193)
Adventitial Arteries	Resident macrophages	Early EMP-derived	Ensan <i>et al.</i> (194) Weinberger <i>et al.</i> (169)
Brain	Microglia	YS primitive macrophage precursor-derived	Sheng <i>et al.</i> (175), Poupot <i>et al.</i> (195), Azzoni <i>et al.</i> (196)
	Border-associated macrophages	YS primitive macrophage precursor-derived	Utz <i>et al.</i> (103)
	Perivascular macrophages	BM-derived	Gordon <i>et al.</i> (176)
	Choroid plexus macrophages	BM-derived	Gordon <i>et al.</i> (176)
	Meningeal macrophages	BM-derived	Gordon <i>et al.</i> (176)
Skin	Dermal resident macrophages	Early EMP-derived Late EMP-derived	Gordon <i>et al.</i> (176), Poupot <i>et al.</i> (195)
	Epidermal Langerhans cells	YS primitive macrophage precursor-derived Late EMP-derived	Sheng <i>et al.</i> (175), Poupot <i>et al.</i> (195), Ghigo <i>et al.</i> (180) Sheng <i>et al.</i> (197)
Skeletal muscle	Resident macrophages	Early EMP-derived Late EMP-derived	Wang <i>et al.</i> (172)
Adipose tissue	Resident macrophages	Early EMP-derived	Chen <i>et al.</i> (198)

Table 1-2. Origins of tissue-resident macrophages.

BM, Bone marrow; DC, Dendritic cells; EMP, Erythromyeloid progenitor; HSC, Haematopoietic stem cells; YS, Yolk sac.

1.2.6 Emergence of endothelial cells during embryonic development

The origin of endothelial cells prenatally and postnatally is complex. During development, endothelial cells contribute to vasculogenesis which gives rise to the aorta and its main branches. The onset of vasculogenesis occurs at E7.0-E7.25 in mice, with the formation of the extra-embryonic blood islands, thought to arise through haemangioblasts (as described above). Although most vascular endothelial cells are thought to arise through mesoderm-derived precursors, there is evidence that in different tissues multipotent progenitors can also give rise to both endothelial and myeloid populations [such as YS EMPs (132)]. Intraembryonic vasculogenesis begins between E9.5-E10.5 with the establishment and migration of strands of angioblasts from the lateral regions of the embryo toward the midline, where they coalesce to form the endocardium, dorsal aorta, and cardinal vein (73). The limb vessels also arise from angioblasts that migrate from mesodermal somites, while vascular connections are formed in many tissue beds between sprouts of the major vessels and locally generated angioblasts.

During this time the embryonic dorsal aorta becomes the source of mesoangioblasts which have been isolated from its posterior and lateral walls (199). These culture-defined progenitor cells express haematopoietic, endothelial and mesodermal markers. While they possess self-renewal capacity and differentiation plasticity for endothelial and mesodermal lineages *in vitro* and *in vivo*, they have not been shown to display *in vivo* haematopoietic repopulating potential (200).

Until now the origins of mesoangioblasts have been uncertain. A lineage-tracing study suggested that in a narrow window of early development (~E8.5-E9.5) a population of progenitors with mesodermal potency arises from CDH5⁺ endothelial cells in the extra-embryonic YS and placenta (201) (**Figure 1-4**). Pulse labelling and reporter gene tracking of these progenitors showed that they give rise to an intraembryonic mesoangioblast-like population capable of haematopoietic and mesenchymal colony

formation, *in vitro* differentiation into endothelial, adipocytes, SMCs, osteocytes, macrophages and chondrocytes and myogenic lineages and *in vivo* muscle regeneration (202). At this stage, little is also known about the role of mesoangioblasts in normal tissue development and whether they are present in postnatal tissues. It has been postulated that they accompany newly branching blood vessels during organogenesis, allowing them to adopt local tissue-specific properties, suggesting they may therefore share an ancestral relationship with postnatal mesenchymogenic pericytes (199). Like pericytes, mesoangioblasts have been used in cell transplantation studies to facilitate tissue repair in animal models of skeletal muscular dystrophy (203).

1.2.7 Origins of postnatal endothelial cells

It has been recognised that in postnatal tissues endothelial cells have the ability to replicate, although the capacity to do so is not evenly distributed throughout the vasculature (204). There are numerous potential mechanisms that have been used to explain how endothelial cells are able to expand during angiogenesis, vasculogenesis and repair of the vascular intima. These include: (1) the proliferation of mature endothelial cells, possibly involving a dedifferentiation process (205); (2) the recruitment and differentiation of BM-derived EPCs via peripheral blood circulation (206) and; (3) the existence of resident EPCs or other endothelial stem cells in tissues (207).

1.2.7.1 Endothelial progenitor cells (EPCs)

In the late 1990s, Asahara *et al.* demonstrated the existence of circulating BM-derived EPCs, expressing CD34 and VEGFR2, which were able to differentiate into endothelial cells (208). They contribute to endothelial progeny in neovessels at sites of tumorigenesis (206), wound healing (209) and ischaemia (210) as well as to intimal re-endothelialisation after vessel wall injury (211). By strict criteria, an EPC must display capacity for clonal expansion, self-renewal and proliferative capacity, and the ability to adhere to matrix molecules and differentiate into functional, mature endothelial cells (73, 212). However, in the last decade, the existence and nature of EPCs have been a source of controversy, with the field notoriously hindered by inconsistencies in their isolation, characterisation and differentiation into a variety of proangiogenic cell subsets. These were all originally labelled under the EPC banner without adherence to the necessary criteria stated above (212, 213). One such mislabelling of a so-called EPC population was actually of circulating monocytes contaminated with platelet microparticles that were derived from injured or ageing endothelial cell shedding from the vessel wall into the blood stream (214).

Through this confusion, it has emerged that EPCs are not a single, homogeneous progenitor population. Culture-based techniques to isolate EPCs have revealed two distinct subtypes: myeloid angiogenic cells (MAC) and endothelial colony-forming cells (ECFC) (6, 215). MACs appear early after one week in cultures of mononuclear cells. They originate from BM-derived haematopoiesis and express both haematopoietic (CD45 and CD14) and endothelial (CD31 and VEGFR2) cell markers. They are also able to take up acetylated-LDL (216). They do not, however, differentiate into mature endothelial cells *in vivo*. On the other hand, ECFCs appear in cultures of mononuclear cells after three weeks, express endothelial markers (CD31, CD34, CDH5 and VEGFR2), the adhesion protein CD146 and the TGF β receptor, endoglin (CD105). ECFCs are able to differentiate into mature endothelial cells both *in vitro* and *in vivo* (217). The origin of ECFCs is somewhat controversial, as it appears that they do not arise from BM (209, 218, 219), but rather from stem cells within the layers of the vessel wall itself including the intima and adventitia (214, 220-223). Therefore, it is postulated that these proangiogenic progenitor subtypes may relate to the continued existence of haemogenic endothelium postnatally, although this remains unproven. Overall, there is a paucity of data concerning the prenatal existence and origin of EPCs, such that is unknown whether circulating EPCs actually contribute to vascular growth during embryonic development.

Interestingly, there is some evidence that EPCs may arise from embryonic YS. Hatzopoulos *et al.* characterised an embryonic cell from murine YS at E7.5 expressing thrombomodulin and GATA4, which suggested they derived from the proximal lateral mesoderm (224). These cells were termed embryonic EPCs (eEPCs) and were able to differentiate into endothelial cells both *in vitro* and *in vivo*. Phenotypically, eEPCs were also TIE2⁺c-Kit⁺Sca-1⁺ during *in vitro* culture, but their expression of VEGFR2 was negligible (224). Another study revealed human placenta contained a bipotent EPC population which were CD45⁻CD34⁺CDH5⁺CD31^{Lo} (225). This population was further found to differentiate into both endothelial and mesenchymal colonies *in vitro* (225). Intriguingly, these bipotent cells were in vessel walls but not in contact with circulating cells, suggesting they do not come from the circulation.

Several studies have also suggested that EPCs are in fact not circulating, but instead resident in different tissues (226-228). Yoder *et al.* isolated a population of clonal, self-renewing EPCs from the adult vascular wall that were LIN⁻CD31⁺CD105⁺Sca-1⁺c-Kit⁺ (226). These EPCs were able to differentiate into mature endothelial cells and when transplanted into a mouse model of melanoma generated functional blood vessels that connected to the host blood circulation. Recently, different lineage-tracing approaches have been used to show that some tissues (skin, aorta, adipose) contain a hierarchy of three subpopulations of endothelial cells that can be distinguished by different levels of expression of CD31, CDH5, Sox18, and VEGFR2. These populations have been characterised as: endovascular progenitors (EVPs) which are CD31^{Lo}VEGFR2^{Lo} and are the ancestral source of both CD31^{Int}VEGFR2^{Lo} transit amplifying cells and CD31^{Hi}VEGFR2^{Hi} mature differentiated endothelial cells (229). The transition from EVPs through to mature endothelium has been shown to be regulated by Sox18/SoxF signalling. Single cell RNA-sequencing has also revealed that EVPs in aorta and skin express high levels of Sox9, *Vegfa*, *Pdgfra* and *Wnt* (9a, 5a, 10b, 16, 11, 2) (230, 231). Interestingly, some of these genes, such as Sox9, were previously associated with fibroblasts and further work on tissue-resident EVPs has suggested that they also have mesenchymal (or fibroblast) potential and may be a source of endothelial-to-mesenchymal transition (232).

Interim Conclusion

Much like macrophages, endothelial cells also arise from diverse origins. Most endothelial cells arise through mesoderm-derived precursors; however, studies have indicated they also develop from other progenitor populations (EMPs, EVPs, EPCs).

1.3 Origins of arterial macrophages and endothelial cells and their pathogenic roles in vascular diseases

1.3.1 Overview of vascular structure

Large and medium sized blood vessels have a trilaminar structure where each of the three layers has their own composition of proteins and cells that contribute to three distinct and important roles (**Figure 1-6**). Their innermost layer is referred to as the intima, which contains an inner lining of endothelial cells and possibly EPCs. This endothelial monolayer rests upon a basement membrane which is replete with structural proteins, such as type IV collagen, laminin and fibronectin (233). The media is the thick middle layer of the vessel. Its muscular nature results from the concentric layers of SMCs interwoven with elastin-rich extracellular matrix (ECM) (233). This layer has also been shown to contain small populations of stem cells (234, 235). The adventitia is the collagen-rich outermost layer of the vessel wall that is composed of heterogeneous populations of cells including fibroblasts and resident inflammatory cells (such as lymphocytes, macrophages, dendritic cells (DCs) and mast cells) (233). It also contains different types of progenitor/stem cells including those with mesenchymal, endothelial, SMC and haematopoietic potential (73, 236). Furthermore, there are nerve endings, lymphatic vessels and an extensive microvascular environment known as the *vasa vasorum*.

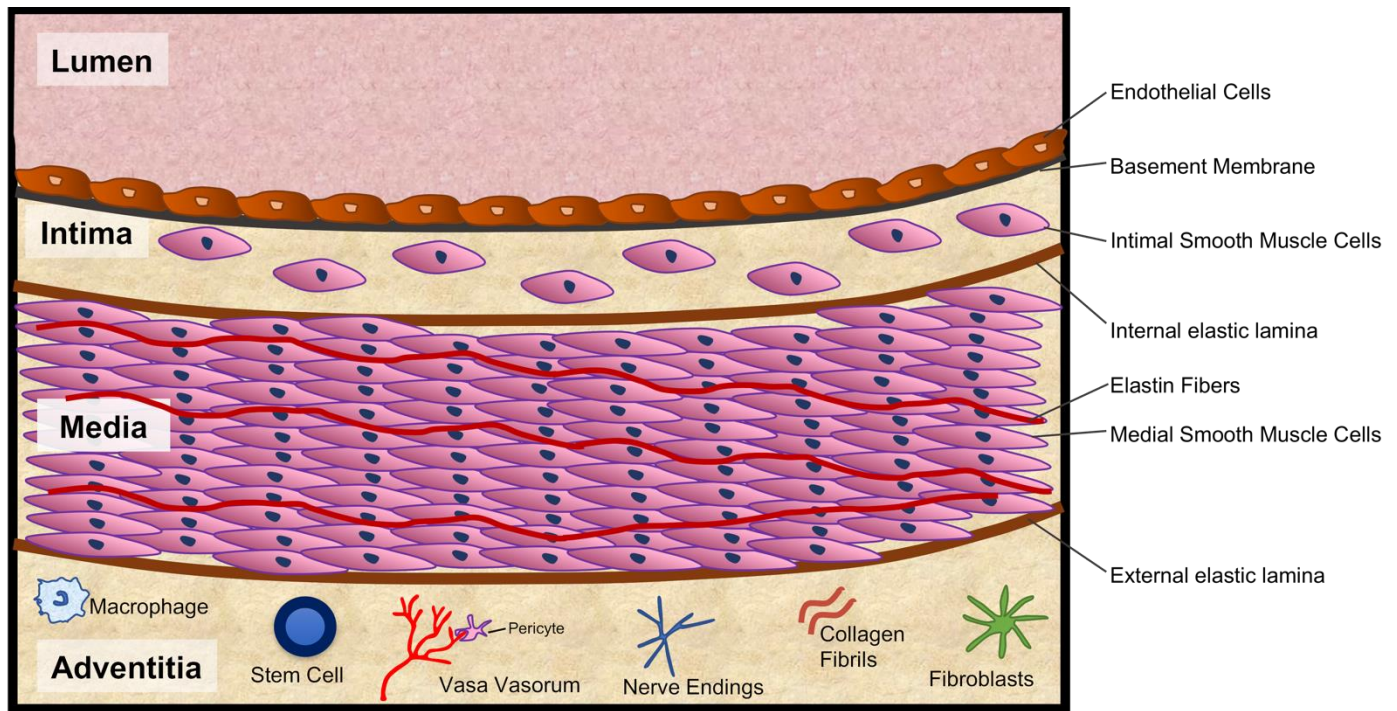


Figure 1-6. Trilaminar structure of a normal vessel wall.

The normal vessel wall is composed of three layers: intima (monolayer of endothelial cells) which resides on a basement membrane, media (concentric layers of SMCs interleaved with elastin fibres) and adventitia (fibroblasts, collagen fibres, nerve endings, *vasa vasorum*, stem cells, macrophages etc). The internal elastic lamina separates the intima from the media and the external elastic lamina separates the media from the adventitia. *Image from Fernando et al. (233)*

1.3.2 Vascular wall-resident progenitor/stem cells (VW-PCs)

Throughout postnatal life, the vasculature is exposed to many potentially harmful influences. It has therefore developed a unique adaptive capacity to respond to different pathophysiological stimuli. This includes a high capacity for rapid cell turnover, growth and repair to maintain normal homeostasis. Cells contained within the three mural layers of postnatal blood vessels were historically considered to be terminally differentiated. Over the past two decades, numerous studies have established the presence of a diverse range of vascular wall-resident progenitor/stem cells (VW-PCs), located within specialised niches in the vessel wall. The presence of these VW-PCs throughout the vascular layers may help to explain the high turnover rate of vascular cells, including both endothelial cells and SMCs after vascular injury (73). Subpopulations of VW-PCs have been shown to be capable of self-renewal and differentiation into different mature cell types, notably endothelial, SMC, pericytes, haematopoietic and mesenchymal cell lineages (73) (**Table 1-3**).

VW-PCs are especially abundant in the adventitia, which is unique in its “niche-like” properties (**Figure 1-7**). Different types of adventitial progenitor cells have been described in the arteries and veins of mice, large animals (e.g. pigs, cows) and foetal, neonatal and adult humans (73). These have been variably characterised based on their expression of different stem cell markers (e.g. Sca-1, c-Kit, CD34) and their differentiation properties under inductive stimuli *in vitro* and after adoptive transfer *in vivo* (73, 220). Although an exhaustive discussion of these is beyond the scope of this thesis, VW-PCs with endothelial and haematopoietic potential will be described in some detail here to provide necessary background for Chapters 3 and 4.

Vessel Wall Layer	VW-PC	Cell Marker	Function	References
Intima or Neointima	Endothelial Lineage	CD34 ⁺ /CD45 ⁻ /VEGFR2 ⁺ /CD31 ⁺ /Sca-1 ⁺ /c-Kit ⁺	Endothelial cell differentiation; Clonogenic potential	Ingram <i>et al.</i> (237), Torsney <i>et al.</i> (238), Naito <i>et al.</i> (228), Tsai <i>et al.</i> (239), Fang <i>et al.</i> (226), Patel <i>et al.</i> (229), Green <i>et al.</i> (240)
	Smooth Muscle Lineage	Sca-1 ⁺ /CD34 ⁺	Smooth muscle cell differentiation	Torsney <i>et al.</i> (238), Tsai <i>et al.</i> (239)
Media	Endothelial Lineage	Sca-1 ⁺ /c-Kit ⁺ /CD34 ⁺ or CD34 ⁻ /VEGFR2 ⁺ /CD31 ⁺ or CD31 ⁻ /Lin ⁻	Endothelial cell differentiation	Sainz <i>et al.</i> (234), Zengin <i>et al.</i> (220), Pasquinelli <i>et al.</i> (241)
	Smooth Muscle Lineage	Sca-1 ⁺ /CD34 ⁺ or CD34 ⁻	Smooth muscle cell differentiation	Sainz <i>et al.</i> (234), Tang <i>et al.</i> (235)
	Mesenchymal Lineage	CD29 ⁺ /CD44 ⁺ /CD105 ⁺ /CD34 ⁻ /CD45 ⁻ /Sca-1 ⁺ or Sca-1 ⁻	Chondrogenic, myogenic, and adipogenic lineage differentiation	Pasquinelli <i>et al.</i> (241, 242), Tang <i>et al.</i> (235), Zaniboni <i>et al.</i> (166, 243), Evans <i>et al.</i> (244)
Adventitia	Endothelial Lineage	CD34 ⁺ / VEGFR2 ⁺ /Sca-1 ⁺ /c-Kit ⁺	Endothelial cell differentiation	Torsney <i>et al.</i> (238), Sawada <i>et al.</i> (245), Toledo-Flores <i>et al.</i> (246)
	Smooth Muscle Lineage	Sca-1 ⁺ /CD34 ⁺ /CD45 ⁻ /Nestin ⁺	Smooth muscle cell differentiation	Wang <i>et al.</i> (247), Hu <i>et al.</i> (248), Torsney <i>et al.</i> (238), Klein <i>et al.</i> (249, 250), Chen <i>et al.</i> (251), Klein <i>et al.</i> (250), Wu <i>et al.</i> (252), Shen <i>et al.</i> (253), Majesky <i>et al.</i> (254), Passman <i>et al.</i> (255)
	Mesenchymal Lineage	CD29 ⁺ /CD44 ⁺ /CD105 ⁺ /CD90 ⁺ /CD45 ⁻ /CD34 ⁺ or CD34 ⁻ /Sca-1 ⁺ or Sca-1 ⁻ /CD31 ⁻ /Nestin ⁺ /Gli ⁺	Chondrogenic, myogenic and adipogenic lineage differentiation; Clonogenic potential	Hoshino <i>et al.</i> (256), Campagnolo <i>et al.</i> (257), Pasquinelli <i>et al.</i> (242), Klein <i>et al.</i> (249)
	Haematopoietic Lineage	CD34 ⁺ /Sca-1 ⁺ /CD45 ⁺ /F4/80 ⁺ /CD115 ⁺ /CD68 ⁺	Myeloid lineage differentiation	Corselli <i>et al.</i> (258), Evans <i>et al.</i> (244), Kramann <i>et al.</i> (259, 260), Zengin <i>et al.</i> (220), Zorzi <i>et al.</i> (261), Psaltis <i>et al.</i> (262, 263), Majesky <i>et al.</i> (254)

Table 1-3. Vascular stem/progenitor cells in the vessel wall.

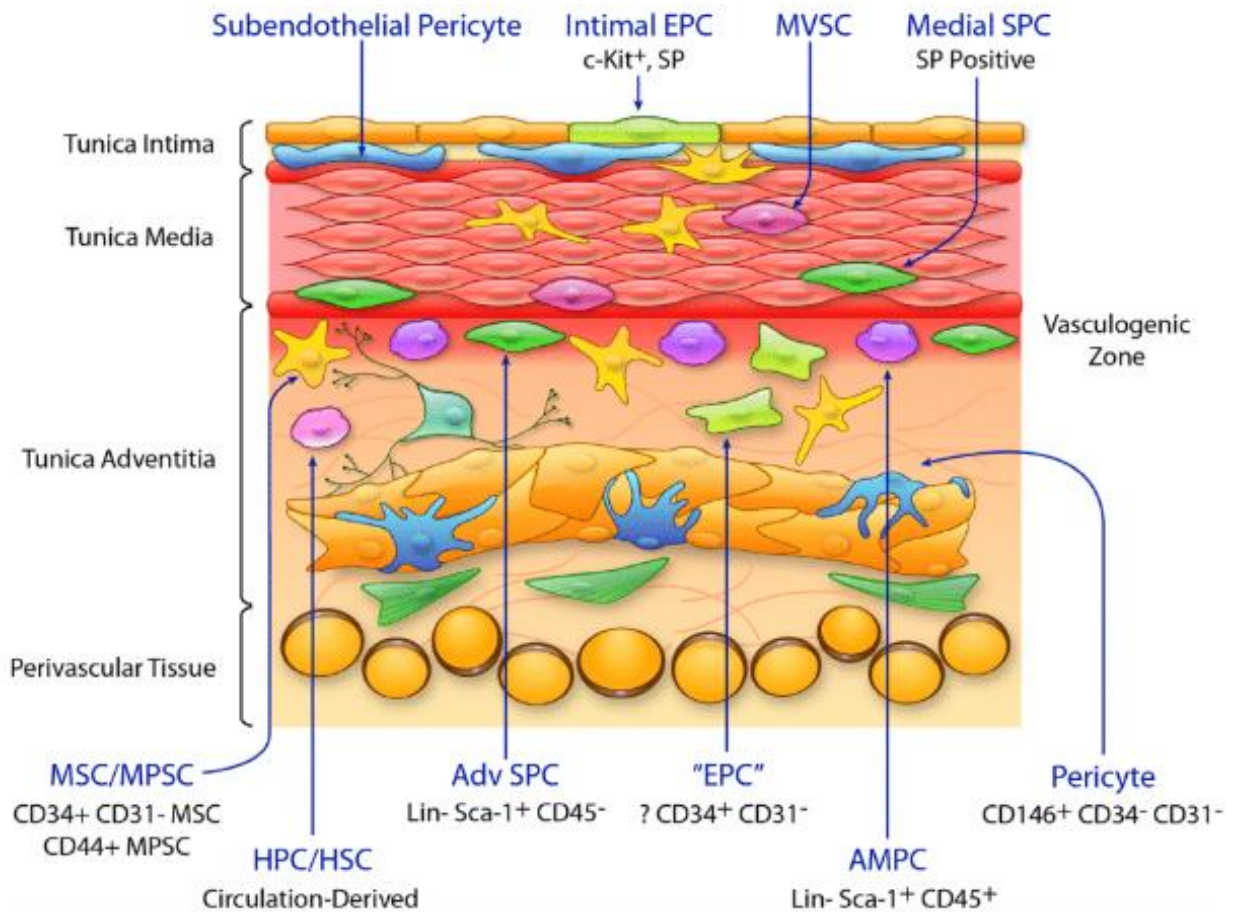


Figure 1-7. Distribution of different types of VW-PCs throughout the vessel wall.

The intima has been shown to contain endothelial progenitor cells (EPCs). The media contains mesenchymal stromal/stem cells and multipotent vascular stem cells (MVSCs). The adventitia hosts EPCs, smooth muscle progenitor cells (SPCs), adventitial macrophage progenitor cells (AMPCs) and mesenchymal stem cells (MSCs) in mice, as well as multipotent stem cells (MPSCs) in humans. *Image from Psaltis et al. (73)*

1.3.2.1 Vascular wall haematopoietic progenitors

The fact that definitive HSCs first arise from embryonic aorta prompts consideration as to whether postnatal blood vessels also harbour ancestral cells of haematopoietic lineage. Robust leukocyte populations, such as resident macrophages, are present in the adventitia of healthy and lesion-prone arteries, while haematopoietic cells are central in the angiogenic responses during all stages in the evolution of vascular diseases, including atherosclerosis and aneurysms.

There have been multiple murine studies showing the existence of postnatal vascular wall haematopoietic progenitor cells through traditional haematopoietic assays (**Table 1-3**). More than a decade ago, Montfort *et al.* interpreted that vascular intima contains proliferative cells with haematopoietic capacity after observing long-term chimers for CD3⁺ lymphocytes when segments of thoracic aorta and inferior vena cava were surgically transplanted beneath the renal capsule of irradiated mice (264). Recently, Psaltis *et al.* subsequently demonstrated that the adventitia of murine arteries, especially aorta, contains a hierarchy of HSCs/HPCs. These were comprised of rare numbers of BM-derived multilineage HSCs and a markedly enriched content of macrophage-dedicated progenitors that form colony forming unit-macrophages (CFU-M) when cultured in haematopoietic methylcellulose assays (262, 263). Lethal irradiation adoptive transfer studies showed that these adventitial macrophage progenitor cells (AMPCs) were maintained locally in the vessel wall after birth and were not reconstituted from a BM or splenic origin. Phenotypically, AMPCs were characterised as lacking surface expression of mature lineage markers (LIN⁻) and were positive for Sca-1 and the pan-leukocyte marker, CD45 (262, 263) (hence LIN⁻Sca-1⁺CD45⁺). This distinguished them from previously described adventitial Sca-1⁺CD45⁻ smooth muscle and endothelial progenitor cells (32, 255). In addition to their proclivity for producing CFU-M, Sca-1⁺CD45⁺ AMPCs showed a predilection for forming macrophages and DCs when stimulated *in vitro* by M-CSF or GM-CSF/IL-4, respectively, and after *in vivo* transfer into the carotid artery adventitia of high cholesterol-diet fed *Apoe*^{-/-} mice (263).

Later work by Ni *et al.* showed the existence of c-Kit⁺ cells in the adventitia (265). These c-Kit⁺ cells were split into two fractions, those that express CD45 and those that do not, suggesting these c-Kit⁺ cells possess multiple fate potentials *in vivo*. This was directly tested through the use of *Kit*^{CreER} x *Rosa*^{tdTomato} mice pulsed with tamoxifen and examined 1 w after the last tamoxifen injection (265). Intriguingly, ~70% of the adventitial tdTomato⁺ cells were Sca-1⁺, while ~40% were CD45⁺, suggesting that Sca-1⁺CD45⁺ cells express c-Kit *in vivo* (266). Furthermore, in an irradiation model, these c-Kit⁺ colonies were shown to be from a non-BM origin, further supporting Psaltis's studies.

1.3.2.2 Vascular wall endothelial progenitors

In the postnatal vasculature there has been data to suggest that some endothelial cells are generated by local vascular-resident progenitor cells. Many studies have now shown that resident non-haematopoietic progenitors isolated from different tissues (muscle, adipose, dermis, liver, and intestine) are able to achieve an endothelial transition. Moreover, several groups have specifically identified VW-PCs with endothelial or angiogenic potential. Although in the majority of cases, these progenitors have been multipotent with plasticity for non-endothelial lineages as well. Hence, few studies have been dedicated to the rigorous characterisation of endothelial-specific VW-PCs (**Table 1-3**).

On the basis of clonal analysis of human umbilical vein endothelial cells (HUVECs) and human aortic endothelial cells (HAECs), Ingram *et al.* observed that the renewal of vessel wall endothelial cells can be attributed to their content of a complete hierarchy of low- and high proliferative endothelial colony-forming cells (267). These intima-derived progenitor cells expressed endothelial surface markers: CD31, CD141, CD105, CD146, CDH5, von Willebrand factor (vWF) and VEGFR2 and, interestingly, displayed similar clonogenic potential to umbilical cord blood EPCs (267). Subsequent data have supported the theory that endothelial cell turnover is the result of a hierarchical organisation of different endothelial subpopulations discriminated by their clonogenic potential, such as the EVP cells described above (229, 231).

By a Hoechst dye exclusion method, stem cells in the luminal surface of pre-existing peripheral blood vessels have also been found to give rise to an endothelial cell (228). This stem cell population is dormant in the steady state but generates a significant number of endothelial cells when transplanted into ischaemic regions and restores blood flow of ischaemic tissue (228). Interestingly, the notion of the presence of resident endothelial progenitor cells with clonal proliferative and self-renewal capacities in the endothelium lining of the vessels was also observed. Fang *et al.* found endothelial colony-forming cells from adult mouse pulmonary vessels contained a rare population of endothelial cells, expressing Lin⁻CD31⁺CD105⁺Sca-1⁺c-Kit⁺ (226). Through *in vivo* transgenic reporter tracing, this clonal subpopulation was shown to be of endothelial origin and independent from haematopoiesis. Interestingly, *in situ* immunostaining revealed these Lin⁻CD31⁺CD105⁺Sca-1⁺c-Kit⁺ endothelial cells were located in the capillaries, arteries and veins of a range of organs (lung, liver, kidney, and subcutaneous tissues). There were also identified in neoangiogenic vessels in subcutaneous MatrigelTM plugs and in murine and human melanoma cells and breast cancer samples (226). Furthermore, the clonal progeny of single c-Kit⁺ endothelial cells formed host-perfused blood vessels *in vivo*, confirming their angiogenic potential (245, 265, 268-271). Recently, Deng *et al.* corroborated these results by demonstrating through scRNA-seq that endogenous non-BM derived-c-Kit⁺ cells in mouse aorta can acquire endothelial cell fate under both physiological and pathological conditions (272). Further studies performed with human coronary arteries showed they contain a small cluster of clonogenic, multipotent c-Kit⁺VEGFR2⁺CD45⁻tryptase⁻ progenitor cells which can differentiate towards becoming endothelial cells, SMCs, and partly, cardiomyocytes (273). These human VW-PCs were able to functionally operate, as they could integrate themselves into newly formed small, intermediate and large-sized blood vessels after *in vivo* transfer in a model of coronary artery stenosis in immunosuppressed dogs (273). Hence, these experiments highlight the potential of VW-PCs to contribute towards endothelial cell populations.

1.3.3 Atherosclerosis

Atherosclerotic cardiovascular diseases are the leading cause of mortality and morbidity around the world, accounting for ~30% of global deaths and imposing an enormous healthcare expenditure (233). Atherosclerotic cardiovascular diseases include myocardial infarction, ischaemic stroke and peripheral vascular disease. It is characterised by the formation of complex inflamed, lipid-rich lesions called plaques in arteries that pose risk by their flow-limiting nature and propensity for rupture or erosion that can lead to thrombotic occlusion (**Figure 1-8**). Atherosclerosis transitions through several major morphological and molecular stages as depicted in **Table 1-4**. Plaques display stability over time in which they are static, grow slowly or even regress or plaques become unstable. The latter plaques have characteristics that make them vulnerable to rupture of their fibrous cap or erosion of their endothelial surface which can be complicated by thrombosis and arterial occlusion. A number of atherogenic pathways play important roles in both the initiation of plaque formation, its progressive growth and ultimately its destabilisation. These include: endothelial cell dysfunction, inflammatory cell recruitment and activation and vascular SMC de-differentiation and proliferation (233). Plaque stabilisation is therefore considered an important objective of anti-atherosclerotic therapies. Currently, treatments for atherosclerosis target causative modifiable risk factors such as hypercholesterolemia and hypertension through cholesterol (e.g. statins) and blood pressure-lowering (e.g. angiotensin-converting enzyme [ACE] inhibitors) medications.

The initiation of atherosclerosis begins with endothelial dysfunction (274). It is characterised by a reduction in production/availability of nitric oxide and an increase in endothelium-derived contracting factors. This imbalance impairs the process of vasodilation of blood vessels. Endothelial cells become chronically activated through a combination of turbulent blood flow, lipid accumulation in the vessel wall and exposure to inflammatory mediators (275, 276). An activated endothelium has increased leukocyte adherence, cell permeability, low-density lipoprotein oxidation, platelet activation and vascular SMC proliferation and migration (277). Moreover, the adhesion, recruitment and trapping of immune cells, such as monocytes/macrophages, within plaque contributes to its expansion and ongoing

inflammation (**Figure 1-8; Figure 1-9**). VW-PCs, along with circulating progenitor cells, have also been implicated in the pathogenesis of atherosclerosis (73) (**Figure 1-9**). Increasingly, it is recognised that a better understanding is required of the regulatory mechanisms and functions of VW-PCs during different phases of atherosclerosis to determine whether they may represent a possible therapeutic target for new anti-atherosclerotic treatment strategies.

Stage	Gross Findings	Mechanisms
Intimal xanthomas / Fatty streaks	Bright yellow minimally raised lesions that demonstrate abundant lipid when stained with oil red O	Endothelial dysfunction, lipoprotein entry and modification, leukocyte recruitment, foam cell formation
Fibroatheroma	Homogenous, white, raised and firmer areas	Migration of SMCs from arterial media to the subintima and proliferation of SMCs, SMC-rich plaque with proteoglycan matrix and focal accumulation of extracellular lipids, formation of the necrotic core
Thin cap fibroatheroma	A thin, unstable fibrous cap ($\leq 65\mu\text{m}$) with an underlying necrotic core	Type 1 collagen, very few SMCs (apoptosis), large necrotic core ($>40\%$ of plaque volume), adventitial inflammation, neovascularisation, spotty calcification, intraplaque haemorrhage
Plaque rupture	Ulcerated plaques showing surface thrombosis	Ruptured fibrous cap, presence of luminal thrombus, larger necrotic core, increased macrophage infiltration, increased expression of MMPs and MPOs
Fibrous calcified plaque	Eruptive nodular calcification with underlying fibrocalcific plaque	Formation of calcified sheets in the collagen-rich matrix
Endothelial erosion	Appear as gross plaque that are visible to the naked eye	Absent endothelium, abundant SMC and proteoglycans, scarcely calcified, less macrophages and less inflammation

Table 1-4. Different stages in the development of atherosclerosis.

Different stages in the development of atherosclerotic plaques are described briefly in their approximate order of occurrence, derived from gross histological and mechanistic findings (278-287). MMP, Matrix metalloproteinases; MPO, Myeloperoxidase; SMC, Smooth muscle cell.

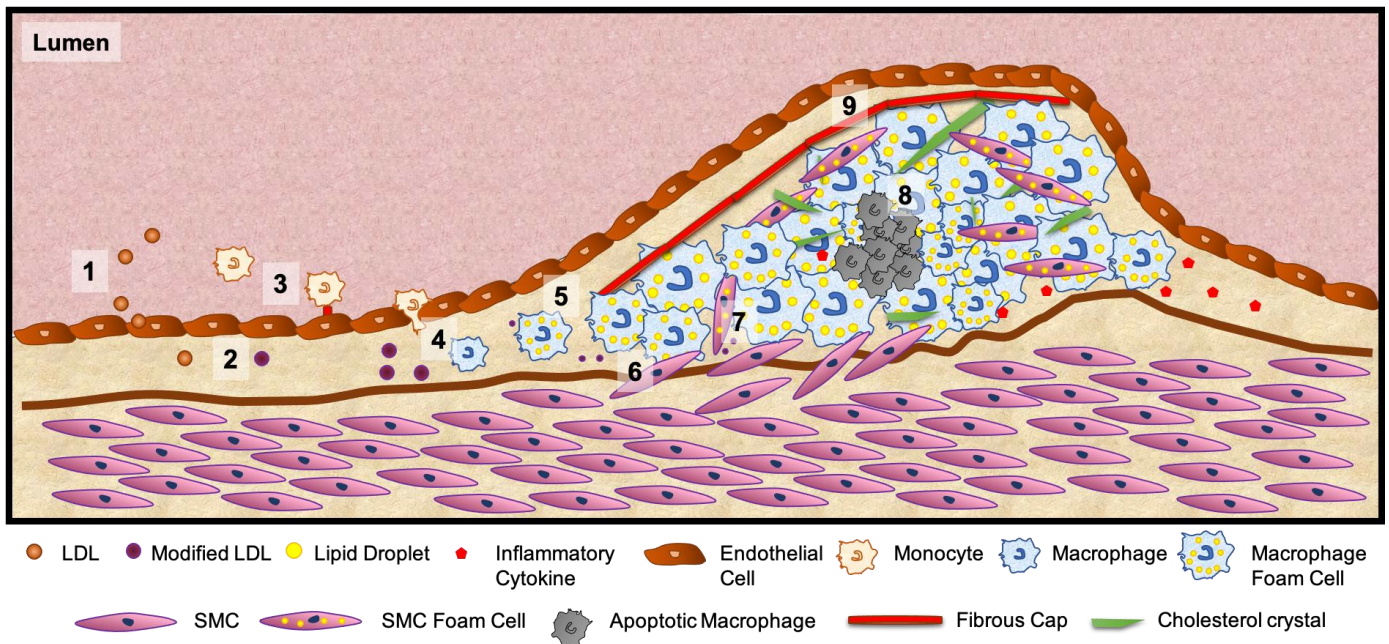


Figure 1-8. Formation of atherosclerotic plaque.

(1) LDL cholesterol enters the subintima and (2) becomes modified. (3) Leukocytes, including monocytes that are circulating in blood, get attached to the adhesion molecules expressed on the endothelial surface and migrate into the subintimal compartment in response to chemokine gradients. (4) Monocytes differentiate into macrophages in the subintima. (5) Macrophages uptake the modified LDL particles and become foam cells. (6) SMCs migrate from the media into the intima and proliferate. (7) SMCs also take up modified LDL particles and form foam cells. (8) Foam cells undergo apoptosis and start forming the necrotic core. (9) The fibrous cap overlying the plaque is formed. LDL, Low density lipoproteins; SMC, Smooth muscle cell. *Image from Fernando et al. (233)*

1.3.4 Macrophages in atherosclerosis

To understand the pathophysiology of atherosclerosis requires knowledge of the cells located in healthy arteries. One abundant immune cell in the arterial wall is the macrophage. Although present throughout each vascular wall layer, they are by far most abundant in the adventitia (194). They play an important role in maintaining vascular wall homeostasis through the clearance of apoptotic cells and mediate local inflammatory processes through the secretion of soluble mediators that interact with adjacent and recruited immune and nonimmune cells. They are highly specialised in sensing the microenvironment and modifying their properties accordingly. Their heterogeneity and plasticity underline their extensive and diverse roles in homeostasis and aortic diseases such as atherosclerosis (**Table 1-5**).

Although it is clear that macrophage phenotypes are difficult to categorise and should be seen as plastic and adaptable, they are often simplified into two extremes: a pro-inflammatory (M1-like) and an anti-inflammatory/pro-resolving (M2-like) profile. M1-like macrophages secrete pro-inflammatory cytokines e.g. interferon-beta ($IL-1\beta$), tumour necrosis factor-alpha ($TNF-\alpha$), reactive oxygen nitrogen species, enzymes as well as many other mediators (plasminogen activators, cathepsins, MMPs), which are known to drive plaque inflammation and growth (288). Alternatively, M2-like macrophages secrete factors such as $TGF-\beta$ and pro-resolving lipids, which favour the resolution of inflammation (160, 289, 290). M1 macrophage polarisation can be induced *in vitro* by stimuli including lipopolysaccharides, $TNF-\alpha$ or TH1 cytokines such as interferon- γ ($IFN-\gamma$), whereas M2 polarisation is typically stimulated by interleukins-4 and -13 (288). The M1 and M2 paradigm of macrophage polarisation is now understood to be a gross over-simplification, with numerous subtypes of macrophages identified in the walls of healthy and diseased blood vessels (**Table 1-5**).

In diseased atherosclerotic aortas, macrophages play key roles in lesion development, progression and destabilisation. Moreover, they are among the key cellular drivers of plaque inflammation. They accumulate rapidly in atheroma where they encounter modified lipoproteins including oxidised LDL (oxLDL) and cholesterol crystals, and transform into lipid-rich foam cells which produce inflammatory chemokines, cytokines and reactive species that advance plaque progression and instability (291, 292). Foam cell development heralds one of the earliest stages in plaque formation (**Figure 1-8**).

Plaque growth and stability are influenced by the accumulation and removal of macrophages and foam cells, the production of pro- (“M1”) and anti-inflammatory (“M2”) cytokines, chemokines, reactive oxygen species (ROS) and matrix degrading enzymes. In early lesions, studies have found a correlation between an increase in macrophage apoptosis and decreased lesion size. However, in advanced lesions, if apoptotic macrophages are not cleared efficiently by efferocytosis, they accumulate and undergo necrosis (233). This, in turn, results in extra-cellular deposits of their lipid-rich cargo into the plaque micro-environment, provoking further inflammation (293, 294) (**Figure 1-8**). Studies have reported observing apoptotic macrophages and SMCs in focal areas surrounding the necrotic core. The presence of the free apoptotic remnants observed in advanced lesions that are not associated with phagocytic cells indicates that impaired efferocytosis contributes to the growth of the necrotic core (290, 295).

In the widely held paradigm of plaque development, the formation of macrophages in atheroma arises from recruitment of BM or spleen-derived circulating monocytes to the vessel wall from a luminal direction. These cells bind to intimal endothelial cells, transmigrate and become macrophages with evolution to foam cells. Hyperlipidaemia stimulates HSCs and progenitor cells (HPCs) and results in increased numbers of monocytes, particularly inflammatory Ly6C^{Hi} cells, which enter developing lesions, purportedly giving rise to M1-like macrophages. The dogma that monocyte infiltration and differentiation dictate macrophage expansion in atheroma has been largely unchallenged and although macrophage proliferation has long been recognised, it has been overlooked as a source of plaque

formation and growth (296, 297). Interestingly, recent re-evaluation of the relative importance of monocyte influx and macrophage proliferation has elucidated a switch in the predominant source of plaque macrophage from monocytes in early lesions, to local macrophage proliferation without monocyte recruitment in established ones. However, in long-term adoptive transfer experiments, their results still indicated that during the course of 5 months lesional aortic macrophages were primarily replenished via the circulation by donor-derived leukocytes. It was further observed that inhibition of local proliferation of macrophages governs plaque regression in response to cholesterol lowering in both an *Apoe*^{-/-} mouse model and in human carotid artery plaques (298).

Macrophage Type	Stimuli	Markers	Cytokines Released	Role in Atherosclerosis
M1	IFN- γ , LPS, TNF- α	CD86, MHCII, iNOS,	IL-1, TNF- α , IL-6, IL-12 High, IL-10 low, IL-23, RNI, ROI, CCL12, CCL18, CCL22, CCL24	Plaque progression, Mediate TH1 responses and Type I inflammation
M2a	IL-4, IL-13	MHCII, MR, CD206, SR-A1, Arg, CD23, CD163	IL-10, Decoy-IL-1RII, IL-1ra, Polyamine	Mediate Th2 responses and Type II inflammation
M2b	Poly-IC + TLR / IL-R ligands	MHCII, CD86, CD80, Arg	TNF, IL-1, IL-6, IL-10 high, IL-12 low	Enriched in regressing plaques, Th2 activation, immunoregulation
M2c	IL-10	SLAM, CD206, MR, CD14, CD150, Arg	IL-10, TGF- β , IL-1ra, CCL16, CCL18, CXCL13, Matrix vesicles	Immunoregulation, Matrix deposition and tissue remodelling
M2d	IL-6, tumour derived factors	VEGF, Arg	IL-10, IL-12, TNF- α , TGF- β	Promote angiogenesis
Mox	oxLDL	VEGF	IL-10, IL-1 β	Lesion development and plaque instability, Redox and anti-oxidant activity, Angiogenesis
Mhem	Heme	CD163, ATF1	LXR- β	Atheroprotective
M-Hb	Haemoglobin, haptoglobin	CD163, MR	ABCA1, ABCG1, LXR- α	Mediate Cholesterol Efflux, Atheroprotective
M4	CXCL4	CD206, CD86, CD45, CD14	TNF- α , IL-6, IL-10, CCL18, CCL22	Minimal foam cell formation, potential pro-atherogenic roles

Table 1-5. Polarised macrophage phenotypes and their role in atherosclerosis.

Monocytes which enter the vascular wall differentiate into macrophages. Different stimuli including growth factors, chemokines, lipoproteins or tissue factors induce specific types of macrophage polarisation. This table summarises some of these macrophage subtypes discovered to date (299-307). ABCA1, ATP binding cassette transporter A1; ABCG1, ATP binding cassette transporter G1; Arg, Arginase; ATF, Activating transcription factor; CCL, CC chemokine ligand; CD, Cluster of differentiation; CXCL, Chemokine (C-X-C motif) ligand; IFN, Interferon; IL, Interleukin; IL-1ra, Interleukin 1 receptor antagonist; iNOS, Inducible nitric oxide synthase; LXR, Liver X receptor; MHC, Major histocompatibility complex; MR, Mineralocorticoid receptor; oxLDL, Oxidised low density lipoprotein; Poly-IC, Polyinosinic-polycytidylic acid; RNI, Reactive nitrogen intermediates; ROI, Reactive oxygen intermediates; SLAM, Signalling lymphocytic activation molecule; SR, Scavenger receptor; Th, T helper cell type; TGF, Transforming growth factor; TNF, Tumour necrosis factor; TLR, Toll-like receptor; VEGF, Vascular endothelial growth factor.

1.3.5 Macrophage subtypes in atherosclerosis

In recent years macrophage research has undergone a renaissance. There have been new insights into their complexity, as well as a new understanding of ontological differences in their development (288). In addition, recent technological advances, such as single-cell RNA sequencing (scRNA-seq) and single-cell mass-cytometry by time-of-flight (CyTOF), have enabled phenotypic and functional analyses of individual immune myeloid cells, identifying for the first-time new clusters of macrophage populations (308), including those in healthy and atherosclerotic vasculature. Broadly speaking, three distinct macrophage subpopulations have been described in atherosclerotic aortas: resident-like, pro-inflammatory and anti-inflammatory foamy TREM2^{Hi} macrophages.

1.3.5.1 Atherosclerotic inflammatory macrophages

The generation of BM monocytes accelerates under inflammatory conditions (309). During atherosclerosis, circulating monocytes infiltrate the arterial wall through a range of chemokine–chemokine receptor interactions and with assistance from endothelial adhesion molecules (287, 310). In the intima's subendothelial space, these infiltrated monocytes differentiate into macrophages in the presence of macrophage colony-stimulating factor (M-CSF/CSF1). These active inflammatory macrophages express major histocompatibility complex class II (MHCII), CD64, CD80 and CD86. They secrete inflammatory cytokines, such as IFN- γ , TNF- α/β , IL-2, and IL-6, and express nitric oxide synthase 2, which activate other immune cells, endothelial cells and SMCs (311, 312). They are often associated with an M1-like phenotype because of their pro-inflammatory nature.

Inflammatory macrophages are primarily present in atherosclerotic and not healthy mouse aorta, where they represent the largest macrophage subset (308, 313–317). They reside in the neointima and are the predominant macrophage population in the shoulder regions of plaque (314). In a scRNA-seq study using atherosclerotic aortas from *Ldlr*^{-/-} mice, Cochain *et al.* showed these inflammatory macrophages are enriched with pro-atherogenic and pro-inflammatory transcripts, *Ccl3*, *Cxcl2*, *Ccl4*, *Il1b*, *Il1a*,

Nlrp3, *Cebpb*, *Egr1*, *Phlda1* and *Tlr2* (313). Similarly, another study revealed that inflammatory macrophages in plaque express *Ccl2*, *Cxcl1*, *Cxcl2*, *Cxcl10*, *Nlrp3* and *Tnf*, along with type 1 interferons, *Ifitm3*, *Irf7*, *Isg15* and *Mnda*, which have pro-atherogenic functions (318). In both studies, these inflammatory macrophage subsets increased with a high cholesterol diet, when compared to chow feeding, confirming a positive association with plaque progression (319, 320).

1.3.5.2 Atherosclerotic resident macrophages

Resident macrophages, as defined by LYVE-1 and CX₃CR1 have been shown to increase in number by two-fold in atherosclerotic mouse aortas (321), although their contribution to plaque progression remains unclear. Through scRNA-seq studies, resident macrophages in atherosclerotic aortas express genes [*Folr2*, *Gas6*, *Sepp1*, *Cbr2*, and *Mrc1* (*Cd206*)] and proteins (LYVE-1, CD206) associated with M2-like macrophages, suggesting that they have anti-inflammatory characteristics (308). Interestingly, mice with depleted LYVE-1⁺ macrophages (*Lyve1*^{WT/Cre}*Csf1r*^{Flox/Flox} mice) have been observed to have increased arterial stiffness and collagen deposition, suggesting that LYVE-1⁺ macrophages play a key role in these processes (322). Through different scRNA-seq studies, there appear to be three macrophage subsets expressing LYVE-1 in atherosclerotic plaques of *Ldlr*^{-/-} mice (313, 323, 324), with only one of these clusters present in healthy murine aorta (308, 313). Notably, resident-like macrophages express higher transcripts of *Lyve1* in healthy aorta compared to higher levels of *Ccr2* in atherosclerosis, with the latter a marker typically associated with recruited macrophages. This has been interpreted to suggest that upon atherosclerosis development resident macrophages transition to a more pro-inflammatory state, upregulating genes usually expressed on recruited, inflammatory macrophages.

Resident-like macrophages in atherosclerotic mouse aorta also display homogenous expression of *Pf4* (encoding CXCL4), a proatherogenic chemokine playing a role in inflammation and wound repair (325, 326), and of thioredoxin-interacting protein (*Txnip*), involved in macrophage and vascular SMCs inflammatory activation (327-330). Another study found a resident-like macrophage population

expressing CD169, which encodes Sialic Acid Binding Ig Like Lectin 1 (SIGLEC-1). SIGLEC-1 interacts with scavenger receptor SR-B1, which mediates cholesterol efflux (331).

Interestingly, studies have shown that most resident macrophages in healthy adventitia are derived from embryonic development. Using multiple fate mapping approaches, Ensan *et al.* showed that adventitial macrophages develop embryonically from early and late CSF1R⁺ and CX₃CR1⁺ EMPs generated in YS (194). These macrophages are especially abundant at the time of birth, making up 93% of all macrophages in neonatal aorta. They are characterised by expression of LYVE-1, CSF1R and CX₃CR1, and have phenotypic and functional differences from macrophages derived from BM HSCs and circulating monocytes (168). Although YS-derived adventitial macrophages are maintained locally by proliferation after birth, their numbers steadily decline with ageing and are replaced in healthy aorta by monocyte-derived macrophages. Importantly, they represent a major population of macrophages in healthy adult mice providing anti-inflammatory and homeostatic functions. Subsequent lineage-tracing studies revealed that these EMP-derived arterial macrophages decline in number during ageing (169). The presence of YS-derived macrophages in arterial adventitia was recently verified by a second study that showed angiotensin II (AngII) infusion caused these cells to accumulate within the inflamed adventitia during the early phase (first 10 days). scRNA-seq revealed that in AngII-treated mice, YS early EMP-derived adventitial macrophages displayed upregulation of signalling pathways related to tissue regeneration, chemotaxis, apoptosis regulation and modulation of distinct secretory proteins (169). In comparison, BM-derived adventitial macrophages showed heterogenous transcriptional profiles associated with both pro-inflammatory and anti-inflammatory processes. These important studies illustrate that the different developmental origins of arterial macrophages are associated with differences in their transcriptional and phenotypic signatures, as well as their functional properties.

How each macrophage subset interacts with each other in diseased aortas is still unknown, although an indirect way to investigate this is to look at the migratory patterns of different macrophage subsets in

an atherosclerotic model. McArdle *et al.* recently used an *Apoe*^{-/-}*Cx3cr1*^{GFP}*CD11c*^{YFP} mouse model (316), in which four macrophage subsets were identifiable by GFP⁺YFP⁻, GFP⁻YFP⁺, GFP⁺YFP⁺ and GFP⁻YFP⁻ signals. Bulk RNAseq data of these four flow-sorted F4/80⁺ macrophage subsets were compared with representative genes of each macrophage subset and found that GFP⁺YFP⁻ cells were most like the inflammatory macrophages, GFP⁺YFP⁺ cells were similar to resident-like macrophages, and GFP⁻YFP⁺ cells were most similar to TREM2^{Hi} macrophages (see below). The double-negative cells were not attributed to any macrophage subset; however, they were enriched with resident-like macrophage genes (*Sepp1*, *Txnip* and *Pf4*) and live-cell microscopy showed they migrate less than other macrophage subsets. Interestingly, this subset upregulated stem-like genes, suggesting may be VW-PCs (315). Cell migration for the double negative population was not observed, although they dominated numerically in more stable plaques. In contrast, both the inflammatory and resident macrophages “danced” on the spot with little net movement, signifying they have non-migratory behaviour in plaque, while the TREM2^{Hi} macrophages were seen to migrate along arteries. This study highlights the functional differences between each macrophage subset.

Along with the McArdle study, there have been other studies which have established a stem-like population in atherosclerotic aortas. Recently, Lin *et al.* identified a proliferative, stem-cell like CX₃CR1⁺ cell cluster in both progression and regression models of aortic atherosclerosis using *Apoe*^{-/-} mice (315). This population expressed high transcripts of cell cycle, monocyte and macrophage genes, and may therefore align with the previous discovery of aortic AMPCs which enter a more proliferative state and expand by up to three-fold with the development of atherosclerosis in *Apoe*^{-/-} and *Ldlr*^{-/-} mice (262, 263). Interestingly, one important property of aortic resident-like macrophages is their ability to proliferate as seen through their high expression of Ki-67 or enrichment of cell cycle gene (164, 314, 315). This could mean that VW-PCs, and specifically AMPCs, contribute to the macrophage pool in both the adventitia and plaque of atherosclerotic arteries, as suggested by Psaltis *et al.* based on adoptive transfer studies into carotid adventitia (263). However, the extent to which this actually occurs and when it occurs during atherosclerosis (e.g. early or late stage) is entirely unknown, as is the effect that

this has on plaque growth and instability. Although these studies provide new insight into VW-PCs and resident macrophage subtypes in different types of plaque, this exciting field of research is still very nascent. Important questions remain unanswered including: 1) is there a stem-like cell in the vessel wall that gives rise to resident-macrophages in plaques? 2) Do resident-macrophages contribute to foam cell formation? 3) How do resident-macrophages contribute to both stable and unstable plaques?

1.3.5.3 Atherosclerotic TREM2^{Hi} macrophages

TREM2 is a myeloid-specific transmembrane glycoprotein that is important for maintaining metabolic stability during physiological stress. It does this by sensing multiple ligands such as lipids, lipoproteins (ApoE and ApoJ) and oligomeric amyloid- β (332-335). It is expressed on macrophages, including microglia, DCs and osteoclast precursors. TREM2 negatively regulates inflammation and reduces macrophage activation (336). Macrophage TREM2 protects against infections, adipocyte hypertrophy, systemic hypercholesterolaemia, body fat accumulation and glucose intolerance in mice and has been shown to decrease plaque stability (337, 338).

Although not found in healthy mouse aorta, scRNA-seq has revealed a cluster of TREM2^{Hi} macrophages in early and advanced atherosclerotic lesions in both *Apoe*^{-/-} and *Ldlr*^{-/-} mice (313, 315). They resemble M2-like cells and express higher transcript levels of *Trem2*, *Cd9*, *Lgals3*, *Spp1*, *Hvcn1*, as well as some cathepsins, *Ctsb*, *Ctsd* and *Ctsz* (313, 315, 316). Cathepsins are known to promote plaque inflammation and vulnerability. Cathepsin Z (*Ctsz*) increases production of pro-inflammatory cytokines, while Cathepsin D (*Ctsd*) controls aspects of apoptosis and modifies LDL in order for macrophages to ingest it and form foam cells (339). The upregulated expression of *Cd9* is also interesting, as *Cd9* encodes the tetraspanin CD9, which is an anti-inflammatory marker of monocytes and macrophages, and interacts with CD36, which triggers foam cell formation in response to oxidised LDL exposure (323, 339, 340). Recently, Kim *et al.* confirmed that TREM2^{Hi} macrophages are lipid-laden foam cells, with increased expression of *Lgals3*, *Abcg1*, *Abca1*, *Mertk* and *Cd36* (314). Moreover,

lesional TREM2^{Hi} macrophages have displayed enrichment in lipid metabolism, regulation of cholesterol efflux, oxidative stress and catabolism (314). Together, these results strongly implicate TREM2^{Hi} macrophages in lipid uptake and foam cell formation.

Thus far, the origin of TREM2^{Hi} macrophages is unknown. Some studies have inferred that they derive from a circulatory source, given that TREM2^{Hi} macrophages are only present in diseased and not healthy aortas. However, more studies are required in this space, including the use of appropriate lineage mapping models.

1.3.6 The adventitia and its *vasa vasorum* as mediators of vascular wall disease

Over the past two decades, our understanding of the inflammatory response to vascular wall insult has been re-examined. The long-held theory was that vascular wall inflammation was primarily an “inside-out” process, whereby inflammation would start from infiltrating cells entering the vessel wall from peripheral blood via its luminal aspect. However, increasingly, it is recognised that following vascular insult or injury, inflammation begins in the adventitia and subsequently extends to the media and intima from the “outside-in” (**Figure 1-9**) (341). Following vascular wall injury and during the development of atherosclerosis, the adventitia becomes an early site of activation and remodelling (342). This response is characterised by myofibroblast migration into the vessel wall (343, 344), accumulation of different inflammatory cells, including macrophages and lymphocytes (345, 346) and importantly, development and expansion of the adventitial microvessels called *vasa vasorum* (347). In normal physiological conditions, the *vasa vasorum* helps transport molecules, nutrients and cells from the blood to the mural layers of the arterial wall to maintain local homeostasis. These microvessels contain a lumen lined by endothelial cells that are surrounded by pericytes or SMCs and express vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and E-selectin, which encourage interactions with circulating immune cells.

The “outside-in” theory of vascular wall inflammation is well supported by several experiments conducted in both animals and humans. One such study by Okamoto *et al.* assessed the local inflammatory response in porcine coronary arteries after overstretch injury induced by balloon angioplasty (**Figure 1-9**) (348). Immunohistochemistry was performed on frozen sections of artery using antibodies directed against human myeloperoxidase (MPO). After injury, MPO-positive neutrophils could be detected in the adventitia 30 minutes after initial insult and only two hours to three days later in the intima and media. Adventitial accumulation of macrophages and upregulation of adhesion molecules on *vasa vasorum* endothelial cells were also observed almost immediately after

balloon injury. This suggests that the early trafficking of inflammatory cells to and from the adventitia is facilitated by the increased expression of these surface proteins. Human studies have also corroborated these findings, demonstrating inflammatory cell infiltration from the adventitia to the site of atherosclerosis (349, 350).

Adventitial *vasa vasorum* expansion is also strongly implicated in the formation of the neointima, although the precise mechanisms for this are incompletely defined. In a collection of studies using non-diseased carotid arteries from rats and rabbits, application of angiogenic growth factors directly to the adventitia caused expansion of the *vasa vasorum* and subsequent neointima formation (351-353). Conversely, inhibition of these growth factors prevented or reversed this process (352). Adding to this, there have now been direct correlations made between the density of adventitial *vasa vasorum* and the burden of neointima or plaque in animal and human studies of atherosclerosis using imaging techniques (347, 354-357).

In addition, the structural integrity and permeability of the *vasa vasorum* has also been associated with plaque complications, including intraplaque haemorrhage, which makes plaque more vulnerable to rupture (342, 358). *Vasa vasorum* integrity relates to the stability of their endothelial cell junctions and the presence and maturity of surrounding pericytes (342, 358, 359). When the *vasa vasorum* expands and infiltrates the vessel wall in response to insult or injury, this integrity is compromised. A post-mortem study of human coronary atherosclerosis specimens by Sluimer *et al.* found that *vasa vasorum* invading the media frequently lacked mural cell (pericyte) coverage, with either absent or incomplete endothelial cell junctions observed in 76% of the samples (359). Friability of expanding *vasa vasorum* makes it prone to haemorrhage, creating a substrate for plaque rupture and atherothrombotic events, such as myocardial infarction (360).

These converging lines of evidence highlight the importance of adventitial and *vasa vasorum* biology in maintaining vascular homeostasis and mediating vascular wall diseases, such as atherosclerosis. This has led to therapeutic efforts to modify *vasa vasorum* growth and stability with both established (e.g. statins) and novel (e.g. angiostatin) pharmacological agents and molecules (361-363). Although preclinical studies have previously shown anti-atherosclerotic and plaque-stabilising benefits from these approaches, *vasa vasorum*-targeted therapies are still far from being translated to the clinic.

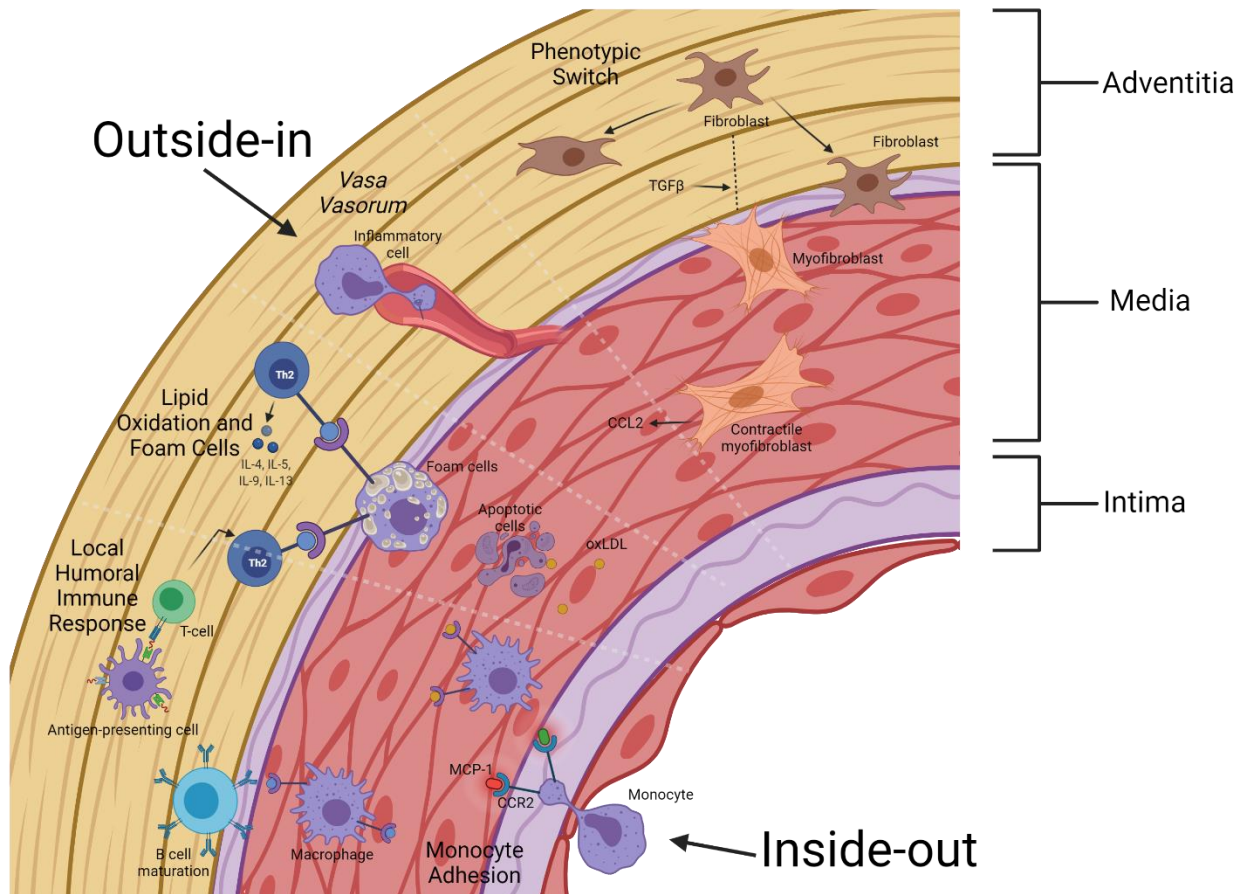


Figure 1-9. The “outside-in” theory of vascular wall inflammation and remodelling.

The “outside-in” theory emphasises that inflammatory cells enter the vascular wall from the outer adventitial layer. These inflammatory cells are then trafficked to the media and intima via expansion and infiltration of the *vasa vasorum*. Image adapted from Mulligan-Kehoe et al. (342, 348)

1.3.7 Macrophage and endothelial interactions in *vasa vasorum* formation

Macrophages are integrally involved in the formation of adventitial *vasa vasorum* and plaque microvessels, which regulate plaque burden and stability (362, 364). Macrophages proliferate and assemble around endothelial-lined *vasa vasorum*, providing paracrine support to endothelial cells by producing pro-angiogenic cytokines and growth factors and supporting endothelial cell anastomosis and vascular branching (342). The origins of the endothelial cells and macrophages that contribute to *vasa vasorum* are still poorly defined.

Interestingly, it has been suggested that locally derived macrophages participate in angiogenesis. One study used treatment with cyclophosphamide to induce BM toxicity and reduce circulating monocyte numbers in a rodent model of hindlimb ischaemia. They demonstrated that this did not negatively affect the accumulation of macrophages in collateralising vessels (365). The authors concluded that these macrophages were from tissue-resident macrophages or their local vascular precursors (365). Similarly, analyses of the *ex vivo* arterial ring sprouting assay have revealed early accumulation and outgrowth of CD45⁺CD68⁺ macrophages and this too was not affected by cyclophosphamide-induced BM depletion. This led to the inference that the vasculogenic zone of adventitia may contain local macrophage progenitor cells that reside in proximity to other progenitor subtypes (73, 365). Furthermore, several human studies have shown an association with CD34⁺CD90⁺ progenitor-like cells and lymphocytic CD79⁺ B and CD3⁺ T cells with the *vasa vasorum* from patients with diffuse coronary artery disease (366, 367).

Moreover, several studies have used the arterial ring model of angiogenesis to show the outer lining of blood vessels contribute to endothelial-fated progenitor cells that undergo vascular sprouting. A study by Zengin *et al.* found CD34⁺CD31⁺VEGFR2⁺TIE2⁺ cells confined to the adventitia of human internal thoracic arteries generated capillary-like outgrowths lined with mature endothelial cells *ex vivo* (368). However, it should be cautioned that MatrigelTM-based sprouting and cord-formation assays are not

specific for cells of endothelial lineage. Outgrowths from vascular explants contain a mixture of fibroblasts, macrophages, endothelial cells and pericytes making it difficult to differentiate *bona fide* endothelial progenitor cells from other proangiogenic progenitors that coexist in the adventitia and also possess mesenchymal or haematopoietic characteristics.

Interim Conclusion

- The tunica adventitia is a niche for stem/progenitor cells including those with macrophage and endothelial forming potential.
- The adventitia remodels early in vascular disease processes, such as atherosclerosis, undergoing both inflammatory cell expansion and growth and infiltration of *vasa vasorum* towards plaque.
- Understanding how these adventitial changes occurs is critical to forming a more complete understanding of atherosclerosis in order to develop new treatments to address its high burden of adverse atherosclerotic events.
- The presence of a bipotent myeloid and vasculogenic stem cell after birth would provide a key link to help explain how macrophages and endothelial cells develop and interact so closely during neovessel formation, including the expansion of *vasa vasorum* in atherosclerosis.

1.4 Origins of macrophages and endothelial cells in healthy and diseased skin

1.4.1 Skin structure and function

As the primary barrier and largest organ, the skin (or integument) maintains the integrity of the body. It is closely integrated to the underlying fascial endoskeleton via retinacular ligaments, nerves, lymphatic and blood vessels (369, 370). Skin is an essential part of the immune, nervous and endocrine systems with extensive cross-talk happening intracutaneously. Broadly, skin has three main functions: protection, regulation and sensation (371).

The skin is made up of three layers: the epidermis, dermis and subcutaneous fat (**Figure 1-1010**). The epidermis, or outermost layer, forms the waterproof exterior layer that is porous and serves as a barrier to infection and other injurious insults. It contains a variety of different cell types, including Merkel cells, keratinocytes, melanocytes, Langerhans cells and inflammatory cells (372). Blood capillaries which connect arterioles and venules, are only found beneath the epidermis, such that the epidermis itself has no blood supply. Rather, it is nourished through diffused oxygen from the surrounding air. The epidermis can be subdivided into four or five layers, depending on the region of skin being considered. Those layers are denoted as: basale, spinosum, granulosum, lucidum and corneum (373). The second layer of skin beneath the epidermis is the dermis, which consists of extracellular connective tissue involving a dense network of tough elastic collagen fibres, hair follicles, sweat glands, sebaceous glands, apocrine glands, lymphatic vessels and blood vessels (373, 374). Among various cell types, the dermis is stocked with fibroblasts, macrophages, adipocytes, mast cells, Schwann cells and stem cells (372). Dermal macrophages consist of several different subsets, including a resident macrophage population (165). The epidermis and dermis are separated by a basement membrane. Deep to the dermis is the subcutaneous fat, the third layer, that contains fat and connective tissue and attaches the skin to the underlying bone and muscle as well as supplying it with blood vessels and nerves (375). The

subcutaneous fat is enriched with fibroblasts, adipose cells and macrophages. This layer serves as padding and insulation for the body (375).

1.4.2 Cellular composition of healthy skin

The adult skin provides spatially distinct microenvironments for cell maintenance and function. Local skin niches contain subgroups of both immune and non-immune cells. For example, the epidermis contains immune cells, namely Langerhans cells, memory T cells that protect against pathogens, keratinocytes which uphold tight junctions vital for barrier function, and pigmented melanocytes that protect against UV radiation (**Figure 1-10**). The dermis contains fibroblasts which allow communication between immune cells and provide supporting extracellular protein scaffolds. Endothelial cells from the innermost lining of dermal blood vessels allow transmigration of immune cells into skin through adhesion molecules and chemoattractants after injury or during infection. Even in skin of healthy individuals, the dermis is enriched with different inflammatory cell subtypes belonging to both the innate and adaptive immune systems. Innate immune cells include dermal DCs, macrophages, monocytes and granulocytes. Adaptive immune cells include T cell subsets and NK cells.

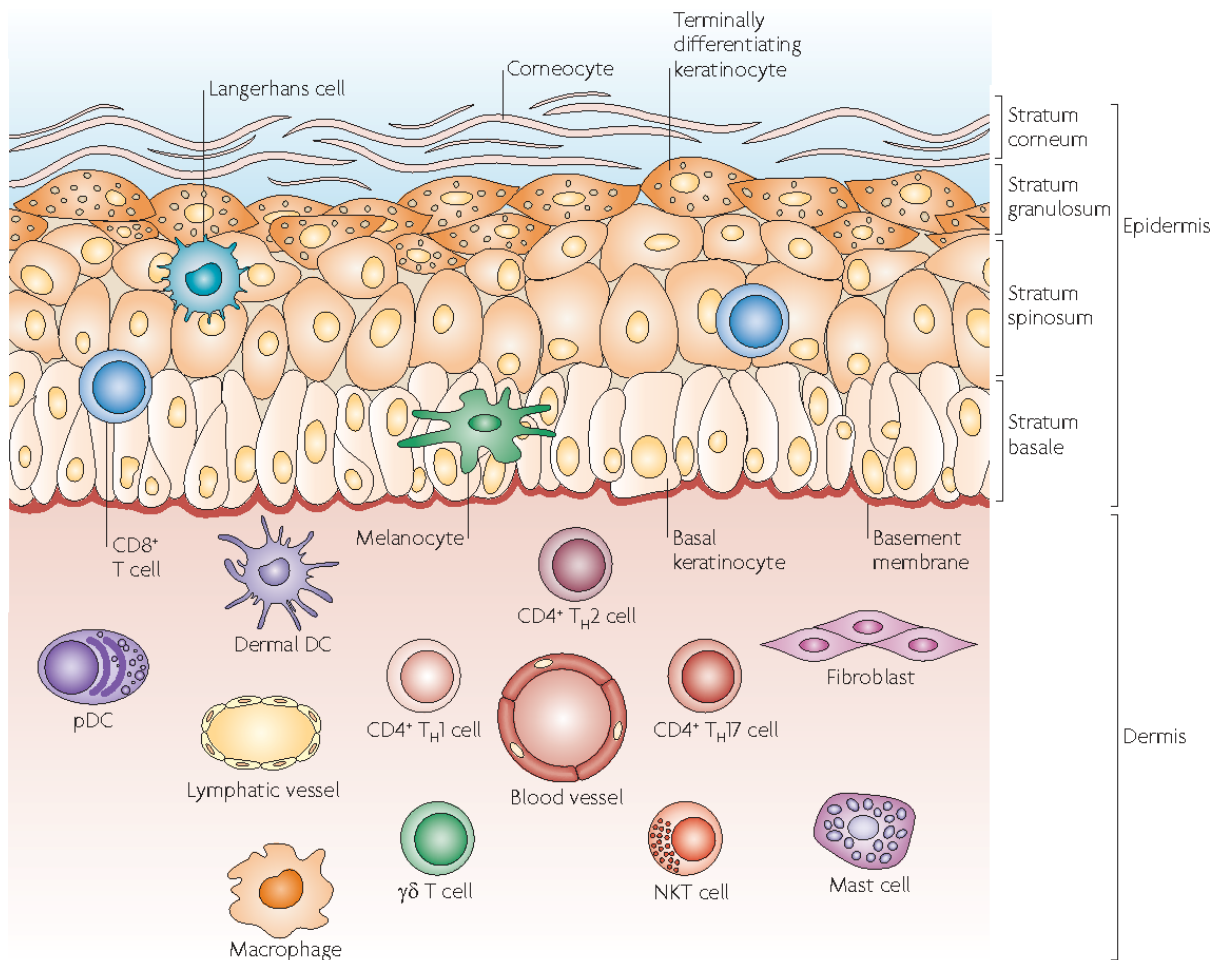


Figure 1-10. Normal structure of the skin.

The epidermis contains four layers: the stratum basale, stratum spinosum, stratum granulosum and the outermost layer, the stratum corneum. Among these layers are melanocytes, which produce melanin, Langerhans cells and rare T cells (e.g. $CD8^+$ cytotoxic T cells). The dermis is composed of collagen, elastic tissue and reticular fibres and contains specialised cells, including DC subsets [e.g. dermal DCs and plasmacytoid DCs (pDCs)], and T cell subsets (e.g. $CD4^+$ T helper 1 (TH1), TH2 and TH17 cells, $\gamma\delta$ T cells) and natural killer T (NKT) cells. In addition, macrophages, mast cells and fibroblasts are present throughout the dermis, along with blood and lymphatic vessels and nerves (not shown). Below the dermis is the subcutaneous fat which is not shown. *Image from Nestle et al. (370)*

1.4.3 Stem cells in healthy skin

The skin provides a well-organised niche for different stem cells that reside within all three layers (**Table 1-6**). Those in the epidermis include hair follicle stem cells which maintain the hair lineages, sebaceous gland stem cells which produce differentiated sebocytes (sebum-producing epithelial cells) and stem cells in the interfollicular epidermis (IFE) that give rise to the outermost barrier layers of the epidermis. The majority of these self-renewing epidermal stem cells can be found in the bulge region of the hair follicle.

Hair follicle stem cells are also found in the dermis, along with mesenchymal stem cells (MSCs) and skin-derived precursor cells (**Table 1-6**). As in other tissues, MSCs occupy perivascular niches within the dermis and have the ability to self-renew and differentiate into different progeny. They are most classically characterised by their trilineage plasticity for bone, cartilage and fat, and in the skin have been shown to differentiate into adipocytes, SMCs, osteocytes and chondrocytes (376-381). Further, MSCs also exert important trophic actions on other cell populations in skin, both via direct contact and via the release of paracrine mediators. These include pro-mitogenic effects on keratinocytes, regulation (often suppression) of immune cell responses and support for angiogenesis (e.g. during wound healing or cancer formation) (382). Skin-derived precursors were also discovered in 2001 by Toma *et al.*, who first described their high proliferative capacity and ability to form sphere-shaped colonies *in vitro* (383, 384). These cells were shown to arise during embryogenesis and are maintained locally into adulthood (385). Individual colonies *in vitro* were able to differentiate into both neuroectodermal and mesodermal lineage, which included neurons, glia, SMCs, melanocytes and adipocytes (383, 384, 386). Like MSCs, these so-called skin-derived precursors have pro-angiogenic properties and are able to generate vascular-like tubes in collagen I-rich MatrigelTM (386).

Moreover, although their properties and function are poorly understood *in vivo*, MSCs and EPCs have also been isolated from subcutaneous fat and have been shown to have adipogenic or endothelial lineage potential, respectively (**Table 1-6**).

Skin Layer	Skin stem/progenitor cells	Cell Marker	Function	References
Epidermis	Epidermal stem cells (hair follicle bulge)	CD34 ⁺ or CD34 ⁻ /CD200 ⁺ /K15 ^{Hi} /E-cadherin ⁺ CD29 ⁺ /c-Kit ⁺ /CD49f ⁺ /Lgr5 ⁺ /Lgr6 ⁺ /Lrig1 ⁺	Hair lineages	Piccione <i>et al.</i> (387), Blanpain <i>et al.</i> (388), Alcolea <i>et al.</i> (389), Cotsarelis <i>et al.</i> (390), Fullgrabe <i>et al.</i> (391) Chen <i>et al.</i> (392)
	Epidermal stem cells (Basal layer); Also known as interfollicular epidermis	CD34 ⁺ or CD34 ⁻ /K15 ^{Lo} /E-cadherin ⁺ / CD29 ⁺ /CD49f ⁺	Specific lineage is unknown however they contribute to the outermost barrier layers of the epidermis	Piccione <i>et al.</i> (387), Blanpain <i>et al.</i> (388), Alcolea <i>et al.</i> (389), Cotsarelis <i>et al.</i> (390)
	Sebaceous Gland Stem Cells	LGR6 ⁺ /Blimp-1 ⁺	Sebaceous gland	Piccione <i>et al.</i> (387), Blanpain <i>et al.</i> (388), Alcolea <i>et al.</i> (389)
	Melanocyte stem cells	CD34 ⁺ /Dct ⁺ /Pax ⁺ /Sox10 ^{Lo}	Provide pigmentation (melanin)	Joshi <i>et al.</i> (393), Lang <i>et al.</i> (394)
Dermis	Hair Follicle Stem Cells	CD44 ⁺ /CD73 ⁺ /CD90 ⁺ /CD34 ⁻ / CD200 ⁺ /K15 ⁺	Hair lineages	Hoogduijn <i>et al.</i> (395), Piccione <i>et al.</i> (387)
	Mesenchymal stem cells (MSC)	K15 ⁺ /CD34 ⁺ /CD44 ⁺ /CD73 ⁺ / CD90 ⁺ /CD105 ⁺ /c-Kit ⁺ /CD271 ⁺	Chondrogenic, myogenic, and adipogenic lineage differentiation	Ferroni <i>et al.</i> (396). Han <i>et al.</i> (397)
	Skin-derived precursors	Nestin ⁺ β3-tubulin ⁺ Fibronectin ⁺	Neuroectodermal and mesodermal lineage	Toma <i>et al.</i> (383) Toma <i>et al.</i> (384)
Subcutaneous fat	Mesenchymal stem cells (MSC)	D29 ⁺ /CD44 ⁺ /CD105 ⁺ /CD90 ⁺ /CD34 ⁻ / CD45 ⁻ /Sca-1 ⁺ or Sca-1 ⁻	Chondrogenic, myogenic, and adipogenic lineage differentiation	Guercio <i>et al.</i> (398), Panina <i>et al.</i> (399), Ritter <i>et al.</i> (400)
	Adipose derived stem cells (a type of MSC)	CD13 ⁺ /CD29 ⁺ /CD34 ⁺ or CD34 ⁻ / CD44 ⁺ /CD90 ⁺ /CD104a ⁺	Adipogenic lineage	Shukla <i>et al.</i> (401), Panina <i>et al.</i> (399), Tang <i>et al.</i> (402)
	Endothelial progenitor cells	CD31 ⁺ /CD34 ⁺ /CD90 ⁻ /CD146 ⁺ /vWF ⁺ /CD45 ⁻	Endothelial lineage	Shukla <i>et al.</i> (401), Panina <i>et al.</i> (399)

Table 1-6. Stem/progenitor cells found in the different layers of skin and their lineage potential.

1.4.4 Macrophages in healthy skin

Macrophages in healthy dermis consist of locally resident macrophages and BM-derived macrophages (403). They play crucial roles in host defence and maintaining steady state homeostasis through the clearance of apoptotic cells, growth factor production, promotion and resolution of inflammation, and trophic support of cell proliferation, tissue restoration and neovascularisation following injury.

As per oath, skin macrophage diversity (or polarity) was classified into the two main subsets described above: classically activated (M1-like) and alternatively activated (M2-like); however, this classification is now known to be oversimplified. A seminal study by Tamoutounour *et al.* revealed the complex heterogeneity of skin macrophages (404). These authors identified a broad population in healthy skin of dermal CD11b⁺ non-DC macrophages, comprising CCR2⁻ and CCR2⁺ subsets. CCR2⁻ macrophages were further classified into Ly6C^{Lo}MHCII⁻ and Ly6C^{Lo}MHCII⁺ subtypes, which were both CD64^{Hi}MerTK⁺ and displayed similar properties to other tissue macrophages, including a foamy cytoplasm, cell cycle kinetics and transcriptional profile (404). CCR2⁺ macrophages comprised Ly6C^{Hi}MHCII⁻, Ly6C^{Hi-to-Lo}MHCII⁺ and Ly6C^{Lo}MHCII⁺ subpopulations, of which the latter two displayed intermediate morphology between DCs and macrophages. The differentiation of these different subtypes was found to occur via distinct CSF1R signalling. Transcriptomic and functional data also demonstrated specialisation of dermal macrophages. For example, those with high phagocytic activity expressed the genes *C4b*, *CD209f*, *Tlr5*, *Pdgfc*, *Itga9* and the scavenger receptors, Stabilin-1 and CD36. Further analyses revealed DCs developed from Flt3-dependent and CCR2-independent pathways. Ly6C^{Hi} blood monocytes generated dermal CCR2⁺Ly6C^{Hi}MHCII⁻, CCR2⁺Ly6C^{Hi-to-Lo}MHCII⁺ and CCR2⁺Ly6C^{Lo}MHCII⁺ cells. Whereas, CCR2⁻Ly6C^{Lo}MHCII⁻ and CCR2⁻Ly6C^{Lo}MHCII⁺ subpopulations consistent of both embryonic and adult haematopoietic cells.

Macrophages present in the skin can arise from the two different developmental pathways described above: namely, YS-derived primitive haematopoiesis and definitive haematopoiesis that arises from AGM/foetal liver embryonically and BM postnatally (2, 92, 109, 165, 175, 404). Epidermal Langerhans

cells were originally classified as archetypal non-lymphoid DCs, however, they are now understood to possess features of both macrophages and DCs (405, 406). Langerhans cells arise from both the embryonic YS and foetal liver, with the foetal liver HSCs contributing to ~80% of Langerhans cells postnatally (175). Although they do not express the macrophage markers, CD64 and MerTK, they do express CD24. Moreover, like DCs, they are involved in cross-presentation of antigens and priming of effector T cells, and migrate to draining lymph nodes, while also playing a tolerogenic role in steady state skin. In mice, their detailed surface marker phenotype has been identified as CD64⁺F4/80⁺MerTK⁻CD11c⁺CD11b⁺EPcam⁺MHCII⁺SIRPα⁺CD207⁺CD24⁺CD26⁻, while in humans they are characterised as CD1a⁺CD14⁺HLA-DR⁺CD207⁺.

1.4.5 Endothelial cells in healthy skin

Healthy skin is a highly vascularised tissue containing blood vessels and lymphatic vessels. These make up its microcirculation, which is located in the dermis (407). In healthy skin, it provides oxygen and nutrients to help maintain homeostasis and thermoregulation (408, 409). Microvascular endothelial cells are the major components of dermal blood vessels and are also centrally involved in cutaneous inflammatory responses. Locally produced cytokines (e.g. TNF-α, IL-1β, IFN-γ) have all been shown to alter microvascular endothelial function (410). Endothelial cells lining both dermal blood and lymphatic vessels express canonical endothelial markers, such as CDH5, CD31, and CD34 (410). Other surface antigens are also present including the major histocompatibility complex class II molecule (HLA-DR) (in mice known as MHCII) and low levels of the CD36 scavenger receptor (410). One distinguishing marker between vascular and lymphatic endothelium in healthy dermis is the expression of LYVE-1 on lymphatic vessels. However, under chronic skin inflammatory conditions, dermal blood vessels also express low amounts of LYVE-1 and podoplanin, along with high levels of E-selectin, ICAM-1 and VCAM-1 expression (411).

1.4.6 Skin function in wound healing and cancer

Upon breach of its barrier function due to insults such as burns, ulcers and lacerations, the skin restores homeostasis by activation of a dynamic, complex repair response, which ultimately enables it to heal. This process comprises four broad stages: 1) haemostasis, 2) inflammation, 3) proliferation and 4) resolution/remodelling (412). These phases can be affected by pathological processes such as infection or hypoxia. Among other disease processes, the skin is also vulnerable to several types of cancer, notably melanoma, basal cell carcinoma, squamous cell carcinoma and Merkel cell cancer, predominantly as a result of ultraviolet light exposure to the sun. Interestingly, the cellular pathogenesis of skin cancers bears some resemblance to poorly healed skin wounds. They share many cellular and molecular mechanisms that essentially differ by being well-regulated during wound healing and dysregulated during cancer growth and metastasis (413-415). Whereas acute wound repair normally has a resolution phase, tumours behave more like chronic wounds that have no resolution phase. Because of these parallels, the study of cutaneous wound healing is thought to provide potentially important insights into the processes that are involved in tumorigenesis and *vice versa* (413-415).

Among various cell types involved in both skin tumorigenesis and wound healing, stem cells, macrophages and endothelial cells play central, intricately linked roles. This is because there is an influx of immune cells, an increase in fibroblast proliferation, extracellular matrix remodelling and the formation of new blood vessels in both conditions via angiogenesis and vasculogenesis. In addition to maintaining normal tissue homeostasis, skin stem cells respond to injury by increasing their proliferation and by reducing differentiation until the cellular content of the skin has been restored (394, 416).

1.4.7 Wound repair

Multicellular organisms such as humans and mice have evolved sophisticated systems to quickly heal epithelial injuries. If the epithelium is damaged severe consequences may happen such as pathogen access and/or fluid loss. The wound healing response involves a refined series of cellular and molecular changes activated by specific signalling pathways.

Following injury, the wound healing response occurs immediately with the sequential phases of inflammation, new tissue formation and tissue remodelling (417). The wound healing process begins with the formation of blood clots that create a hypoxic microenvironment. Following this, local recruitment and activation of immune cells occur at the wound site to prevent infection and to remove debris (417, 418). Blood monocytes arrive and subsequently differentiate into macrophages, while resident immune cells such as Langerhans cells, NK and T cells proliferate and are activated. Both resident and recruited immune cells release a large number of cytokines and chemokines to combat the injury and fight off possible infection (**Figure 1-11a**) (417).

During the mid-stage response to wound healing, neovascularisation and re-epithelialisation occur as keratinocytes around the wound edge initiate hyperproliferation and migrate into the wound bed to refill it with granulation tissue (417). Granulation tissue also includes an influx of endothelial cells that promote angiogenesis, as well as the accumulation of macrophages and proliferation of fibroblasts (419). Interestingly, re-epithelialisation during wound healing closely resembles tumour initiation and metastasis (**Figure 1-11b**) (413-415). Once the epidermis is restored, keratinocytes and fibroblasts synthesise ECM proteins that are incorporated into the new basement membrane.

1.4.8 Stem cells in wound healing

Under normal homeostatic conditions, the stem/progenitor cells in both the dermis and epidermis self-renew; however, each stem/progenitor cell niche behaves differently to replenish its own compartment. Those stem cells in the interfollicular epidermis and sebaceous gland undergo constant self-renewal, whereas those in the hair follicles undergo cycles of phases including resting, growth and involution (420). During wound healing, stem/progenitor cells undergo rapid activation to differentiate into progeny that are critical for re-epithelialisation (420, 421). Each stem/progenitor cell type responds differently to injury, depending not only on its specific niche but also on how close it is to the wound site (420).

During wound healing, stem/progenitor cells are activated and recruited from different skin regions when spatial confinement and lineage restriction are transiently lost, allowing them to contribute to the formation of different mature cells (422). For example, when hair follicle stem cells migrate toward the epidermis, they lose their specific markers and adopt a phenotype similar to that of interfollicular epidermis stem cells. However, once in the epidermis, these cells are short-lived and disappear soon after the damaged tissue is repaired, suggesting that hair follicle stem cells act as an intermediate solution before interfollicular epidermis stem cells maintain long-term repair.

1.4.9 Macrophages in wound healing

During wound healing, important roles are performed by both pro-inflammatory and anti-inflammatory macrophages. Pro-inflammatory macrophages assist in the breakdown of ECM by producing cytokines, such as matrix metalloproteinases. Conversely, anti-inflammatory macrophages help mediate the conversion of fibroblasts into myofibroblasts and increase collagen and ECM deposition during granulation tissue formation (417, 418, 423, 424). When re-epithelialisation is complete, neutrophils that were initially recruited to the wound undergo apoptosis and granulation tissue attenuates further proliferation, neovascularisation and inflammation. Macrophages assist in inducing neutrophil apoptosis and efferocytosis, the process by which apoptotic cells are recognised and ingested, thus

helping resolve wound inflammation (417, 418, 423, 424). The importance of macrophages in wound healing was systematically demonstrated in a mouse study in which conditional depletion of macrophages was induced during sequential stages of wound healing (417, 418, 423, 424). Macrophage depletion in the early phase caused a significant decrease in the formation of granulation tissue, impaired epithelialisation and minimised scar formation. In the middle stage it was associated with severe haemorrhage and impaired wound closure. Interestingly, in the final stage, macrophage depletion resulted in no significant differences in wound healing. In considering the implications of this study to clinical wound healing, it must be remembered that the cutaneous repair response in mice differs considerably to that of humans (365, 425).

1.4.10 Endothelial cells in wound healing

During wound healing, endothelial cells migrate into the surrounding dead tissue and/or clot where they proliferate and rearrange to form a hollow cord, creating microcapillaries (419, 426, 427). These endothelial cells have a dynamic interaction with angiogenic cytokines such as fibroblast growth factor (FGF), PDGF, VEGF, TGF- β , angiopoietin and mast cell tryptase produced by macrophages, fibroblasts, MSCs, and the ECM environment (426). The proliferation, migration and angiogenic properties of these endothelial cells are stimulated by molecules secreted from the hypoxic wound environment. This endothelial response comprises four stages: 1) production of proteases for degradation of the basal lamina of the parent vessel in order to crawl through the ECM, 2) chemotaxis, 3) proliferation and 4) remodelling and differentiation (427). New vessels are formed in the process of angiogenesis. Angiogenic capillary sprouts create a microvascular network throughout the granulation tissue to enable blood flow (427). Over time, as the granulation tissue transforms into a scar, the density of blood vessels diminishes. The endothelial cells involved in wound healing have been shown in different studies to originate either from resident endothelial cells already in the skin, local EVPs or the recruitment and differentiation of circulating BM-derived EPCs (229, 428).

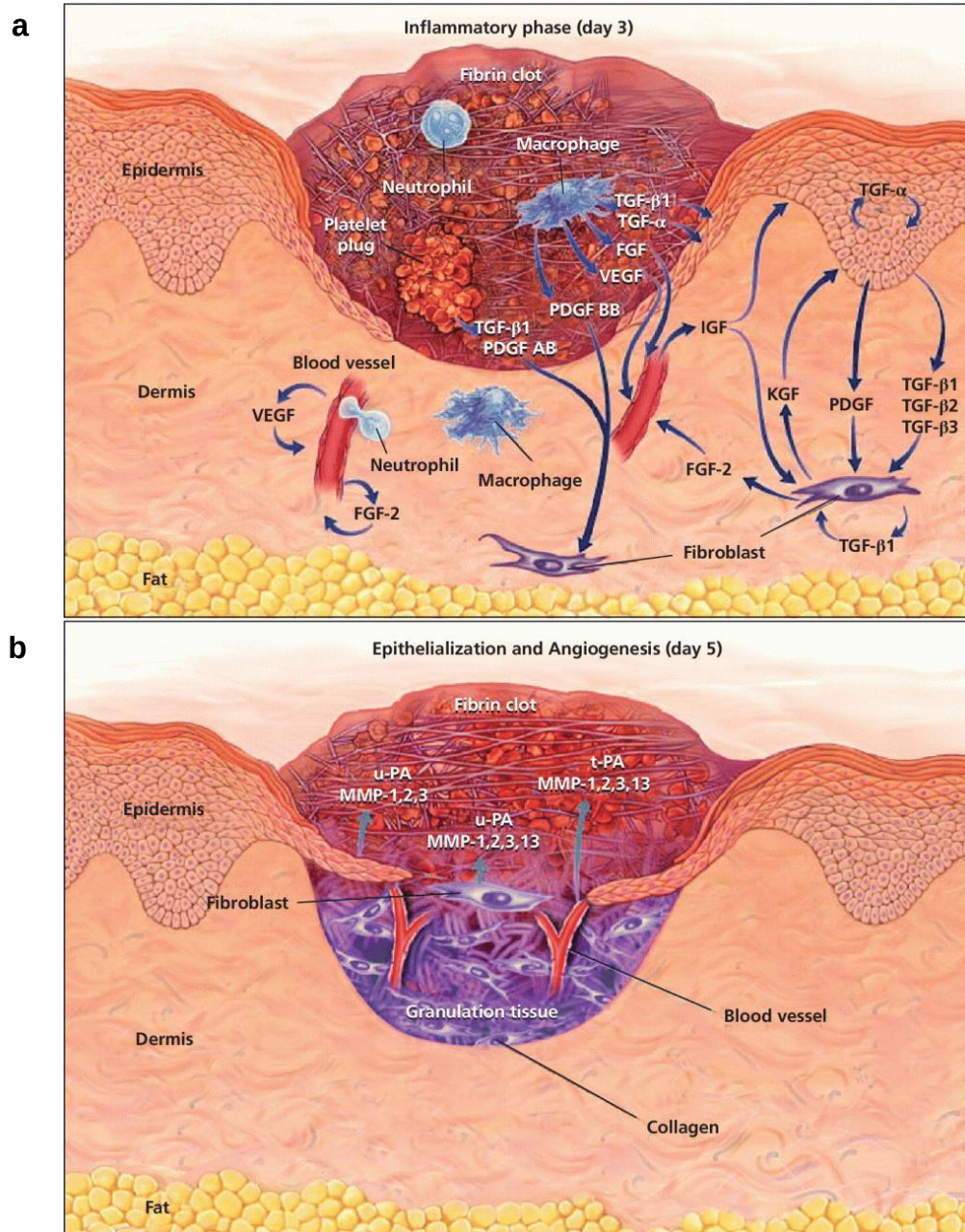


Figure 1-11. Role of macrophages and endothelial cells in the sequential phases of wound healing.

(a) Inflammatory phase occurs three days after skin wound injury. Macrophage activation leads to the release of growth factors, which mediate important interactions with other cell types in the wound (e.g. keratinocytes, endothelial cells, fibroblasts).

(b) Re-epithelialisation and neovascularisation of the cutaneous wound occurs five days after injury in which there is blood vessel formation and epithelial cell accumulation that form a scab.

FGF, Fibroblast growth factor; IGF, Insulin-like growth factor; KGF, Keratinocyte growth factor; MMP, Matrix metalloproteinase; PDGF, Platelet-derived growth factor; TGF, Transforming growth factor; VEGF, Vascular endothelial growth factor. *Image from Childs et al. (417)*

1.4.11 Cancer (melanoma)

Skin cancers, such as melanoma, basal cell cancer and squamous cell carcinoma represent abnormal growth of skin cells. Of these, melanoma is the most aggressive. In Australia alone, it is responsible for 75% of all skin cancer deaths (429). Once melanoma starts to spread, the majority of cases are resistant to treatment with cytostatic chemotherapy and radiotherapy. It is therefore imperative to find novel cellular and molecular mechanisms involved in melanomagenesis and tumour progression that enhance targeted, more effective therapies (429). At the heart of melanomagenesis are genetic mutations in melanocytes, most frequently involving the *B-Raf* proto-oncogene, serine/threonine kinase, *N-Ras* proto-oncogene, GTPase and phosphatase and tensin homolog (430). However, it is the interaction between mutated melanocytes and their microenvironment that is central to cancer progression and metastasis (**Figure 1-12**). The melanoma microenvironment involves many different types of cells including fibroblasts, macrophages, lymphocytes, DCs, adipocytes, endothelial cells and stem cells along with the ECM (431). These interact with cancer cells both through direct contact and the release of active protein substances such as metalloproteinases, osteonectin and various growth factors like TGF- β , Wnt, Hedgehog, epidermal growth factor (EGF), hepatocyte growth factor (HGF) and PDGF (432).

1.4.12 Cancer stem cells

Skin carcinogenesis is typically divided into three major stages: initiation, promotion and progression (416). These stages are induced by, or are associated with, the accumulation of irreversible alterations in growth control and/or differentiation in cell-targeted populations. In order for the tumour to grow, its cells must have a high proliferative and self-renewal capability so that they can divide and accumulate (433). Emerging evidence has indicated a subpopulation of resident stem-like cells within tumours called cancer stem cells. These exhibit characteristics of both stem cells and cancer cells (434). Cancer stem cells commonly express CD133, CD44 and CD90 (416). There have been ongoing debates as to how they originate in the skin, whether they obtain their replicative and self-renewal capacity from mutations in existing skin stem cells or if genetic alterations cause the dedifferentiation and

dysregulation of mature cells (434). Often after metastatic tumours have been treated (e.g. by chemotherapy) the cancer relapses to a more aggressive form. This is thought to be due to the persistence of subpopulations of stem-like cells with resistance properties that are capable of re-initiating tumorigenesis and metastasis (434).

Due to the self-renewal and differentiation capacity of cancer stem cells that helps drive tumour growth, they are hypothesised to derive from normal stem /progenitor cells resident within the tumour-affected tissue (435, 436). In the case of skin malignancies, it has been observed that more severe cases originate from the hair follicles where many stem cells are located (394). This has led to speculation that these tumours are likely to contain cancer stem cells, which arise from dysregulated proliferation and activation of hair follicle stem cells (437). This was supported by evidence from radioactive tracing of benz(a)pyrene, which is maintained in hair follicles and the interfollicular epidermis in dormant stem cells (438). Under physiological conditions, normal CD34⁺ stem cells located in the bulge of hair follicles were found to be in a dormant state. However, when exposed to cancer cells, CD34⁺ stem cells become activated and able to divide and proliferate, leading to their identification as cancer stem cells (439). Another type of skin stem cell that has been shown to contribute to the progeny of cancer stem cells, are the Lgr6⁺ proliferating cells found in the bulge of hair follicles (440). Lineage-tracing models were able to determine the presence of Lgr6⁺ cells in chemically induced tumours, contributing to the tumour's expansion (440).

Research into skin cancer stem cells has also revealed the roles of stemness transcription factors in cancer development. In a mouse model of skin melanoma, the transcription factor Sox2 had a key role in the initiation and progression of tumour development (441). Interestingly, Sox2 is not expressed in normal skin, but rather appears in the early stages of tumour formation. Consistent with this, in Sox2-deleted mice, tumour initiation is prevented (441). In addition, upon their transplantation, Sox2⁺ cells acted as tumour proliferating cells, while the removal of Sox2⁺ cells from established tumours led to

tumour regression (442). However, it is worth noting, that in subsequent experiments, genetic deletion of *Sox2* has not been found to affect either the initiation or progression of melanoma (443, 444).

1.4.13 Tumour-associated macrophages (TAM ϕ s*)

One significant population contributing to the tumour microenvironment are tumour-associated macrophages (TAM ϕ s*). TAM ϕ s form part of the stromal cell milieu in solid tumours. Their pleiotropic functions help to promote tumour growth, invasion and metastasis by: 1) enhancing angiogenesis to vascularise the growing tumour, 2) suppressing the defensive responses of other immune cells, 3) inducing chemotherapeutic resistance in tumour cells and 4) remodelling the ECM and stimulating tumour cell migration (445-447). They especially accumulate around the tumour edge, the stromal border and in both hypoxic/necrotic regions and perivascular areas (448).

Within the heterogeneous TAM ϕ population are numerous phenotypic subsets with specialised roles. Cancer cells produce cytokines which influence the plasticity of TAM ϕ s, namely CSF-1, C-C motif chemokine ligand 2 (CCL2), IL34, VEGFA and C-X-C motif chemokine ligand 12 (CXCL12) (448). These help TAM ϕ s to switch from one phenotype to another for example, from anti-tumour M1-like to pro-tumour M2-like phenotypes under different conditions within the tumour microenvironment (85). Generally, the M1-like phenotype tends to predominate in the early stages of tumour development, while M2-like cells increase with tumour progression (449-451).

Until now, there is very little knowledge about the ontogeny of TAM ϕ s. One important technical limitation contributing to this is the lack of specific markers to discriminate and selectively target different TAM ϕ subtypes (446, 452). Fate-mapping models and fluorescent reporters have shown that TAM ϕ s include both tissue-resident and BM-derived macrophages (446). Limited information is also available about the distinct functions driven by different origins of TAM ϕ s and the interaction between macrophages from different origins in the tumour milieu. In a murine model of pancreatic cancer,

resident macrophages were shown to promote tumour progression with fibrosis-modulating functions, while impairment of circulating monocytes alone had little effect on tumour progression (453). BM-derived TAM ϕ s were also shown to be more potent at antigen presentation, whereas resident macrophages had a higher pro-fibrotic transcriptional profile, suggesting a role in the production and remodelling of the ECM. Another mouse study of lung cancer showed that resident macrophages contributed to tumour cell growth *in vivo*, while accumulation of BM-derived macrophages led to tumour spreading throughout the lung (454). Therefore, these studies suggest that the contributions of resident and BM-derived TAM ϕ s to tumorigenesis probably differ from one another and evolve throughout the disease process. They have not, however, examined what phenotypic subsets of TAM ϕ s they specifically contribute to. Further elucidation of the developmental origins, transcriptional regulation and functional properties of different subsets of TAM ϕ s will be important to determine which of these can be selectively targeted to develop new anti-cancer therapies.

1.4.14 Endothelial cells in tumorigenesis

Neovascularisation is a crucial mediator of tumour progression as blood vessels provide nutrition and oxygen to the developing tumour. The endothelium also assists to translocate invasive cancer cells into the blood vessel lumen, ultimately providing the conduit by which they can circulate systemically and metastasise to other organs. Moreover, endothelial cells are one of the main sources of cancer-associated fibroblasts through the process of endothelial-to-mesenchymal transition (455), which also support cancer cell migration and invasion. As such, endothelial cells are integrally involved in cancer metastasis (456, 457). Importantly, new blood vessels that develop within growing tumours are abnormal morphologically. They present themselves in a chaotic network of twisting endothelium that lacks the normal hierarchical arrangement of artery-arteriole-capillary (458). This also contributes to immature vessels, regressing vessels, transport vessels undergoing arteriogenesis and peritumor vessels (459). These neovessels are also very unstable and leaky, in part due to low pericyte coverage (460), which affects tumour blood flow and increases the risk of intra-tumour haemorrhage.

Interim Conclusion

- Skin contains immune and non-immune cells, including macrophages, fibroblasts, Langerhans cells, endothelial cells and stem cells.
- Macrophages and endothelial cells play integral, linked roles in adaptive skin wound repair, however, can be maladaptive in contributing to other skin diseases, including cancers.
- Understanding how the interactions between skin-resident macrophages and endothelial cells develop is important to developing new treatments for skin wounds and/or skin malignancies, such as melanoma.

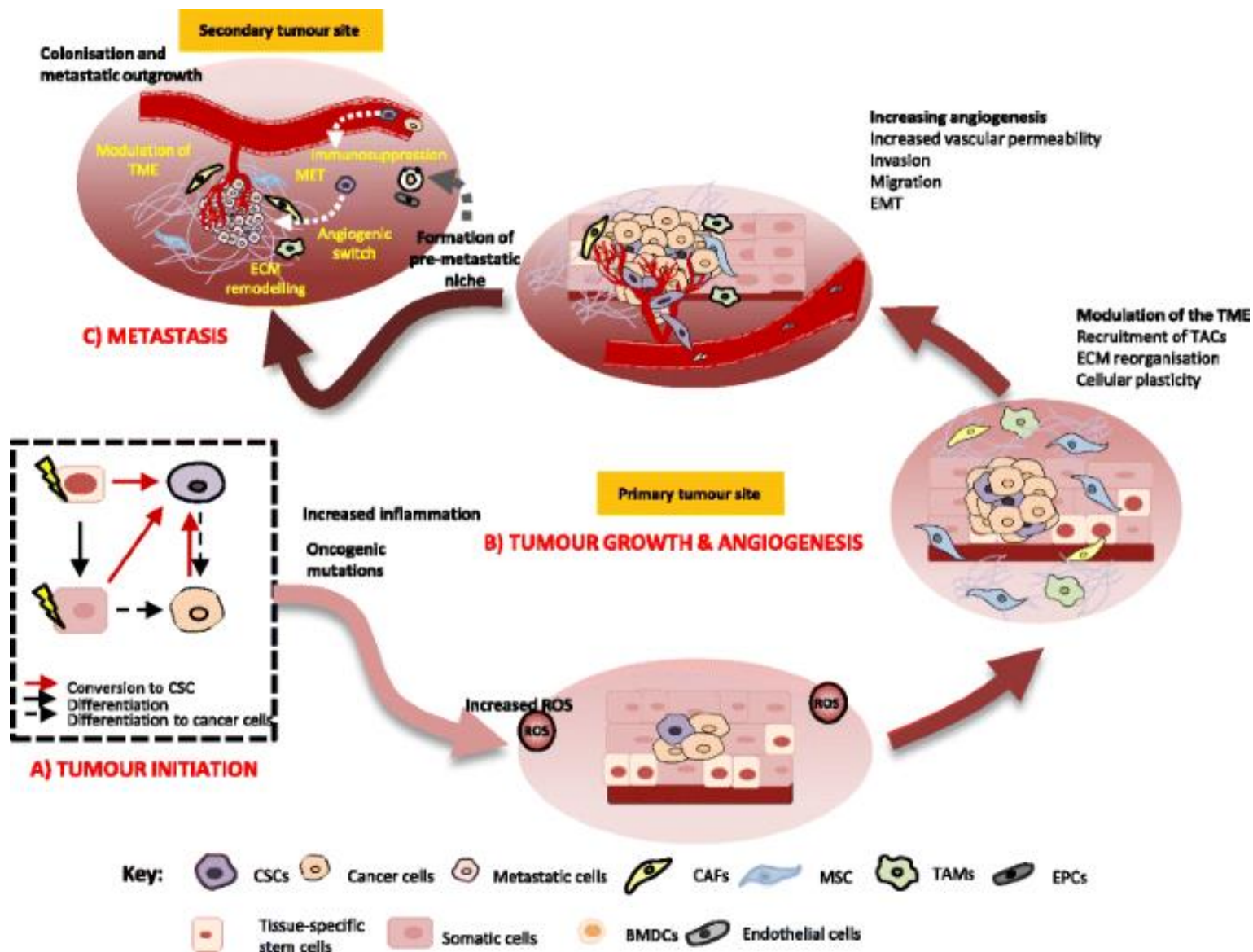


Figure 1-12. Summary of key cellular events during the progression of cancer from tumour initiation, tumour growth, angiogenesis and metastasis.

BMDCs, Bone-marrow derived cells; CAFs, Cancer-associated fibroblasts; CSC, Cancer stem cells; ECM, Extracellular matrix; EMT, Epithelial mesenchymal transition; EPCs, Endothelial progenitor cells; MET, Mesenchymal-epithelial transition; MSC, Mesenchymal stem cells; ROS, Reactive oxygen species; TAC, Tumour-associated cells; TAM, Tumour-associated macrophages. *Image from Ayob et al. (416)*

1.5 Origins and role of macrophages and endothelial cells in skeletal muscle

The skeletal muscle accounts for about 40% of the human body mass and is one of three significant muscles in the human body. It is vital for functions such as locomotion, postural support and breathing. Moreover, among other functions, it: 1) acts as a major site for storage of amino acids which are used by other organs for organ-specific protein synthesis, 2) maintains thermostasis and 3) acts as an energy source during starvation.

1.5.1 Skeletal muscle structure

Each skeletal muscle contains countless muscle fibres bound together by connective tissue and connected by tendons to bone (**Figure 1-13**). Each muscle is surrounded in a sheath of connective tissue known as the epimysium (461). The epimysium is vital to maintaining muscle structural integrity during muscle contraction. The individual bundles of muscle fibres in a skeletal muscle, called fasciculi (or plural, fascicles), cover the connective tissue sheath, known as the perimysium, and the innermost sheath surrounding muscle fibre, known as the endomysium (462, 463). Each muscle fibre is comprised of a bundle of myofibrils containing numerous myofilaments, organised in a unique pattern, and sarcomeres, that are fundamental for contraction. The two most significant myofilaments are actin and myosin which are arranged in a distinctive way that forms various bands on the skeletal muscle (463). Moreover, the endomysium contains the extracellular fluid and nutrients to support the muscle fibre that are supplied by blood vessels in the muscle (461). The stem cells in skeletal muscle, known as satellite cells, are able to differentiate into mature muscle fibres and are found between the basement membrane and the muscle fibre cell. A large number of cells, including resident macrophages, are present in the interstitial spaces of skeletal muscle and together, with satellite cells, help maintain the life-long presence of muscle tissue.

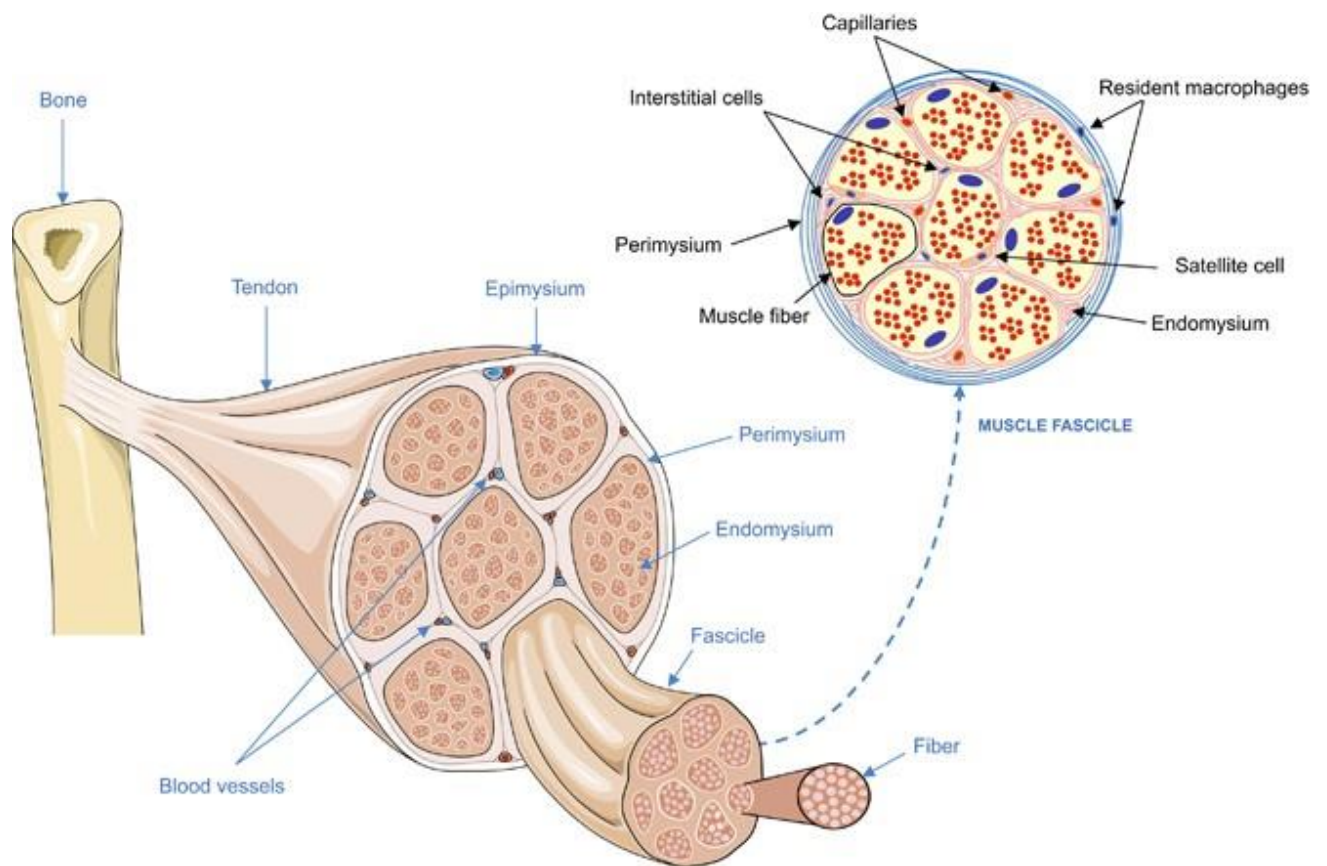


Figure 1-13. Structural composition of skeletal muscle.

Image from Sciorati (461)

1.5.2 Muscle stem cells: “muscle interstitial cells (MICs)”

The term muscle interstitial cells (MICs) is given to a range of different cell types found in the interstitium of skeletal muscle (464). The role of these cells in muscle homeostasis and regeneration remains relatively unclear. Most MICs originate from the mesoderm, including a large number that arise from the vasculature during embryonic development (464). MICs are typically divided into two main groups: cells that support muscle regeneration and those that may directly contribute to myonuclei (Table 1-7).

Of particular interest, are a subtype of MICs that have been found to have haematopoietic potential. These were first identified by Gussoni *et al.* in the late 1990s (465) who found a subpopulation of cells able to efflux Hoechst 33342 - a characteristic of haematopoietic side-population stem cells - among healthy murine muscle digests (465). They also characterised these HSC-like cells as forming haematopoietic colonies in methylcellulose, and as expressing Sca-1 and CD45 but no other haematopoietic lineage markers such as CD4, CD5, CD8, CD11 and Gr-1 (465-467). Further evaluation concluded that these haematopoietic cells in muscle came from the peripheral circulation based on their close proximity to CD31⁺ endothelial cells lining blood vessels (468, 469). Of note, most of the research on these cells was carried out between 1999-2003, with no recent updates using new, more sophisticated experimental techniques, such as lineage-tracing or scRNA-seq.

Another noteworthy subtype of MICs are myoendothelial cells, that express both satellite (CD56) and endothelial cell (CD34, CDH5) markers, whilst being negative for CD45 (465, 467). These are located between myofibers, but outside of the basal lamina. Clonal cultures of CD34⁺CD45⁻ sorted myoendothelial cells showed that they can differentiate into myogenic and endothelial lineages *in vitro* (469, 470), as well as osteogenic and chondrogenic cells (471). Moreover, they have also been demonstrated to efficiently regenerate injured skeletal muscle after transplantation *in vivo* (471).

Muscle interstitial cell types	Cell Marker	Direct contribution to myogenesis	Differentiation potential	References
Fibro-adipogenic precursors (FAP)	CD45 ⁻ /CD31 ⁻ /LIN ⁻ /Sca-1 ⁺ / CD34 ⁺ (low Osr1)	No	Adipocytes, osteoblasts smooth muscle cells	Joe <i>et al.</i> (472)
	CD45 ⁻ /CD31 ⁻ /LIN ⁻ /Sca-1 ⁺ / $\alpha 7^{\text{int-}}$	No		Joe <i>et al.</i> (472)
	CD31 ⁻ /CD45 ⁻ /SM/C-2.6 ⁻ / PDGFR α^+ /PDGFR β^+	No		Uezumi <i>et al.</i> (473)
Side population	Sca-1 ⁺ /CD4 ⁻ /CD5 ⁻ / CD8 ⁻ / CD11 ⁻ / Gr-1 ⁻ / c-Kit ⁺ /CD45 ⁺ or CD45 ⁻	Yes	Haematopoietic potential	Gussoni <i>et al.</i> (465); Kapoor <i>et al.</i> (467)
Myoendothelial cells	CD34 ⁺ /Sca-1 ⁺	Yes	Myogenic, endothelial	Tamaki. <i>et al.</i> , (469, 470)
	CD56 ⁺ /CD34 ⁺ / CDH5 ⁺	Yes	Myogenic, endothelial osteogenic, and chondrogenic cells	Zheng <i>et al.</i> (471)
PICs (FAP subpopulation?)	CD34 ⁺ /Sca-1 ⁺ /Pax7 ⁻ /PDGFR α^+	Yes	Myofibroblasts, muscle fibres, adipocytes	Besson <i>et al.</i> (474); Yao <i>et al.</i> (475), Pannerec <i>et al.</i> (476)
	CD34 ⁺ /Sca-1 ⁺ /Pax7 ⁻ /PDGFR α^+	No	Myogenic potential	
Unknown	Twist2 ⁺ /Pax7 ⁻	Maintenance/regeneration of fast muscle fibres only	Myofibers	Liu <i>et al.</i> (477)

Table 1-7. Muscle interstitial stem/progenitor cells in the skeletal muscle.

FAP, Fibro-adipogenic precursors; PICs, Cells positive for Paternally-expressed gene 3 (PEG3) and negative for Paired box protein Pax-7 (Pax7).

1.5.3 Macrophages in healthy skeletal muscle

Until recently the origin of intramuscular macrophages was thought to be only from BM-derived cells. A study by Wang *et al.* has now demonstrated through different murine lineage-tracking approaches that skeletal muscle macrophages originate from both extra-embryonic YS and foetal liver-derived progenitors and BM-derived monocytes (172). They identified five distinct macrophage clusters from scRNA-seq: 1) a *Ccr2* cluster, 2) a proliferating cluster, 3) a *Cd206* cluster, 4) a *Klf2* cluster and 5) “cluster 0”. BM-derived macrophages that were phenotypically MHCII^{Hi}LYVE-1^{Lo} were associated with the *Ccr2* cluster, while resident YS-derived macrophages that were MHCII^{Lo}LYVE-1^{Hi} were found in the non-*Ccr2* clusters. Interestingly, the *Cd209* cluster was only identified in the quadricep muscles of mice and had high expression of M2-like genes such as *Mrc1*, *Fcgrt*, *Folr2*, *Cd163*, *Fcna*, *Timd4*, and *Tslp* and the C-type lectin receptor (CLR) genes. Meanwhile, the *Klf2* cluster was only seen in the diaphragm and displayed high expression of stress-response genes (e.g. *Egr1*, *Fosb*, *Ier3*). The *Ccr2* cluster uniquely was enriched in antigen expressing genes *H2-Ab1*, *Cd74* and *Cd52*. Notably, the transcriptional characteristics of non-*Ccr2* clusters in muscle resemble those of other embryonically-derived tissue-resident macrophages found in aorta, lung and heart (172). It was speculated that although they probably share a common developmental origin, the differences in the four non-*Ccr2* clusters were due to microenvironmental regulatory cues in their different muscle sites.

1.5.4 Endothelial cells in healthy muscle

Postnatally, the role of the skeletal muscle microcirculation is to support muscle contraction. Interestingly, myogenic and endothelial cells in muscle share a common embryonic mesoangioblast origin in the form of the dermomyotome, a transient epithelial structure of the somite formed in the mouse at gestational age E9. The dermomyotome gives rise to myoblasts, dermis, endothelial cells and SMCs (72). It also generates a contingent of distinct skeletal muscle endothelial cells, named somite-derived endothelial cells (SDECs), as shown in avian, murine and zebrafish studies (478). These SDECs act as a secondary input to the haemogenic progenitors that form HSCs during embryogenesis.

1.5.5 Skeletal muscle injury

The skeletal muscle has a rapid repair mechanism which maintains day-to-day functions. However, after larger injuries this innate repair mechanism can become overwhelmed, which results in skeletal muscle dysfunction and incomplete functional repair after mechanical trauma, thermal stress, myotoxic agents, ischaemia and neurological damage (479). Skeletal muscle injuries occur from traumatic incidents such as those caused by car accidents or surgical resections, or from injuries sustained in sports, which result in the loss of functionality of the injured muscle (480). Moreover, ageing skeletal muscle has reduced muscle mass and strength (called sarcopenia), and diminished muscle regenerative capacity (464, 481).

Muscle regeneration is a complex biological process, which is regulated by immune cells, satellite cells and endothelial cells. It is divided into several stages: necrosis of the injured muscle cell, activation of muscle stem cells, proliferation of the activated muscle stem cells, differentiation of the muscle stem cells, maturation of the newly formed muscle fibres and the remodelling of muscle fibres (**Figure 1-14**) (479). In almost all these stages, acute inflammation and immune cells play critical roles.

1.5.6 Satellite cells in muscle injury

Satellite cells are the major contributor to muscle regeneration. Under normal conditions these cells are dormant. Following muscle injury, after exercise or during muscle growth, dormant satellite cells become activated, briefly proliferate and differentiate into new myotubes or fuse to the damaged myofibers. Satellite cells become activated by physiological stimuli such as HGF and FGF, which are produced by inflammatory cells, endothelial cells and fibroblasts or released from the ECM via specialised proteases (482). Proliferating satellite cells start to express the *MyoD* regulatory factor (**Figure 1-14**). After they undergo multiple cell divisions, they differentiate into myoblasts which fuse and form small centro-nucleated fibres that gradually grow in size to achieve complete muscle

regeneration. Subsequently, these muscle stem cells return to their dormant state and replenish the muscle stem cell niche.

Ageing has been shown to affect the regenerative capacity of satellite cells. This has been seen in the decline of *Notch*-dependent activation of p53 in satellite cells with age which results in impaired self-renewal leading to satellite cell death. Interestingly, if p53 is stabilised in aged satellite cells, their viability is preserved and their regenerative potential improves (483). Similarly, reduced activation of the AMPK/p27Kip1 pathway in aged satellites leads to dysfunctional autophagy and higher susceptibility to apoptotic cell death (484, 485). Genetic or pharmacological restoration of AMPK or p27Kip1 activity was able to effectively suppress the expression of senescence markers and apoptosis in aged satellite cells. These studies highlight both Notch/p53 and AMPK/p27Kip1 pathways as potential targets for satellite cell regeneration.

1.5.7 Macrophages in injured skeletal muscle

Intramuscular macrophages are important effectors and regulators of muscle inflammation, fibrosis and regeneration, and contribute to acute skeletal muscle injury repair and muscular dystrophy (486, 487). In response to muscle injury, immune cells such as macrophages are activated to rapidly remove necrotic tissue and release chemokines that regulate the activation, proliferation and differentiation of satellite cells to mediate muscle repair and regeneration (**Figure 1-14**) (479, 488).

Recruitment of circulating monocytes into the site of muscle trauma is thought to initiate the first phase of this inflammatory response. These are recruited by neutrophil production of IL-1 and IL-8 at the site of injury, and the secretion of CCL2 from injured muscle fibres (489). Depletion of monocytes and their macrophage progeny in *Ccr2*^{-/-} mice and after administration of liposomal clodronate has been shown to impair the regenerative response to muscle injury (489).

The initial stage of muscle injury involves an influx of pro-inflammatory M1-like macrophages (490). After this initial stage, there is an expansion of anti-inflammatory, pro-angiogenic and pro-regenerative M2-like macrophages (491, 492). This switch between the pro-inflammatory to the anti-inflammatory macrophage subtype is critical to enable a healthy regenerative response (492). M1-like macrophages secrete TNF- α , IL-1 β , IFN- γ and reactive oxygen species to facilitate myoblast proliferation, while anti-inflammatory cytokines from M2-like macrophages promote myoblast differentiation (481, 493). Inhibition of this M1 to M2 switch has been shown to reduce muscle regeneration and muscle fibre growth (484, 494, 495).

Skeletal muscle macrophages mediate their trophic support of satellite cells via production of ADAM Metallopeptidase With Thrombospondin Type 1 Motif 1 (ADAMTS1), which suppresses the dormant regulator of satellite cells (Notch1) and causes satellite activation (496). Another important macrophage-secreted factor is Growth Differentiation Factor-3 (GDF3), that enables the fusion of myoblasts (after satellite differentiation) and myotube formation (495). Moreover, during muscle injury and ageing, macrophage-derived glutamine activates mTOR signalling in satellite cells, which promotes their proliferation and differentiation (497). The discovery of these macrophage-derived signals to promote satellite cell activation further confirms the importance of macrophages during muscle repair and regeneration. However, this area of research is still relatively new and questions remain unanswered concerning the relative contributions that different BM- and embryonic-derived macrophages and their subtypes make toward these important regenerative functions.

1.5.8 Endothelial cells in injured muscle

In a similar way to macrophages, endothelial cells also undergo important changes during the response to muscle damage, especially when ischaemia is present (498). Muscle injury itself leads to local hypoxia, which induces expression of VEGF and other pro-angiogenic factors. Angiogenesis helps to

restore oxygen and nutrient delivery to the regenerating muscle tissue, while angiocrine growth factors such as VEGF, IGF and PDGF also promote the proliferation of local resident progenitor cells and fibroblasts (498-500).

Endothelial cells interact very closely with both macrophages and satellite cells during muscle regeneration. They regulate macrophage differentiation and maturation through angiocrine Notch signalling (*Dll1*), which promotes arteriogenesis during recovery from muscle ischaemia (490). Studies have shown that activated satellite cells are always in close proximity to endothelial-lined blood vessels, and they express VEGF-A, which helps maintain Delta-like ligand 4 (DLL4), a ligand important in endothelial sprouting (501). Co-culture of human endothelial cells and muscle satellite cells has revealed the bi-directional nature of their interaction, increasing both the proliferation of satellite cells as well as the formation of capillaries (502-504).

Interim Conclusion

- Neovascularisation after skeletal muscle ischaemia involves accumulation of non-circulation derived macrophages which give structural and trophic support to endothelial-lined neovessels.
- Understanding the basis of how muscle-resident macrophages and endothelial cells interact is central to: 1) normal muscle development; 2) postnatal muscle homeostasis and ageing; 3) mechanisms of muscle repair and regeneration.

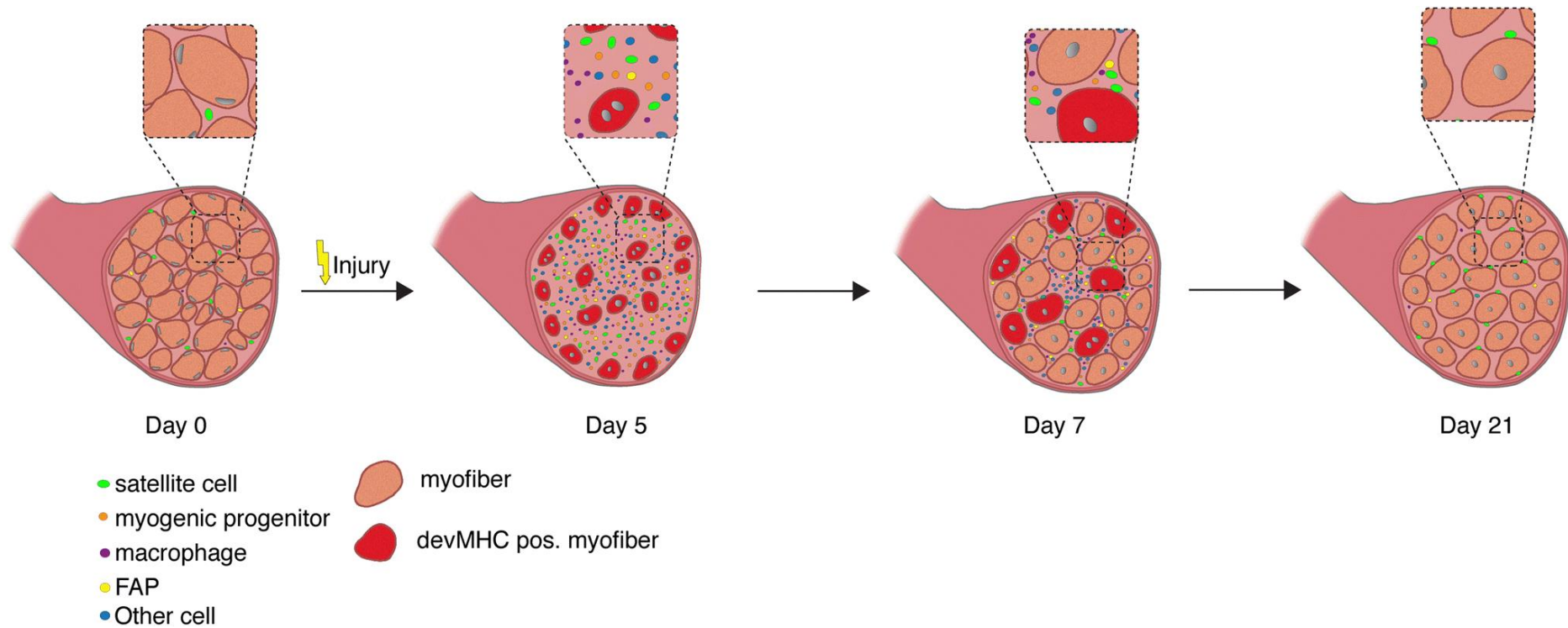


Figure 1-14. Muscle repair and regeneration.

Time course of changes in cellular composition during skeletal muscle regeneration following muscle injury. Satellite cells (in green) are quiescent in resting skeletal muscle. Five days after injury, regenerating muscles contain mostly satellite cells, immune cells [including macrophages (purple)] and other progenitors [including myogenic progenitor (orange) and fibro-adipogenic precursors (FAP, yellow)], however by day 7 blood vessels and myotubes (red) begin to form, which later become mature myofibers (brown). *Image from Schmidt et al. (505)*

1.6 Project scope

Macrophages and endothelial cells perform intimately linked functions in different tissues during prenatal development and postnatal maintenance of steady state homeostasis. Their well-orchestrated interactions are integral as tissues mount inflammatory responses and neovessel formation to mediate repair and/or regeneration following injury or ischaemic insult. However, these processes can also be maladaptive, especially if they become dysregulated and unchecked in pathological conditions, like atherosclerosis and tumorigenesis. It follows that a better understanding of how the intricate relationships between macrophages and endothelial cells come about may open up new therapeutic opportunities to target these major disease processes. One important clue may lie in the ontogenies of both cell lineages, which increasingly appear to overlap, at least during early prenatal life. Converging lines of evidence indicate that macrophages and endothelial cells share a common source from c-Kit⁺CSF1R⁺ EMPs in extra-embryonic YS, as shown by murine lineage mapping studies (93, 131, 132). These recent observations align well with historical descriptions of bipotent haemangioblasts in YS and haemogenic endothelium in both the YS and AGM during embryogenesis. Current expert consensus contends that YS-derived macrophages and endothelial cells are seeded in tissues prenatally and are maintained after birth by local self-renewal in their mature, differentiated state. However, an alternative, largely neglected explanation is that the YS also releases EMP-like progenitors to different tissues that persist postnatally to sustain local myeloid and endothelial populations under steady-state and mediate their expansion in disease.

This thesis, therefore, explores the existence of a common YS-derived, bipotent progenitor cell for macrophages and endothelial cells in different postnatal murine tissues. Extending on our laboratory's previous discovery of Sca-1⁺CD45⁺ AMPCs in postnatal mouse arteries (262, 263), we first set out to investigate whether this adventitial progenitor population also has angiogenic potential, as relevant to the development and expansion of *vasa vasorum* in atherosclerosis (Chapter 3). This leads to a rigorous

interrogation of CFU-M-forming progenitor cells in murine aorta, to uncover their precise immunophenotypic fingerprint and study their bipotent capacity for myeloid and endothelial lineages in the context of their developmental origins (Chapter 4). Discovery that these postnatal Endothelial-Macrophage (EndoMac) progenitors are in fact derived from a $\text{CSF1R}^+ \text{CX}_3\text{CR1}^+$ source in the YS and are also seeded in other tissues (heart, lungs), prompts exploration of their presence in mouse skeletal muscle (Chapter 5) and skin (Chapter 6), leading to interrogation of their actions in peripheral ischaemia, skin wound healing and melanoma.

Chapter 2

Materials and methods

2.1 General materials

2.1.1 Electronic equipment and software used

Table 2-1. Electronic equipment and software used and, their specifications.

Item name	Specifications and Supplier
<u>Electronic Equipment</u>	
Anaesthetic Machine	Stinger anaesthetic gas machine, DarvallVet, NSW, Australia
Bead steriliser	FST, Fine Science Tools, Vancouver, Canada
Cell-strainer 40 µm	#542040, Greiner Bio-One, Interpath Services, Victoria, Australia
Centrifuges	Cell Culture/ Flow Cytometry – Eppendorf 5810R RNA – Eppendorf 5424R Giemsa stain-Thermo Shandon cytospin 4
CFX96 Touch Real-Time PCR Detection System	BIO-RAD Laboratories, CA, USA
Chemical Fume Hood	LabSystems, VIC, Australia
ChemiDoc™ MP Imaging system	#170-8280 BIO-RAD Laboratories, CA, USA
CO₂ asphyxiation machine	Quietek CO ₂ Induction System, Next Advance, NY, USA
Confocal microscopy	Leica TCS SP8, Leica, Wetzlar, Germany
Cryotome	Shandon Cryotome E, Thermo Scientific, MA, USA
Digital dry Bath	Corning LSE™, NY, USA
Embedder	HistoStar™ Embedding Workstation, ThermoFisher Scientific, MA, USA
Flow Cytometer	LSRFortessa™, BD Biosciences, CA, USA FACSCanto II™, BD Biosciences, CA, USA FACSARIA™ Fusion Cell Sorter, BD Biosciences, CA, USA
Freezer	-20°C – Haier Biomedical, China -80°C – 89000 series, Thermo Scientific, MA, USA
Fridge	4°C – GPS series, Thermo Scientific, MA, USA
IKAMAG™ multi-position magnetic stirrer	Sigma-Aldrich Inc., MO, USA
Incubator	Binder, LabFriend, Tuttlingen, Germany
Laser Doppler Imager	#MOORLDI2-IR, moor instruments, Devon, UK
Microscope Camera	AxioCam ERc 5s, Carl Zeiss Microscopy, Germany
Microscopes	<i>Dissecting</i> – Zeiss Stereo Discovery.V8, Carl Zeiss Microscopy, Germany <i>Inverted</i> – Olympus CKX31, Olympus Life Science, Tokyo, Japan <i>Light</i> – Zeiss Lab.A1, Carl Zeiss Microscopy, Germany

NanoDrop™ Spectrophotometer	8000	ThermoFisher Scientific, CA, USA
NanoZoomer HT Digital Scanner		Hamamatsu Photonics, Japan
Scales		#XS105, Mettler Toledo, OH, USA
T100 Thermal Cycler		BIO-RAD Laboratories, CA, USA
Tissue Culture Hood		CellGard™ ES (Energy Saver) NU-480 3ft Class II, Type A2 Biological Safety Cabinet, NuAire, MN, USA
Tissue Processor		Excelsior Tissue Processor™, ThermoFisher Scientific, CA, USA
Vortex Mixer		Corning LSE™, NY, USA
Water bath		TW20 Julabo® Water bath, Sigma-Aldrich Inc., MO, USA
<u>Software</u>		
Adobe Photoshop		2018 – 2021, Creative Cloud, Adobe, CA, USA
Fiji (Image J)		Versions available in 2018-2021, NIH, MD, USA
FlowJo		Versions available between 2018-2021, BD Biosciences, CA, USA
Graph Pad Prism		Versions available in 2018-2021, GraphPad Software, LLC, CA, USA
Image Lab		BIO-RAD laboratories, CA, USA
moorLDL measurement		moorLD12-IR, Moor Instruments, Devon, UK
Microsoft Excel		Microsoft Excel® 2018 – 2021 versions, Microsoft Corporations, WA, USA
Microsoft Word		Microsoft Word® 2018 – 2021 versions, Microsoft Corporations, WA, USA
NDP.view 2		Hamamatsu Photonics, Tokyo, Japan

2.1.2 Materials

Table 2-2. Materials used and their suppliers.

Material	Catalog number (#) and Supplier
8-well Chamber Slides (removable)	#80841, ibidi, Gräfelfing, Germany
ALZET osmotic pumps	2001 #0000292, BioScientific, CA, USA 1003D #0000289, BioScientific, CA, USA
Biopsy punch (6mm)	#LIVBIOPUNCH06, Livingstone, NSW, Australia
Bottle-top vacuum filters polyethersulfone membrane, pore size 0.22 µm	#CLS431118-12EA, Sigma-Aldrich Inc., MO, USA
Brightline™ Haemocytometer	#Z359629, Sigma-Aldrich Inc., MO, USA
Castroviejo Callipers	#REF4050, Katalyst Surgical, MO, USA
Cautery Handle	#HIT1, Dalcross Medical Equipment, NSW, Australia
CellBIND® Surface Cell Culture	25cm ² - #CLS3289, Corning Incorporated, ME, USA 75cm ² - #CLS3290, Corning Incorporated, ME, USA
CoolCell® Freezer Containers	#432000, Corning, NY, USA
Corning tissue-culture treated culture dishes 60mm x 15mm	#CLS430166, Invitro Technologies, VIC, Australia
Costar® Multiple well CellBIND® Cell Culture Plates	6 wells – #3516, Corning Incorporated, ME, USA 12 wells – #3513, Corning Incorporated, ME, USA 24 wells - #3738, Corning Incorporated, ME, USA
Costar® Ultra-Low Attachment Multiple Well Plates	6 wells – #3471, Corning Incorporated, ME, USA 24 wells - #3473, Corning Incorporated, ME, USA
Countess cell counter chamber slides	#C10228, Life Technologies, CA, USA
Cryogenic vials	#431386, Corning, NY, USA
Easy strainer Green	#542040, Interpath, VIC, Australia
Embedding Cassette	#40634001, Techno Plas, SA, Australia
Eppendorf Tubes	1.5mL safe-lock tubes – #T9661, Sigma-Aldrich Inc., MO, USA 0.6mL tubes – # T5149, Sigma-Aldrich Inc., MO, USA 1.5mL tubes – #T6649, Sigma-Aldrich Inc., MO, USA 2.0mL tubes – #T3031, Sigma-Aldrich Inc., MO, USA
Falcon® Tubes	5mL Round bottom – #352003, Corning Incorporated, ME, USA 15mL – #352096, Corning Incorporated, ME, USA 50mL – #352070, Corning Incorporated, ME, USA
Filters	#SLGV033RS, 0.22µm, Millex® GV, Merk Millipore Ltd, Ireland
Fine science tools	Dumont Forceps #11254-20, FST, Vancouver, Canada Fine scissors 10.5cm #14094-11, FST, Vancouver, Canada Tough Cut Pattern Scissors #14054-13, FST, Vancouver, Canada Mini-perforated spoon #10370-19, FST, Vancouver, Canada Needle holder #12003-15, FST, Vancouver, Canada

Forceps	#14114, 15cm, 0.3mm tip, Coherent Scientific, SA, Australia
Hair removal cream	#2485516, Veet, Chemist Warehouse, SA, Australia
Iris scissors, 420SS. straight, 115mm	#TS0984, ProSci Tech, QLD, Australia
Needles	18G 1 1/2in - #302032, BD Biosciences, NJ, USA 19G 1 1/2in - #305187, BD Biosciences, NJ, USA 27 G x 1 1/2 in - #301629, BD Biosciences, NJ, USA
Opsite™	#4967, Smith & Nephew, London, UK
PAP pen for immunostaining, 2mm tip	#Z672548-1EA, Sigma-Aldrich Inc., MO, USA
Pipette Gun	Thermo Fisher Scientific, Australia
Pipettes 0.5-10µl, 2-20µl, 20-200µl, 100-1000µl	For flow tubes – Eppendorf Research Plus For everything else – Corning Lambda™ Plus
Pointed tweezers, economy, style 5	#T05-811, ProSci Tech, QLD, Australia
Polyallomer Quick-Seal Centrifuge tubes	6.0mL - #58594, Beckman Coulter®, USA 40mL - #342414, Beckman Coulter®, USA
polypropylene round-bottom capped tubes	#14-959-11A, Thermofisher Scientific, CA, USA
Polystyrene Round-Bottom Tube with Cell-strainer cap	#FAL35225, Invitro Technologies, VIC, Australia
Polystyrene Test Tube without Cap, 12 x 75 mm	#FAL352008, Invitro Technologies, VIC, Australia
Scalpels	#0501, Swann-Morton®, Sheffield, England
Scintillation Vials	#Z376817, Sigma-Aldrich Inc., MO, USA
Shaver	#41590-0438, WAHL BravMini+, IL, USA
Silicone (0.5mm thick)	#P18178, Life technologies, CA, USA
Stripette® Serological Pipets	5mL – #4487, Corning Incorporated, ME, USA 10mL – #4488, Corning Incorporated, ME, USA 25mL – #4489, Corning Incorporated, ME, USA
Sutures	6-0 PROLENE™, 639G, Ethicon LLC, San Lorenzo, Puerto Rico 7-0 SILK, 768G, Ethicon LLC, San Lorenzo, Puerto Rico 8-0 PERMAHAND™, 818W, Ethicon LLC, San Lorenzo, Puerto Rico
Syringes	1mL - #SS+01T, Terumo, Tokyo, Japan 5mL - #SS05S, Terumo, Tokyo, Japan 10mL - #SS-10S, Terumo, Tokyo, Japan 20mL - #SS-20S2, Terumo, Tokyo, Japan 50mL – #SS*50ESE, Terumo, Tokyo, Japan
Tissue-tek® Cryomold for OCT	#4565, Sakura Finetek, C.A., USA
Transfer Pipets	#222, Samco Scientific, CA, USA
Vannas Scissors, delicate, straight, 3mm	#EMS72932-01, ProSci Tech, QLD, Australia

2.1.3 Reagents

Table 2-3. Reagents used and their suppliers.

Reagent	Catalog # and Supplier
Ammonium Chloride Solution	#07850, StemCell Technologies, Vancouver, Canada
Angiotensin II	#A9525, Sigma-Aldrich, Inc., St Louis, MO, USA
Antibiotic Antimycotic Solution (100x)	#A5955, Sigma-Aldrich Inc., MO, USA
APC BrdU Flow Kit	#557892, BD Biosciences, NJ, USA
Bovine Serum Albumin (BSA)	#A7906, Sigma Aldrich Inc., MO, USA
Brilliant Buffer Stain	#566349, BD Biosciences, NJ, USA
Buprenorphine	#TEMVET, Lyppard Australia Pty Ltd., Vic, Australia
Bupivacaine	#BUPIV1, Lyppard Australia Pty Ltd., Vic, Australia
Carprofen	#ZENE, Lyppard Australia Pty Ltd., Vic, Australia
Chloroform	#AJA152-2.5L GL, Ajax Finechem, NSW, Australia
Clodronate control liposomes	clodronateliposomes.org, Vrije Universiteit, Netherlands
Compensation Beads (Rat/Hamster)	#552843, BD Biosciences, NJ, USA
Cytofix Fixation Buffer	#554655, BD Biosciences, NJ, USA
Dimethyl sulphoxide (DMSO)	#D8418, Sigma Aldrich Inc., MO, USA
Dubelcco's Phosphate-buffered Saline 10x	#D1408, Sigma-Aldrich Inc., MO, USA
Dubelcco's Phosphate-buffered Saline 1x	#D8537, Sigma-Aldrich Inc., MO, USA
Dulbecco's Modified Eagle's Medium, High Modified (DMEM)	#D5030, Sigma Aldrich Inc., MO, USA
Endothelial Cell Growth Media (EGM-10)	#CC-5035, Lonza, Basel, Switzerland
Enalapril	#E6888, Sigma-Aldrich, Inc., St Louis, MO, USA
Ethanol	#E7023, Sigma Aldrich Inc., MO, USA
Ethyl alcohol, Pure, for molecular biology	#E7023, Sigma Aldrich Inc., MO, USA
Fixable Viability Stain 700	#564997, BD Biosciences, NJ, USA
Foetal Bovine Serum (FBS)	#AU-FBS/PG, CellSera, NSW, Australia
Formaldehyde	#C004, ProSciTech, QLD, Australia
Formalin	#HT501128, Sigma Aldrich Inc., MO, USA
Fractalkine	#F2302, Sigma-Aldrich Inc., MO, USA
Hanks' Balanced Salt Solution (HBSS)	#H9394, Sigma Aldrich Inc., MO, USA
High Cholesterol Diet	#SF00219, Specialty Feeds, WA, Australia
Hydrogen peroxide solution	#216763, Sigma Aldrich Inc., MO, USA
Iscoe's Modified Dulbecco's Medium (IMDM)	Sigma Aldrich Inc., MO, USA
iScript cDNA Synthesis Kit	#1708841, Bio-Rad Laboratories, CA, USA
Isopropanol	#I9516, Sigma-Aldrich Inc., MO, USA
L-glutamine	#G7513, Sigma-Aldrich Inc., MO, USA

Liberase™ Research Grade	#5401127001, Sigma-Aldrich Inc., MO, USA
Losartan	#61188, Sigma-Aldrich, Inc., St Louis, MO, USA
Matrigel™	#354234, Corning Inc, NY, USA
MethoCult GF M343, 100mL	#3434, StemCell Technologies, Vancouver, Canada
Mouse Inflammation CBA kit	#552364, BD Biosciences, NJ, USA
Mouse Inflammation Standards	#620290, BD Biosciences, NJ, USA
Murine M-CSF	#315-02-500, PeproTech, NJ, USA
Normal Horse Serum	#S2000, Vector Laboratories, CA, USA
Nuclease free water	#P1193, Promega, WI, USA
O.C.T. Compound	#4583, Sakura Finetek, C.A., USA
Opti-MEM™ I Serum Reduce Media	#31985070, Life technologies, CA, USA
Paraformaldehyde (PFA)	#C004, ProSciTech, QLD, Australia
PBS control liposomes	clodronateliposomes.org, Vrije Universiteit, Netherlands
PD123,319	#PD186, Sigma Aldrich Inc., MO, USA
Penicillin-Streptomycin	#P433, Sigma-Aldrich Inc., MO, USA
Percoll	#P4937, Sigma-Aldrich Inc., MO, USA
Pierce™ Streptavidin Poly-HRP	#21140, Life Technologies, CA, USA
Prolong Gold Antifade Mountant with DAPI	#P36935, Life Technologies, CA, USA
Propidium Iodide	#P4170, Sigma-Aldrich Inc., MO, USA
Proteinase K	#17916, ThermoFisher Scientific, MA, USA
RNase A, DNase and protease-free	#G9545, Sigma-Aldrich Inc., MO, USA
Roswell Park Memorial Institute (RPMI) 1640 Medium	#R7388, Sigma-Aldrich Inc., MO, USA
Sodium Azide	#SA189, Chem-supply, SA, Australia
Sodium Bicarbonate	#S5761, Sigma-Aldrich Inc., MO, USA
Sodium Hypochlorite 8-12.5%	#DT055-5L, ChemSupply, SA, Australia
SsoAdvanced™ Universal SYBR® Green Supermix	#1725275, Bio-Rad Laboratories, CA, USA
Stain Buffer FBS	#554656, BD Biosciences, NJ, USA
Sucrose	#S0389, Sigma-Aldrich Inc., MO, USA
Tamoxifen	#T5648, Sigma-Aldrich Inc., MO, USA
TRI® Reagent	#T9424, Sigma-Aldrich Inc., MO, USA
Tris hydrochloride	#T3253, Sigma-Aldrich Inc., MO, USA
Triton X-100	#X100, Sigma-Aldrich Inc., MO, USA
Trypan Blue solution	#T8154, Sigma-Aldrich Inc., MO, USA
Trypsin-EDTA	#15400054, ThermoFisher Scientific, MA, USA
Tween®-20	#P9416, Sigma-Aldrich Inc., MO, USA
Type IV collagenase	#C4-BIOC, Sigma-Aldrich Inc., MO, USA

2.2 General solutions and media

For the names of suppliers/manufacturers of reagents, materials and equipment, please refer to the preceding tables.

2.2.1 Cell line media

Roswell Park Memorial Institute (RPMI-1640), 10% foetal bovine serum (FBS), 1% antimycotic/antibiotic solution, 1% L-glutamine. Stored at 4°C.

2.2.2 Dulbecco's modified eagle's medium, high modified (DMEM)

DMEM, 10% FBS, 1% antimycotic/antibiotic solution. Stored at 4°C.

2.2.3 Endothelial cell growth media (EGM-10)

EGM-10, 10% FBS, 1% antimycotic/antibiotic solution. EGM-10 contains 20 ng/mL vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF). Stored at 4°C.

2.2.4 Freezing media

FBS with 10% DMSO. Filtered and stored at 4°C.

2.2.4 Iscove's modified dulbecco's medium (IMDM)

IMDM supplemented with 10% FBS and 1% antimycotic/antibiotic solution. Stored at 4°C.

2.3 Mice

2.3.1 Mouse strains

Cx3cr1^{GFP/+} (B6.129P-Cx3cr1^{tm1Litt}/J), *Ccr2*^{-/-} (B6.129S4-Ccr2^{tm1Ifc}/J), *Flt3*^{Cre} (Tg.Flt3^{cre}/ERT2), *Rosa*^{mT/mG} (Gt(ROSA)26Sor^{tm4}(ACTB-tdTomato,-EGFP)Luo/J), *Csf1r*^{Mer-iCre-Mer} (FVB-Tg(Csf1r-cre/Esr1*)1Jwp/J), *Cx3cr1*^{YFP-creER} (B6.129P2(Cg)-Cx3cr1^{tm2.1}(cre/ERT2)Litt/WganJ), *UBI-GFP* (C57BL/6-Tg(UBC-GFP)30Scha/J), *ApoE*^{-/-} (B6.129P2-ApoE^{tm1Unc}/J) and *Ldlr*^{-/-} mice (B6.129S7-Ldlr^{tm1Her}/J) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). C57BL/6 (C57BL/6J) mice were from the South Australian Health and Medical Research Institute (SAHMRI). A breeding colony of *Rosa*^{tdTomato} mice was kindly provided by Dr Daniel Worthley (Precision Medicine Theme, SAHMRI). *Cx3cr1*^{GFP/+} mice were crossed to obtain *Cx3cr1*^{GFP/GFP} mice. *Flt3*^{Cre} mice were crossed with *Rosa*^{mT/mG} to obtain a *Flt3*^{Cre} x *Rosa*^{mT/mG} genotype mice. *Csf1r*^{Mer-iCre-Mer} were crossed with *Rosa*^{mT/mG} to obtain *Csf1r*^{MerCreMer} x *Rosa*^{mT/mG} mice. *Cx3cr1*^{YFP-creER} were crossed with *Rosa*^{tdTomato} to obtain *Cx3cr1*^{CreER-YFP} x *Rosa*^{tdTomato} mice. *Csf1r*^{Mer-iCre-Mer} were maintained in a mixed background of FVB and C57BL/6J. Both males and females were used as specified throughout the text and figure legends with all mice models, with the exception of *Flt3*^{Cre} x *Rosa*^{mT/mG} where only males were used as the Flt3-Cre modification is located on the Y chromosome.

Mice with *ApoE*^{-/-} and *Ldlr*^{-/-} genotypes were used for atherosclerosis studies, and fed a high cholesterol diet (HCD) composed of 0.15% cholesterol and 21% fat (Specialty Feeds, Perth, Australia) as specified in Chapters 3, starting from 8 weeks (w) of age.

In Chapters 4-6, gestational embryonic age was defined on the basis of the date of vaginal plug formation, which was set at E0.5. Plug was detected by examining the female mice vaginal opening for a whitish mass (506). To achieve a Cre-Lox recombination in *Cx3cr1*^{CreER-YFP} x *Rosa*^{tdTomato} and *Csf1r*^{MerCreMer} x *Rosa*^{mT/mG} mice, 75 µg/g of tamoxifen (TAM, Sigma-Aldrich) dissolved in corn oil (Sigma-Aldrich) was intraperitoneally injected into pregnant dams at either E8.5 or E9.5, as specified.

For the purpose of analysing the cell cycle status of different cell populations in different tissues, 1 mg of bromodeoxyuridine (BrdU) was injected intraperitoneally before euthanasia and tissue harvest at times specified in Chapters 4-6.

In all instances, euthanasia was performed under isoflurane anaesthesia by cardiac puncture or CO₂ asphyxiation (following SAHMRI SOP-0491 and SOP-0501 for humane killing methods for mice). Decapitation using sharp scissors was performed to ensure death for P1 mice (neonates). Death of an animal was determined by loss of breathing, changes in eye colour and toe-pinch tests. Blood was collected by cardiac puncture using a 100 µL syringe with a 27G needle and aliquoted into ethylenediamine tetra acetic acid tubes (EDTA). The heart and aorta were flushed with cold phosphate buffered saline (PBS) before harvesting and processing tissues as described below.

2.3.2 Animal ethics

All experimental procedures and animal care were performed in accordance with the National Health and Medical Research Council (NHMRC) research guidelines and the Australian Code for the Care and Use of Animals for Scientific Purposes.

Ethics approval for Chapter 3's collaborative studies was obtained from SAHMRI (ID SAM118 and SAM155) and the Mayo Clinic animal ethics committee. Ethics approvals for Chapters 4-6 were obtained from SAHMRI (ID SAM155, SAM117, SAM155, SAM244, SAM308 and SAM432.19).

2.4 Generic methods

2.4.1 Cryopreservation of cells

The following technique of cryopreservation was universally applied for all cell types. Cells were suspended in freezing media. The cell suspension was aliquoted quickly into cryoampoules (1.6 x 10⁶ cells per 2 mL ampoule) which were placed in the rate cooling Mr Frosty container for 24 h at -80°C. Once frozen, ampoules were stored in liquid nitrogen (-196°C).

2.4.2 Thawing of cryopreserved cells

Cell ampoules were removed from liquid nitrogen and thawed rapidly in a 37°C water bath for approximately 2 min. The thawed cell suspension was transferred into a 50 mL Falcon® tube filled with respective cell media (warmed to 37°C). Cells were pelleted by centrifugation at 400 *g*, 22°C for 5 min. The supernatant was aspirated and cells were washed a further two times with media to remove any residual DMSO.

2.4.3 Cell counts and viability

Cell concentration was determined by diluting the cell suspensions in media, while viability was assessed by diluting samples with 0.1% trypan blue solution. 10 µL of these suspensions were transferred to a haemocytometer counting chamber. The average number of cells per square in the middle large square was counted to calculate the concentration of cells as follows:

$$\text{Cell concentration (cells/mL)} = \text{average number of cells per square} \times \text{dilution factor} \times 10^4$$

2.5 Preparation of single cells

2.5.1 Preparation of aortic, heart, lung, skin, skeletal muscle and liver cells

Tissues were harvested from mice immediately after euthanasia to prepare single cell disaggregates of aorta (or aortic adventitia), heart, lung, skin, skeletal muscle and liver. Aortas were dissected out intact, along their entire length from aortic valve to iliac bifurcation and flushed extensively with PBS, before microscopic dissection of surrounding perivascular fat and, where indicated, careful separation of the adventitia. Once all tissues were harvested, each was cut into smaller pieces with sterile surgical blades and digested with 50 µg/ml of Liberase™ (50 µg/ml) (Roche Applied Science, Mannheim, Germany) for up to 1.5 h at 37°C (vortexing every 30 min). Tissue digests were then passed through a 40 µm nylon mesh (Greiner Bio-One, Interpath Services, Victoria, AU) and neutralised in IMDM supplemented with 10% FBS and 1% antimycotic/antibiotic solution and washed before performing cell counts.

2.5.2 Preparation of brain cells

The brain was carefully removed from cephalic mesenchyme and meninges, dissected out, minced, and incubated in Liberase™ (50 µg/ml) for 2 h and passed through a 40 µm nylon mesh (507). Digests of brain were resuspended in 37% isotonic Percoll and underlaid with 70% isotonic Percoll (GE Healthcare) and centrifuged at room temperature, 600 *g* for 25 min. Cells in the interface were collected and cell counts were performed.

2.5.3 Preparation of blood

Blood was collected by cardiac puncture in EDTA coated blood collection tubes. For any blood sample that did not reach 1 mL, PBS was added to make up the remaining volume. Erythrocytes were lysed in a 1:10 dilution of ammonium chloride (StemCell Technologies) at 4°C for 10 min following which the

resulting suspension was washed and resuspended in IMDM supplemented with 10% FBS and 1% antimycotic/antibiotic solution, before performing total white cell counts.

2.5.4 Preparation of bone marrow (BM) and spleen cells

Following sacrifice, BM cell suspensions were prepared by snipping femurs and tibias from mice at each end, then centrifuging at 800 *g* for 2 min at 22°C using a perforated (made with a 19 G needle) 2 mL microcentrifuge, containing 500 µL of PBS. The flow-through was collected and resuspended in an additional 500 µL of PBS to make a total volume of 1 mL. This BM cell suspension was incubated in a 1:10 dilution of ammonium chloride at 4°C for 8 min, following which the cells were washed and resuspended in IMDM supplemented with 10% FBS and 1% antimycotic/antibiotic solution, before performing cell counts. Single cell suspensions from spleens were prepared by forceful filtering of the intact spleen against a 40 µm cell strainer, following which the cells were processed as per BM cells.

2.5.5 Preparation of peritoneal macrophages

Macrophages were harvested from the peritoneal cavity of mice by injecting 5 mL of PBS intraperitoneally (508). The mouse abdomen was massaged for 30 s, after which the peritoneal cavity was opened slightly and up to 5 mL of peritoneal fluid was collected using sterile syringes. Peritoneal isolates were centrifuged at 400 *g* for 5 min at 22°C. The cells were resuspended in IMDM supplemented with 10% FBS and 1% antimycotic/antibiotic solution, before performing total cell counts.

2.5.6 Preparation of embryonic tissues

To obtain single cell suspension from embryos, pregnant females were euthanised by CO₂ exposure. Each embryo was carefully dissected from the uterus under light microscope (Carl Zeiss, Dissection Light Microscope); tissues harvested were YS (E8.5-E15.5), whole embryo (E8.5-E9.5), AGM (E10.5-E15.5), liver (E11.5-E15.5), head (E10.5-E15.5), lung (E11.5-E15.5), heart (10.5-E15.5) and skin (E15.5) (509). Tissues were washed with PBS and placed in Hanks balanced-salt solution (HBSS,

Sigma) supplemented with 2% FBS and digested with 1 mg/mL of collagenase II for 15 min at 37°C. Tissue suspensions were filtered using Polystyrene Round-Bottom Tubes with Cell-strainer cap strainers (*In vitro* Technologies). They were then centrifuged at 400 *g* for 5 min and resuspended in IMDM supplemented with 10% FBS and 1% antimycotic/antibiotic solution, before performing total cell counts.

2.6 *In vivo* specific techniques

2.6.1 Adventitial transfer of cells in carotid atherosclerosis

The *in vivo* fate of adventitial progenitor cells was studied in atherosclerotic arteries for Sca-1⁺CD45⁺ progenitor cells in Chapters 3. Aortic adventitial cells from 12 w GFP donor mice were freshly isolated and separated by magnetic activated cell supporting into the four Sca-1/CD45 fractions, as described previously (263). Cells were resuspended in MatrigelTM (5×10⁵ in 20 µL) and injected (27 G needle) into the adventitia of the left carotid artery of recipient 8 w *Apoe*^{-/-} mice. These mice then commenced an atherogenic HCD which was maintained until euthanasia 16 w later. A control group of mice received adventitial injection of the equivalent volume of MatrigelTM without cells. At study completion, peripheral blood and both carotid arteries were harvested, fixed and processed to investigate the fate of donor GFP⁺ cells by immunofluorescence staining and confocal microscopy.

2.6.2 *In vivo* angiotensin II (AngII) studies

Installation of osmotic minipumps was performed to administer PBS or AngII to *Cx3cr1*^{CreER-YFP} x *Rosa*^{tdTom} and *Flt3*^{Cre} x *Rosa*^{mT/mG} mice aged 10-14 w (510) in Chapter 4. Osmotic minipumps were positioned through a small lateral incision (~7 mm) between the shoulder blades. A small skin pocket was created at the incision via blunt dissection, into which the minipump containing AngII (0.7 mg/kg/d), or equivalent volume of PBS, was inserted. The wound was closed with wound clips. Mice were euthanised after 12h, 48h or 168h of exposure to AngII/PBS. BrdU was administered *i.p.* as indicated, either 24 h before euthanasia, or for the purpose of pulse-chase studies at a fixed time of 23 h from commencement of AngII/PBS.

2.6.3 Clodronate monocyte depletion

To deplete circulating monocytes, clodronate (clodronateliposomes.org, Vrije Universiteit, Netherlands) was injected *i.p.* into 12 w C57BL/6 mice daily for two days, while vehicle (PBS) control liposomes were used as control (Chapter 4 and 6).

2.6.4 Peritoneal transfer assay

Peritoneal transfer assays were used in Chapter 4. Progenitor cells were isolated and checked for purity from CFU-M grown from 12 w ubiquitous GFP aortas. 1×10^4 GFP⁺ progenitors were injected into the peritoneal cavity of 12 w C57BL/6 mice, which then received daily *i.p.* injections of either PBS, 0.7 mg/kg AngII or 0.05 µg M-CSF. Mice were euthanised after 72 h and cells harvested from the peritoneal cavity by injecting 5 mL of PBS *i.p.*. Following gentle massage of the peritoneum, the peritoneal fluid was collected and centrifuged (400 *g*, 5 min) to obtain single cell suspensions for immunostaining and analysis of GFP⁺ cell fate by flow cytometry.

2.6.5 Hindlimb ischaemia

Hindlimb ischaemia surgeries were performed in Chapters 3, 4 and 5 using a well-established model (511). For the purpose of adoptive cell transfer studies, we used male and female adult C57BL/6 mice as recipients and ubiquitous GFP mice as donors. Surgeries were performed under general anaesthesia. To induce hindlimb ischaemia, the proximal portion of the left femoral artery and the distal saphenous artery were both sutures ligated, as well as the popliteal artery and all branches in between, and an atherectomy performed to remove the intermediate segment of vessel. Blood flow through the ischaemic (left) and non-ischaemic (right) limbs was immediately determined and compared to pre-surgical baseline using laser Doppler perfusion imaging (moorLD12-IR, Moor Instruments, Devon, UK). Once adequate ischaemia was confirmed, the surgical site was closed. In Chapter 3, freshly sorted aortic Sca-1⁺CD45⁺, Sca-1⁺CD45⁻, Sca-1⁻CD45⁺ and Sca-1⁻CD45⁻ cells from 12 w GFP donor mice (1.5×10^4 in 60 µL MatrigelTM) were administered as a three intramuscular injection (27 G needle) into the gastrocnemius of the ischaemic limb. In Chapter 4, pure EndoMac progenitor cells were used from CFU-M grown from either adult (12 w) aortic cells or E9.5 YS from GFP donor mice (1.5×10^4 in 60 µL MatrigelTM). In Chapter 5, we used skeletal muscle EndoMac progenitor cells harvested from CFU-M grown from adult GFP gastrocnemius muscle (1.5×10^4 in 60 µL MatrigelTM). In each chapter, a control group received injections of MatrigelTM without cells (60 µL). Mice were recovered, and then

Laser Doppler perfusion scans were repeated at days 7 and 14 under general anaesthesia to determine the perfusion ratio between the ischaemic and non-ischaemic limbs. Euthanasia was performed after the final scan on day 14. Peripheral blood was taken by cardiac puncture, while muscle from both limbs was harvested and processed for the purpose of 1) haematopoietic colony forming unit (CFU) assays, 2) flow cytometry or 3) immunofluorescence staining and confocal microscopy, to detect engraftment of donor GFP⁺ cells, their cellular fate and vasculogenic capacity.

Additional hindlimb ischaemia experiments were performed in Chapter 5 without cell transfer, to study the prevalence, cell cycle status and developmental origins of EndoMac progenitor cells, macrophages (and their subtypes) and endothelial cells in gastrocnemius muscle at different time-points after hindlimb ischaemia. These experiments used adult *Flt3^{Cre}* x *Rosa^{mT/mG}* or E8.5 TAM-induced *Csf1r^{MerCreMer}* x *Rosa^{mT/mG}* mice. Mice were euthanised at day 1, 3, 7 or 14 and different tissues (muscle from both limbs, brain, blood, BM) were harvested and processed for CFU assays, flow cytometry or immunofluorescence staining and confocal microscopy. BrdU was administered *i.p.* either 24 h before euthanasia, or for the purpose of pulse-chase studies at a fixed time of 23 h after surgery with mice then sacrificed at 24 h, 168 h or 336 h from the time of surgery.

2.6.6 Wound healing

Chapter 6 involved a wound healing model. Briefly, 12 w male C57BL/6 and *Csf1r^{MerCreMer}* x *Rosa^{mT/mG}* mice were used in three separate wound healing experiments, using a model previously established by Ass/Prof Christina Bursil (512). Mice were anaesthetised with isoflurane and their upper dorsal surface was removed of hair using clippers and depilatory cream. Two full thickness excision wounds that included the panniculus carnosus were created on either side of the dorsal midline. A 0.5mm thick silicone splint (Life Technologies, CA, USA) was placed to prevent healing via contraction using an adhesive and secured with interrupted 6-0 PROLENETM sutures (Ethicon LLC, San Lorenzo, Puerto Rico). All wounds were covered with OpsiteTM dressings (Smith & Nephew, London, UK). Mice were recovered from anaesthesia and given 0.1mg of Buprenorphine via subcutaneous injection on the day

of surgery and 24 h later for analgesia. For studies involving C57BL/6 mice, skin CFU-M from 12 w GFP donor mice (1.5×10^4 in 60 μ L MatrigelTM), was administered into the open wound, along with a MatrigelTM, cell-free control. Wound sizes were measured daily for 14 days, using a standard Vernier calliper in two perpendicular dimensions. Blood perfusion of each wound was analysed under anaesthesia by Laser Doppler Imaging (moorLD12-IR, Moor Instruments) on days 0, 3, 7 and 10. On day 10 mice were euthanised by CO₂ asphyxiation and the skin from each wound site harvested. Half of the wound was used to prepare skin digests for flow cytometry, and the remainder fixed in 30% sucrose and 10% formalin for embedding in optimal cutting temperature (O.C.T) compound (Sakura Finetek USA, Inc., Torrance, CA, USA) and later staining with immunofluorescence confocal microscopy. *Csf1r*^{MerCreMer} x *Rosa*^{mT/mG} mice received BrdU (1-2mg) either 24 h before euthanasia.

2.6.7 Skin melanoma

A well-established murine model (513) was used to study the origins of macrophages and endothelial cells in melanoma, through the lens of YS-derived skin EndoMac progenitor cells in Chapter 6. Briefly, B16-F10 melanoma cells (provided by Prof Claudine Bonder, University of South Australia) (5×10^5 cells in 100 μ L MatrigelTM) were subcutaneously injected into the flanks of either male or female 8-12 w *Flt3*^{Cre} x *Rosa*^{mT/mG} mice, C57BL/6 or E8.5 TAM-induced *Csf1r*^{MerCreMer} x *Rosa*^{mT/mG} mice. Tumour volume (in mm³) was measured three times weekly with a Vernier calliper and calculated by the following formula: volume = (width)² $\times$ length/2. Mice were sacrificed between days 7 and 14 post tumour cell inoculation, or when tumours reached 1cm³ or between days 7-14 post-implantation mice were sacrificed. Tumours were carefully dissected out and processed for the purpose of flow cytometry or immunofluorescence staining and confocal microscopy.

For studies involving *Flt3*^{Cre} x *Rosa*^{mT/mG} mice, mice were also treated every two days with *i.p.* injections of PBS or clodronate lysosomes (20 mg/kg/day) from day 0 to 12, to study the effect of monocyte depletion on tumour growth. For studies involving cell transfer, EndoMac progenitor cells were isolated and purified from CFU-M grown from skin digests of GFP donor mice and administered subcutaneously

in a mixture with B16-F10 melanoma cells to C57BL/6 recipients (1.5×10^4 progenitor cells per injection). In the experiments involving *Flt3*^{Cre} x *Rosa*^{mT/mG} and *Csf1r*^{MerCreMer} x *Rosa*^{mT/mG} mice, BrdU was administered before euthanasia and tissue harvest, as described in Chapter 6.

2.7 *In vitro* methods

2.7.1 Cell lines culture

2.7.1.1 Cancer cell line (B16-F10)

B16-F10 mouse melanoma cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and antibiotics with $2-4 \times 10^5$ cells/mL, 5% CO₂, 37°C in 75 cm² CellBIND® Surface Cell Culture flasks. All cells were cultured in a humidified atmosphere of 5% CO₂ at 37°C.

2.7.1.2 Human cell lines (HUVEC)

HUVEC (ATCC, Manassas, VA) were maintained using cell line media with $2-4 \times 10^5$ cells/mL, 5% CO₂, 37°C in 75 cm² CellBIND® Surface Cell Culture flasks. Medium was replaced every 2 to 3 days. Cells were passaged at 80% confluency.

2.7.2 Primary cultures

2.7.2.1 Preparation of colony-forming unit (CFU) assays

Clonogenicity was assessed by performing primary CFU cultures. Briefly, 2×10^5 freshly isolated cells from different tissues were plated in methylcellulose (#3434, StemCell Technologies, Vancouver, Canada), in duplicate. Where specified, cultures were performed in the presence of absence of Fractalkine (CX₃CL) (100 nM; #F2302, Sigma-Aldrich Inc., MO, USA), M-CSF (50 nM; #315-02-500, PeproTech, NJ, USA), AngII (1000 nM; Sigma-Aldrich, St Louis, USA), Enalapril (100 nM; #E6888, Sigma-Aldrich, Inc., St Louis, MO, USA), Losartan (100 nM; #61188, Sigma-Aldrich, Inc., St Louis, MO, USA) or PD123,319 (50 nM; #PD186, Sigma Aldrich Inc., MO, USA h), which were replenished every three days. After 14 days, CFUs were counted under light microscopy and classified by colony subtype (e.g. CFU-M) and size, using published morphological criteria (262, 263). By definition, CFUs comprised a minimum of 30 cells.

Self-renewal CFU assays were performed in one of two ways. For bulk self-renewal, the methylcellulose of primary cultures was liquefied to retrieve all the cells contained in the well. After pipetting up and down and vortexing to obtain a single cell suspension, cells were re-cultured in methylcellulose at a density of 2×10^5 per well, for another 14 days before colony counting. Further passages (tertiary, quaternary, quinary) were conducted in the same way, where specified. Single colony self-renewal assays were performed by individually isolating primary CFU-M from methylcellulose under light microscopy with a 10 μ L pipette. The cells contained in each individual CFU-M were disaggregated by vortexing, and re-plated in methylcellulose in 96-well plates, with each well seeded with cells from only one CFU-M. Wells were inspected the following day (day 1) to quantify their content of single cells and rule out cell couplets or clumps. They were then imaged daily until day 14 to document and quantify the emergence of new secondary colonies from individual cells.

2.7.3 *In vitro* culture-based differentiation.

Freshly isolated C57BL/6 aortic adventitial cells were fractionated into different Sca-1/CD45 subpopulations which were seeded in collagen-coated glass chamber slides (Nalge Nunc International, Naperville, IL) at 2×10^4 cells in 200 μ L in one of four different media conditions: (1) RPMI-1640 (Sigma-Aldrich) supplemented with 10% FBS; (2) RPMI-10 with 20 ng/mL M-CSF (PeproTech Inc., Rocky Hill, NJ); (3) RPMI-10 with 20 ng/mL IL-4 and 20 ng/mL GM-CSF; (4) endothelial growth medium (Lonza, Walkersville, MD) supplemented with 10% FBS (EGM-10). Medium was changed every three days until day 10, at which time the wells were fixed with cold methanol and permeated with 0.1% triton before immunostaining (as described above) to detect endothelial (Isolectin, CD31) markers, with Hoechst nuclear counter-staining.

2.7.4 Aortic and skin rings outgrowth model

Aortic and skin ring assays were used to study the involvement of aortic EndoMac progenitor cells in adventitial and skin angiogenesis in Chapter 4 and 6. Aortic explants were carefully flushed to remove

blood and dissected free of surrounding adipose. For studies from *Csf1r*^{MerCreMer} x *Rosa*^{mT/mG} and *Cx3cr1*^{CreER-YFP} x *Rosa*^{tdTom} mice which required intact adventitia, aortas were used with all three mural layers intact, while skin was also used intact. For studies in which GFP⁺ aortic EndoMac progenitors were added, C57BL/6 aortas were dissected to completely remove the adventitia. Rings of 1 mm thickness were then cut from thoracic aorta and skin, embedded in MatrigelTM and overlaid with EGM containing 5% FBS and 20 ng/mL vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) (EGM-10, CC-4542, Lonza, Switzerland). Medium was changed after two days and sprouts were imaged on day 5 for quantification of sprout length using Image J software (Versions available in 2018-2021, NIH, MD, USA). The cellular content of adventitial and skin sprouts was then retrieved by scraping the Matrigel and then digested in type IV collagenase (0.2 mg/mL in PBS) (Sigma-Aldrich Inc., St Louis, MO, USA) for 45 min. After neutralisation with Iscove's Modified Dulbecco's Medium (Sigma-Aldrich) supplemented with 10% FBS (Cell Sera, NSW, Australia), aortic and skin ring suspensions were filtered using Polystyrene Round-Bottom Tubes with Cell-strainer cap strainers (*In vitro* Technologies). Single cell suspensions were then immunostained for flow cytometry, as described below.

2.7.5 MatrigelTM-based tube formation

FACS-sorted cells from aortic digests (Chapter 3 and 4) and progenitor cells isolated from day 14 CFU-M (Chapter 4-6) were used for *in vitro* MatrigelTM-based angiogenesis assays. Single cell suspensions were prepared in Endothelial Cell Growth Medium (EGM-10, CC-4542, Lonza, Switzerland) and plated on growth factor-reduced MatrigelTM (BD Biosciences, San Jose, CA) at 2x10⁴ cells per cm² in 80 µL of media, to study their intrinsic capacity to form vascular-like cords. Cultures were inspected and photographed under light microscopy daily until day 7 to detect the presence of cords, which were identified as cellular extensions linking cell masses or branch points. Images were captured at x10 objective magnification in five different regions (upper centre, centre, lower centre, left centre, right centre) to encompass the entire well. Total tube length and number of branching points were quantified from each image using Image J software. The cellular content of these cultures was retrieved by scraping

the Matrigel and then digested in type IV collagenase (0.2 mg/mL in PBS) (Sigma-Aldrich Inc., St Louis, MO, USA) for 45 min. After neutralisation with Iscove's Modified Dulbecco's Medium (Sigma-Aldrich) supplemented with 10% FBS (Cell Sera, NSW, Australia), single cell suspensions were then immunostained for flow cytometry, as published.

In some experiments in Chapter 3 and 6, clodronate or PBS control liposomes (clodronateliposomes.org, Vrije Universiteit, Netherlands) were added to wells at a concentration of 0.45 mg/mL alternate daily from day 3 until day 7. In Chapter 4, 50 µg/mL of Dil-oxLDL and acLDL were added to wells on day 6. In another experiment in Chapter 6, supernatant was collected from cultured cancer B16-F10 cells and added to wells with skin CFU-Ms growing in MatrigelTM.

2.8 Staining

2.8.1 Immunofluorescence and confocal microscopy

Intact tissue samples were harvested from mice and placed in 30% sucrose overnight, followed by 10% formalin fixation for 24 hours and then embedded in Optimal Cutting Temperature (O.C.T.) compound (Sakura Finetek USA, Inc., Torrance, CA, USA). Five μm thick frozen sections were cut, fixed and then placed in methanol with 0.3% H_2O_2 before heat-mediated citrate antigen retrieval. Blocking was performed with either 10% normal goat or donkey serum, or 3% normal horse serum. Primary antibodies were left to incubate overnight before application of secondary antibodies that are detailed in **Table 2-4**. Nuclei were counterstained with DAPI. Microscopy was performed with a Leica TCS SP8X/MP laser scanning confocal microscope system (Leica Microsystems Pty Ltd, Macquarie Park, Australia).

2.8.2 Method for quantifying capillary and αSMA density

Fresh frozen 5 μm thick frozen sections of gastrocnemius muscle from ischaemic and non-ischaemic hindlimbs of mice at 14 days post-ischaemia induction were stained to detect the number of CD31^+ capillaries and αSMA^+ arterioles per myocyte (laminin). All sections were imaged at 10x magnification and analysed with Image-Pro Premier software and ImageJ to determine capillary and arteriolar density per myocyte.

2.8.3 Wright-Giemsa staining

FACS sorted cells were resuspended in 200 μl of IMDM containing 2% FBS. Cells were cytopun onto cytoslides (Thermo Shandon) using a cytopsin centrifuge (Thermo Shandon cytopsin 4, 300 g , 5 min). They were then stained with Wright-Giemsa reagent (Sigma Aldrich) followed by imaging under light microscopy (Carl Zeiss, Axio light microscope). 60-100 cells were randomly photographed per donor mouse and analysed.

Table 2-4. Reagents for immunohistochemistry.

Antibody	Catalog # Manufacturer
<u>Controls</u>	
<i>Goat IgG, polyclonal</i>	#AB-108-C, R&D Systems, MN, USA
<i>Rat IgG, polyclonal</i>	#NBP1-71666, Novus Biologicals, USA
<i>Rabbit IgG, polyclonal</i>	#ab172730, Abcam, Cambridge, UK
<i>Mouse IgG₁, monoclonal</i>	#MAB002R, R&D Systems, MN, USA
<u>Primary antibodies</u>	
<i>Chicken anti-GFP</i>	#ab13970, Abcam, Cambridge, UK
<i>Goat anti-mouse Sca-1</i>	#AF1226, R&D Systems, MN, USA
<i>Rabbit anti-CD31 (polyclonal)</i>	#ab28364, Abcam, Cambridge, UK
<i>Rabbit anti-Ki67</i>	#ab16667, Abcam, Cambridge, UK
<i>Rat anti-mouse CD68</i>	#MCA1957GA, Bio-Rad, Laboratories, CA, USA
<i>Rat anti-mouse CD45</i>	#550539, BD Pharmingen™, NJ, USA
<i>Rat anti-mouse MOMA-2</i>	#ab33451, Abcam, Cambridge, MA
<i>Rabbit anti-human vWF</i>	#GA52761-2, Dako, CA, USA
<i>Rabbit anti-mouse Lyve-1</i>	#ab218535, Abcam, Cambridge, UK
<i>Griffonia (Bandeiraea) Simplicifolia Lectin I</i>	#B-1205, Vector Labs, Burlingame, CA
<i>Isolectin B4 Biotinylated</i>	
<i>Rat anti-mouse CD31</i>	#DIA-310 Dianova, Hamburg, Germany
<i>Rat anti-mouse Laminin $\alpha 1/\beta 1$</i>	#MAB1905, Merck Millipore NJ, USA
<i>Mouse anti-Actin, α-Smooth Muscle (FITC conjugate)</i>	#F3777-2ML, Merck, NJ, USA
<i>Goat anti-mouse c-kit</i>	#AF1356, R&D Systems, MN, USA
<i>Rabbit anti-RFP (polyclonal)</i>	#600-401-379, Rockland Immunochemicals, PA, USA
<u>Secondary antibodies</u>	
<i>Goat anti-Chicken AF488</i>	#A32931, Life Technologies, CA, USA
<i>Goat anti-Rat AF488, AF555, AF594</i>	#A11006, #A21434, #A11007, Life Technologies, CA, USA
<i>Goat anti-Rabbit AF488, AF594, AF647</i>	#A32731, #A32740, #A32733, Life Technologies, CA, USA
<i>Donkey anti-Goat AF488, AF647</i>	#A32814, #A32849, Life Technologies, CA, USA
<i>Donkey anti-Rat AF488</i>	#A21208, Life Technologies, CA, USA
<i>Donkey anti-Rabbit AF647</i>	#A32795 Life Technologies, CA, USA
<i>Streptavidin AF647</i>	#S32357 Life Technologies, CA, USA
<u>Other</u>	
<i>Wright-Giemsa Stain</i>	#WG16, Sigma-Aldrich Inc., MO, USA
<i>Donkey serum</i>	#D9663, Sigma-Aldrich Inc., MO, USA
<i>Goat serum</i>	#G9023, Sigma-Aldrich Inc., MO, USA
<i>Horse serum</i>	#H1138, Sigma-Aldrich Inc., MO, USA
<i>DAPI</i>	#P3935, Life Technologies, CA, USA

2.9 Protein analysis

2.9.1 Flow cytometry

Single cell suspensions from tissue digests or cultured-derived CFU-M were resuspended in aliquots of $\leq 10 \times 10^6$ cells per 1ml of PBS / 5% FBS / 0.2% sodium azide (#S2002, Sigma-Aldrich). 1 μ l of fixable viability stain 700 (BD Horizon, San Diego CA, USA) was added to each sample and incubated for 15 min on ice. After washing, samples were blocked for 5 min on ice with purified rat anti-mouse CD16/CD32 (BD Pharmingen, San Diego CA, USA). Cells were then incubated for 30 min with primary antibodies and subsequently with secondary antibodies when required (**Table 2-5**). Samples were then washed and fixed in FACS fix (PBS containing 2% glucose (w/v) (#G8270, Sigma-Aldrich) and 1% formaldehyde (v/v) (#F8775, Sigma-Aldrich, 0.02% sodium azide) for analysis with either BD LSRFortessa™ X-20 or BD FACs Diva Software (BD Biosciences). Data files were analysed using FlowJO version X software (Tree Star Inc., Ashland, OR, USA). For the purpose of analysis, gating was performed based on FSC-A (to exclude cell debris), single cells and viability stain selected cells, and used FMO controls to determine the positive percentage expression of different surface markers. For cell sorting, a FACSFusion cell sorter (BD Bioscience) was used, and the sorted cells were used for primary cultures.

For cell cycle analysis, BD Pharmingen BrdU Flow Kit was used. After cells were stained with surface antigens cells were fixed and permeabilised with BD Cytfix Cytoperm Buffer and BD Cytoperm Permeabilization Buffer Plus as per manufacturer's instructions. Cells were then treated with DNase to expose incorporated BrdU and BrdU was stained. 7-AAD was added 15 min prior to data acquisition.

Table 2-5. Reagents for flow cytometry.

<i>Reagents</i>	<i>Catalog # Manufacturer</i>
<u><i>Conjugated primary antibodies</i></u>	
<i>AF488 Mouse anti-mouse CD140β (PDFGRβ)</i>	#558427, BD Phosflow™, CA, USA
<i>AF488 Mouse anti-mouse Nanog</i>	#560278, BD Pharmingen™, NJ, USA
<i>AF488 Mouse anti-mouse Tie2</i>	#560053, BD Phosflow™, CA, USA
<i>AF647 Mouse anti-mouse CX3CR1</i>	#149004, BioLegend, CA, USA
<i>AF647 Mouse anti-mouse Sox2</i>	# 656108, BioLegend, CA, USA
<i>AF647 Rat anti-mouse CD105</i>	#562761, BD Pharmingen™, NJ, USA
<i>AF647 Rat anti-mouse CD150</i>	#562647, BD Pharmingen™, NJ, USA
<i>AF647 Rat anti-mouse CD206 (MMR)</i>	#141712 BioLegend, CA, USA
<i>APC Anti-BrdU</i>	#555627, BD Pharmingen™, NJ, USA
<i>APC Goat anti-human/mouse Brachyury</i>	#IC2085A, R&D Systems, MN, USA
<i>APC Rat anti-mouse CD115</i>	#17-1152-82, eBioscience, CA, USA
<i>APC Rat Anti-mouse CD36</i>	#562744, BD Pharmingen™, NJ, USA
<i>APC Rat anti-mouse CD93 (AA4.1)</i>	#136509, BioLegend, CA, USA
<i>APC Rat anti-mouse MerTK</i>	#FAB5912A, R&D System, MN, USA
<i>APC-Cy™7 Rat anti-CD11b</i>	#557657, BD Pharmingen™, NJ, USA
<i>BUV395 Mouse anti-mouse CD45.2</i>	#564616, BD Horizon™, CA, USA
<i>BUV395 Rat anti-mouse CD41</i>	#564056, BD Horizon™, CA, USA
<i>BUV737 Rat anti-mouse CD24</i>	#565308, BD Horizon™, CA, USA
<i>BV421 Mouse anti-mouse CD64</i>	#139309, BioLegend, CA, USA
<i>BV421 Rat anti-mouse CD115 (CSF1R)</i>	#135513, BioLegend, CA, USA
<i>BV421 Rat anti-mouse CD31</i>	#563356, BD Horizon™, CA, USA
<i>BV421 Rat anti-mouse CD34</i>	#562608, BD Horizon™, CA, USA
<i>BV421 Rat anti-mouse CD5</i>	#562739, BD Horizon™, CA, USA
<i>BV421 Rat anti-mouse Ly-6A/E</i>	#562729, BD Horizon™, CA, USA
<i>BV510 Mouse anti-mouse Oct4</i>	#563524, BD Horizon™, CA, USA
<i>BV510 Rat anti-mouse Gr-1 (Ly6G/Ly6C)</i>	#108438, BioLegend, CA, USA
<i>BV605 Rat anti-mouse CD73</i>	#127215, BioLegend, CA, USA
<i>BV605 Rat anti-mouse Flt-1 (VEGFR2)</i>	#745220, BD OptiBuild™, CA, USA
<i>BV605 Rat anti-mouse Ly-6C</i>	#563011, BD Pharmingen™, NJ, USA
<i>BV650 Rat anti-mouse CD146</i>	#740636, BD OptiBuild™, CA, USA
<i>BV650 Rat anti-mouse CD86</i>	#564200, BD Horizon™, CA, USA
<i>BV785 Hamster anti-mouse CD103</i>	#121439, BioLegend, CA, USA
<i>BV785 Mouse anti-mouse CX3CR1</i>	#149029, BioLegend, CA, USA
<i>BV785 Rat anti-mouse F4/80</i>	#123141, BioLegend, CA, USA
<i>BV785 Rat Anti-mouse Ly-6A/E</i>	#563991, BD Horizon™, CA, USA

eFluoro®660 Rat anti-mouse Lyve1	#50-0443-82, ThermoFisher
FITC Anti-mouse F4/80	#123108, BioLegend, CA, USA
FITC Hamster anti-rat CD29	#561796, BD Pharmingen™, NJ, USA
FITC Rat anti-mouse CCR2	#150608, BioLegend, CA, USA
FITC Rat anti-mouse CD16/CD32	#553149, BD Pharmingen™, NJ, USA
FITC Rat anti-mouse F4/80	#123108, BioLegend, CA, USA
PE Mouse anti-mouse CD36	#562702, BD Pharmingen™, NJ, USA
PE Mouse anti-mouse c-Myc	#NB600-302PE, Novus Biologicals, USA
PE Rat anti-mouse CD106	#105713, BD Biosciences, CA, USA
PE Rat anti-mouse CD115 (CSF1R)	#12115281, eBioscience™, CA, USA
PE Rat anti-mouse CD90.2	#105308, BioLegend, CA, USA
PE Rat anti-mouse LDLR	#FAB2255P, R&D Systems, MN, USA
Pe/Cy7 Anti-mouse CD206 (MMR)	#141720, BioLegend, CA, USA
Pe/Cy7 Anti-mouse F4/80	#123141, BioLegend, CA, USA
Pe/Cy7 Rat anti-mouse CD117 (c-Kit)	#558163, BD Pharmingen™, NJ, USA
Pe/Cy7 Rat anti-mouse CD144 (CDH5)	#138016, BioLegend, CA, USA
Pe/Cy7 Rat anti-mouse CD43	#562866, Rat Pharmingen™, NJ, USA
Pe/Cy7 Rat anti-mouse CD44	#560569, BD Pharmingen™, NJ, USA
PE-CF594 Hamster anti-mouse CD11c	#562454, BD Pharmingen™, NJ, USA
PE-CF594 Rat anti-mouse CD117 (c-Kit)	#562417, BD Horizon™, CA, USA
PE-CF594 Rat anti-mouse CD140a (PDGFRα)	#562775, BD Biosciences, CA, USA
PerCp-Cy™5.5 Mouse anti-mouse NK-1.1	#55114, BD Pharmingen™, NJ, USA
PerCp-Cy™5.5 Rat anti-mouse CD19	#551001, BD Pharmingen™, NJ, USA
PerCp-Cy™5.5 Rat anti-mouse CD3	#560527, BD Pharmingen™, NJ, USA
PerCp-Cy™5.5 Rat anti-mouse CD31	#562861, BD Pharmingen™, NJ, USA
PerCp-Cy™5.5 Rat anti-mouse CD45R/B220	#552771, BD Pharmingen™, NJ, USA
PerCp-Cy™5.5 Rat anti-mouse Ly-6G	#560602, BD Pharmingen™, NJ, USA
PerCp-Cy™5.5 Rat anti-mouse Ter-119	#560512, BD Pharmingen™, NJ, USA
V500 Rat anti-mouse I-A/I-E (MHC-II)	#562366, BD Horizon™, CA, USA
<u>Non conjugated primary antibodies</u>	
Anti-ACE	#PA578711, ThermoFisher Scientific, MA, USA
Rabbit anti-human/mouse ATR1	#AAR-011, Alomone labs, JBP, Israel
Rabbit anti-human/mouse ATR2	#AAR-011, Alomone labs, JBP, Israel
<u>Conjugated secondary antibodies</u>	
Alexa-Fluor™ 488 Goat anti-rabbit IgG	#A11034, Life Technologies™, CA, USA
Alexa-Fluor™ 594 Goat anti-mouse IgG2a	#A21135, Life Technologies™, CA, USA
<u>Others</u>	
7-AAD	#559925, BD Pharmingen™, NJ, USA
BD™ Comp beads anti-mouse Ig, κ	#51-90-9001229, BD Biosciences, CA, USA

<i>BDTM Comp beads anti-rat and anti-hamster Ig, κ</i>	#51-90-9000949, BD Biosciences, CA, USA
<i>BDTM Comp beads negative control</i>	#51-90-9001291, BD Biosciences, CA, USA
<i>Brilliant Stain Buffer</i>	#566349, BD Biosciences, CA, USA
<i>Cytofix</i>	#554655, BD Pharmingen TM , NJ, USA
<i>Di oxLDL</i>	#L34358, Life technologies, CA, USA
<i>Di acLDL</i>	#L3484, Life technologies, CA, USA
<i>Fixable Viability Stain 700</i>	#5649997, BD Horizon TM , CA, USA
<i><u>Mitox Red Mitochondrial</u></i>	#M36008, ThermoFisher Scientific, MA, USA
<i><u>MitoTrackerTM Deep Red FM</u></i>	# M22426, ThermoFisher Scientific, MA, USA
<i><u>MitoTrackerTM Green FM</u></i>	#M7514, ThermoFisher Scientific, MA, USA
<i>Propidium Iodide (PI)</i>	#P4170, Sigma-Aldrich Inc., MO, USA
<i>Purified Rat Anti-mouse CD16/CD32 (Mouse BD Fc Block)</i>	#553142, BD Pharmingen TM , NJ, USA
<i>Stain Buffer (FBS)</i>	#554656, BD Pharmingen TM , NJ, USA

2.10 Molecular biology techniques

2.10.1 RNA microarray

For the purpose of RNA isolation for microarray and RT-qPCR, aortas from six 12 w C57BL/6 mice per donor experiment were pooled to allow MACS-based separation of the Sca-1⁺CD45⁺, Sca-1⁻CD45⁺ and Sca-1⁺CD45⁻ adventitial fractions. Total RNA was isolated and purified immediately using an RNeasy® Plus Mini Kit (Qiagen, Germany), as per manufacturer's instructions.

RNA microarray was performed to compare the transcriptional profiles of adventitial Sca-1⁺CD45⁺ and Sca-1⁻CD45⁺ cells from three donor experiments. The quality of all total RNA samples was assessed according to manufacturer's instructions for the Agilent 2100 Bioanalyser (Santa Clara, CA, USA). High-quality samples were labelled according to instructions for the Illumina Direct Hyb labelling method (San Diego, CA, USA). Briefly, 100 ng of total RNA was reverse transcribed with T7 Oligo d(T) to create second strand cDNA in concordance with the protocol for the Illumina TotalPrep RNA Amplification kit (Ambion/Life Technologies, Foster City, CA). Subsequently, the products were column purified (Ambion/Life Technologies) and then *in vitro* transcribed to generate biotin-labelled cRNA. cRNA products were column purified and hybridised onto Illumina MouseWG-6 Beadchips for 16h at 58°C. Following hybridisation, the arrays were washed, stained with streptavidin-cy3 conjugate, and scanned in an Illumina BeadArray Reader. All quality assessment parameters were determined to be within normal ranges before proceeding to the final data reduction.

The log-2 of the gene expression data was normalised using the non-linear normalisation fastlo. To assess differential expression between groups of interest, the LIMMA package in R was utilised to implement the empirical Bayes method of Smyth to shrink the gene-wise sample variances towards a common value. The false discovery rate (FD), which is the expected proportion of false discoveries amongst the rejected hypotheses, was also calculated.

Pathway analysis by Ingenuity IPA (<http://www.ingenuity.com>) was conducted to identify significantly enriched canonical pathways, functional groups or biological processes, with focus for this study on genes involved in angiogenesis or vasculogenesis. Selected angiogenesis/vasculogenesis-related genes that were differentially regulated between Sca-1⁺CD45⁺ and Sca-1⁻CD45⁺ cells and had an FDR <0.05 from microarray were validated using hydrolysis (Taqman) probe-based RT-qPCR (MIQE reference - <http://miqe.gene-quantification.info/>). All assays and reagents were ordered from Life Technologies (Foster City, CA, USA). Cq values were calculated using the Viia7 software for individual qPCR run. The data were imported and analysed in the Expression Suite Software (Life Technologies) and the reported delta-delta Ct was plotted as log-fold difference.

2.10.2 RNA extractions

AurumTM Total RNA Mini Kit (Bio-Rad, Hercules, CA, USA) was used to extract RNA from aortic single cell suspensions. Briefly, 300 µl of lysis solution was added to pelleted cells. 700 µl of 60% ethanol was added to lysed cells. RNA binding column was inserted onto capless wash tube and homogenised lysate was pipetted into it. Samples were centrifuged for 30 sec at 15,000 rpm and the filtrate from the wash tube was discarded. Low stringency wash solution was added into RNA binding column, centrifuged for 30 sec and discarded after passing through the column. DNase I was added to the RNA binding column and incubated at 37°C for 10 min. 700 µl of high stringency solution was added to the RNA binding column, centrifuged for 30 sec and solution from the wash tube was discarded. This was repeated one more time with the low stringency solution. Samples were centrifuged for additional 2 min to remove residual wash solution. RNA binding column was transferred to a capped microcentrifuge tube and 30 µl of pre-warmed nuclease-free water was added onto the membrane stack at the bottom of each column and centrifuged for 15 min to elute the total RNA. RNA was quantified using NanoDrop 8000 (Thermo Scientific, Waltham, MA, USA).

2.10.3 RNA quantification

The quantity of RNA in each sample was determined using a spectrophotometer. Absorbance was measured at 260 nm (A260) and 280 nm (A280), and the purity of the extracted RNA was determined from the absorbance ratio (A260/A280), with a value between 1.7 to 2.0 considered acceptable purity. All RNA samples were normalised to a concentration of 200 ± 5 ng/ μ L and stored at -80°C until further use.

2.10.4 Reverse transcription

All steps were performed on ice. Complementary DNA (cDNA) was synthesised by reverse transcription of 100 ng total RNA from each sample using the iScript cDNA Synthesis Kit, per the component volumes outlined in **Table 2-6**. The reactions were performed with a T100 Thermo Cycler, using a pre-set protocol for first-strand cDNA synthesis: annealing at 25°C for 5 min, extension at 42°C for 30 min, and inactivation at 95°C for 5 min. Triplicate cDNA samples were pooled and diluted 1:5 in nuclease-free water, then stored at -20°C until further use.

Table 2-6. Reverse transcription reagents and quantities used.

<i>Reagent</i>	<i>Volume / reaction (μL)</i>
<i>Iscript</i>	4
<i>Water</i>	3
<i>RNA (200 ng/μL)</i>	5

2.10.5 Quantitative polymerase chain reaction (q-PCR)

The mRNA expression levels of the genes of interest and reference housekeeper gene were measured in triplicate by quantitative real-time PCR (q-PCR) using the SYBR Green fluorophore in a Bio-Rad CFX96 thermocycler. All amplicons were amplified according to the reaction mix outlined in **Table 2-7**, which includes iQ SYBR Green Supermix and 20 pmol/L each of the forward and reverse primers (**Table 2-8**). All plates were centrifuged at 800 rpm for 1 min to ensure all contents were spun down

prior to commencing real-time PCR. The PCR cycling protocol was comprised of initial denaturation and enzyme activation at 95°C for 3 min, followed by 40 cycles comprised of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 30 sec. Relative changes in target gene expression were normalised using the $\Delta\Delta C_t$ method to the housekeeping gene *Actb* (β -actin) and the experimental control condition (514). Melt curve analysis was performed to confirm a single product in each reaction.

Table 2-7. Components required for SYBR master mix to perform q-PCR.

<i>Component</i>	<i>Volume / reaction (μL)</i>
<i>Forward Primer (10 μM)</i>	0.6
<i>Reverse Primer (10 μM)</i>	0.6
<i>SYBR Green</i>	7.5
<i>RNase-free water</i>	3.3
<i>cDNA (from 1:5 dilution)</i>	3.0

Table 2-8. Interest and reference housekeeper gene sequences used for q-PCR.

All primers were acquired from Sigma-Aldrich or Life Technologies.

<i>Primer</i>	<i>Sequence</i>
<i>Actb - F</i>	5'- CTT TTC CAG CCT TCC TTC TTG G -3'
<i>Actb - R</i>	5'- CAG CAC TGT GTT GGC ATA GAG G -3'
<i>Adgre1-F</i>	5'- CTT TGG CTA TGG GCT TCC AGT C -3'
<i>Adgre1-R</i>	5'- TTC TGA GCT GAC ACT GCA GA -3'
<i>Agtr1-F</i>	5'- CCA AAG TCA CCT GCA TCA TC -3'
<i>Agtr1-R</i>	5'- CAC AAT CGC CAT AAT TAT CCT A -3'
<i>Agtr2-F</i>	5'- CTT CAA TCT GGC CTT GGC -3'
<i>Agtr2-R</i>	5'- GCA TAT TTC TCG GGT GGG -3'
<i>Arg1-F</i>	5'- CAT TGG CTT GCG AGA CGT AGA C-3'
<i>Arg1-R</i>	5'- GCC TTT TCT TCC TTC CCA GCA G-3'
<i>Cca1-F</i>	5'- CGC ACA GAG ACC CTG TAC TT -3'
<i>Cca1-R</i>	5'- TTG GAA CGG TCA GAT CAA AT-3'
<i>Cca2-F</i>	5'- CCG CGA TGC CGG GCA CCT -3'
<i>Cca2-R</i>	5'- TCA CAC ACT TAG TGT CTC TG -3'

<i>Ccb1-F</i>	5'- AGC AAA TAT GAG GAG ATG TAC C -3'
<i>Ccb1-R</i>	5'- CGA CTT TAG ATG CTC TAC GGA- 3'
<i>Ccd2-F</i>	5'- GCT CTG TGC GCT ACC GAC TT -3'
<i>Ccd2-R</i>	5'- CAC GCT TCC AGT TGC AAT CA -3'
<i>Cce1-F</i>	5'- GAG CCT CCCCAC TTC CCG TCT T-3'
<i>Cce1-R</i>	5'- CCG GAG CAA GCG CCA TCT GT -3'
<i>CD248-F</i>	5'- GGG CCC CTA CCA CTC CTC AGT -3'
<i>CD248-R</i>	5'- AGG TGG GTG GAC AGG GCT CAG 3'
<i>Cdh5-F</i>	5'- ACG GAC AAG ATC AGC TCC TC-3'
<i>Cdh5-R</i>	5'- TCT CTT CAT CGA TGT GCA TT-3'
<i>Cebpb-F</i>	5'- CTT CAG CCC CTA CCT GGA -3'
<i>Cebpb-R</i>	5'- GGC TCA CGT AAC CGT AGT CG -3'
<i>Flt1-F</i>	5'- GCG GGC TCC TGA CTA CAC -3'
<i>Flt1-R</i>	5'- CCA AAT GCT CCA CCA ACT CTG -3'
<i>Irf8-F</i>	5'- CAG ATC CTC CCT GAC TGG TG -3'
<i>Irf8-R</i>	5'- GCT TGC CCC CGT AGT AGA AG -3'
<i>Isyna1-F</i>	5'- ACA TCG ACC CGT CCA CAG TAT -3'
<i>Isyna1-R</i>	5'- CAG AGG GGT AGG CTT GTC TC-3'
<i>Itgam-F</i>	5'- TGA CTC TTA AAG CTC TTC TGG TCA CA -3'
<i>Itgam-R</i>	5'- AGC TGG ACC ACA CTC TGT CC -3'
<i>Kit-F</i>	5' -TGG GGA TCA TTG TGA TGG T-3'
<i>Kit-R</i>	5'-ATG GCA GCA TCC GAC TTA AT -3'
<i>Klf4-F</i>	5'- CAG GCT GTG GCA AAA CCT AT -3'
<i>Klf4-R</i>	5'- CGG TAG TGC CTG GTC AGT TC -3'
<i>Ly6a-F</i>	5'- GAG GAT GGA CAC TTC T -3'
<i>Ly6a-R</i>	5'- GGT CTG CAG GAG GAC TGA GC -3'
<i>Myc-F</i>	5'- GCT GTA GTA ATT CCA GCG AGA GAC A -3'
<i>Myc-R</i>	5'- CTC TGC ACA CAC GGC TCT TC -3'
<i>Nanog-F</i>	5'- TGG GAT TTA CAG GCG TGA GCC AC -3'
<i>Nanog-R</i>	5'- AAG CAA AGC CTC CCA ATC CCA AAC -3'
<i>Nr4a1-F</i>	5'- AGC TTG GGT GTT GAT GTT CC -3'
<i>Nr4a1-R</i>	5'- AAT GCG ATT CTG CAG CTC TT -3'
<i>Spi1-F</i>	5'- AGA GCA TAC CAA CGT CCA ATG C -3'
<i>Spi1-R</i>	5- GTG CGG AGA AAT CCC AGT AGT G -3'
<i>Tek-F</i>	5'- GAT TTT GGA TTG TCC CGA GGT CAA G -3'
<i>Tek-R</i>	5'- CAC CAA TAT CTG GGC AAA TGA TGG -3'

2.10.6 Primer efficiency testing

Primer pairs were assessed for amplification efficiency by generating a dilution series of cDNA covering four dilution points (undiluted, 1:5, 1:25, 1:125) and performing q-PCR analysis, as detailed above. Average Ct values were plotted against quantity (1, 0.2, 0.04, 0.008) on an X-log scale. Linear regression was performed, and the slope of the curve was then used to determine amplification efficiency according to:

$$Efficiency (\%) = (10^{\frac{-1}{slope}} - 1) \times 100$$

Primers with efficiencies between 90-110% were considered efficient.

2.11 Statistical analysis

Where relevant, data analysis was performed blinded to study group. Statistical analyses were performed using Prism (GraphPad version 9). Data sets were tested for normality of distribution by Shapiro-Wilk test. Statistical comparisons were performed with parametric or non-parametric unpaired or paired two-sample *t*-tests or ANOVA (with post-test multiple comparisons), as specified. Results are expressed as mean $\pm$ standard deviation of multiple experiments. In all cases, statistical significance was established at two-tailed $p < 0.05$. The statistical parameters and the number of mice used per experiment are found in the figure legends.

Chapter 3

Vasculogenic properties of adventitial
Sca-1⁺CD45⁺ progenitor cells in mice: a
potential source of *vasa vasorum* in
atherosclerosis

3.1 Introduction

The formation of neovessels by angiogenesis and/or vasculogenesis is central to many physiologic and pathologic processes (515). In angiogenesis, new vessels form as outgrowths from the endothelium of existing blood vessels, through the coordinated involvement of endothelial cell migration, proliferation and cord formation. In contrast, vasculogenesis occurs through the activity and differentiation of endothelial progenitor cells (EPCs) rather than established endothelium. Historically, much of this was thought to occur by the homing of circulating and/or bone marrow (BM)-derived EPCs to sites of tissue neovascularisation (213, 516); however, local tissue-resident progenitor cells that are not of BM origin have also been shown to contribute endothelial progeny to newly developing microvessels (220, 229, 517). Angiogenesis and vasculogenesis are also supported by the production of soluble cytokines and growth factors by neighbouring cell populations that are not of endothelial lineage. These include myeloid angiogenic cells, such as M2-like (alternatively activated) macrophages (518-520). Although different myeloid angiogenic cells undergo phenotypic changes in response to their surrounding microenvironment, they are generally not known to have endothelial differentiation capacity, and their involvement in neovascularisation is therefore regarded as paracrine.

The specialised microvascular network that supplies oxygen and nutrients to the walls of large blood vessels is known as the *vasa vasorum*. Arterial *vasa vasorum* comprise two subtypes, denoted as “interna” and “externa”, based on whether they arise from the intima/media or adventitia respectively (342). Multiple lines of preclinical and clinical evidence have demonstrated intimate relationships between *vasa vasorum* density and atherosclerosis (342). It follows that a better understanding of the cellular origins of *vasa vasorum* expansion is an important pursuit in the study of vascular physiology and atherogenesis. Among its cellular content, the adventitia contains different progenitor cell populations, which may be a local source of *vasa vasorum* formation (73). One of the markers commonly used to identify progenitor cells in mouse adventitia, is stem cell antigen-1 (Sca-1) (73). Associate Professor Peter Psaltis recently identified that postnatal mouse arteries contain an adventitial Sca-1⁺CD45⁺ subpopulation that is enriched with adventitial macrophage progenitor cells (AMPCs)

(262, 521). Given that resident macrophages are known to expand rapidly during neovessel formation in aortic ring studies (220, 518) and other angiogenic processes (365), this chapter investigated whether adventitial Sca-1⁺CD45⁺ progenitors may also have angiogenic or vasculogenic potential and contribute to *vasa vasorum* growth. For the purpose of transparency in this thesis, some of these experiments were conducted by my supervisor, Associate Professor Peter Psaltis (Mayo Clinic, MN, USA) and a post-doctoral fellow, Dr Deborah Toledo-Flores (SAHMRI) and have been labelled as “collaborative” experiments in this chapter. All other experiments were performed exclusively by me.

3.2 Methods in brief (also see Chapter 2)

3.2.1 Overview

Breeding pairs of C57BL/6 (C57BL/6J), GFP (C57BL/6-Tg(UBC-GFP)30Scha/J), *Apoe*^{-/-} (B6.129P2-*Apoe*^{tm1Unc}/J), GFP (C57BL/6-Tg(UBC-GFP)30Scha/J), *Ly6a*^{GFP} transgenic (B6.Cg-Tg(Ly6a-EGFP)G5Dzk/J), *Ldlr*^{-/-} mice (B6.129S7-*Ldlr*^{tm1Her}/J) and *Cx3cr1*^{GFP/+} (B6.129P-Cx3cr1^{tm1Litt}/J) mouse strains were acquired from The Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed in the animal care facilities at Mayo Clinic or SAHMRI and were maintained on standard chow or an atherogenic high cholesterol diet (HCD) (Specialty Feeds, Australia), as specified. Aortas were prepared and used for methylcellulose colony forming-unit (CFU) assays, MatrigelTM-based aortic ring and cord-forming assays, tissue immunofluorescence staining and confocal microscopy, and multi-colour flow cytometry, as detailed in Chapter 2. Bulk-RNA microarray sequencing and RT-qPCR validation were performed on freshly sorted adventitial Sca-1⁺CD45⁺ and Sca-1⁻CD45⁺ cells. Adventitial Sca-1⁺CD45⁺ and Sca-1⁻CD45⁺ cells from GFP donor mice were also injected into two different models to assess their differentiation potential and vasculogenic capacity *in vivo*: 1) gastrocnemius injection into C57BL/6 recipients following surgery to induce hindlimb ischaemia; and 2) carotid adventitial injection into *Apoe*^{-/-} recipients that were then maintained on HCD for up to 24 weeks. All animal experiments were performed in accordance with the standards stated in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Academy of Sciences, Bethesda, MD, USA) and were approved by the Mayo Clinic Animal Care and Use Committees, and from SAHMRI (ID SAM118 and SAM155) animal ethics committee. All experimental procedures and animal care were performed in accordance with the National Health and Medical Research Council of Australia research guidelines and Australian Code for the Care and Use of Animals for Scientific Purposes.

3.2.2 Statistical analysis

Data sets were tested for normality of distribution by D'Agostino-Pearson omnibus test. Statistical comparisons were performed with parametric or non-parametric unpaired or paired two-sample *t*-tests or ANOVA (with post-test comparisons), as specified. Results are expressed as mean $\pm$ standard deviation (SD) of multiple experiments, unless otherwise specified. In all cases, statistical significance was established at two-tailed $P < 0.05$.

3.3 Results

Dr Deborah Toledo-Flores contributed to results in **Figure 3-3a-b, 3-5, 3-7 and 3-14**, and Associate Professor Peter Psaltis **Table 3-3 and Figure 3-9**.

3.3.1 Sca-1⁺CD45⁺ cells express endothelial markers in atherosclerotic but not healthy aorta

We first used multi-colour flow cytometry to compare expression of endothelial markers in four subpopulations of aortic cells gated based on Sca-1 and CD45 (**Figure 3-1a, b**). CD31, CDH5, TIE2, VEGFR2, CD106 (vascular cell adhesion molecule 1, VCAM-1) and LYVE-1 were all expressed at low levels (<5% positive cells) overall in aortic digests from 12 week-old (12 w) C57BL/6 mice, with highest expression seen in the Sca-1⁺CD45⁻ subpopulation, which has previously been reported to contain endothelial and smooth muscle progenitor cells (522, 523). By comparison, the Sca-1⁺CD45⁺ population displayed very low co-expression of each of these markers, with <1% positive cells for each of CD31, CDH5 and TIE2 (**Figure 3-1a, Table 3-1**). The overall expression of each endothelial marker was substantially increased in aortic digests from atherosclerotic *Apoe*^{-/-} mice compared to C57BL/6 mice, with the Sca-1⁺CD45⁺ fraction containing much higher percentages of cells positive for CD31, TIE2 and VEGFR2 especially (all *p*<0.05) (**Figure 3-1b, Table 3-1**).

Using immunofluorescence staining and confocal microscopy, Sca-1⁺CD45⁺ cells were predominantly located in the adventitia of C57BL/6 aortas; these mice displayed a paucity of adventitial *vasa vasorum*, although microvessels were present in perivascular fat and connective tissue (**Figure 3-1d**). In contrast, *Apoe*^{-/-} mice maintained on HCD for 16 w demonstrated transmural distribution of Sca-1⁺CD45⁺ cells across all three layers of their atherosclerotic aortas (**Figure 3-1e, Figure. 3-2**), and Sca-1 and CD45 were frequently co-expressed on *Griffonia simplicifolia* I-B4 isolectin⁺ (ISL⁺) and von Willebrand Factor⁺ (vWF⁺) *vasa vasorum*, and adventitial LYVE-1⁺ lymphatics (**Figure 3-1e, f and 3-2**). These

observations in *Apoe*^{-/-} aortas provided the first indication that Sca-1⁺CD45⁺ cells may have endothelial capacity and be involved in the formation of *vasa vasorum* when atherosclerosis is induced.

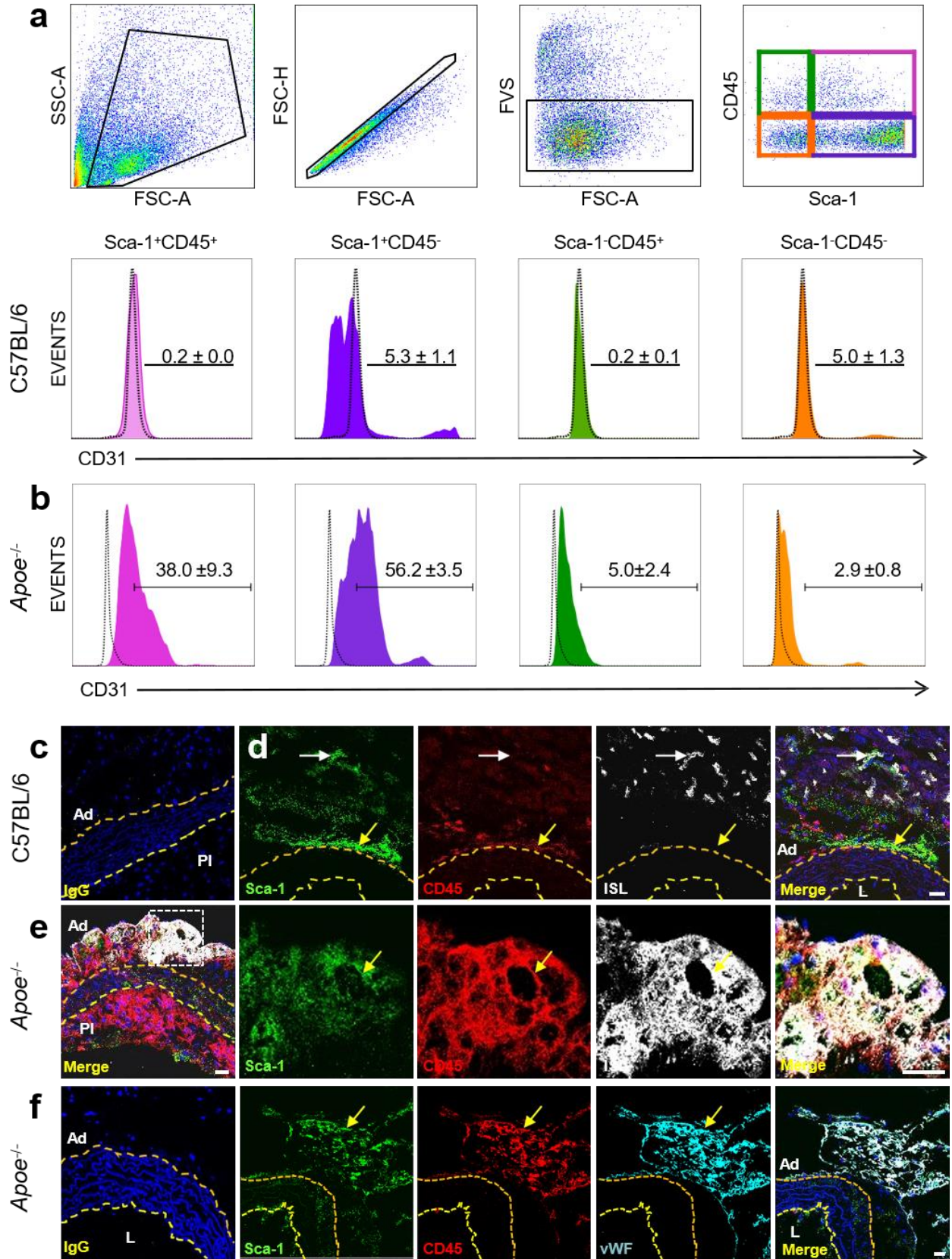


Figure 3-1. Expression of endothelial markers on Sca-1/CD45 subpopulations in C57BL/6 and *Apoe*^{-/-} aortas.

- (a)** Flow cytometry gating strategy to identify four Sca-1/CD45 subpopulations of cells in aortic digests and their respective surface expression of CD31, from 12 w C57BL/6 mice on chow diet. N=3 mice. Percentage of CD31⁺ expression, relative to FMO negative control.
- (b)** Flow cytometry histograms showing CD31 expression in the corresponding Sca-1/CD45 fractions from digests of 24 w *Apoe*^{-/-} aortas after 16 w of HCD to induce atherosclerosis. N=3 mice.
- (c)** Merged image from IgG isotype control staining for Sca-1 (green), CD45 (red), ISL (white).
- (d-f)** Immunofluorescence staining and confocal microscopy of sections of aortic arch from **(d)** 12 w C57BL/6 mouse on chow diet and **(e-f)** 24 w *Apoe*^{-/-} mice on 16 w HCD. Scale bar, 20 μ m (white). White arrow indicates a peri-adventitial Sca-1⁺CD45⁻ cell with binding to ISL. Yellow arrow indicates an adventitial Sca-1⁺CD45⁺ cell closely apposed to the external elastic lamina, with no ISL binding.
- (e-f)** Dense co-localisation of **(e)** Sca-1 and CD45 with ISL⁺ (white) and **(f)** vWF⁺ (cyan) microvessels in adventitia of atherosclerotic *Apoe*^{-/-} aortas, with examples denoted by yellow arrows. Inset boxes in low magnification images correspond to adjacent high magnification images. IgG isotype control staining is also shown. Nuclei are counterstained blue with Hoechst. Broken yellow and orange lines indicate internal and external elastic lamina respectively.

Ad, Adventitia; L, Lumen; Pl, Plaque.

Data summarised as mean $\pm$ SD.

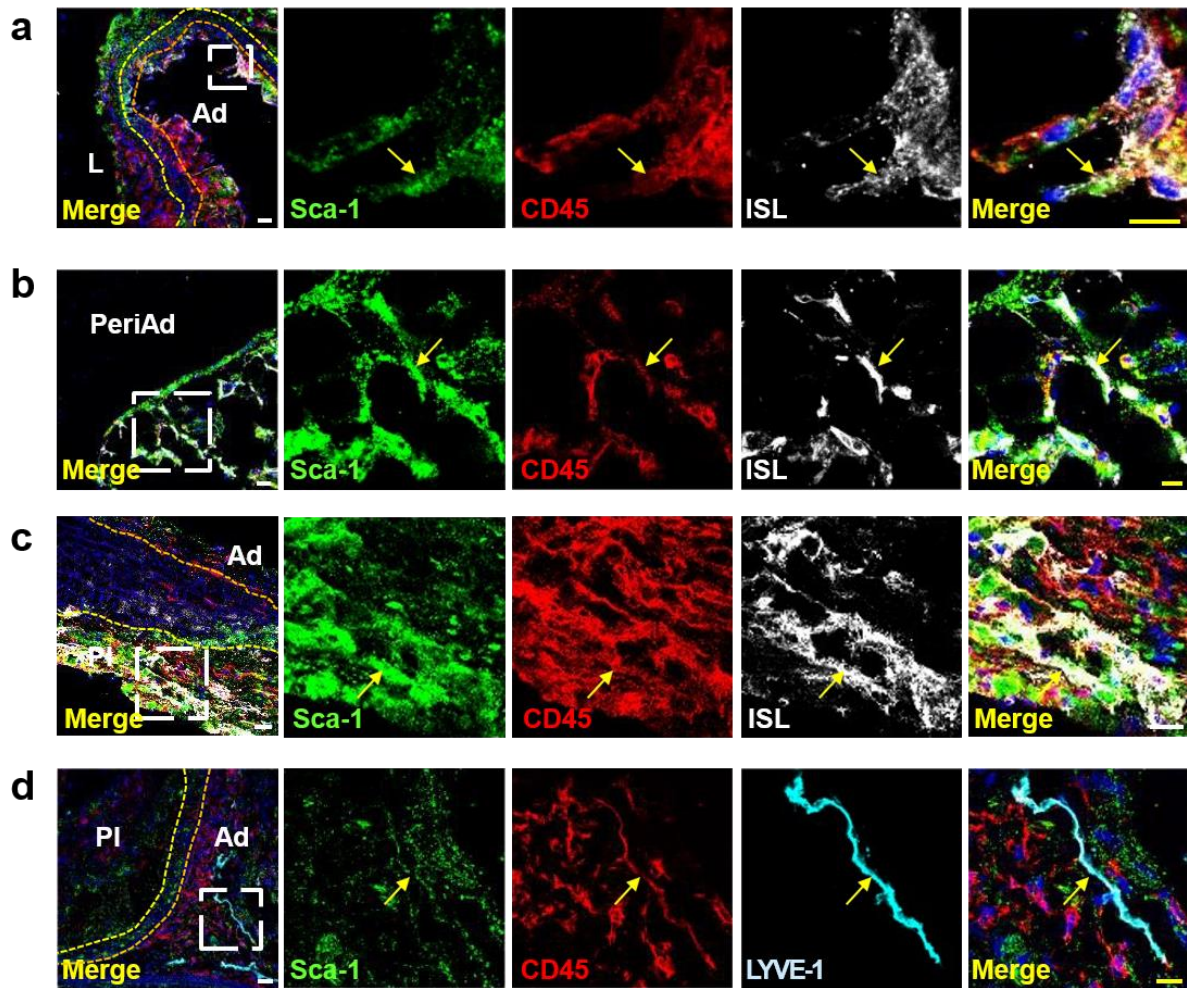


Figure 3-2. Co-expression of Sca-1 and CD45 on microvessels in *Apoe*^{-/-} aortic arch.

Immunofluorescence staining and confocal microscopy of different sections of aortic arch from 24 w *Apoe*^{-/-} mice on 16 w of HCD.

(a-c) Co-localisation of Sca-1 and CD45 with ISL⁺ microvessels in **(a)** adventitia, **(b)** peri-adventitial tissue and **(c)** neointimal plaque, with examples indicated by yellow arrows. Scale bars, 10 μm (yellow), 20 μm (white).

(d) Staining of adventitial LYVE-1⁺ lymphatic vessels, with an example of co-staining for Sca-1 and CD45 denoted by the yellow arrow. Higher magnification images correspond to preceding inset box. Nuclei are counterstained blue with Hoechst. Internal and external elastic laminae are demarcated by broken yellow and orange lines respectively. Scale bars, 10 μm (yellow), 20 μm (white).

Ad, Adventitia; L, Lumen; PeriAd, Peri-adventitial tissue; Pl, Plaque.

Surface marker	Total	Sca-1 ⁺ CD45 ⁺	Sca-1 ⁺ CD45 ⁻	Sca-1 ⁻ CD45 ⁺	Sca-1 ⁻ CD45 ⁻
C57BL/6					
CD31 ⁺	1.9 (1.6–2.0)%	0.3 (0.2–0.3)%	5.2 (3.4–7.3)%	0.2 (0.1–0.3)%	4.8 (3.0–7.4)%
CDH5 ⁺	0.4 (0.4–0.5)%	0.8 (0.6–1.2)%	3.2 (2.5–6.2)%	0.6 (0.3–1.0)%	2.7 (1.3–6.7)%
TIE2 ⁺	0.2 (0.1–0.3)%	0.1 (0.1–4.4)%	0.5 (0.4–0.5)%	0.5 (0.1–2.9)%	0.0 (0.0–0.0)%
VEGFR2 ⁺	3.5 (3.4–5.2)%	3.1 (1.4–16.9)%	21.6 (20.9–23.4)%	2.5 (0.9–10.5)%	1.4 (1.1–1.9)%
CD106 ⁺	3.9 (3.6–4.9)%	2.8 (1.5–10.5)%	19.4 (18.8–22.3)%	2.1 (1.0–6.5)%	2.0 (1.7–2.6)%
LYVE-1 ⁺	1.0 (0.7–1.2)%	1.1 (0.5–6.7)%	3.6 (2.9–3.8)%	0.7 (0.2–2.9)%	0.6 (0.5–0.7)%
<i>Apoe</i> ^{-/-}					
CD31 ⁺	26.6 (20.7–41.2)%	47.2 (19.4–47.4)%	52.9 (52.6–63.1)%	5.3 (0.6–9.0)%	2.4 (2.0–4.4)%
CDH5 ⁺	3.3 (3.0–10.5)%	10.2 (3.2–10.5)%	4.2 (3.3–19.7)%	3.3 (1.3–5.4)%	1.1 (0.5–4.4)%
TIE2 ⁺	2.9 (1.9–3.5)%	9.3 (8.2–15.4)%	1.0 (0.6–2.0)%	4.5 (1.4–5.3)%	0.0 (0.0–0.1)%
VEGFR2 ⁺	21.9 (14.9–31.4)%	40.0 (26.3–41.5)%	25.8 (23.8–52.7)%	21.7 (10.2–24.7)%	4.8 (1.2–6.2)%
CD106 ⁺	13.2 (11.7–18.9)%	21.0 (6.7–27.4)%	27.0 (18.0–27.2)%	6.7 (6.1–21.9)%	7.3 (2.9–7.8)%
LYVE-1 ⁺	13.5 (12.9–16.5)%	15.8 (10.3–32.2)%	10.0 (9.8–25.8)%	35.2 (1.1–41.8)%	2.6 (1.9–6.8)%

Table 3-1. Endothelial marker expression on Sca-1/CD45 subpopulations in C57BL/6 and *Apoe*^{-/-} aortas.

Shown are the median and range values for percent surface expression assessed by flow cytometry of six endothelial-related markers in aortic cell digests from n=3 male 12 w C57BL/6 mice fed chow and n=3 male 24 w *Apoe*^{-/-} mice fed HCD for 16 weeks, gated from the total viable population and the four Sca-1/CD45 subpopulations as shown in Figure. 1. Statistical comparisons were performed between corresponding cell populations from C57BL/6 and *Apoe*^{-/-} mice by Mann-Whitney test with all P-values being non-significant.

3.3.2 Adventitial Sca-1⁺CD45⁺ cells display endothelial plasticity and angiogenic capacity *in vitro*

We next studied aortas harvested from *Ly6a*^{GFP/+} (Sca-1-green fluorescence protein) transgenic mice (524). GFP⁺ cells were predominantly located in the adventitia, and methylcellulose-based culture of aortic digests produced macrophage colony forming units (CFU-M) that were exclusively GFP⁺, consistent with our previous discovery that AMPCs are contained within the adventitial Sca-1⁺(CD45⁺) population (262, 521) (**Figure 3-4c**). *Ex vivo* aortic ring studies performed in MatrigelTM from these mice demonstrated that GFP⁺ cells of Sca-1⁺ origin participate in the process of angiogenic sprouting (**Figure 3-3a, b**). We then confirmed that adventitial integrity is a prerequisite for this by showing that removal of the adventitia from C57BL/6 aortic rings completely eliminated sprouting, unlike intimal denudation which had no significant effect (**Figure 3-3c-e**). To quantify the cellular composition of adventitial sprouts, we scraped the MatrigelTM and performed collagenase digestion to separate the cellular outgrowths from the ring itself, and then analysed the resulting single cell suspensions by flow cytometry. In keeping with their failure to form angiogenic sprouts, aortic ring studies performed without adventitia had a much lower content of both Sca-1⁺ and CD31⁺ cells than those with intact adventitia (**Figure 3-3f**). Approximately 80% of the cellular make-up of aortic ring outgrowths was Sca-1⁺, with the majority of these cells lacking CD45 (69.8±8.1% Sca-1⁺CD45⁻ and 11.3±0.9% Sca-1⁺CD45⁺ of all viable cells, n=3 donor mouse experiments with each using ≥3 aorta rings) (**Figure 3-3g**). However, we found that CD31 was expressed on a much higher percentage of outgrowing Sca-1⁺CD45⁺ cells than in the Sca-1⁺CD45⁻ subpopulation (**Figure 3-3h**). This aligned with our previous observation that although endothelial markers (e.g. CD31, CDH5) were virtually absent from the adventitial Sca-1⁺CD45⁺ fraction in C57BL/6 aorta *in situ*, they became strongly co-expressed on these cells with *vasa vasorum* formation in atherosclerosis.

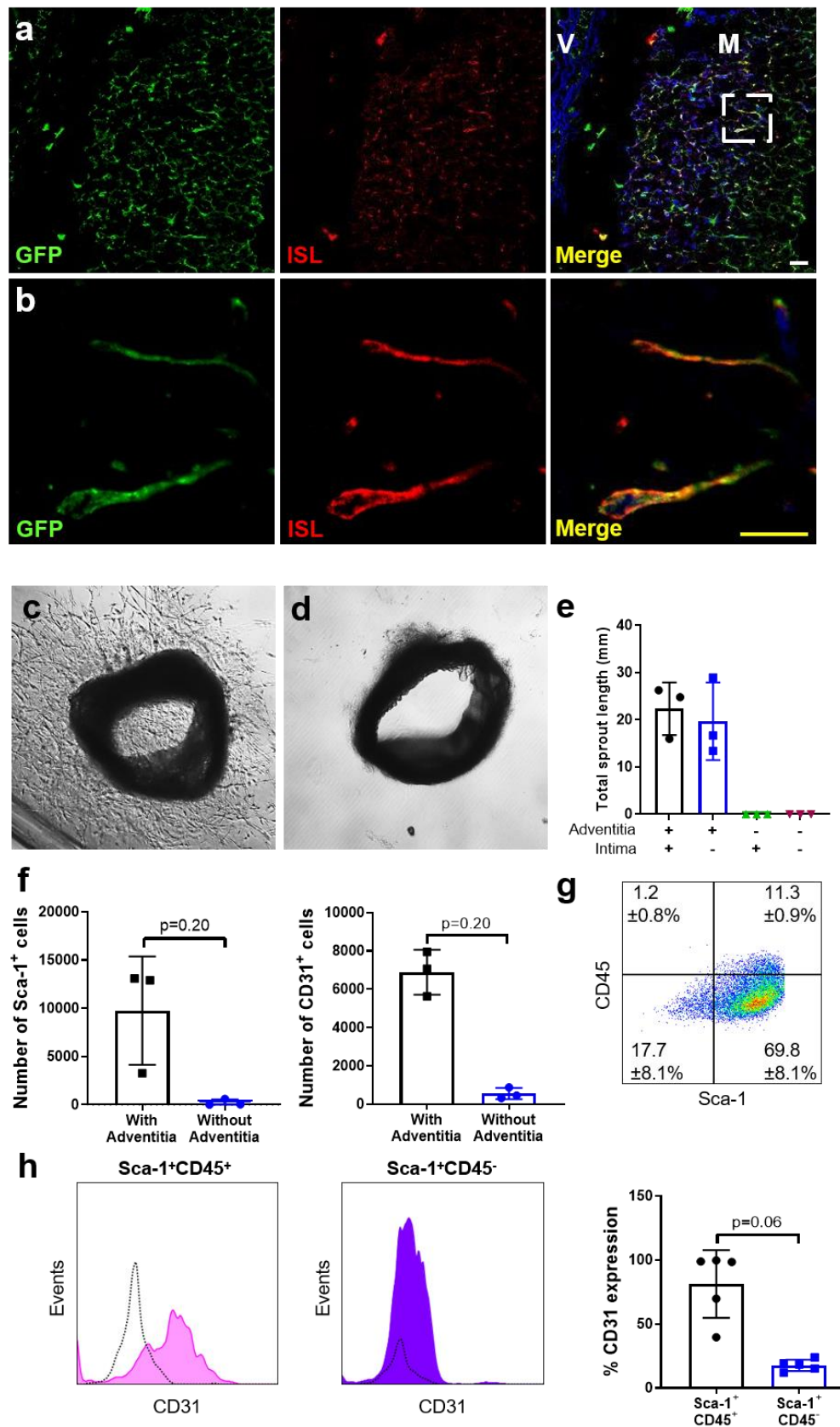


Figure 3-3a-b are collaborative results led by Dr Deborah Toledo-Flores

Figure 3-3. Contribution of adventitial Sca-1⁺ cells to *ex vivo* aortic ring sprouts.

(a-b) Confocal microscopy images showing the binding of GFP⁺ (green) cells to ISL (red) following adventitial sprouting from aortic rings harvested from *Ly6a*^{GFP/+} mice. Inset box in **(a)** corresponds to high magnification images in **(b)** nuclei are counterstained blue with Hoechst. V, Vessel wall; M, Extra-vascular MatrigelTM. Scale bars, 10 μ m (yellow), 20 μ m (white).

(c-d) Light microscopic images (x40) of sprouting from aortic rings with **(c)** adventitia intact and **(d)** adventitia removed.

(e) Graph showing the total length of adventitial sprouts grown from aortic rings from 12 w C57BL/6 mice where the adventitia and/or intima were left intact (+) or removed/denuded (-). N=3 mice/gp. One-way ANOVA: p=0.03.

(f) Results from flow cytometry for the total number of outgrowing Sca-1⁺ and CD31⁺ cells in C57BL/6 aortic ring studies with and without adventitia. N=3 mice/gp. Unpaired *t*-test.

(g) Flow cytometry density plot for Sca-1 and CD45 expression from aortic ring adventitial outgrowths.

(h) Representative histograms and graph depicting CD31 expression within the Sca-1⁺CD45⁺ and Sca-1⁺CD45⁻ populations growing out from C57BL/6 aortic rings. N=5 mice/gp. Paired *t*-test.

Data summarised as mean $\pm$ SD.

To interrogate more directly the vasculogenic potential of adventitial Sca-1⁺CD45⁺ cells, we sorted this population to high purity from digested C57BL/6 aortas (**Figure 3-4a**). In keeping with our previous studies (262, 263), we firstly verified that the CFU-M (macrophage colony) forming capacity of aortic cells is entirely restricted to its Sca-1⁺CD45⁺ population. (**Figure 3-4b-c**). In contrast to the Sca-1⁺CD45⁻ fraction, freshly isolated Sca-1⁺CD45⁺ cells again displayed negligible CD31 expression (**Figure 3-4d**). Moreover, they did not acquire CD31 when cultured *in vitro* for 10 days under basal conditions (RPMI/10% Foetal bovine serum) or in the presence of interleukin-4 (IL-4) and granulocyte-macrophage colony-stimulating factor (GM-CSF), which we previously have shown induces their differentiation into CD11c⁺ myeloid cells (**Figure 3-5a-b**) (521). Macrophage colony-stimulating factor (M-CSF), which directs these cells to an F4/80⁺CD115⁺ macrophage phenotype (521), also resulted in only limited CD31⁺ expression (**Figure 3-5c**). However, culture in endothelial growth medium (EGM) supplemented with vascular endothelial growth factor (VEGF) resulted in the appearance of endothelial-like colonies after 7-10 days, which formed a homogeneous, confluent cell layer with cobble-stone morphology between 14 and 21 days. These cells stained uniformly for CD31 and displayed binding to *Griffonia simplicifolia* I-B4 isolectin (**Figure 3-5e-g**).

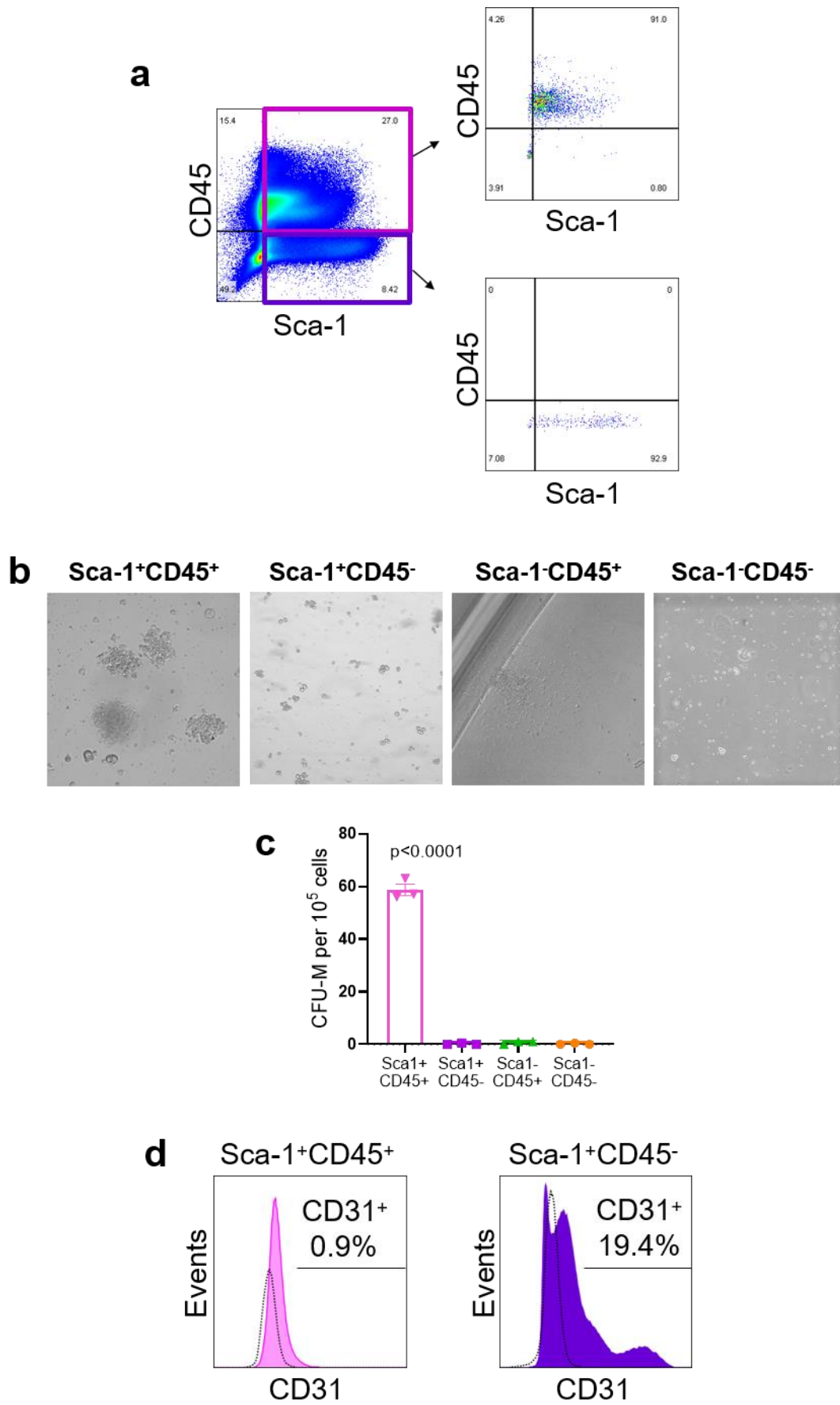


Figure 3-4. Flow cytometry of freshly sorted aortic digests from C57BL/6 mice.

(a) Representative flow cytometry dot plot is shown on the left for Sca-1 and CD45 from aortic cell digests pooled from n=10 C57BL/6 mice, immediately prior to fluorescence activated cell sorting. To the right are representative dot plots following sorting of the Sca-1⁺CD45⁺ (top right) and Sca-1⁺CD45⁻ (bottom right) subpopulations.

(b) Representative light microscopy images (x40 magnification) of methylcellulose-based cultures of different aortic Sca-1/CD45 subpopulations after 14 days, showing formation of CFU-M only from the sorted Sca⁺CD45⁺ populations.

(c) CFU-M yield from different aortic Sca-1/CD45 fractions. Repeated measures ANOVA with post-hoc comparison. $p < 0.0001$.

(d) Histograms are shown for CD31 expression in the freshly isolated Sca-1⁺CD45⁺ (pink) and Sca-1⁺CD45⁻ (purple) populations, with FMO control histogram shown as the underlying dotted line.

Data are summarised as mean $\pm$ SD from n=3 different experiments, each performed from pooled aortas of ≥ 6 12 w C57BL/6 mice.

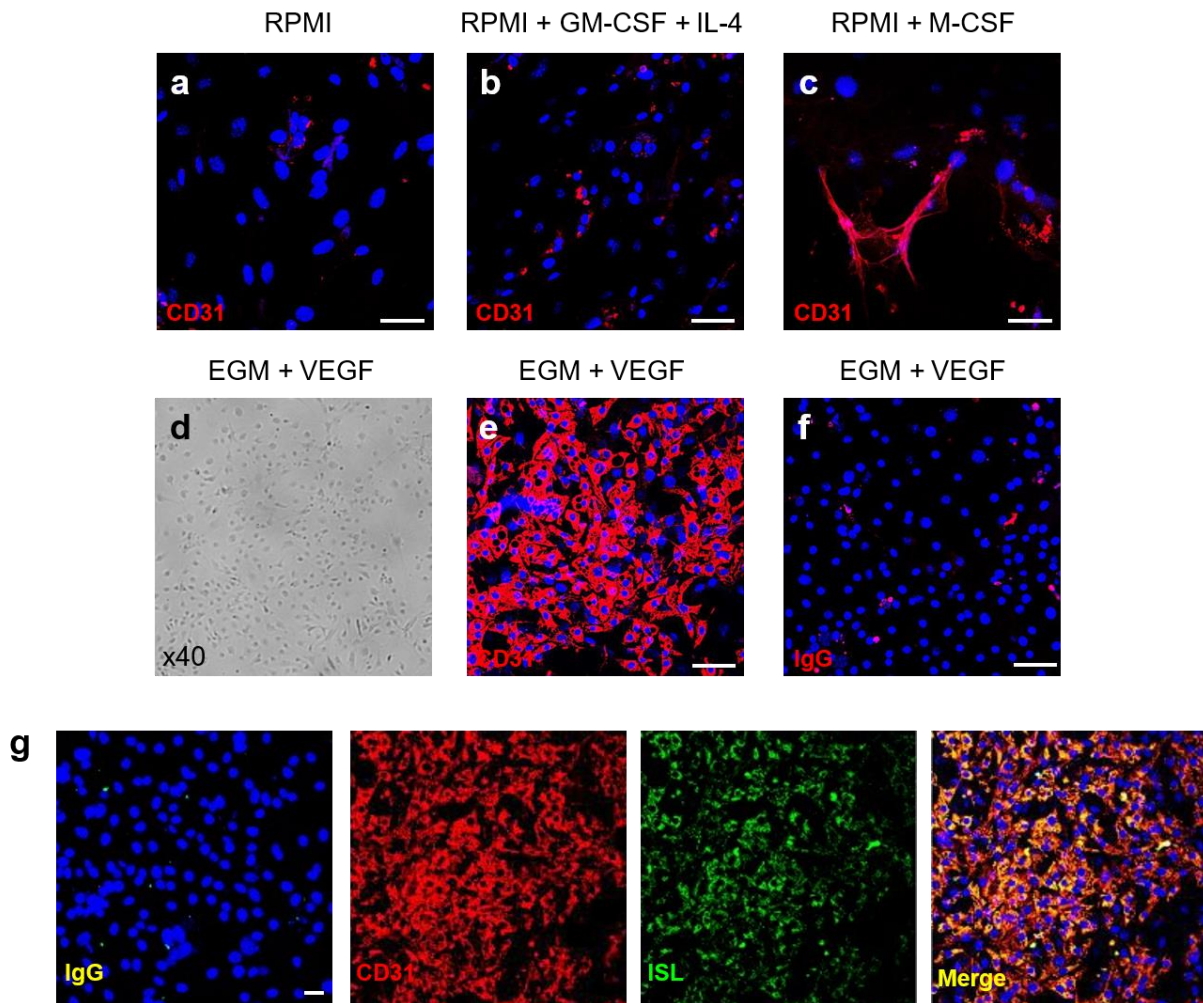


Figure 3-5 are collaborative results led by Dr Deborah Toledo-Flores

Figure 3-5. Endothelial differentiation of adventitial Sca-1⁺CD45⁺ cells.

(a-c) Representative immunofluorescence staining for CD31 expression is shown for C57BL/6 adventitial Sca-1⁺CD45⁺ cells cultured for 10 days in media containing different growth factors. CD31 staining was negligible when cells were cultured in **(a)** basal RPMI media, **(b)** RPMI supplemented with IL-4/GM-CSF and **(c)** modest in the presence of M-CSF. Scale bar, 50 μm.

(d) Endothelial growth medium (EGM) supplemented with VEGF induced cobble-stone endothelial-like morphology on light microscopy. Scale bar, 50 μm.

(e) Uniform CD31 expression. Scale bar, 50 μm.

(f-g) IgG control staining for CD31. **(g)** Immunofluorescence staining for CD31 and binding to ISL, of adventitial Sca-1⁺CD45⁺ cells after culture EGM containing VEGF. Images are representative of n=2-3 separate experiments for each condition. Each experiment involved the pooling of aortas from n=6

C57BL/6 mice. Nuclei are counterstained blue with Hoechst. Scale bars, 50 μm for **(f)** and 20 μm for **(g)**.

In MatrigelTM-based assays, freshly isolated Sca-1⁺CD45⁺ cells showed a time-dependent, intrinsic capacity to produce angiogenic cords, to a significantly greater extent than their Sca-1⁺CD45⁺ and Sca-1⁺CD45⁻ counterparts (**Figure 3-6a-c**). These cords took several days to form and displayed dense micro-sprouting at branch-points, not typical of the smooth cords produced within one day by human umbilical vein endothelial cells (HUVECs) (**Figure 3-7**). MatrigelTM-based co-culture delineated the ability of Sca-1⁺CD45⁺ cells to align with the early cords formed by HUVECs, before integrating into these structures and ultimately forming their own consolidated, linear connections after HUVEC cords had become fragmented (**Figure 3-7**). Transmission electron microscopy was used to more closely examine the cord networks produced by aortic Sca-1⁺CD45⁺ cells in MatrigelTM. This revealed mixed presence of phagocytic cells and cells that were interconnected by adhesions (**Figure 3-6d**), suggesting that they had produced heterogeneous progeny comprising macrophage and endothelial-like progeny.

We therefore performed further analysis of the cellular composition of the cord networks. Day 7 cords were retrieved from MatrigelTM by scraping, digested with collagenase and then the resulting single cell suspensions were immunostained for flow cytometry (**Figure 3-6e-h**, **Table 3-2**). This firstly revealed that the majority of Sca-1⁺CD45⁺ cells lost CD45 expression as they formed cords, suggesting that they largely transformed away from the myeloid lineage. Notably, the Sca-1⁺CD45⁺ fraction gave rise to a high percentage of Sca-1⁺CD45⁻ cells (**Figure 3-6f**), but the converse did not occur to any extent (**Figure 3-6h**). The Sca-1⁺CD45⁺ population was again found to generate CD31⁺ cells consistent with *de novo* endothelial transdifferentiation (**Figure 3-6g**), along with a small percentage of cells expressing CD146, a marker associated with both endothelium and pericytes (**Table 3-2**). However, very few PDGFR β ⁺ (CD140 β ⁺) pericytes were produced by either Sca-1⁺ fraction under the culture conditions used (**Table 3-2**).

As expected, Sca-1⁺CD45⁺ cells did produce a small population of macrophages in MatrigelTM (**Figure 3-6g**), which were not observed from Sca-1⁺CD45⁻ cells (**Figure 3-6h**): median % of LIN⁻CD45⁺CD11b⁺F4/80⁺ macrophages were 3.4% (range 2.4-3.5%) from Sca-1⁺CD45⁺ and 0.1% (0.0-0.1%) from Sca-1⁺CD45⁻ fractions (n=3 experiments). To determine the distribution of macrophages

relative to cords, we performed additional experiments using sorted adventitial cells from *Cx3cr1*^{GFP/+} mice, in which GFP signal enabled detection of CX₃CR1⁺ progeny, that included macrophages. As shown in **Figure 3-8a**, GFP⁺ cells were present in the cord networks formed from the Sca-1⁺CD45⁺ but not the Sca-1⁺CD45⁻ fraction, specifically at the branch-point intersections of cords but not in or along the cords themselves. To establish the importance of macrophage progeny to their cord-forming capacity, Sca-1⁺CD45⁺ cells were also treated with liposomal clodronate which was added to the MatrigelTM every second day to deplete macrophage numbers. Although this had no effect on the total length of cords formed, it did result in reduction of cord branching (**Figure 3-8b**), consistent with the known ability of macrophages to support endothelial tip cell anastomosis (525).

Collectively our observations from both *ex vivo* aortic ring sprouting and *in vitro* cord formation indicate that adventitial Sca-1⁺CD45⁺ progenitors possess intrinsic endothelial plasticity and vasculogenic potential.

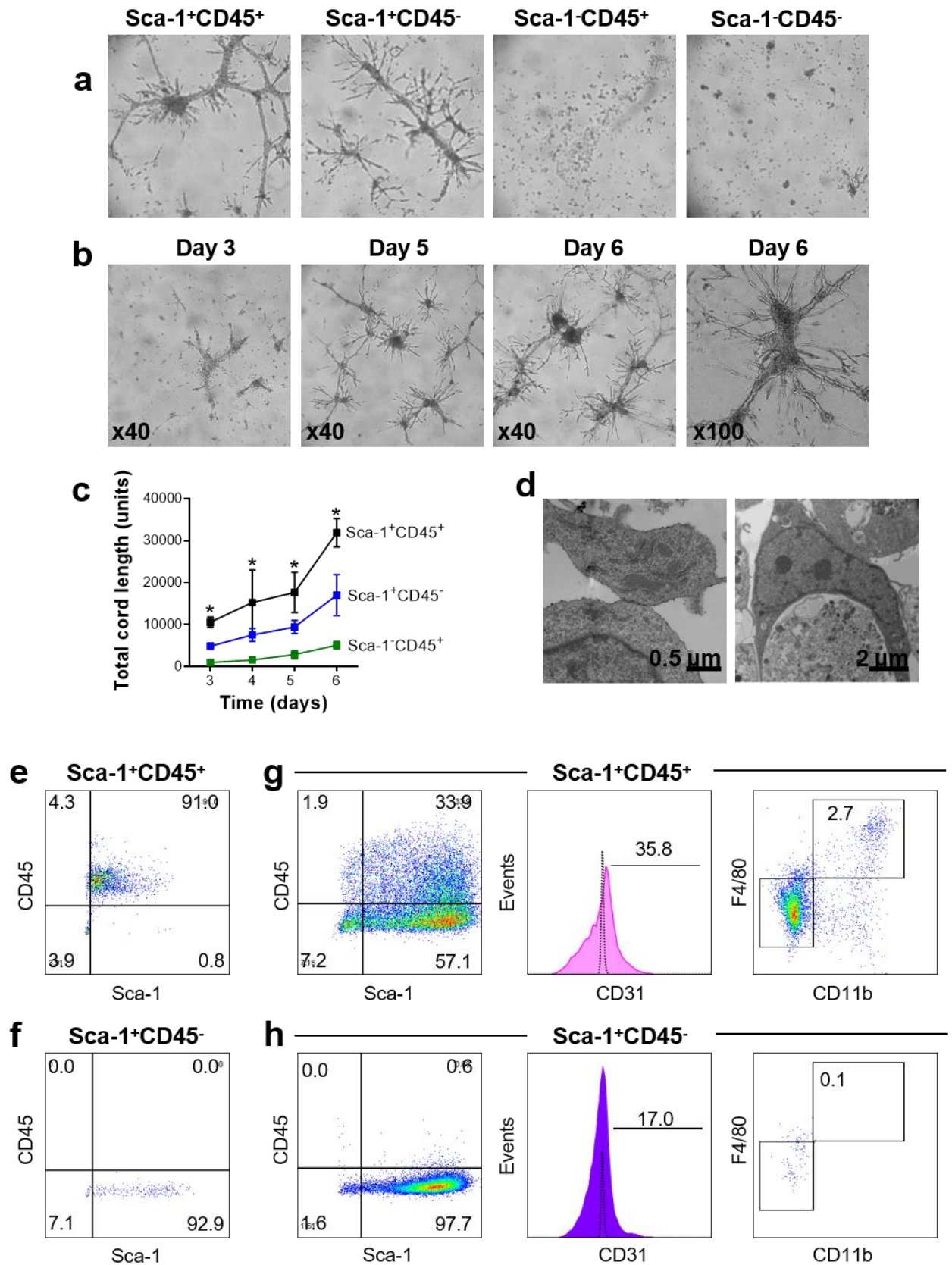


Figure 3-6. Endothelial plasticity and cord forming capacity of adventitial Sca-1⁺CD45⁺ cells.

(a) Light microscopic images (x40) taken on day 5 after culturing different adventitial Sca-1/CD45 subpopulations in MatrigelTM and endothelial growth medium. Note absence of cords from both Sca-1⁻ populations compared to elaborate cord connections formed from both Sca-1⁺CD45⁺ and Sca-1⁺CD45⁻ cells.

(b) Time course of cord formation after plating Sca-1⁺CD45⁺ cells in MatrigelTM.

(c) Graph summarises results from 3-4 independent experiments comparing cord formation from different Sca-1/CD45 subpopulations. One-way ANOVA with Bonferonni post-hoc comparisons, *p<0.05 compared to Sca-1⁻CD45⁺ cells.

(d) Transmission electron microscopy images from day 6 Sca-1⁺CD45⁺ well showing examples of intercellular adhesion (left) and phagocytosis (right).

(e-f) Flow cytometry dot plots showing purity of freshly sorted **(e)** Sca-1⁺CD45⁺ and **(f)** Sca-1⁺CD45⁻ fractions immediately before plating in MatrigelTM.

(g-h) Representative dot plots and histograms showing expression of Sca-1, CD45, CD31, CD11b and F4/80 from cells obtained after cords had formed from starting **(g)** Sca-1⁺CD45⁺ and **(h)** Sca-1⁺CD45⁻ populations.

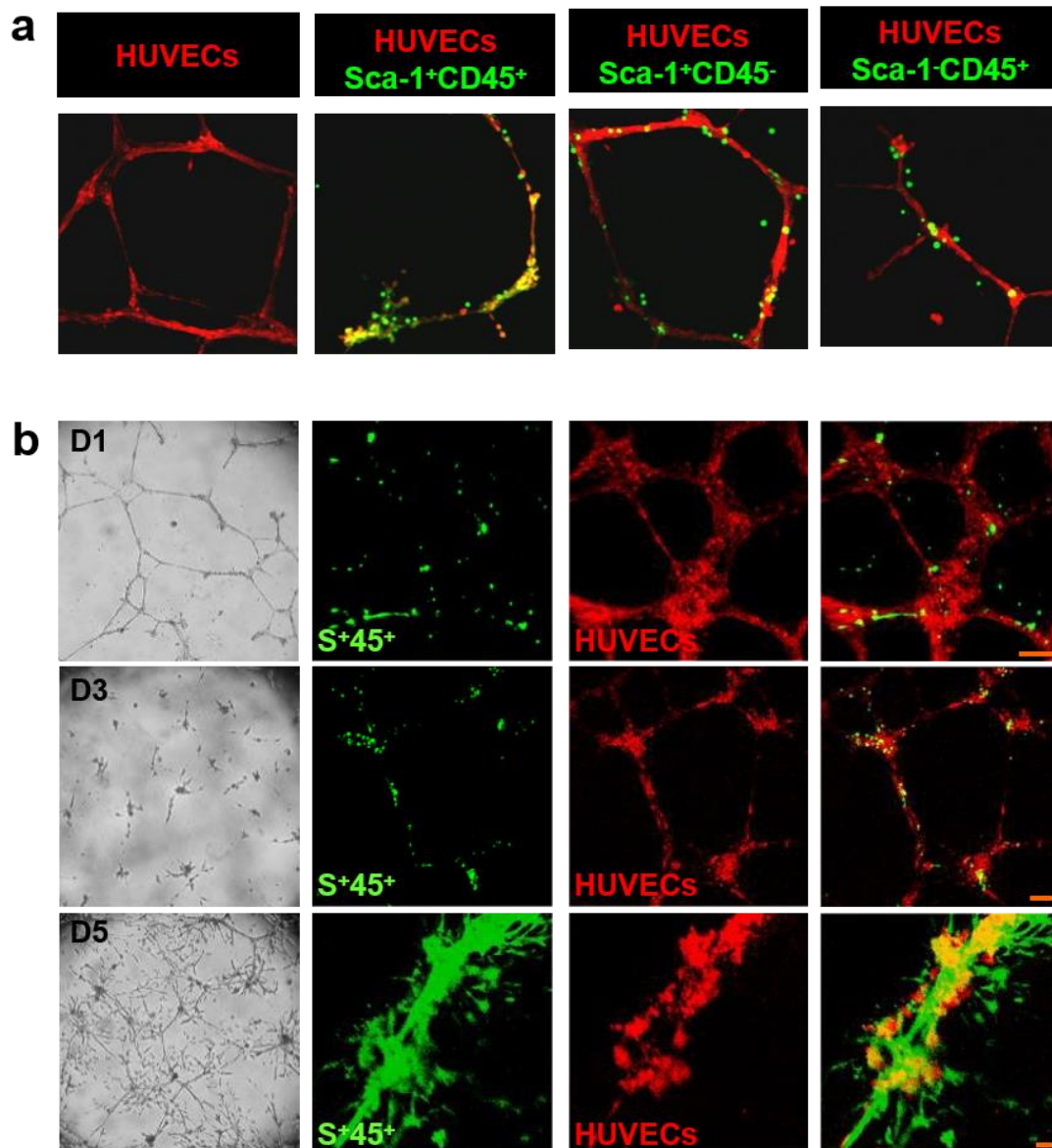


Figure 3-7 are collaborative results led by Dr Deborah Toledo-Flores

Figure 3-7. Matrigel™-based cord-forming capacity of different adventitial Sca-1/CD45 subpopulations.

(a) Merged confocal images taken on day 3 after 1:1 co-culture of HUVECs (red) and different adventitial GFP⁺ Sca-1/CD45 fractions (green) in Matrigel™. Sca-1⁺CD45⁺ cells integrated and contributed to cords to a greater extent than Sca-1⁺CD45⁻ and Sca-1⁻CD45⁺ cells. Scale bar, 50 μ m (orange).

(b) Time course of cord formation after 1:1 co-culture of GFP⁺Sca-1⁺CD45⁺ cells and HUVECs labelled with orange cell-tracker. D, Day. Images are representative of n=2-4 independent experiments. Each experiment involved the pooling of aortas from n $\geq$ 6 C57BL/6 donor mice. Scale bar, 50 μ m (orange).

Surface marker	Sca-1 ⁺ CD45 ⁺	Sca-1 ⁺ CD45 ⁻	P-value
Sca-1	95.6% (92.0–98.1)	86.0% (62.7–98.1)	0.250
CD45	26.1% (16.3–29.1)	3.0% (0.6–4.7)	0.125
c-Kit	14.4% (5.1–26.3)	5.3% (1.4–5.7)	0.250
CD31	35.8% (11.3–43.9)	17.0% (6.8–17.2)	0.500
CD146	5.3% (0.7–28.0)	2.7% (0.7–8.8)	0.625
PDGFR β	1.8% (0.2–2.8)	0.0% (0.0–0.1)	0.125

Table 3-2. Surface marker expression on cells isolated from vascular-like cords formed from Sca-1⁺CD45⁺ and Sca-1⁺CD45⁻ aortic cells in MatrigelTM.

Shown are the median and range values for percent expression of different surface markers expressed by cells that were obtained from the MatrigelTM cord assay, seven days after culturing freshly sorted Sca-1⁺CD45⁺ or Sca-1⁺CD45⁻ aortic cells from 12 w C57BL/6 mice. N=3-4 different experiments, with each using cells sorted from n=8-10 pooled aortas. Wilcoxon matched-pairs signed ranks test.

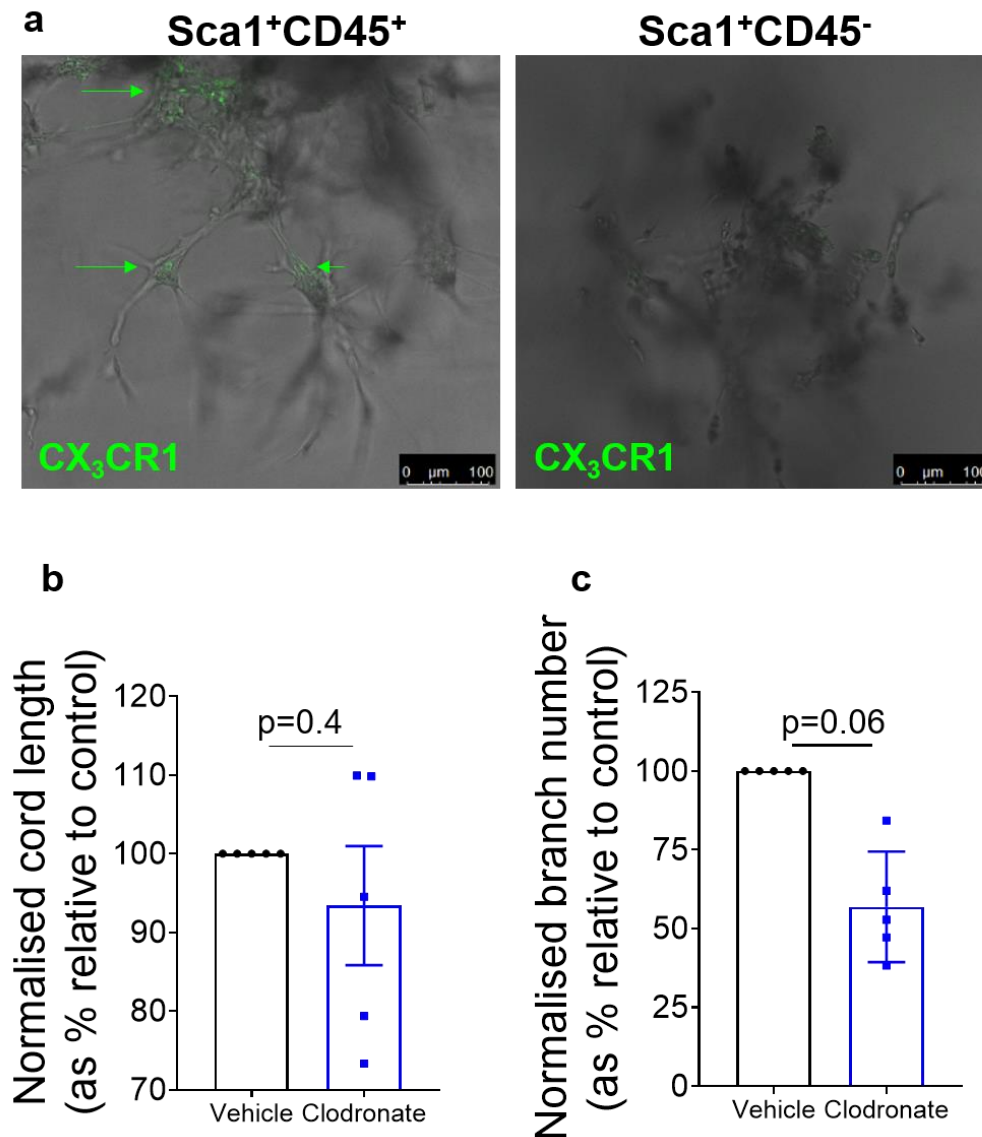


Figure 3-8. Distribution of CX₃CR1⁺ cells in cord networks formed from adventitial Sca-1⁺CD45⁺ cells and the effect of clodronate on cord formation.

(a) Merged images from phase contrast and immunofluorescence microscopy of cord networks from Sca-1⁺CD45⁺ (left) and Sca-1⁺CD45⁻ (right) aortic cells sorted from *Cx3cr1*^{GFP/+} mice. CX₃CR1⁺ (GFP⁺) cells, including macrophages, were observed at the connecting intersections of cords grown from the Sca-1⁺CD45⁺ (green arrows) but not Sca-1⁺CD45⁻ fraction.

(b-c) Normalised data for **(b)** total cord length and **(c)** number of branch points after adventitial Sca-1⁺CD45⁺ cells were cultured in Matrigel[™] for seven days in the presence or absence of clodronate liposomes which were used to deplete their macrophage progeny. N=5 mice.

Data summarised as mean±SD.

3.3.3 Sca-1⁺CD45⁺ cells express angiogenic and endothelial progenitor genes

Previously we reported the results of unbiased mRNA microarray analysis that compared Sca-1⁺CD45⁺, Sca-1⁺CD45⁻ and Sca-1⁻CD45⁺ cells from C57BL/6 aortas (521). Re-interrogation of this data-set revealed that compared to Sca-1⁻CD45⁺ cells, Sca-1⁺CD45⁺ cells expressed higher transcript levels of at least fifty genes that are known to regulate angiogenesis and/or vasculogenesis (**Table 3-3, Figure 3-9**). Notable among these were: *Cd248* (endosialin), which is involved in developmental and tumour-associated neovascularisation (526), and has been shown to be upregulated in atherosclerotic vessels of *Apoe*^{-/-} mice (527); *Mmp2*, *Mmp3*, *Mmp14*, *Mmp23*, *Egfr*, *Pdgfra*, *Il33*, *Sox9* and *Cd34*, which were recently reported to be more highly expressed in tissue-resident endovascular progenitors (EVPs) than in mature endothelial cells (229); *Ace* and *Agt*, which are members of the angiotensin-renin system; and various well-known growth factors, cytokines and chemokines that are pro-angiogenic (*Cxcl12*, *Csf1*, *Vegfa*, *Il6*) or lymphangiogenic (*Vegfc*). The same genes were not differentially expressed between Sca-1⁺CD45⁺ and Sca-1⁺CD45⁻ progenitor cells, indicating that both of these Sca-1⁺ subpopulations are transcriptionally primed to promote neovascularisation. Importantly, mRNA transcripts of mature endothelial genes (e.g. *Cd31*, *Vwf*, *Tie1* or *2*, *Enos*) were not significantly higher in Sca-1⁺CD45⁺ compared to Sca-1⁻CD45⁺ cells, in keeping with our earlier results from flow cytometry analysis of C57BL/6 aortas (**Figure 3-1a, Table 3-1**).

Gene	Fold difference	FDR	Gene	Fold difference	FDR
<i>Cd248</i>	11.9	1.3E-05	<i>Rnase4</i>	2.5	8.3E-04
<i>Angptl1</i>	10.9	2.5E-05	<i>Angpt4</i>	2.4	2.2E-04
<i>Ccl11</i>	8.2	2.4E-05	<i>Serpinf1</i>	2.4	6.0E-04
<i>Cxcl12</i>	5.6	4.2E-04	<i>Tgfb3</i>	2.4	8.1E-04
<i>Timp1</i>	5.6	2.9E-05	<i>Timp3</i>	2.4	1.6E-03
<i>Pdgfra</i>	4.7	6.8E-05	<i>Ace</i>	2.4	1.2E-03
<i>Mmp3</i>	4.3	2.3E-04	<i>Thbs2</i>	2.3	2.5E-04
<i>Fst</i>	4.1	2.3E-04	<i>Angptl2</i>	2.2	7.7E-04
<i>Cd34</i>	4.0	5.8E-05	<i>Tek</i>	2.2	1.9E-02
<i>Il33</i>	3.8	7.7E-05	<i>Efnal</i>	2.1	2.0E-02
<i>Mmp2</i>	3.7	2.9E-04	<i>Igf1</i>	2.1	3.2E-04
<i>Ntrk2</i>	3.6	1.1E-04	<i>Adamts1</i>	2.0	3.6E-02
<i>Mmp23</i>	3.6	2.0E-04	<i>Angptl4</i>	1.9	2.9E-02
<i>Sox9</i>	3.5	2.4E-05	<i>Smo</i>	1.9	3.2E-03
<i>Csf1</i>	3.4	2.8E-05	<i>Ephb4</i>	1.8	3.3E-02
<i>Egfr</i>	3.4	1.4E-04	<i>Timp2</i>	1.8	3.8E-03
<i>Vegfc</i>	3.3	2.1E-04	<i>Col4a3</i>	1.7	1.5E-02
<i>Agt</i>	2.9	2.4E-04	<i>Tgfb2</i>	1.7	3.8E-03
<i>Angptl7</i>	2.8	8.8E-04	<i>Ang</i>	1.6	1.1E-02
<i>Anpep</i>	2.8	5.1E-04	<i>ErbB2</i>	1.6	1.5E-02
<i>Mmp14</i>	2.8	4.0E-03	<i>Il6</i>	1.6	3.1E-02
<i>Fn1</i>	2.6	8.1E-03	<i>Jag1</i>	1.6	3.1E-03
<i>Hey1</i>	2.6	8.6E-03	<i>Ptgs1</i>	1.5	5.0E-02
<i>Mdk</i>	2.6	3.1E-04	<i>Ptk2</i>	1.5	6.0E-03
<i>Cxcl1</i>	2.5	7.7E-04	<i>Vegfa</i>	1.5	2.0E-02

Table 3-3 are collaborative results led by A/Prof Peter Psaltis

Table 3-3. Angiogenic and vasculogenic gene expression in adventitial Sca-1⁺CD45⁺ progenitor cells.

Shown are fifty genes that are known to be involved in angiogenesis and/or vasculogenesis and were found by microarray analysis to be more highly expressed by a factor of 1.5 or more in aortic Sca-1⁺CD45⁺ cells compared to Sca-1⁻CD45⁺ cells. FDR, False discovery rate. N=3 donor experiments, with each experiment pooled from n=6 12 w C57BL/6 mice.

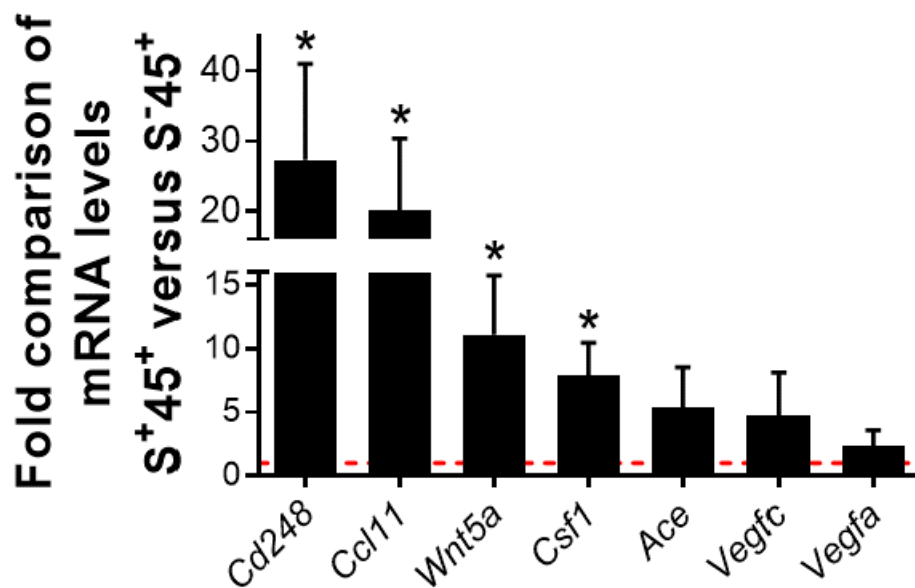


Figure 3-9 are collaborative results led by A/Prof Peter Psaltis

Figure 3-9. mRNA expression of selected angiogenic/vasculogenic genes.

RT-qPCR validation was performed for seven genes that are known to be involved in angiogenesis or vasculogenesis and were found by unbiased microarray to be more highly expressed in adventitial Sca-1⁺CD45⁺ cells than Sca-1⁻CD45⁺ cells from 12 w C57BL/6 aortas. Graph summarises the mean±SD fold comparison of mRNA expression between Sca-1⁺CD45⁺ and Sca-1⁻CD45⁺ cells, with the red dotted line corresponding to equivalence. N=3 different experiments. Each experiment involved the pooling of aortas from n=6 12 w C57BL/6 donor mice. *p<0.05 by paired *t*-test.

3.3.4 Adventitial Sca-1⁺CD45⁺ cells adopt endothelial fate and form new vessels *in vivo*

Next, we used a model of hindlimb ischaemia to study the vasculogenic potential of adventitial Sca-1⁺CD45⁺ cells outside of their native environment of the artery wall. Recipient C57BL/6 mice were subjected to permanent ligation surgery of their left iliac artery and distal vessels before receiving injections of cell-free MatrigelTM, GFP⁺ Sca-1⁺CD45⁺ cells, or other GFP⁺ Sca-1/CD45 subpopulations into the gastrocnemius muscle. Two weeks later, doppler imaging revealed that perfusion in the ischaemic limb was 50% higher in recipients of Sca-1⁺CD45⁺ progenitor cells than MatrigelTM control and this was accompanied by trends toward better clinical health and limb movement (n=5-6 per group) (**Figure 3-10a-c**). Notably, injection of Sca-1⁺CD45⁺ leukocytes resulted in no significant benefit to hindlimb perfusion.

Although donor Sca-1⁺CD45⁺ cells were retained in the connective tissue of injected hindlimbs, they were generally distributed as clusters of individual cells, which was also the case for injected Sca-1⁺CD45⁻ and Sca-1⁻CD45⁻ cells (**Figure 3-10d**). In contrast, each mouse that received Sca-1⁺CD45⁺ cells displayed robust and complex GFP⁺ networks and lumen-containing tubular structures, that expressed endothelial (CD31) and lymphatic (LYVE-1) markers, bound to isolectin and were proliferative (Ki67⁺) (**Figure 3-11b-d**). Furthermore, we found evidence of Ter119⁺ erythrocytes inside the lumen of GFP⁺CD31⁺ neovessels, suggesting their connection to host vasculature (**Figure 3-11a**). Some tissue sections also contained isolated clusters of individual GFP⁺ cells that expressed the macrophage marker, MOMA-2, although these were not in particularly close proximity to GFP⁺ neovessels (**Figure 3-11b**). Despite resulting in significant improvement in perfusion and the formation of new donor-derived blood vessels in the recipient hindlimbs, we were unable to demonstrate an overall increase in either CD31⁺ capillary or CD31⁺SMA⁺ arteriolar density in the tissue sections of Sca-1⁺CD45⁺ recipients compared to the MatrigelTM control group (**Figure 3-12a-d**).

These results from this adoptive transfer study confirm that in addition to its content of macrophage progenitors (521), the adventitial Sca-1⁺CD45⁺ subpopulation also produces mature endothelial cell progeny, that contributes to the formation of functional neovessels *in vivo*.

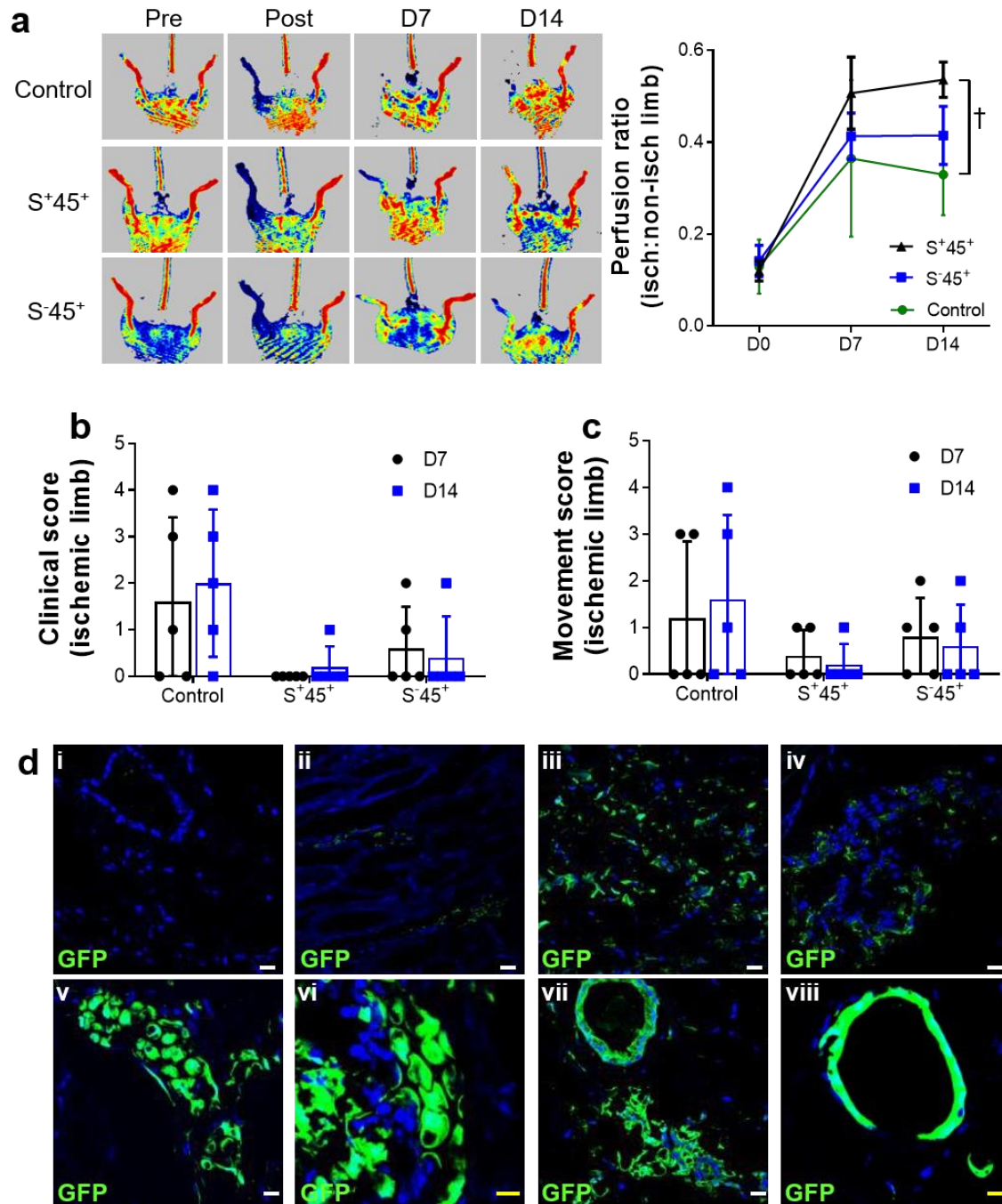


Figure 3-10. Vasculogenic properties of adventitial Sca-1⁺CD45⁺ cells in hindlimb ischaemia model.

(a) Representative doppler perfusion images of C57BL/6 mice before and after hindlimb ischaemia surgery with intramuscular injection of cell-free Matrigel™, aortic adventitial GFP⁺Sca-1⁺CD45⁺ (S⁺45⁺) or GFP⁺Sca-1⁻CD45⁺ (S-45⁺) cells. Graph shows perfusion ratios of ischaemic:non-ischaemic

limb two weeks of recovery. N=5-6 per group. One-way ANOVA with Bonferonni post-hoc comparison: †p<0.01

(b) Clinical and **(c)** movement scores for the ischaemic hindlimb at day 7 and 14 after arterial ligation and injection of cell-free MatrigelTM (control) or freshly-isolated aortic adventitial Sca-1⁺CD45⁺ (S⁺45⁺) or Sca-1⁻CD45⁺ (S⁻CD45⁺) cells. N=5-6 mice/gp. One-way ANOVA.

(d) GFP detection in gastrocnemius sections from ischaemic limb 14 days after injection of (i) MatrigelTM, (ii) Sca-1⁺CD45⁻, (iii) Sca-1⁻CD45⁺, (iv) Sca-1⁻CD45⁻ or (v-viii) Sca-1⁺CD45⁺ cells (four different recipient mice shown). Nuclei are counterstained blue with Hoechst. Scale bars, 10 µm (yellow) and 20 µm (white).

Data are shown as mean±SD

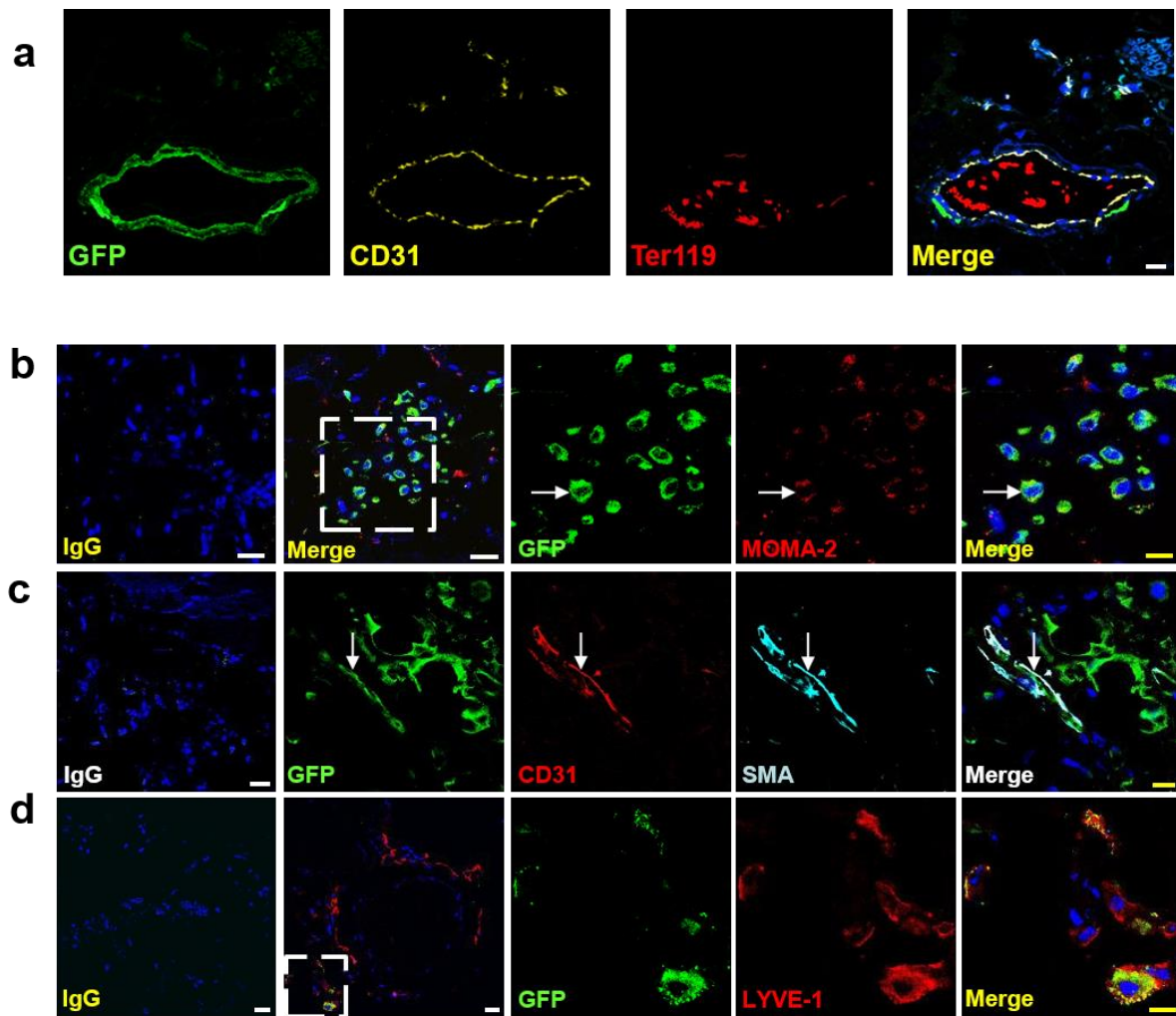


Figure 3-11. Fate of adventitial Sca-1⁺CD45⁺ cells in hindlimb ischaemia.

(a) Example of a GFP⁺CD31⁺ blood vessel containing Ter119⁺ erythrocytes in its lumen, 14 days after ischaemic surgery and injection of GFP⁺Sca-1⁺CD45⁺ cells. Scale bars, 20 μm (white).

(b-d) Co-staining of GFP with **(b)** macrophage (MOMA-2), **(c)** endothelial (CD31) and smooth muscle (SMA), and **(d)** lymphatic endothelial (LYVE-1) markers, 14 days after ischaemic surgery and intramuscular injection of aortic GFP⁺Sca-1⁺CD45⁺ cells. In each case the merged image from the relevant IgG control antibodies is also shown. Higher magnification images correspond to the preceding inset box. Nuclei are counterstained blue with Hoechst. Scale bars, 10 μm (yellow) and 20 μm (white).

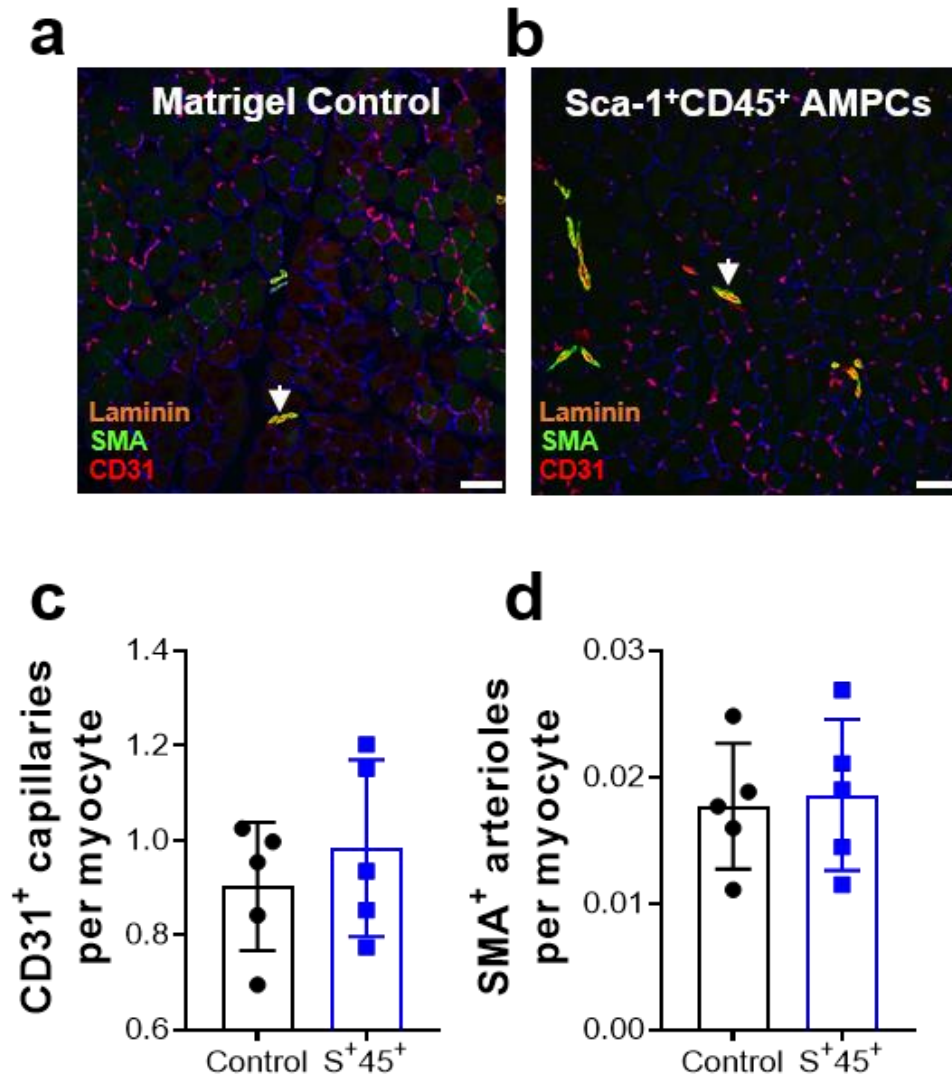


Figure 3-12. Capillary and arteriolar density after cell transfer in hindlimb ischaemia model.

(a- b) Examples of immunofluorescence images taken from sections of gastrocnemius muscle that had received injections of **(a)** cell-free MatrigelTM control or **(b)** Sca-1⁺CD45⁺ cells at the time of hindlimb ischaemia surgery. Examples of CD31⁺SMA⁺ arteriolar staining are indicated by arrows. Counter-staining was performed with an antibody to laminin to help delineate myocytes and DAPI for nuclei. Scale bar, 100 μ m (white).

(c- d) Quantification of the **(c)** capillary and **(d)** arteriolar density from gastrocnemius sections for the two groups. N=5 mice/gp. Paired *t*-test. *p*=ns for both.

Data are shown as mean $\pm$ SD

3.3.5 Vasculogenic fate of adventitial Sca-1⁺CD45⁺ cells in atherosclerosis

Although *vasa vasorum* are a well-established mediator of atherogenesis, their cellular origins remain ill-defined (342). Given their endothelial and vasculogenic potential, we considered whether adventitial Sca-1⁺CD45⁺ progenitor cells may be a source of neovascularisation in atherosclerotic arteries. We firstly observed that compared to chow-fed C57BL/6 mice, CFU-M yield was significantly increased from aortic digests of *Apoe*^{-/-} mice that had been fed HCD for 4 or 16 w (from 8 weeks of age) to induce early or established atherosclerosis, respectively (**Figure 3-13a**). Similar results were obtained for *Ldlr*^{-/-} mice after 16 w HCD. Of note, there was no increase in the frequencies of other non-M CFU subtypes (data not shown).

Sca-1⁺CD45⁺ cells from *Apoe*^{-/-} mice maintained on HCD for 16 w, showed a non-significant trend for increased formation of angiogenic cords compared to age-matched C57BL/6 mice (**Figure 3-13b-c**). Adventitial sprouting was also significantly increased from aortic rings of *Apoe*^{-/-} mice after 16 w HCD compared to chow-fed, age-matched C57BL/6 mice (**Figure 3-13d-e**). This was accompanied by higher content of Sca-1⁺CD45⁺ cells in *Apoe*^{-/-} rings (**Figure 3-13f-g**). These results show an expansion of CFU-M forming Sca-1⁺CD45⁺ progenitor cells in atherosclerotic mouse aorta coupled with an increase in adventitial angiogenic capacity.

We next directly tracked the fate of Sca-1⁺CD45⁺ cells during atherogenesis by isolating them from aortas of GFP donor mice and then injecting them into the carotid artery adventitia of 8 w *Apoe*^{-/-} recipients (521). Following 16 w of HCD, GFP⁺ cells were found in and around the injected artery of all recipient mice (n=6), but not in peripheral blood, remote tissues or contralateral carotid artery. In addition to giving rise to GFP⁺ macrophages [see previous publication (521)], we also detected networks of interconnected GFP⁺ cells and lumen-containing GFP⁺ structures in the carotid adventitia and peri-adventitia (**Figure 3-14a**). Some of these displayed preserved expressions of Sca-1 (**Figure 3-14b**) and also stained for vWF (**Figure 3-14c**) and LYVE-1 (**Figure 3-14d**), indicating that donor Sca-1⁺CD45⁺ cells had produced new microvessels and lymphatics. As seen in **Figure 3-14b**, the presence of a GFP⁺

(host) CD45⁺ leukocyte adherent to the luminal surface of a GFP⁺ vessel was consistent with connection to the host circulation, while the clustering of host CD45⁺ cells just outside the same structure suggested that it may have supported leukocyte transmigration. Interestingly, we also found examples of complex adventitial and peri-adventitial GFP⁺ networks that expressed F4/80 (indicating macrophage content), but these were not found to stain for vWF (**Figure 3-14e**).

Together these findings show that Sca-1⁺CD45⁺ cells produce durable neovessels in atherosclerotic arterial adventitia *in vivo*, consistent with newly formed *vasa vasorum*.

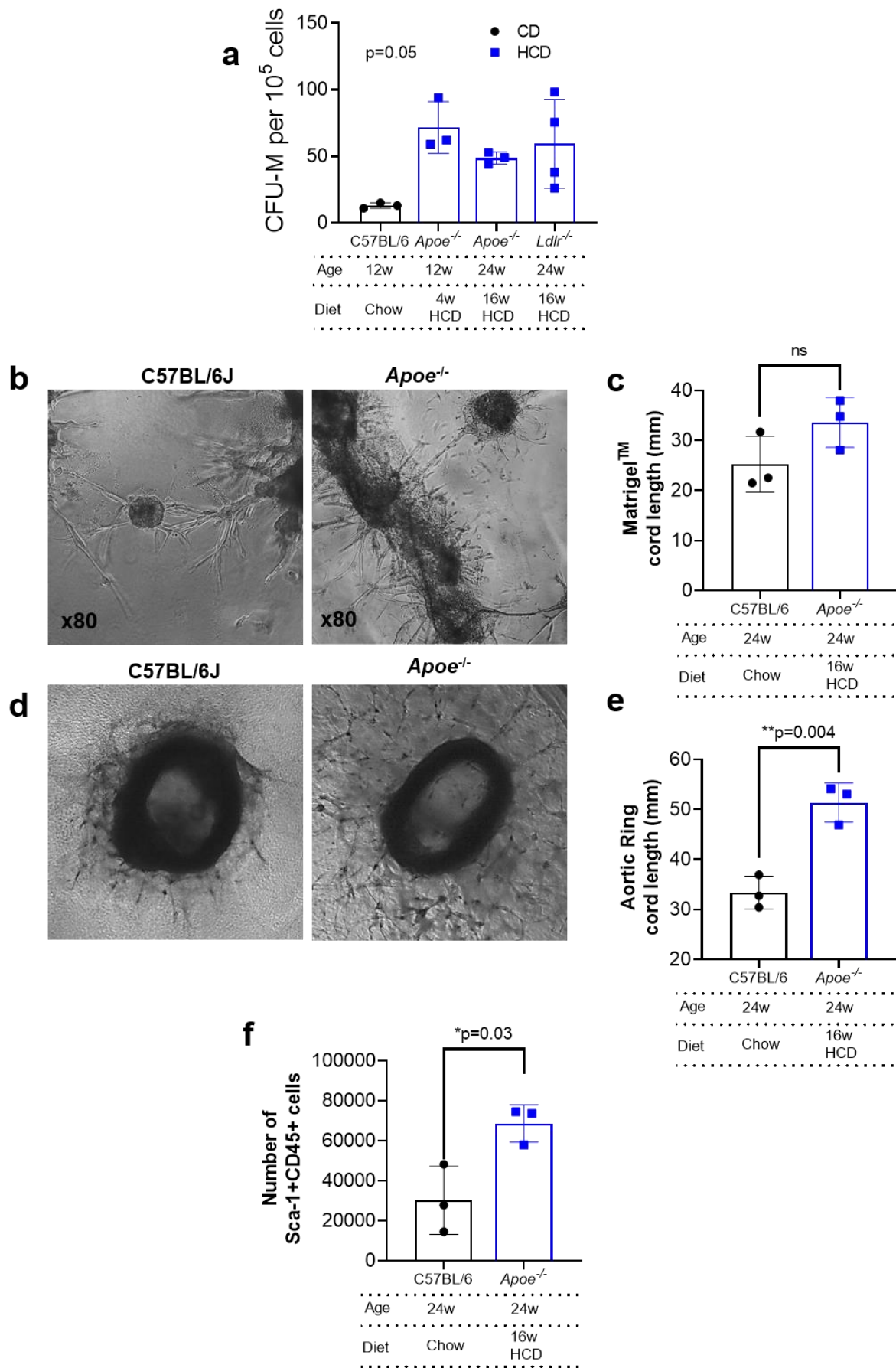


Figure 3-13. Adventitial Sca-1⁺CD45⁺ cells in atherosclerotic mice.

(a) Day 14 CFU-M yield from single cell digests of whole aorta harvested from 12 w chow-fed C57BL/6 mice, 12 w *Apoe*^{-/-} mice (4 w of HCD), 24 w *Apoe*^{-/-} mice (16 w of HCD) and 24 w *Ldlr*^{-/-} mice (16 w of HCD). N=3-4 mice/gp. Kruskal-Wallis: p=0.05.

(b) Light microscopy images (x80) showing cord formation in MatrigelTM (day 7) from Sca-1⁺CD45⁺ aortic cells obtained from 24 w C57BL/6 (chow diet) and 24 w *Apoe*^{-/-} (16 w of HCD) mice.

(c) Cord length from C57BL/6 or *Apoe*^{-/-} Sca-1⁺CD45⁺ cells in MatrigelTM (day 7). N=3 mice/gp. Unpaired *t*-test.

(e-d) Light microscopic images (x80) of sprouting from **(d)** aortic rings with adventitia intact and **(e)** graph showing the total length of adventitial sprouts grown from aortic rings from 24 w C57BL/6 mice on chow diet compared to 24 w *Apoe*^{-/-} mice on a 16 w HCD. N=3 mice/gp. Unpaired *t*-test.

(f) Results from flow cytometry for the total number of outgrowing Sca-1⁺CD45⁺ cells in C57BL/6 and *Apoe*^{-/-} aortic ring studies with adventitia. N=3 mice/gp. Unpaired *t*-test.

Data summarised as mean±SD.

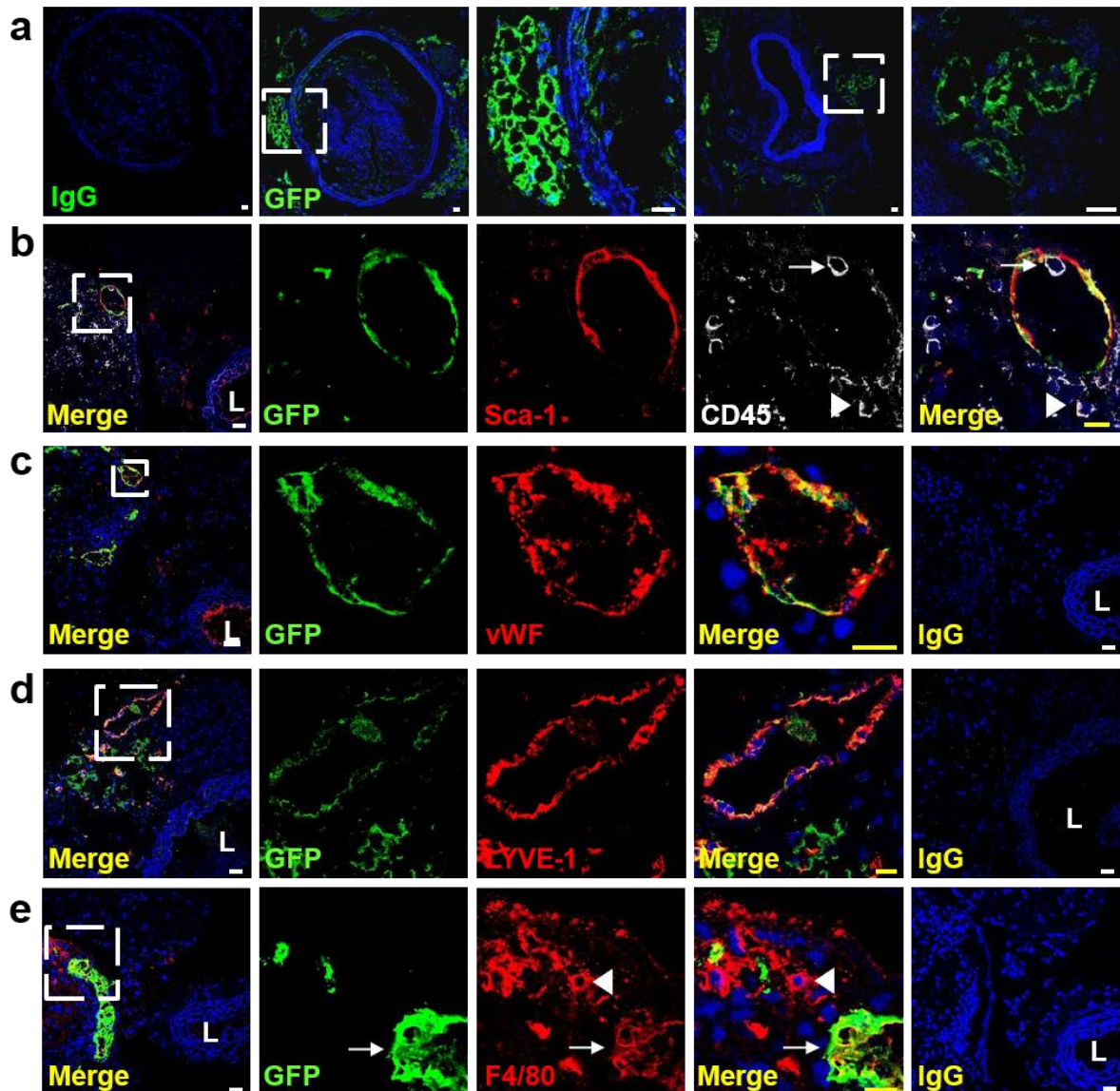


Figure 3-14 are collaborative results led by Dr Deborah Toledo-Flores

Figure 3-14. Formation of adventitial and peri-adventitial microvessels by Sca-1⁺CD45⁺ cells in atherosclerosis.

(a) Confocal images of *Apoe*^{-/-} carotid arteries 16 w after adventitial injection of aortic GFP⁺ Sca-1⁺CD45⁺ cells and HCD. Note the presence of GFP⁺ cells forming lumen-containing microvascular structures and networks in adventitia and peri-adventitia. Scale bars, 20 μ m (white).

(b-e) Co-staining for GFP with **(b)** Sca-1 and CD45, **(c)** vWF, **(d)** LYVE-1 and **(e)** F4/80 showing that donor cells produced durable endothelial-lined microvessels in adventitia and peri-adventitia of atherosclerotic carotid artery, and also formed macrophages. Inset boxes correspond to adjacent high magnification images. In **(b)** the white arrow points to a GFP⁻ (host-derived) CD45⁺ leukocyte inside

the lumen and adherent to the luminal surface of a well-formed GFP⁺ vascular structure suggesting integration with the host circulation. The white arrowhead in the same image indicates a cluster of host leukocytes around the outside of this neovessel, suggesting possible transmigration across it. IgG control staining is also shown for each set of images. Nuclei are counterstained blue with Hoechst. L, Lumen. Scale bars, 10 μ m (yellow) and 20 μ m (white).

3.4 Discussion

The importance of the adventitia, and more specifically its *vasa vasorum* network, to vessel wall health and disease is well established (73, 342, 354, 364, 528, 529). Although knowledge of the developmental basis of *vasa vasorum* remains incomplete, existing data point to integral roles for different populations of mature and ancestral cells located within the adventitia. On the one hand, the angiogenic proliferation and sprouting of existing *vasa vasorum* endothelial cells are supported structurally and via paracrine regulation by pericytes, fibroblasts and haematopoietic cells, especially macrophages (518, 530-532). On the other, adventitial EPCs may provide a local ancestral source of endothelial cells for postnatal vasculogenesis (220, 229). Having already discovered that mouse arteries contain AMPCs that are Sca-1⁺CD45⁺ (521), we embarked on this study in the expectation that this population would also have pro-angiogenic capacity to support adventitial neovascularisation. Our findings as summarised below, were both anticipated and unanticipated.

We first confirmed that in C57BL/6 mouse aorta, the adventitial Sca-1⁺ compartment is heterogeneous. As expected in the normal adventitial environment, the Sca-1⁺CD45⁺ subpopulation displayed negligible expression of mature endothelial surface markers, with most of these antigens present on the numerically more abundant Sca-1⁺CD45⁻ cells, that were already known to have endothelial plasticity (229, 522). Despite this, adventitial Sca-1⁺CD45⁺ cells were found to possess striking angiogenic and vasculogenic capacity across a range of *in vitro*, *in vivo*, and gene profiling studies. This markedly exceeded that of adventitial Sca-1⁻CD45⁺ leukocytes when tested in MatrigelTM assays and the hindlimb ischaemia model. Although we had speculated that the angiogenic properties of the Sca-1⁺CD45⁺ subpopulation would be mediated indirectly through formation of paracrine supportive macrophages, the results of our study support *bona fide* endothelial plasticity and vasculogenesis. Sca-1⁺CD45⁺ cells transformed *in vitro* into a CD31⁺ state specifically under endothelial inductive, but not other culture conditions. Most remarkably they also generated intact vWF⁺ and CD31⁺ microvessels, as well as LYVE-1⁺ lymphatic vessels *in vivo*, with complementary evidence for direct contributions to adventitial and peri-adventitial microvessels in the carotid transfer experiment, and functional tissue perfusion in

the hindlimb model of ischaemia. Notably, the aortic Sca-1⁺CD45⁺ population increases in atherogenic mice after HCD (263), and this is accompanied by both an increase in aortic CFU-M yield and augmented adventitial angiogenic sprouting. Pending further work, these results provide some explanation for our other observation that Sca-1 and CD45 were co-expressed on the endothelium of adventitial and plaque microvessels in atherosclerotic *Apoe*^{-/-} mouse aortas and suggest the involvement of Sca-1⁺CD45⁺ cells in atherosclerotic *vasa vasorum* formation.

Since their original discovery from BM and peripheral blood (533), the definition, cell lineage and hierarchy of EPCs have remained controversial (534). Myeloid cells, mesenchymal stem cells and other cell types that are capable of promoting angiogenesis through indirect mechanisms rather than true endothelial differentiation, have notoriously been mislabelled as EPCs, despite being unable to form endothelial layers of vessels *de novo*. Within the existing literature about vascular-resident progenitor cells, Ingram *et al.* were the first to identify a clonogenic hierarchy of EPCs from isolates of human vascular endothelial cells, without localising the distribution of these cells to a mural layer of the vessel wall (267). Subsequently Zengin and co-workers located a CD34⁺ progenitor population in the inner adventitial lining of human internal thoracic arteries and inferred the contribution of these cells to capillary-like sprouts from *ex vivo* ring assays that expressed endothelial markers and were surrounded by CD45⁺ cells (220). Importantly, they noted that it was not possible to determine the exact source or fate of these adventitial CD45⁺ cells and found no evidence that CD45⁺ cells participated in capillary formation or co-expressed endothelial antigens. This is a key point of difference from the current study, where we directly compared different subpopulations of adventitial cells, and elucidated marked differences between the vasculogenic capacity and endothelial fate of the Sca-1⁺ (known to contain AMPCs) and Sca-1⁻ fractions [known to have a higher content of monocytes/macrophages but no AMPCs (521)] of the CD45⁺ compartment.

Stem cell antigen-1 is best known as an identifying marker of BM haematopoietic stem cells and different types of tissue-resident stem/progenitor cells in mice (535). We and others have reported the abundance of Sca-1 expression in the adventitia of postnatal mouse arteries, where it has been used to

identify and isolate vascular wall-resident stem cells (73, 262, 521-523, 536, 537). Other groups have ascribed plasticity for endothelial, smooth muscle and mesenchymal progeny, including fibroblasts, to murine adventitial Sca-1⁺CD45⁻ cells (522-524, 537, 538) and to corresponding adventitial progenitor cells in human vasculature (539, 540). Lineage-tracing has revealed that adventitial Sca-1⁺CD45⁻ cells do not originate from BM (229, 522, 536), nor do they possess any haematopoietic potential (521). Recently Majesky *et al.* used *in vivo* fate-mapping approaches, including tamoxifen-inducible *Myh11*-Cre transgenic mice, combined with a smooth muscle cell epigenetic lineage mark, to show that 30-60% of adventitial Sca-1⁺ cells are derived from differentiated smooth muscle cells (537). Almost 99% of these cells were Sca-1⁺CD45⁻, with the vast majority of adventitial Sca-1⁺CD45⁺ cells therefore originating from an alternative source.

Sca-1 has also been found on mature endothelial cells, which may themselves display endothelial plasticity in disease (229, 535, 541). Patel *et al.* recently identified a novel endothelial hierarchy present in different postnatal mouse tissues, including normal mouse aorta (229). Three subpopulations of endothelial cells were identified among CDH5⁺CD45⁻ cells, comprising CD31^{Lo}VEGFR2^{Lo} endovascular progenitors (EVPs), CD31^{Int}VEGFR2^{Lo} transit amplifying precursors and definitive differentiated CD31^{Hi}VEGFR2^{Hi} endothelial cells. Notably, each of these was identified only after excluding the CD45⁺ fraction, and each expressed Sca-1 (i.e. Sca-1⁺CD45⁻). In keeping with this and flow cytometry analysis reported in the Majesky study (537), we also observed that mature endothelial markers were exclusively expressed on the CD45⁻ subset of cells in non-atherosclerotic C57BL/6 aortas. However, this was not the case in atherosclerotic aortas from *Apoe*^{-/-} mice, nor in the adventitial vascular sprouts induced during *ex vivo* aortic ring assays. In both instances we found a surprisingly high level of Sca-1⁺CD45⁺ co-expression with a range of endothelial markers, including CD31, CDH5, VEGFR2 and TIE2.

In the study by Patel *et al.*, the self-renewing, clonal capacity of EVPs was established in a MatrigelTM-based assay (229), whereby EVPs from mouse aorta or tumour microenvironment required at least three days to form endothelial colonies, before producing branching outgrowths which increased in length

after day 4 through to day 7. We observed a similar time-frame of *de novo* cord formation from both Sca-1⁺CD45⁺ and Sca-1⁺CD45⁻ adventitial cells in Matrigel™, although there was a trend for greater capacity to do this for Sca-1⁺CD45⁺ cells. Our study clearly demonstrates the ability of aortic Sca-1⁺CD45⁺ progenitors to transdifferentiate away from the CD45⁺ myeloid lineage in the process of forming endothelial cells under vasculogenic conditions. Moreover, with loss of CD45 expression, the Sca-1⁺CD45⁺ fraction gave rise to a high percentage of Sca-1⁺CD45⁻ cells in Matrigel™, whereas the reverse did not occur. Interestingly, many of the angiogenic/vasculogenic genes found to be expressed highly in Sca-1⁺CD45⁺ cells, were also among those that were upregulated in EVPs compared to mature endothelial cells in the Patel study (229): *Mmp2*, *Mmp3*, *Il33*, *Sox9*, *Cd34*, *Egfr*. Therefore, it is evident that both the CD45⁺ and CD45⁻ subsets of the adventitial Sca-1⁺ population have potential to produce endothelial-fated progeny and participate in vasculogenesis. Questions now remain as to how these two subpopulations inter-relate developmentally, with our findings suggesting for the first time that Sca-1⁺CD45⁺ progenitors may be hierarchically ancestral to at least a subpopulation of Sca-1⁺CD45⁻ cells present in murine adventitia.

In combination with our previous results (521), this study has revealed the bipotent nature of the adventitial Sca-1⁺CD45⁺ population, with capacity to produce both myeloid (macrophage) and endothelial progeny. Although we did not add macrophage-specific growth factors to the Matrigel™ cord-forming assay, Sca-1⁺CD45⁺ cells still produced a small percentage of macrophages which selectively clustered at cord branch points. It followed that their depletion by clodronate impacted significantly on cord branching, indicating that the macrophage progeny of Sca-1⁺CD45⁺ progenitors acted as vascular fusion cells to support endothelial tip cell anastomosis, as has been described by others (525). Although our carotid and hindlimb adoptive transfer experiments demonstrated the ability of Sca-1⁺CD45⁺ cells to produce endothelial-lined neovessels *in vivo*, it should be noted that we did not quantify the proportion of donor cells that were able to do this nor their relative predisposition to producing macrophages versus endothelium.

The lack of a significant increase in capillary or arteriolar density 14 days after Sca-1⁺CD45⁺ cells were injected into ischaemic hindlimbs, was at odds with the significant augmentation in perfusion ratio that these cells achieved compared to cell-free control. There are several possible reasons for this discrepancy. Firstly, C57BL/6 mice have an inherent ability to quickly undergo spontaneous angiogenesis-mediated recovery after artery ligation, even in the absence of treatment, which may make it difficult to detect subtle differences in capillary density. Secondly, other mechanisms in addition to angiogenesis and arteriogenesis are involved in the reperfusion response to hindlimb ischaemia. These include skeletal muscle regeneration and necrotic tissue clearance which were not measured here. Finally, although we quantified capillary and arteriolar density from three randomly selected 5 µm-thick sections of gastrocnemius muscle obtained from each animal, these measures may be vulnerable to a degree of sampling bias.

It is important to emphasise that like other studies that have reported on the multipotency of different adventitial progenitor cells (522, 537, 539, 540), our findings still relate to population biology, and do not prove multipotency at the level of individual cells. We therefore cannot yet claim to have proven the presence of haemangioblasts in postnatal vasculature. The notion that such cells exist was introduced in the 1930s, borne out of the intimate relationship between developmental vasculogenesis and primitive haematopoiesis in extra-embryonic YS (542). Yolk sac EMPs are now well known to give rise to self-renewing macrophages that reside in different tissues, including those found in murine adventitia especially in early postnatal life (168). Most recently, they have also been shown to generate vascular endothelium in both developing and postnatal mouse brain (132). We have already determined that the AMPC subset of adventitial Sca-1⁺CD45⁺ cells are not derived from BM or splenic haematopoiesis (521). Although we did not exclude a BM source for the vasculogenic Sca-1⁺CD45⁺ progenitor cells described here, results from studies of other types of vascular wall EPCs suggest that this is unlikely (229).

Directions for future research are therefore to determine at a clonal or single cell level whether postnatal adventitial Sca-1⁺CD45⁺ progenitors contain *bona fide* haemangioblasts, and if so, to elucidate their

embryonic origins. Other objectives will be to determine whether the vasculogenic properties of these cells are adaptive or maladaptive in diseases such as atherosclerosis; whether similar cells are present in human arteries, where Sca-1 cannot be used as a candidate marker for their identification; and whether there are corresponding populations of CD45⁺ progenitors residing around the microvasculature of other tissues, that might participate in vasculogenic responses to wound healing, ischaemia and cancer.

3.5 Conclusion

In conclusion, this chapter identified that in addition to serving as a local source of macrophage expansion in the mouse vasculature, adventitial Sca-1⁺CD45⁺ progenitor cells are also vasculogenic, with potential relevance to the origins of *vasa vasorum* formation in atherosclerosis.

Chapter 4

Yolk sac-derived bipotent Endothelial-Macrophage progenitor cells are a self-renewing tissue-resident source of inflammation and neovascularisation postnatally

4.1 Introduction

Among diverse roles, macrophages are integral to development of blood and lymphatic vessels during normal organogenesis and responses to tissue injury, ischaemia and other diseases (525, 543-545). They proliferate and assemble around neovessels, producing angiogenic factors and supporting endothelial anastomoses and vascular remodelling (525). In return, endothelial cells help regulate the self-renewal of haematopoietic stem cells (HSCs) and their differentiation into macrophages (546). Understanding how macrophage-endothelial interactions arise is important to more effectively target inflammation and neovascularisation in different pathophysiological conditions.

Historically, circulating monocytes were thought to be the source of tissue macrophages (547). Monocytes derive from definitive haematopoiesis, which originates embryonically with emergence of HSC clusters from the endothelium of the aorta-gonad-mesonephros (AGM) at around embryonic day (E) 10.5 in mice (548-550). These HSCs seed foetal liver before colonizing bone marrow (BM) perinatally, the main haematopoietic organ after birth (550). Numerous studies have now established that HSC-monocyte ancestry does not account for all tissue macrophages (98, 168, 551-555). During embryogenesis, extra-embryonic yolk sac (YS) is the first site to produce macrophages via distinct developmental programs. This begins with primitive macrophage precursors in YS blood islands between E7.0 and E8.25 (109, 556), followed by erythromyeloid progenitors (EMPs) which bud from specialised YS haemogenic endothelium (557) and comprise two waves (109). From E7.5, early c-Kit⁺CSF1R⁺ EMPs produce erythrocytes, megakaryocytes and YS macrophages in a process that is independent of the transcription factor, Myb (551, 555, 558). This does not involve monocyte intermediates but rather sequential differentiation into CX₃CR1⁺ pre-macrophages and mature F4/80^{Hi} macrophages (24, 30, 556, 559). Macrophage progenitors, in both multipotent EMP and pre-macrophage stages, expand in YS and circulate to embryonic tissues to complete their maturation (560). This migration peaks around E10.5 and is mostly complete by E12.5. From E8.5, late Myb⁺c-Kit⁺CSF1R^{Lo} EMPs also differentiate into YS macrophages and traffic to liver, where they expand and

generate lineage-specific haematopoietic progenitors and foetal blood cells, including monocytes (30, 561).

Long-lived populations of YS-derived macrophages persist in adult tissues, including brain, skin, liver, heart, lung and aortic adventitia (98, 168, 551-555, 562). These are maintained independently of BM haematopoiesis through local proliferation and seemingly, by self-renewal (178). With the exception of brain microglia that originate from the initial pool of YS-derived macrophages (98), controversy remains as to whether other tissue macrophages are seeded from early EMPs (without monocyte intermediates) (555) or late EMPs (via foetal liver monocytes) (561).

Recent studies indicate that YS early EMPs also contribute endothelial cells to the blood and lymphatic vessels of some organs (132, 545). Although disputed (563), the possibility that EMPs produce both haematopoietic and endothelial cells is intriguing, especially given long-standing speculation around the embryonic existence of mesoderm-derived, bipotent haemangioblasts (542). While haemangioblasts have been shown to emerge during haemato-endothelial differentiation of pluripotent stem cells *in vitro* (564, 565), their presence in postnatal tissues remains unproven (73). In Chapter 3, we identified that the adventitia of adult mouse arteries contains Sca-1⁺CD45⁺ progenitors, which selectively generate macrophage colony-forming units (CFU-M) in methylcellulose and produce macrophages and endothelial-lined neovessels after *in vivo* transfer (262, 521, 566). As the adventitial Sca-1⁺CD45⁺ population is heterogeneous, our studies stopped short of claiming the bipotency of these progenitors at a clonal level. Here, we establish the identity of postnatal tissue-resident CFU-M progenitors as self-renewing, bipotent endothelial-macrophage (EndoMac) progenitor cells, that originate from an early YS source and are seeded in aorta, heart and lung during foetal development.

4.2 Methods overview (also see Chapter 2)

Breeding pairs of C57BL/6 (C57BL/6J), *Cx3cr1*^{GFP/+} (B6.129P-Cx3cr1^{tm1Litt}/J), *Ccr2*^{-/-} (B6.129S4-Ccr2^{tm1Ifc}/J), *Flt3*^{Cre} (Tg.Flt3^{cre}/ERT2), *Rosa*^{mT/mG} (Gt(ROSA)26Sor^{tm4}(ACTB-tdTomato,-EGFP)^{Luo}/J), *Csf1r*^{Mer-iCre-Mer} (FVB-Tg(Csf1r-cre/Esr1*)1Jwp/J), *Cx3cr1*^{YFP-creER} (B6.129P2(Cg)-Cx3cr1^{tm2.1}(cre/ERT2)^{Litt}/WganJ), *UBI-GFP* (C57BL/6-Tg(UBC-GFP)30Scha/J) mouse strains were acquired from The Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed in the animal care facilities at SAHMRI, and were maintained on standard chow diet. In embryonic experiments, date of embryonic development was defined on the basis of the date of vaginal plug formation, which was set at E0.5, as detailed in Chapter 2. Aorta, heart and lung from these mice were prepared and used for *in vitro* various assays including, methylcellulose colony forming assay, Matrigel-based aortic rings and tube-forming assays, tissue immunofluorescence staining and confocal microscopy, and multi-colour flow cytometry. Murine models of hindlimb ischaemic and AngII-induced osmotic minipumps were used for an *in vivo* model and tissue immunofluorescence staining was performed on the respective tissues from these models. All animal experiments were performed in accordance with the standards stated in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Academy of Sciences, Bethesda, MD, USA) and were approved by SAHMRI (ID SAM117, SAM155, SAM244, SAM308 and SAM432.19) animal ethics committee. All experimental procedures and animal care were performed in accordance with the National Health and Medical Research Council of Australia research guidelines and Australian Code for the Care and Use of Animals for Scientific Purposes.

4.2.1 Statistical analysis

Where relevant, data analysis was performed blinded to study group. Statistical analyses were performed using Prism (GraphPad version 9). Data sets were tested for normality of distribution by Shapiro-Wilk test. Statistical comparisons were performed with parametric or non-parametric unpaired or paired two-sample *t*-tests or ANOVA (with post-test multiple comparisons), as specified. Results are expressed as mean $\pm$ standard deviation of multiple experiments. In all cases, statistical significance was established at two-tailed $p < 0.05$. The statistical parameters and the number of mice used per experiment are found in the figure legends.

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4.3 Results

The results have been prepared as a single manuscript, which has been uploaded to BioRxiv (identifier doi: <https://doi.org/10.1101/2021.10.18.464001>) and is being prepared for submission to a journal for publication. The figures in this Chapter have been re-formatted to accommodate both the main (**Figures 4-1, 4-4, 4-7, 4-9, 4-10, 4-12, 4-13**) and supplementary figures (**Figures 4-2, 4-3, 4-5, 4-6, 4-8, 4-11, 4-14**) of this manuscript.

4.3.1 c-Kit, CX₃CR1 and CSF1R identify highly proliferative, self-renewing CFU-M progenitors in postnatal aorta

To establish the precise identity of CFU-M forming cells from mouse aorta, we cultured aortic digests from 12 week-old (w) C57BL/6J mice in methylcellulose (MethoCult GF M343, StemCell Technologies) for 14 days (d). This generated 17.0 ± 12.0 CFU-M/ 10^5 cells (n=10), with colonies further classified based on size as ~72% small (~30-100 cells), ~27% medium (~100-1000) and <1% large (>1000) (**Figure 4-1a**). Re-culturing the content of primary (1°) cultures for another 14 d resulted in secondary (2°) CFU-M yield that was more than ~12-fold that of 1° cultures, indicating colony renewal and expansion (**Figure 4-1b**). To study this further, we isolated individual 1° CFU-M and disaggregated each colony into single cell suspensions that were re-plated in separate wells. Importantly, this showed that CFU-M renewed from single cell origins, with 1 new colony produced per ~15 cells (**Figure 4-1c**).

Having established that CFU-M renew clonally, we used flow cytometry to identify their cellular composition. 1° CFU-M contained a homogeneous population that was negative for lineage (LIN) and monocyte/macrophage markers (CD11b, F4/80), but positive for CD45, Sca-1, c-Kit, CX₃CR1 and CSF1R (**Figure 4-1d**). This immunophenotype was similarly enriched in 2° CFU-M, confirming self-renewal (**Figure 4-1e**). CFU-M also expressed the haematopoietic progenitor and EMP markers, CD34, CD16/32, CD93 (AA4.1) and CD43 (567), but not CD41 (very early-stage haematopoiesis), Flt3

(CD135) (definitive haematopoiesis) or Brachyury (haemangioblast) (**Figure 4-2**). The stem cell marker, c-Myc, which regulates self-renewal was expressed on $25.0 \pm 5.4\%$ cells, while Sox2, Nanog and Oct4 which are also associated with pluripotency, were negative. Apart from CX₃CR1 and CSF1R, other mature myeloid markers were negative (CCR2, CD206, CD86, MerTK, MHCII, Ly6C, Gr-1) or lowly expressed (CD64, CD24). CFU-M were also negative for the lymphatic and macrophage marker, LYVE-1, the erythroid marker, Ter119, and the endothelial markers, CDH5 (CD144), VEGFR2 and TIE2, although they did express CD31, which is also found on myeloid progenitors (556). Importantly, we identified LIN⁻CD45^{+/Lo}CD11b⁻F4/80⁻c-Kit⁺Sca-1⁺CX₃CR1⁺CSF1R⁺ progenitors in digests of C57BL/6 aorta ($0.3 \pm 0.3\%$ or 3270 ± 2937 cells per aorta, n=20), mirroring the cell surface phenotype of cultured CFU-M (**Figure 4-1f**). Bromodeoxyuridine (BrdU) uptake revealed that these are actively proliferating, with three-fold more of these progenitors in S-phase of cycle than CD11b⁺F4/80⁺ macrophages (**Figure 4-1g**).

Fractalkine receptor, CX₃CR1, has been used to characterise tissue-resident macrophages, including those in adventitia, and to trace their embryonic origins (168, 552, 554). As CFU-M progenitors expressed CX₃CR1, we studied *Cx3cr1*^{GFP/+} mice, which express green fluorescent protein (GFP) under control of the *Cx3cr1* locus (568). CFU-M from *Cx3cr1*^{GFP/+} aortas were GFP⁺ under fluorescence microscopy and flow cytometry (**Figure 4-3a**). Whereas other studies have focused on CX₃CR1 expression by aortic macrophages (168), we also identified CX₃CR1⁺c-Kit⁺ progenitors in aortic adventitia (**Figure 4-1h**) and determined by flow cytometry that they account for $18.9 \pm 4.7\%$ of CX₃CR1⁺ cells in the aortic wall (n=6) (**Figure 4-3b**). Fluorescence activated cell sorting (FACS) of *Cx3cr1*^{GFP/+} aortic cells showed that CFU-M forming capacity was restricted to GFP⁺ (especially GFP^{Hi}) cells (**Figure 4-3c**), and specifically GFP⁺CD11b⁻F4/80⁻ progenitors, not GFP⁺CD11b⁺F4/80⁺ macrophages (**Figure 4-1i, 4-3d**).

As evidence of the functional importance of CX₃CR1, addition of its ligand, CX₃CL1, resulted in 1.6-fold higher CFU-M yield from aortic cells (**Figure 4-1j**), similar to the effect of macrophage colony-stimulating factor (M-CSF), the ligand for CSF1R (**Figure 4-1k**). We also compared *Cx3cr1*^{GFP/+} mice

to *Cx3cr1*^{GFP/GFP} littermates, that lack both functional *Cx3cr1* alleles. *Cx3cr1*^{GFP/GFP} aortas had almost four-fold lower CFU-M yield (**Figure 4-1l**) and contained ~60% fewer progenitors by flow cytometry (**Figure 4-1m**). Therefore, CX₃CR1 is both a marker of self-renewing CFU-M progenitors and promotes their clonogenicity and prevalence in aorta.

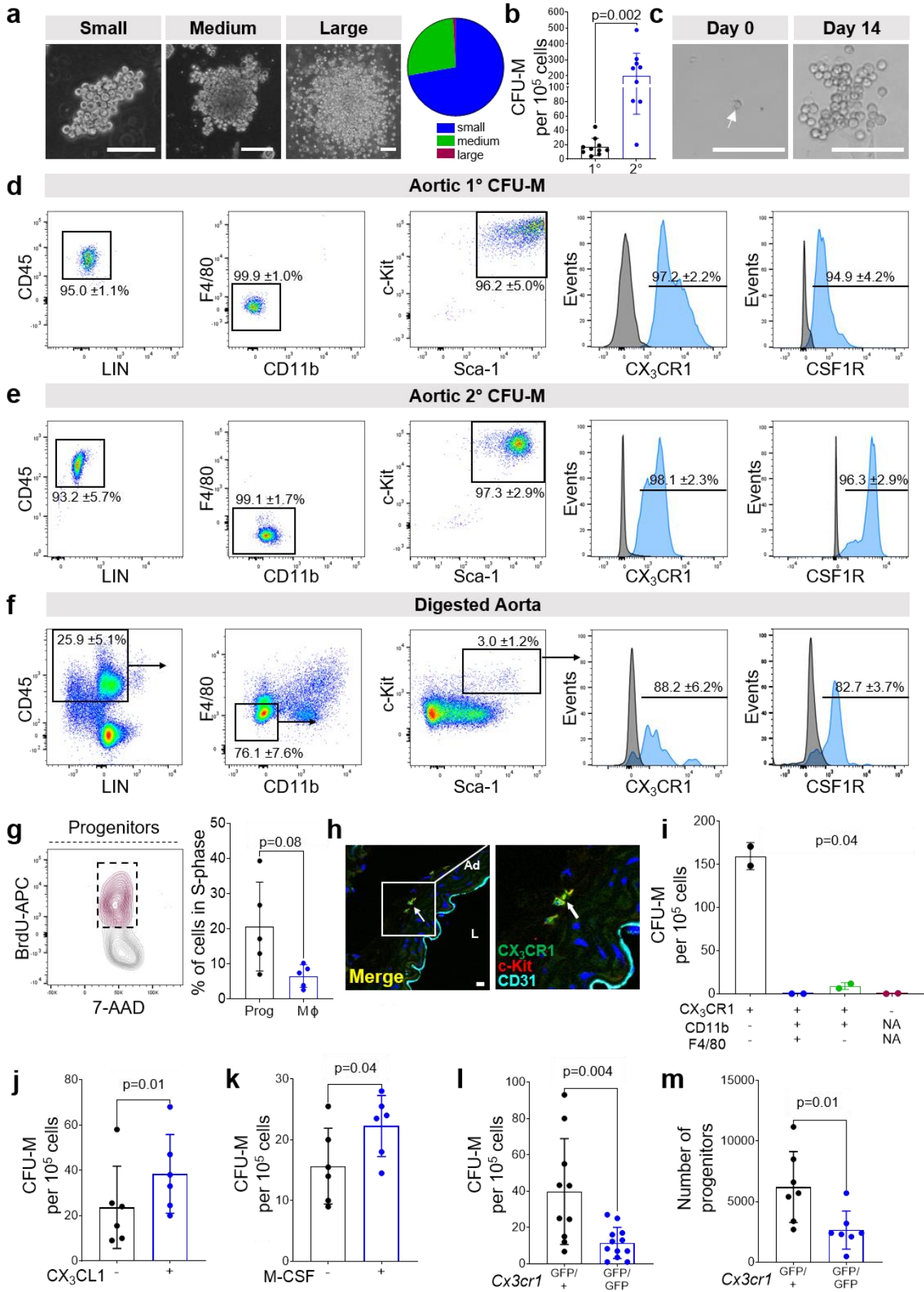


Figure 4-1. c-Kit, CX₃CR1 and CSF1R identify self-renewing aortic CFU-M progenitors.

(a) Examples of small, medium and large CFU-M from 12 w C57BL/6 aortic cells. Pie chart shows breakdown of CFU-M by colony size. N=5 mice. Scale bar, 100 μ m.

(b) Donor-matched primary (1°) and secondary (2°) CFU-M yield from 12 w C57BL/6 aorta. N=10 mice. Paired *t*-test.

(c) Self-renewal of 2° CFU-M from single cell obtained from 1° CFU-M. Scale bar, 100 μ m.

(d, e) Flow cytometry of cells from **(d)** 1° (n=3 mice) and **(e)** 2° aortic CFU-M (n=4 mice). Blue histogram, Sample. Dotted, FMO control.

(f) Flow cytometry of aortic digest shows progenitor population. N=4.

(g) Flow cytometry of bromodeoxyuridine (BrdU) versus 7-aminoactinomycin (7-AAD) gated from aortic progenitors. Dotted box, S-phase. Percentages of progenitors (Prog) and macrophages (M ϕ) in S-phase from 12 w C57BL/6 aorta. N=5 mice. Paired *t*-test.

(h) Confocal microscopy of descending aorta from 12 w *Cx3cr1*^{GFP/+} mouse shows adventitial (Ad) CX₃CR1⁺c-Kit⁺ progenitors. L, Lumen. Scale bar, 20 μ m. See **Appendix 1** for bigger images.

(i) CFU-M yield from FACS-sorted aortic populations from 12 w *Cx3cr1*^{GFP/+} mice (two experiments, six aortas each). N/A, Not applicable.

(j, k) CFU-M yield from 12 w C57BL/6 aortic cells in presence of **(j)** 100 nM CX₃CL1 or **(k)** 50 nM M-CSF. N=6 mice. Paired *t*-tests.

(l, m) Frequency of **(l)** CFU-M and **(m)** progenitors from aortas of 12 w *Cx3cr1*^{GFP/+} and *Cx3cr1*^{GFP/GFP} mice. N=7-10 mice/gp. Unpaired *t*-tests.

Data summarised as mean $\pm$ SD.

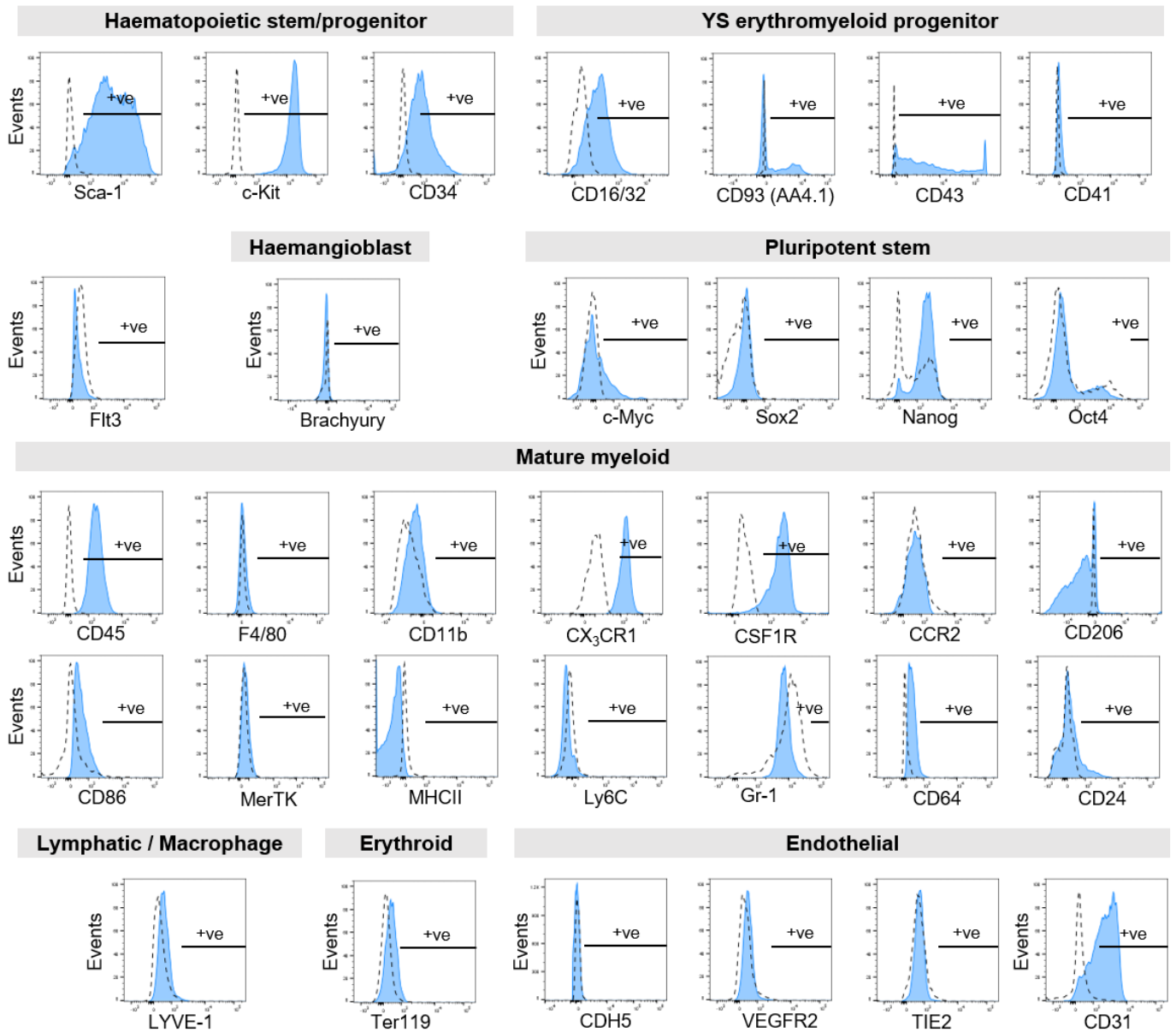


Figure 4-2. Surface marker expression of progenitors contained within 1° aortic CFU-M.

Flow cytometry histograms depict expression of different types of surface markers on cells from primary (1°) aortic CFU-M (blue histogram). Dotted histogram, FMO control. Representative of n=3 12 w C57BL/6 mice.

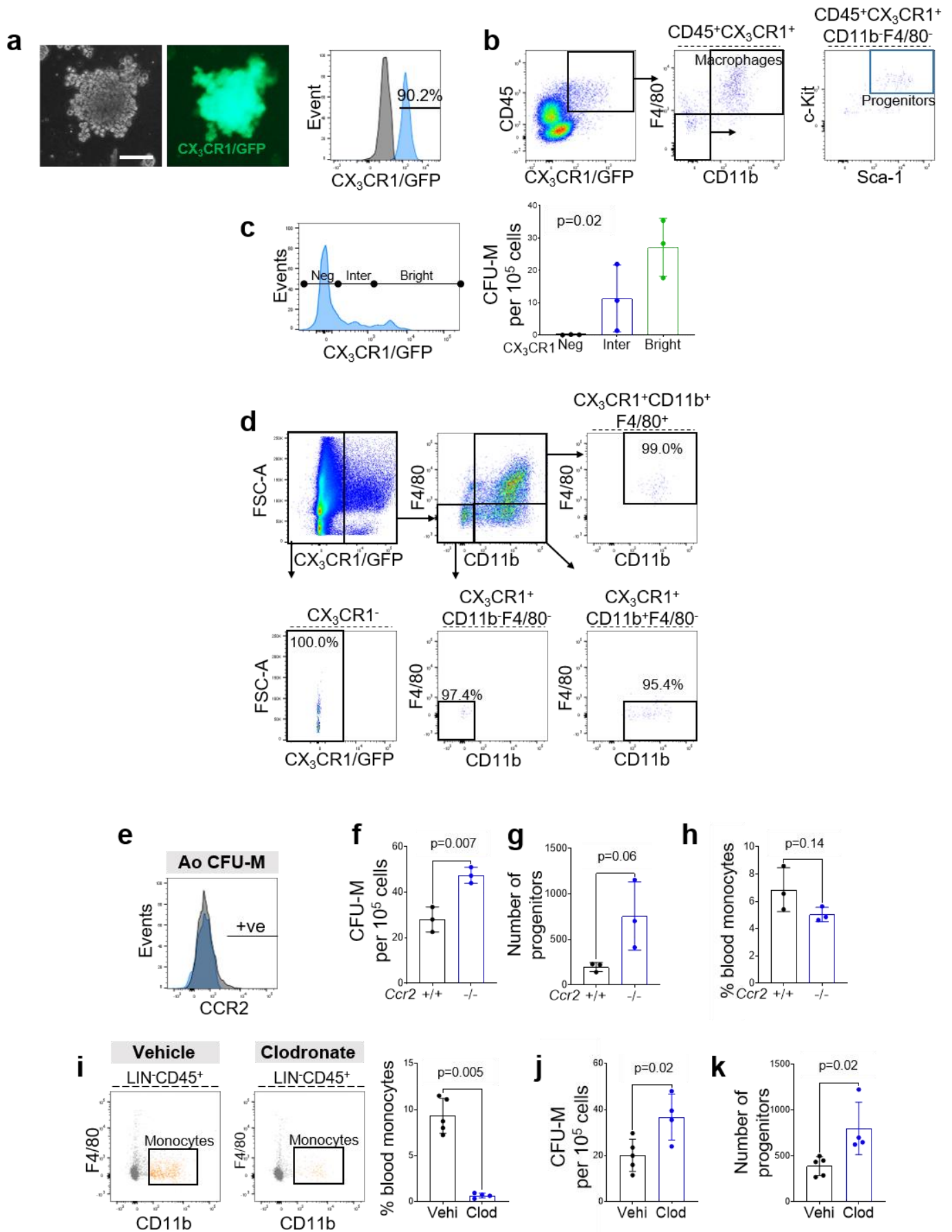


Figure 4-3. Aortic CFU-M progenitors express CX₃CR1 and are not dependent on blood monocytes.

(a) Fluorescence microscopy and flow cytometry (blue histogram) show GFP⁺ (CX₃CR1⁺) expression of cells contained in CFU-M from 12 w *Cx3cr1*^{GFP/+} aortas. Grey histogram, C57BL/6 control. N=4 mice. Scale bar, 100 μ m.

(b) Flow cytometry shows presence of CD11b⁺F4/80⁻Sca-1⁺c-Kit⁺ progenitors and CD11b⁺F4/80⁺ macrophages (M ϕ) among CD45⁺CX₃CR1⁺ cells in aortas of 12 w *Cx3cr1*^{GFP/+} mice. Representative of N=6 mice.

(c) FACS isolation of CX₃CR1^{Neg}, CX₃CR1^{Interm} and CX₃CR1^{Bright} populations from 12 w *Cx3cr1*^{GFP/+} aortas. Graph shows 1^o CFU-M yield from each population (three experiments, six aortas each). Repeated measures one-way ANOVA.

(d) Gating strategy and purity checks for FACS isolation of aortic cells from 12 w *Cx3cr1*^{GFP/+} mice into CX₃CR1⁺CD11b⁺F4/80⁻, CX₃CR1⁺CD11b⁺F4/80⁺, CX₃CR1⁺CD11b⁺F4/80⁻ and CX₃CR1⁻ populations, used in **Figure 4-1i**.

(e) Absence of CCR2 expression on surface of progenitor cells from 1^o aortic CFU-M (blue histogram). Grey histogram, FMO control. N=3 mice/gp.

(f-h) Graphs show comparisons between 12 w *Ccr2*^{+/+} and *Ccr2*^{-/-} mice for **(f)** aortic CFU-M yield, **(g)** number of progenitors in aorta and **(h)** percentage of blood monocytes, as measured by flow cytometry. N=3 mice/gp. Unpaired *t*-tests.

(i-k) 12 w C57BL/6 mice were administered *i.p.* clodronate (Clod) or vehicle (Vehi) liposomes for three days. Graphs show comparisons for **(i)** blood monocytes, **(j)** aortic CFU-M yield and **(k)** number of progenitors in aorta. N=4-5 mice/gp. Unpaired *t*-tests.

Data summarised as mean $\pm$ SD.

4.3.2 Tissue CFU-M progenitors are independent of definitive haematopoiesis

C-C chemokine receptor 2 (CCR2), the cognate receptor for C-C chemokine ligand 2 (CCL2), has been used to differentiate between monocyte-derived (CCR2⁺) and locally maintained, embryonically derived (CCR2⁻) macrophages in some tissues (569). Unlike CX₃CR1, CCR2 was not expressed on aortic CFU-M progenitors (**Figure 4-3e**). Aortic cells from *Ccr2*^{-/-} mice formed more CFU-M than wildtype littermates, with a trend toward higher progenitor counts, despite fewer circulating monocytes (**Figure 4-3f-h**). Similarly, a disconnect between blood monocyte and aortic progenitor numbers was also seen when clodronate liposomes were used to deplete monocytes, suggesting independence between the two (**Figure 4-3i-k**).

These results led us to examine the relationship between CFU-M progenitors and definitive BM haematopoiesis by using *Flt3*^{Cre} x *Rosa*^{mT/mG} mice. BM HSCs transiently upregulate the receptor tyrosine kinase Flt3 during haematopoietic differentiation (570). In *Flt3*^{Cre} x *Rosa*^{mT/mG} mice, cells that originate from definitive haematopoiesis express GFP (Flt3-Cre⁺), while those that do not are GFP⁻ and express tdTomato (tdTom) (Flt3-Cre⁻). Using these and C57BL/6 mice, we identified that digests of heart and lung also produce CFU-M that contain progenitors with the same immunophenotype as aorta (**Figure 4-5a-c**). CFU-M from all three tissues from *Flt3*^{Cre} x *Rosa*^{mT/mG} mice were >95% Flt3-Cre⁻, while BM produced a mixture of CFU-M and non-macrophage colonies that were >90% Flt3-Cre⁺ (**Figure 4-4a,b**). Similarly, although blood monocytes and LIN⁻c-Kit⁺Sca-1⁺ BM HSCs were Flt3-Cre⁺ (**Figure 4-5d,e**), aorta, heart and lung contained LIN⁻CD45^{+/Lo}CD11b⁻F4/80⁻Sca-1⁺c-Kit⁺ progenitors that were predominantly Flt3-Cre⁻ (**Figure 4-4c**). In keeping with other studies (168, 554), these tissues also contained both Flt3-Cre⁺ and Flt3-Cre⁻ macrophages, whereas BM macrophages were predominantly Flt3-Cre⁺ and brain microglia Flt3-Cre⁻ (**Figure 4-5f**). Together, these results show that postnatal tissue CFU-M progenitors are not derived from definitive haematopoiesis.

4.3.3 Tissue CFU-M progenitors are seeded embryonically

To elucidate the origins of tissue CFU-M progenitors, we next performed age profiling after birth. Aortic CFU-M yield was ~10-fold higher from P1 (postnatal day 1) than 3 w and 12 w mice, and lowest from 52 w mice (**Figure 4-4d**). P1 CFU-M formed more quickly, comprised more medium and large colonies and had greater capacity for self-renewal (**Figure 4-6a,b**). Flow cytometry supported the higher abundance of progenitors in P1 aorta and their declining prevalence as mice age (**Figure 4-4e**). Similar results were obtained for heart and lung (**Figure 4-6c-g**).

As tissue CFU-M progenitors are present at birth, we examined their emergence during embryonic development. CFU-M grew from digests of YS, beginning around E7.5 and with higher yield from E8.5 onwards (**Figure 4-4f**). They made up 99% of all colonies grown from YS at E7.5, 90% at E8.5 and 85% at E9.5 (**Figure 4-6h**). YS CFU-M from *Cx3cr1^{GFP/+}* embryos expressed the same surface marker phenotype as CFU-M from postnatal tissues (**Figure 4-4g**) and were negative for Flt3 (**Figure 4-6i**). Compared to adult aortic CFU-M, YS CFU-M were larger in size (**Figure 4-6g**), with higher self-renewal capacity (**Figure 4-6b**) and higher expression of Sox2 and c-Myc, suggesting a more primitive state (**Figure 4-6i**). Although CFU-M did not grow from digests of whole embryo at E8.5 or E9.5, they formed from AGM, heart and brain from E10.5 onwards (**Figure 4-4f**). Coinciding with the emergence of definitive HSCs from dorsal aorta at ~E10.5-11.5 (550), we also observed non-macrophage colonies (G, granulocyte; GM, granulocyte-macrophage; GEMM, granulocyte-erythrocyte-monocyte-megakaryocyte; BFU-E, burst-forming units-erythroid) from AGM during this gestational window (**Figure 4-6j**). However, unlike CFU-M these disappeared by E12.5. Colony growth from liver increased between E10.5 and E11.5 and consisted mostly of non-macrophage colonies through to E15.5, consistent with seeding of HSCs and multipotent progenitors from AGM and YS (30, 561) (**Figure 4-6k**).

Flow cytometry showed a similar time-course for the appearance of CD45^{+Lo}c-Kit⁺CX₃CR1⁺ cells in YS from E8.5 and AGM from E10.5 (**Figure 4-4h-i**), while confocal microscopy identified c-Kit⁺CX₃CR1⁺ progenitors in aortic adventitia at E12.5 and E15.5 (i.e. after disappearance of definitive HSCs from AGM) (**Figure 4-4j**), as well as in heart and lung (**Figure 4-6l**).

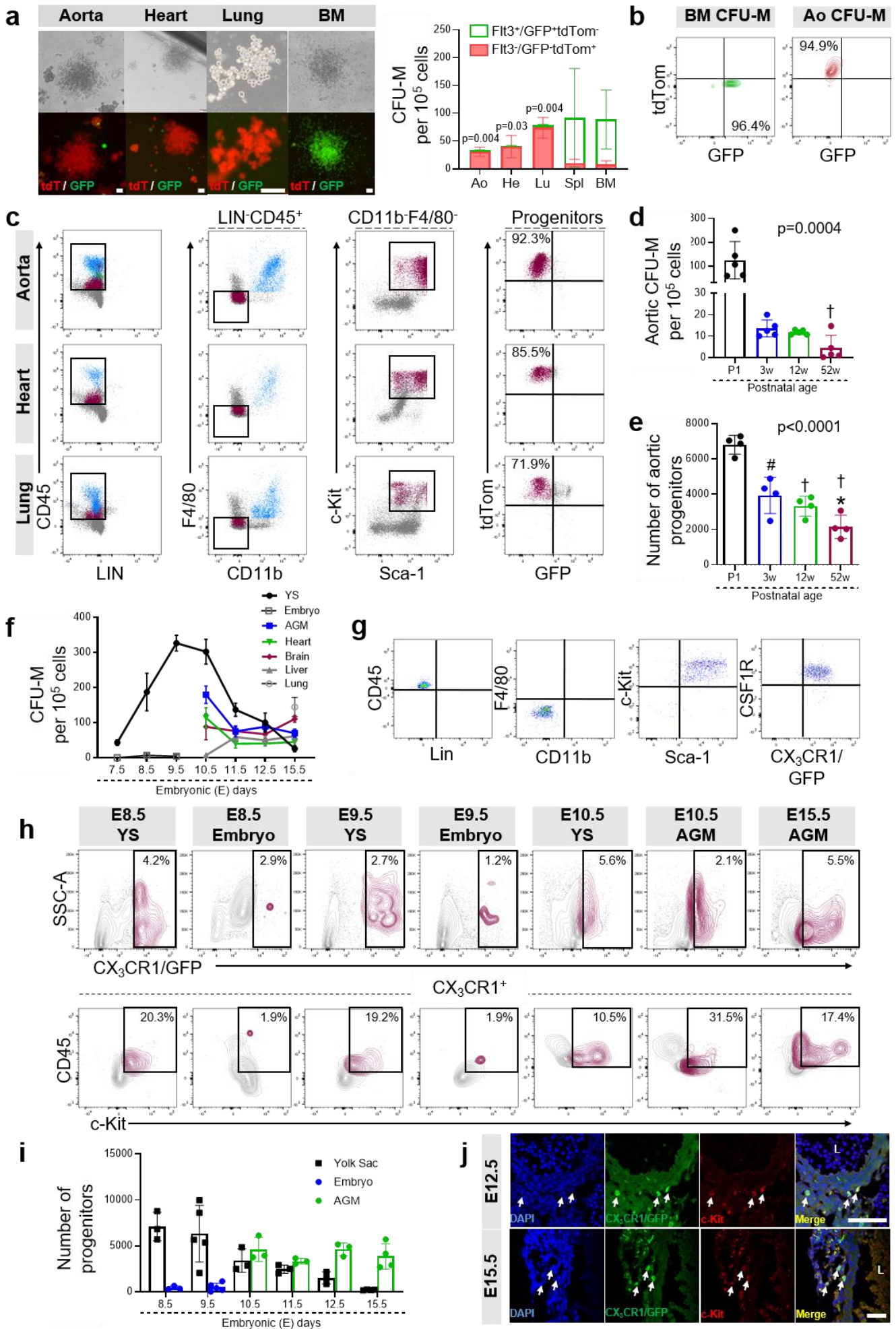


Figure 4-4. Tissue CFU-M progenitors are seeded embryonically and are independent of Flt3⁺ haematopoiesis.

(a) Light and fluorescence microscopy of CFU-M from aorta, heart, lung and BM of adult *Flt3^{Cre}* x *Rosa^{mT/mG}* mice. Graph of Flt3⁺ and Flt3⁻ CFU-M yield from different tissues. N=5 mice. Spl, Spleen. Paired *t*-tests for each tissue. Scale bar, 100 μ m.

(b) GFP and tdTom expression of cells in CFU-M from *Flt3^{Cre}* x *Rosa^{mT/mG}* BM and aorta.

(c) Flow cytometry plots show Flt3⁻ progenitors (maroon) in aorta, heart and lung of *Flt3^{Cre}* x *Rosa^{mT/mG}* mice. Blue dots, Macrophages. Percentages represent mean of n=4 mice.

(d, e) Age comparisons of **(d)** CFU-M yield (Kruskal-Wallis) and **(e)** number of progenitors (one-way ANOVA) for C57BL/6 aortas. N=3-6 mice/gp. Multiple comparison tests: **p*<0.05 for 3 w vs 52 w; †*p*<0.01 and #*p*<0.0001 for comparison to P1.

(f) CFU-M yield from different tissues of *Cx3cr1^{GFP/+}* mice at different embryonic ages. N=3-9 mice/gp.

(g) Flow cytometry of cells from YS CFU-M. N= 3 mice.

(h) Flow cytometry of digests of YS, whole embryo and AGM from *Cx3cr1^{GFP/+}* mice at different embryonic ages show CX₃CR1⁺CD45^{+/Lo}c-Kit⁺ progenitors. N=3-5 mice/gp.

(i) Number of progenitors in YS, whole embryo or AGM at different embryonic ages. N=3-5 mice/gp.

(j) Confocal microscopy of E12.5 and E15.5 AGM from *Cx3cr1^{GFP/+}* mice shows adventitial CX₃CR1⁺c-Kit⁺ progenitors (arrows). L, Lumen. Scale bar, 50 μ m. See **Appendix 2** for bigger images.

Data summarised as mean or mean $\pm$ SD.

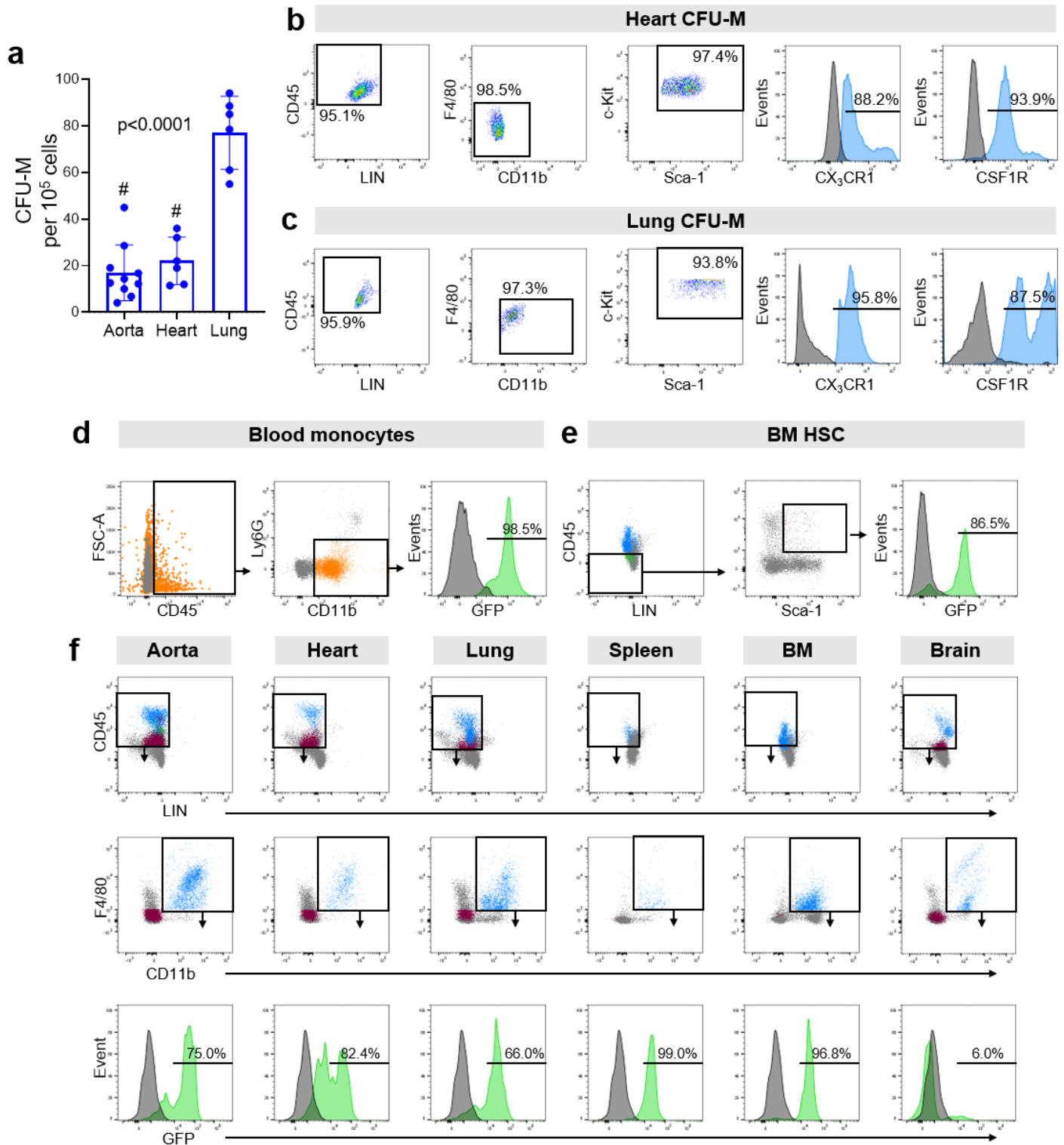


Figure 4-5. Identification of CFU-M progenitors in murine heart and lung and Flt3 status of macrophages in different postnatal tissues.

(a) 1° CFU-M yield from aorta, heart and lung from 12 w C57BL/6 mice. N=6-9 mice. One-way ANOVA. Multiple comparisons test: # p<0.0001 vs lung.

(b-c) Surface marker expression of cells in **(b)** heart and **(c)** lung CFU-M. N=3 mice. Blue histograms, Sample. Grey, FMO control.

(d-f) Flow cytometry profiling of GFP⁺ (Flt3-Cre⁺) expression on **(d)** blood monocytes, **(e)** LIN⁻c-Kit⁺Sca-1⁺ BM haematopoietic stem cells (HSCs) and **(f)** LIN⁻CD45⁺CD11b⁺F4/80⁺ macrophages from different tissues of adult *Flt3*^{Cre} x *Rosa*^{mT/mG} mice. Orange dots, Monocytes; Blue, Macrophages; Maroon, Progenitors; Green histogram, Sample; Grey, *Flt3*^{Cre} control. N=4 mice.

Data summarised as mean±SD in **(a)** and as mean percentage expression in **(b-f)**.

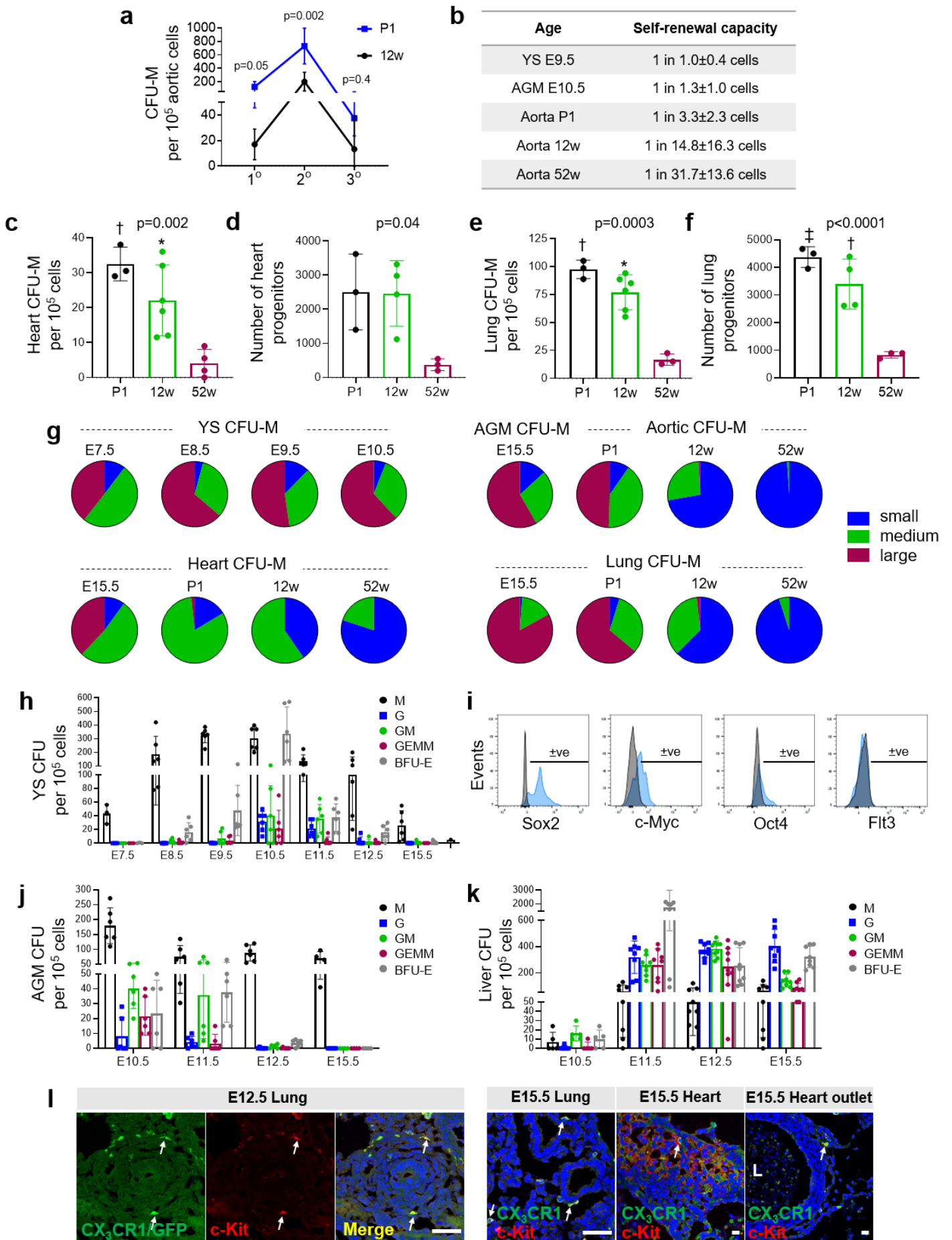


Figure 4-6. Profiling of CFU-M progenitors according to postnatal and embryonic age.

(a) CFU-M yield from primary (1°), secondary (2°) and tertiary (3°) cultures of aortic cells from P1 and 12 w C57BL/6 mice. N=5-10 mice/gp. p-values are from unpaired *t*-tests comparing P1 and 12 w results for each passage.

(b) CFU-M self-renewal capacity from YS, AGM and aorta at different ages, expressed as the number (mean±SD) of 1° CFU-M progenitors required to generate one 2° CFU-M. N=3-5 mice/age.

(c-d) Age comparisons of the **(c)** 1° CFU-M yield and **(d)** number of progenitors for C57BL/6 heart. N=3-6 mice/age. One-way ANOVA for **(c)** and Kruskal-Wallis test for **(d)**. Multiple test comparisons: **p*<0.05 and †*p*<0.01 for comparison to 52 w.

(e-f) Age comparisons of the **(e)** 1° CFU-M yield and **(f)** number of progenitors for C57BL/6 lung. N=3-6 mice/age. One-way ANOVA for both. Multiple test comparisons: **p*<0.05, †*p*<0.01 and ‡*p*<0.001 for comparison to 52 w.

(g) Pie charts show classification of CFU-M based on colony size for YS, AGM, heart and lung at different embryonic ages. N=3-6 for each.

(h) Frequency of different CFU types from YS at different embryonic ages post conception. M, Macrophage; GM, Granulocyte-macrophage; GEMM, Granulocyte-erythrocyte-monocyte-megakaryocyte; G, Granulocyte; BFU-E, Burst-forming units-erythroid.

(i) Flow cytometry expression of select surface markers for cells contained within YS CFU-M. N=3 mice. Blue histogram, Sample. Grey, FMO control.

(j-k) Frequency of different CFU types from **(j)** AGM and **(k)** liver at different embryonic ages.

(l) Confocal microscopy of CX₃CR1⁺c-Kit⁺ progenitor cells (arrows) in lung and heart at E12.5 and E15.5 from *Cx3cr1*^{GFP/+} embryos. L, Lumen. Scale bar, 50 μm. See **Appendix 3** for bigger images.

Data summarised as mean±SD.

4.3.4 Tissue CFU-M progenitors originate from CX₃CR1⁺ and CSF1R⁺ YS progenitors

Murine aortic adventitia has been shown to contain locally maintained macrophages derived from CX₃CR1⁺ and CSF1R⁺ YS progenitors (168, 571). As CFU-M progenitors appear in YS before AGM, we used timed fate-mapping approaches to confirm their YS origins. Female *Cx3cr1*^{CreER-YFP} mice, which express Cre recombinase under control of the *Cx3cr1* promoter upon exposure to 4-hydroxytamoxifen (TAM), were crossed to male *Rosa*^{tdTom} mice (168). Pregnant dams were administered TAM at E8.5 or E9.5 to induce irreversible expression of the tdTom reporter in YS CX₃CR1⁺ cells and their progeny. Tissues from offspring were examined at E15.5, P1 and 12 w for tdTom⁺ labelling, which was normalised to brain microglia that are YS-derived (551) (**Figure 4-7a**). Although TAM induction at E8.5 resulted in minimal tdTom⁺ expression (not shown), induction at E9.5 led to robust tdTom⁺ labelling of microglia (**Figure 4-8a**). Unlike colonies from E15.5 liver and adult BM that were tdTom⁻, CFU-M from aorta, heart and lung were tdTom⁺ and exclusively derived from an E9.5 CX₃CR1⁺ source (**Figure 4-7a** and **8b**). Whereas previous studies have focused on the macrophage (168, 551, 554) or endothelial fate (132, 563) of YS progenitors, we tracked both lineages together with CFU-M progenitors. E9.5 CX₃CR1⁺ cells gave rise to all three cell types in aorta, heart and lung (**Figure 4-7c-d** and **8b-f**). Progenitors made up the highest number of tdTom⁺ cells in E15.5 AGM, followed by endothelial cells and lastly macrophages. After birth, the proportions of YS-derived endothelium and macrophages in aorta both increased, with reduction in progenitors (**Figure 4-7c,d**). By comparison, endothelial cells were the predominant tdTom⁺ population at each age studied in heart and in 12 w lung (**Figure 4-8d-f**).

Complementary fate-mapping was performed by giving TAM to *Csf1r*^{MerCreMer} x *Rosa*^{mT/mG} mice at E8.5 to induce GFP expression in YS CSF1R⁺ cells, including EMPs and their progeny (554) (**Figure 4-7e**). CFU-M from 12 w aorta, heart and lung showed 100% GFP labelling normalised to microglia, as distinct from BM colonies that were GFP⁻ (**Figure 4-7f** and **8g,h**). Flow cytometry identified GFP⁺

progenitors, macrophages and endothelial cells in adult aorta, heart and lung, with endothelial cells again being most prevalent in each tissue (**Figure 4-7g,h** and **8j,k**). Confocal microscopy also demonstrated GFP⁺ YS-derived cells in adult aorta (**Figure 4-7i**), comprising each of these three cell types (**Figure 4-3j**). We then used FACS to isolate GFP⁺ progenitors, macrophages and endothelial cells to high purity from the aortas of these 12 w mice (**Figure 4-7k**). Wright-Giemsa staining showed that progenitors had rounded morphology with smooth surface membrane and lacked the protrusions and intracellular vacuolations of macrophages (**Figure 4-7l**), thus resembling the morphology reported for c-Kit⁺CD93⁺CD45^{Lo} EMPs and CX₃CR1⁺ pre-macrophages in YS (559). Although they had similarly sized nuclei as macrophages, progenitors had less cytoplasm and a higher nuclear: cell area ratio (**Figure 4-7l**). Critically, CFU-M were only produced by progenitors and not YS-derived macrophages or endothelial cells after culture in methylcellulose (**Figure 4-7m**). These data establish that tissue-resident CFU-M progenitors originate from YS EMPs and are seeded embryonically.

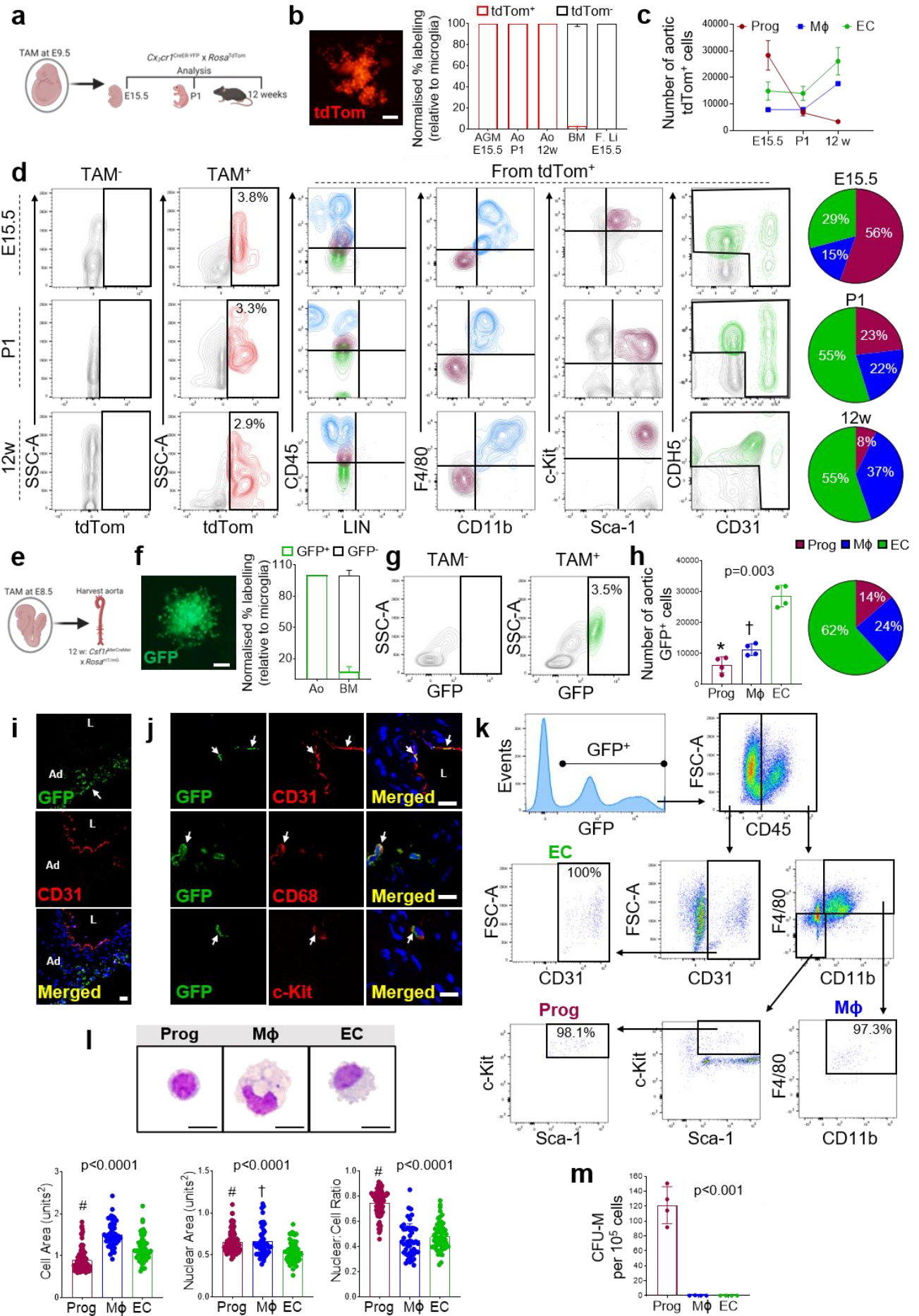


Figure 4-7. CX₃CR1⁺ and CSF1R⁺ YS cells give rise to progenitors, macrophages and endothelial cells in aorta.

- (a) E9.5 4-hydroxytamoxifen (TAM)-induced labelling of *Cx3cr1*^{CreER-YFP} x *Rosa*^{tdTom} mice, with analysis at E15.5, P1 or 12 w.
- (b) tdTom⁺ CFU-M from 12 w aorta. Graph shows tdTom⁺ vs tdTom⁻ status of CFU-M from different tissues and ages, with results normalised to brain microglia. N=4 mice/age. Ao, Aorta. F. Li, Foetal liver. Scale bar, 100 µm.
- (c) Numbers of tdTom⁺ progenitors (prog), macrophages (Mφ) and endothelial cells (EC) in AGM/aorta at different ages (n=3 for E15.5 and 12 w and n=4 for P1). Mixed effects two-way ANOVA: p=0.02 for time; p=0.01 for cell type; p<0.0001 for time x group.
- (d) Flow cytometry plots show tdTom⁺ progenitors (maroon), Mφ (blue) and EC (green) in AGM/aorta at different ages. Pie charts show lineage breakdown of tdTom⁺ cells. N=3-4 mice/age.
- (e) *Csf1r*^{MerCreMer} x *Rosa*^{GFP} mice were induced with TAM at E8.5 and analysed at 12 w.
- (f) GFP⁺ CFU-M from 12 w aorta. Graph shows GFP⁺ vs GFP⁻ status of CFU-M from aorta and BM, with results normalised to microglia. N=4 mice. Scale bar, 100 µm.
- (g) GFP⁺ expression in 12 w aorta (mean % from n=4). No TAM control also shown.
- (h) Graph shows numbers of GFP⁺ progenitors, Mφ and EC in 12 w aorta. Pie chart shows lineage breakdown of GFP⁺ cells. N=4 mice. Repeated measures ANOVA. Multiple test comparisons: *p<0.05 and †p<0.01 vs EC.
- (i, j) Confocal microscopy of 12 w aorta shows (i) GFP⁺ cells in adventitia (Ad) and (j) magnified examples of GFP⁺ cells co-expressing CD31 in intima and CD68 and c-Kit in adventitia (arrows). L, Lumen. Scale bar, 20 µm. See **Appendix 4** for bigger images.
- (k) FACS isolation and purity checks of YS-derived GFP⁺ progenitors, Mφ and EC from 12 w aorta.
- (l) Wright-Giemsa staining of sorted GFP⁺ populations, with results for total cell area, nuclear area and nuclear:cell area ratio. N=47-100 per cell type. Kruskal-Wallis tests. Multiple test comparisons: #p<0.0001 vs Mφ and EC; †p<0.01 vs EC.

(m) CFU-M yield from GFP⁺ progenitors, M ϕ and EC (four experiments, six aortas each). Friedman test.

Data summarised as mean $\pm$ SD.

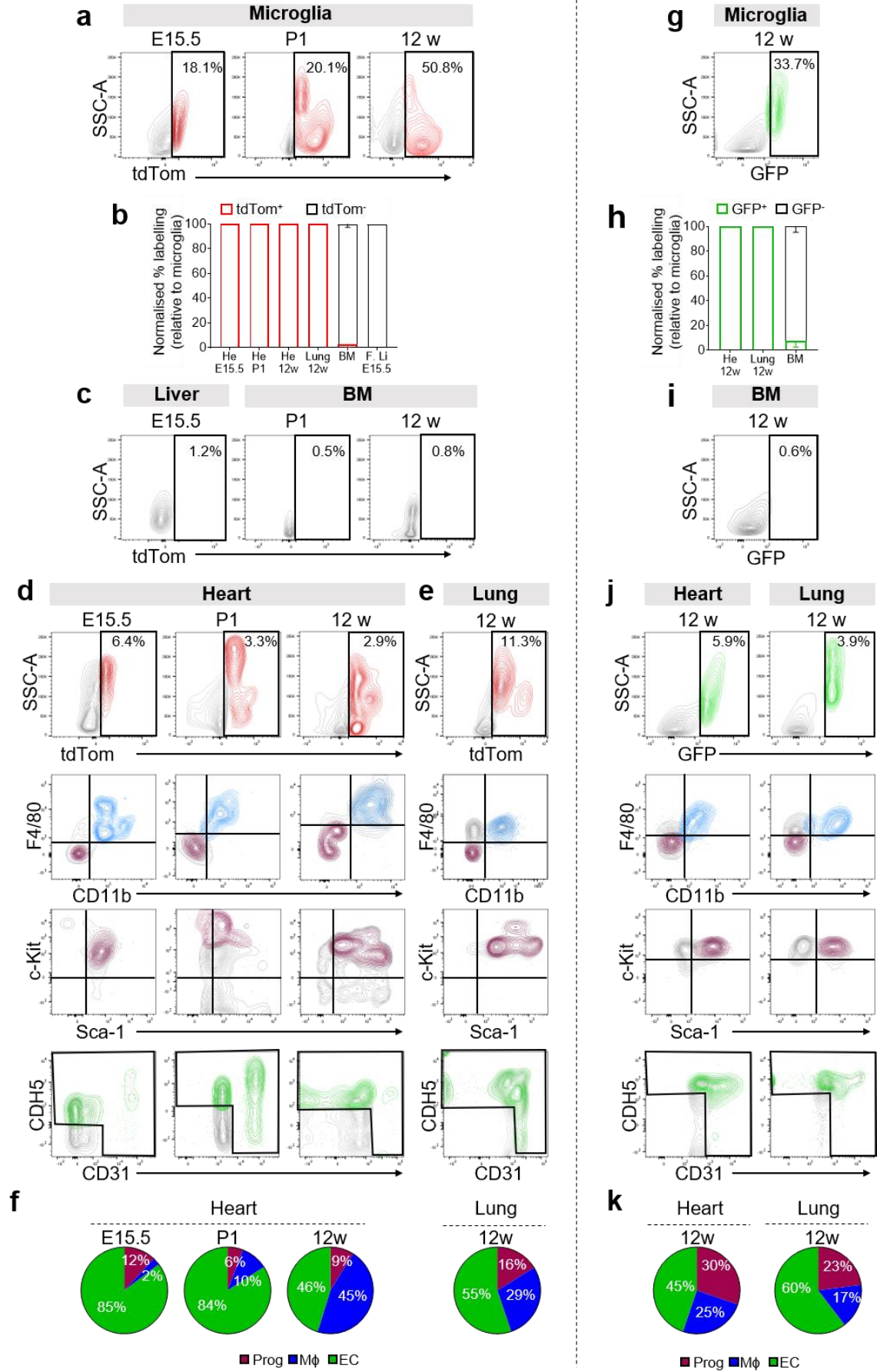


Figure 4-8. CX₃CR1⁺ and CSF1R⁺ YS cells give rise to progenitors, macrophages and endothelial cells in heart and lung.

(a-f) E9.5 4-hydroxytamoxifen (TAM)-induced labelling of *Cx3cr1*^{CreER-YFP} x *Rosa*^{tdTom} mice, with tissues analysed at E15.5, P1 or 12 w.

(a) Flow cytometry of tdTom labelling of CD11b⁺F4/80⁺ microglia at different ages. Mean percentages shown from N=3-4 mice.

(b) tdTom⁺ vs tdTom⁻ status of CFU-M from different tissues and ages, with results normalised to brain microglia. N=4 mice. He, Heart. BM, Bone marrow. F. Li, Foetal liver.

(c) Flow cytometry of tdTom expression in foetal liver and BM at different ages. Mean percentages shown from N=3-4 mice.

(d-e) Flow cytometry examples of tdTom⁺ progenitors (maroon), macrophages (blue) and endothelial cells (green) in **(d)** heart at different ages and **(e)** 12 w lung. Mean percentages for tdTom labelling are shown from N=3-4 mice.

(f) Pie charts show lineage breakdown of tdTom⁺ cells in heart and lung. N=3-4 mice.

(g-k) *Csf1r*^{MerCreMer} x *Rosa*^{mT/mG} mice were induced with TAM at E8.5 and analysed at 12 w.

(g) GFP labelling of brain microglia. Mean percentage from N=4 mice.

(h) GFP⁺ vs GFP⁻ status of CFU-M from heart, lung and BM, with results normalised to microglia. N=4 mice.

(i) GFP expression in BM. Mean percentage from N=4 mice.

(j) Flow cytometry examples of GFP⁺ progenitors (maroon), macrophages (blue) and endothelial cells (green) in adult heart and lung. Mean percentages for GFP labelling from N=4 mice.

(k) Pie charts show lineage breakdown of GFP⁺ cells in heart and lung. N=4 mice.

Progenitors, CD45^{+/Lo}CD11b⁻F4/80⁻c-Kit⁺Sca-1⁺. Macrophages (Mφ), CD45⁺CD11b⁺F4/80⁺. Endothelial cells, CD45⁻CD31⁺ and/or CD45⁻CDH5⁺.

4.3.5 CFU-M progenitors have bipotent endothelial-macrophage plasticity

The diminution in YS-derived progenitors in aorta after birth countered by increases in YS-derived macrophages and endothelial cells (**Figure 4-7c**), led us to examine whether progenitors differentiate into these lineages to contribute to their postnatal expansion and maintenance. FACS-isolated progenitors, macrophages and endothelial cells from aorta were cultured in Matrigel™ to compare their angiogenic capacity *in vitro*. While macrophages produced no vascular-like cords, progenitor and endothelial cells formed cord networks of different morphologies, but similar length cords (**Figure 4-9a**). Progenitors assembled in clusters by day 3 (data not shown) before producing complex interconnected cords by day 7. Whereas flow cytometry showed that cord networks from endothelial cultures only contained endothelial cells (**Figure 4-9b**), networks produced by progenitors consisted of macrophages and endothelial cells, with some residual progenitor cells (**Figure 4-9c**).

The Matrigel™ assay was repeated using progenitors from culture-derived aortic CFU-M. Progenitors from individual colonies again formed similar networks comprising both endothelial cells and macrophages, confirming that they are bipotent at a clonal level (**Figure 4-9d,e**). Newly produced macrophages were mostly LYVE-1⁺, MHCII⁻, CX₃CR1⁺ and CCR2⁻ (**Figure 4-9e**, **Table 4-1**), consistent with the surface marker profile of YS-derived tissue macrophages (168, 554). Macrophage and endothelial transformation of progenitors was also confirmed by showing new capacity to take up oxidized and acetylated LDL, respectively (**Figure 4-9f,g**). Angiogenic capacity was highest for CFU-M progenitors from E9.5 YS and P1 aorta and lower from 12 w and 52 w aorta (**Figure 4-9h**).

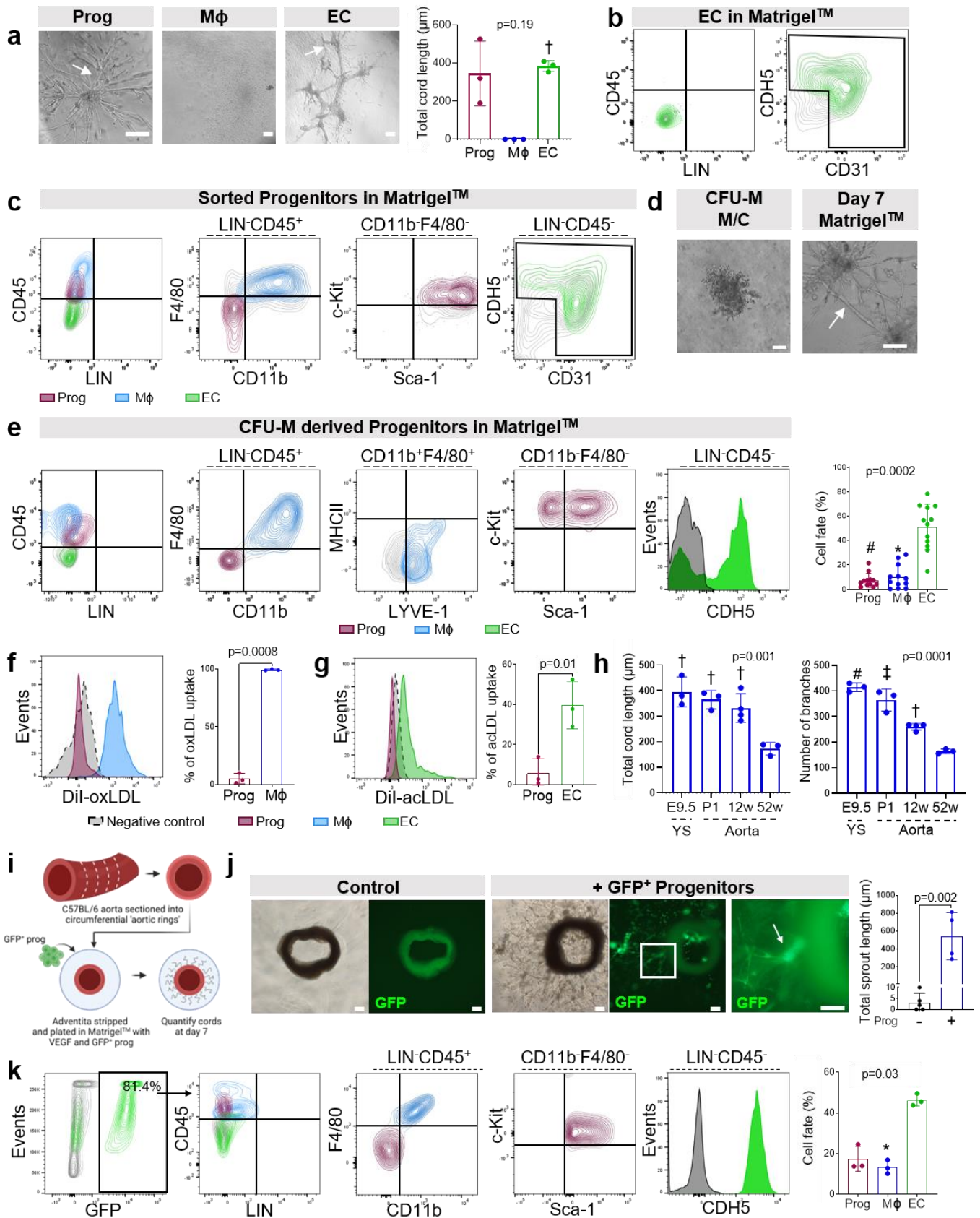


Figure 4-9. Aortic CFU-M progenitors have endothelial and macrophage potential.

(a) Day 7 images after FACS sorted progenitors (Prog), macrophages (M ϕ) or endothelial cells (EC) from 12 w aorta were cultured in MatrigelTM. Arrows indicate cords. Graph shows total cord length (three experiments, six aortas each). Friedman test. Scale bar, 100 μ m.

(b, c) Flow cytometry plots show fate of **(b)** EC and **(c)** progenitors in MatrigelTM. Maroon, progenitors; blue, M ϕ ; green, EC.

(d) Example of aortic CFU-M from which progenitors produced cord networks (arrow) in MatrigelTM. Scale bar, 100 μ m.

(e) Flow cytometry plots and graph show fate of CFU-M derived progenitors in MatrigelTM. N=12 mice. Green histogram, Sample; Grey, FMO control. Friedman test. Multiple comparison tests: * $p < 0.05$ and # $p < 0.0001$ for comparison to EC.

(f, g) Uptake of **(f)** DiI-oxLDL or **(g)** DiI-acLDL by aortic progenitors or their M ϕ or EC progeny produced in MatrigelTM. N=3 mice. Paired *t*-tests.

(h) Total cord length and number of branches produced in MatrigelTM by progenitors from YS or aortic CFU-M from different aged C57BL/6 mice. N=3-4 mice/age. One-way ANOVA. Multiple test comparisons: * $p < 0.05$, † $p < 0.01$, ‡ $p < 0.001$ and # $p < 0.0001$ for comparison to 52 w.

(i) Adventitial sprouting assay using aortic progenitors from GFP mice added to adventitia-less aortic rings from C57BL/6 mice.

(j) Light and fluorescence microscopy and summarised results for adventitial sprouting with (+) and without (-) addition of GFP⁺ progenitors. N=4-5 mice. Unpaired *t*-test. Scale bar, 100 μ m.

(k) Flow cytometry plots and graph show fate of GFP⁺ aortic progenitors after 7 d of aortic ring assay (n=3). Friedman test. Multiple comparisons test: * $p < 0.05$ for comparison to EC.

Data summarised as mean $\pm$ SD.

We next examined whether aortic CFU-M progenitors mediate adventitial neovascularisation, as occurs during *vasa vasorum* expansion. Aortic ring assays were performed from E9.5 TAM-induced 12 w *Cx3cr1*^{CreER-YFP} x *Rosa*^{tdTom} mice and produced YS-derived tdTom⁺ sprouts (**Figure 4-11a, b**), with seven-fold higher content of tdTom⁺ cells compared to whole aorta, as measured by flow cytometry (**Figure 4-11c**). tdTom⁺ sprouts contained endothelial cells, progenitors and macrophages (mostly CX₃CR1⁺LYVE-1⁺MHCII⁻) in decreasing order of abundance (**Figure 4-11d, Table 4-2**). Similar results were obtained from E8.5 TAM-induced 12 w *Csf1r*^{MerCreMer} x *Rosa*^{mT/mG} mice (**Table 4-2**). Finally, we used aortic rings from C57BL/6 mice, in which adventitia had been removed to eliminate sprouting (**Figure 4-9i**). Seeding aortic CFU-M progenitors from ubiquitous GFP mice rescued adventitial angiogenesis with formation of GFP⁺ sprouts, that contained endothelial cells and predominantly LYVE-1⁺MHCII⁻ macrophages (**Figure 4-9j,k and Table 4-1**). Therefore YS-derived progenitors participate in adventitial neovascularisation, by producing both endothelial cells and macrophages.

To study the fate and vasculogenic capacity of CFU-M progenitors *in vivo*, we performed adoptive cell transfer experiments. Surgery was performed on 12 w C57BL/6 mice to induce hindlimb ischaemia (566), before injecting the quadriceps and gastrocnemius with CFU-M-derived progenitors from 12 w aorta or E9.5 YS from GFP mice (~1.5x10⁴ cells) or cell-free MatrigelTM (**Figure 4-10a, 4-11e**). Laser Doppler imaging showed that aortic progenitors improved perfusion recovery over 14 d compared to control (**Figure 4-10b**), accompanied by increased capillary and arteriolar density in injected muscle (**Figure 4-11e**). At day 14, GFP⁺ cells were detected in recipient muscle but not peripheral blood, with flow cytometry revealing that donor progenitors had produced new endothelial cells and macrophages (**Figure 4-10c, Table 4-1**). Confocal microscopy identified host-perfused, GFP⁺ endothelial-lined neovessels, with adjacent clusters of GFP⁺ CD68⁺ macrophages (**Figure 4-10d**). Similar results for perfusion recovery and endothelial and macrophage fate were obtained after injection of YS progenitors (**Figure 4-11f-h**).

Collectively, the above results identify CFU-M progenitors as vasculogenic bipotent EndoMac progenitors. We next examined their self-renewal capacity and the durability of their progeny *in vivo*. Digests of quadriceps and gastrocnemius were prepared from C57BL/6 mice 14 d after injection of GFP⁺ aortic progenitors. They generated GFP⁺ CFU-M in methylcellulose-based culture from which GFP⁺ progenitors were isolated and used in 2° hindlimb transfer studies (**Figure 4-10e**). After another 14 d, engrafted GFP⁺ cells had mostly transformed into endothelial cells or macrophages, with few remaining progenitors (**Figure 4-10f**). Similar results were obtained in 1° transfer studies that were followed for eight weeks instead of two (**Figure 4-10g, 4-10h**). These results show that while aortic progenitors produce durable endothelial and macrophage progeny, their own numbers diminish over time indicating that their capacity for self-renewal is finite.

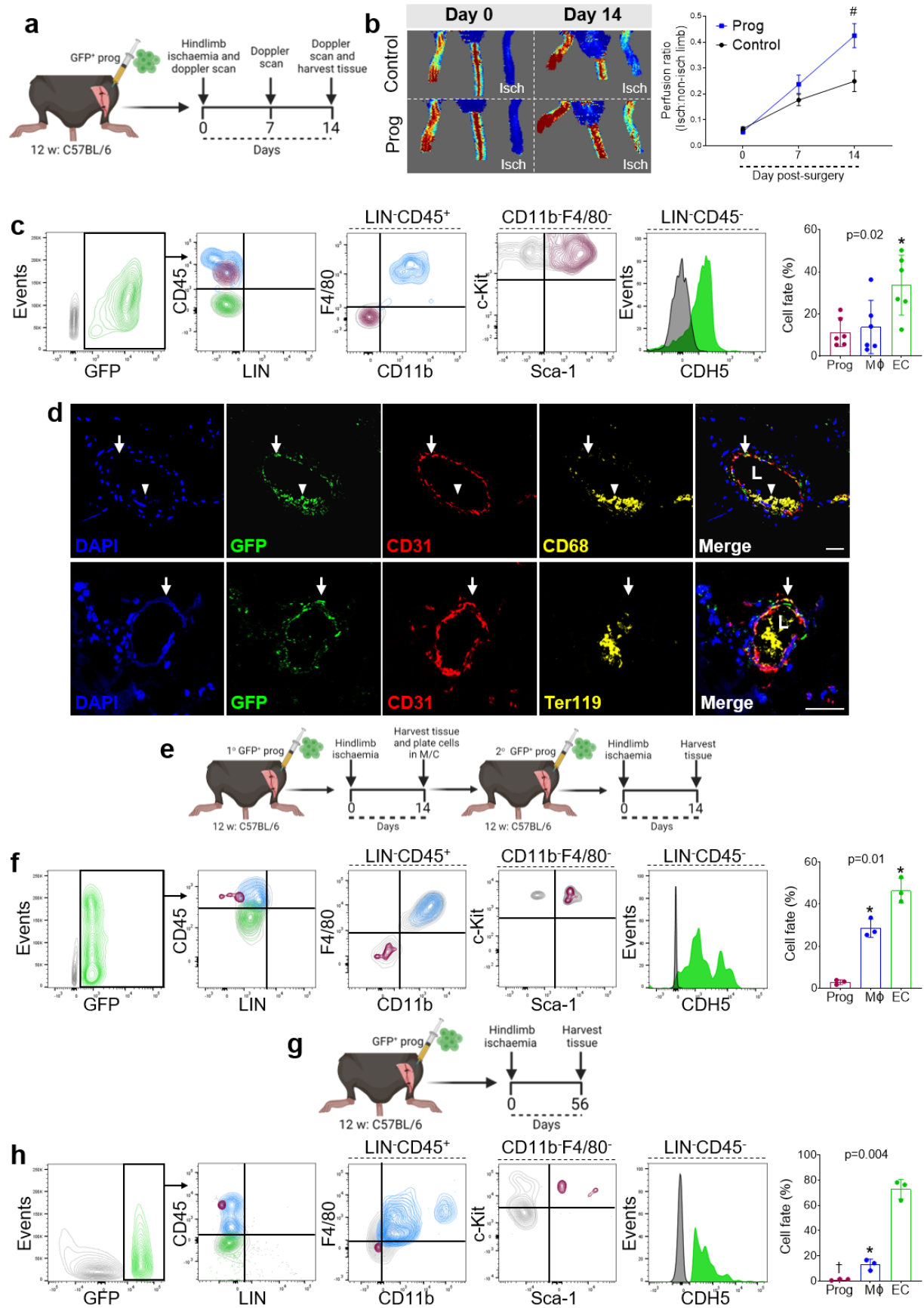


Figure 4-10. CFU-M progenitors have endothelial-macrophage plasticity and vasculogenic capacity *in vivo*.

- (a) 1° transfer of GFP⁺ aortic progenitors after hindlimb ischaemia surgery with two-week follow-up.
- (b) Laser Doppler perfusion images on D0 and D14 after ischaemia in mice receiving cell-free control (above) and progenitors (below). Graph shows results n=6 mice/gp. Mixed effects two-way ANOVA: $p < 0.0001$ for time; $p = 0.03$ for group; $p = 0.005$ for time x group. Multiple comparison test: $\#p < 0.0001$ for prog vs control.
- (c) Flow cytometry plots and graph show fate of GFP⁺ donor cells in recipient muscle. N=6 mice. Maroon, Progenitors (Prog); Blue, Macrophages (M ϕ); Green, Endothelial cells (EC). Green histogram, Sample; Grey, FMO control. Repeated measures ANOVA. Multiple comparison test: $*p < 0.05$ vs M ϕ .
- (d) Confocal microscopy of ischaemic muscle shows neovessels lined by GFP⁺CD31⁺ EC (arrows) with cluster of GFP⁺CD68⁺ M ϕ (arrowhead) (above) and perfused with host Ter119⁺ erythrocytes (below). L, Lumen. Scale bar, 20 μ m. See **Appendix 5** for bigger images.
- (e) 2° transfer of GFP⁺ progenitors after hindlimb ischaemia.
- (f) Flow cytometry plots and graph show fate of GFP⁺ donor cells after 2° transfer. N=3 mice. Repeated measures ANOVA. Multiple comparison test: $*p < 0.05$ vs prog.
- (g) 1° transfer of GFP⁺ progenitors after hindlimb ischaemia with eight-week follow-up.
- (h) Flow cytometry plots and graph show fate of GFP⁺ donor cells 8 w after 1° transfer. N=3 mice. Repeated measures ANOVA. Multiple comparison test: $*p < 0.05$ and $\dagger p < 0.01$ vs EC.
- Data summarised as mean $\pm$ SD.

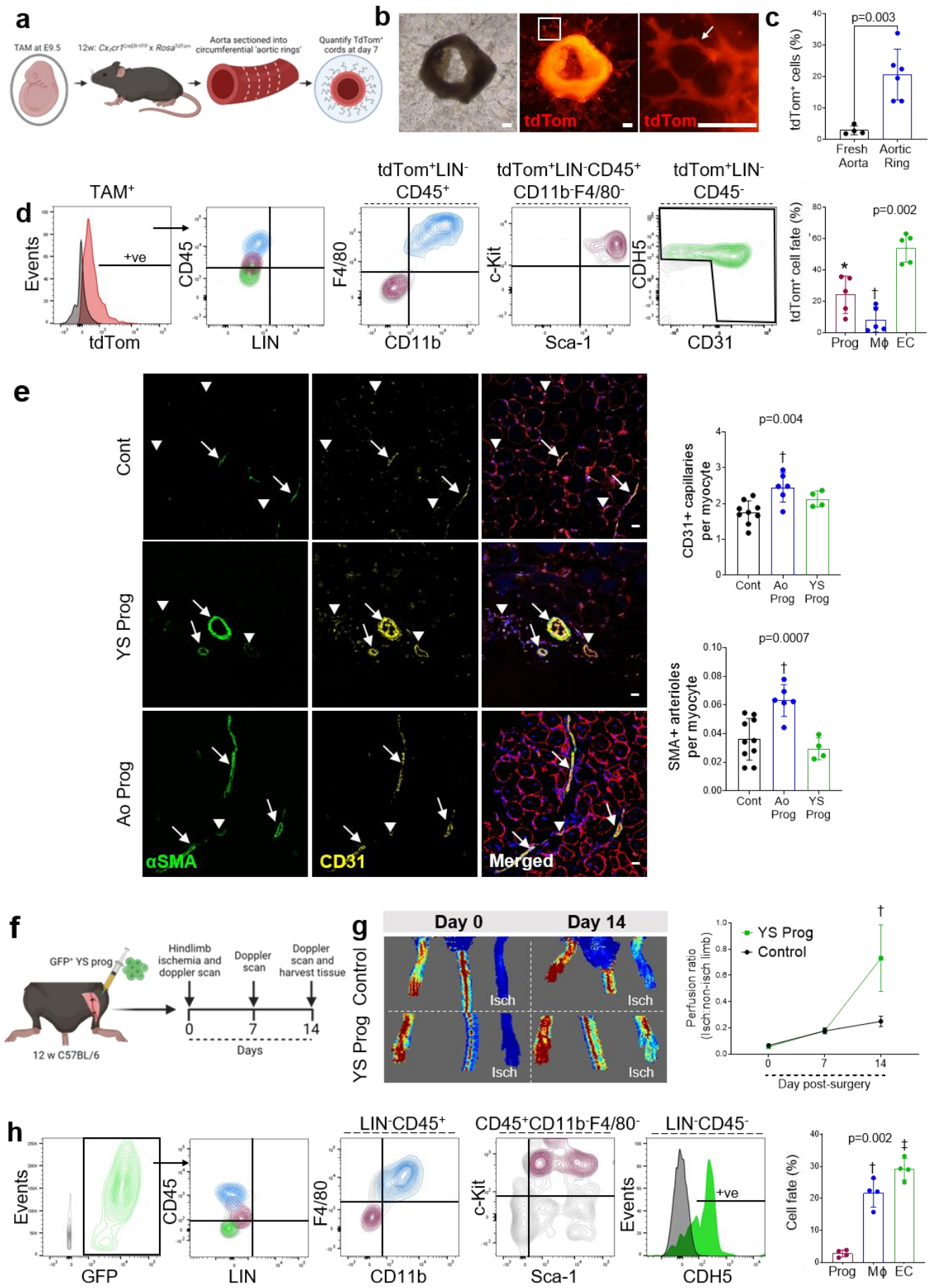


Figure 4-11. Endothelial and macrophage differentiation capacity of YS-derived progenitors.

(a) Schematic for aortic ring assay performed using aortas from E9.5 TAM-induced adult *Cx3cr1*^{CreER-}YFP x *Rosa*^{tdTom} mice to label YS-derived cells tdTom⁺.

(b) Light and fluorescence microscopy of aortic ring shows tdTom⁺ adventitial sprouting.

(c) Graph compares % tdTom⁺ expression (not normalised to microglia) in day 7 aortic ring assays with fresh digests of whole aorta. N=4-6 mice/gp. Unpaired *t*-test. Scale bar, 100 µm.

(d) Flow cytometry of aortic ring sprouting assay from 12 w *Cx3cr1*^{CreER-YFP} x *Rosa*^{tdTom} mouse with TAM-induction at E9.5. Histogram for tdTom expression (red) is overlaid with no TAM Cre negative control (grey). Graph shows lineage fate of YS-derived tdTom⁺ cells. N=5 mice. Maroon, tdTom⁺ progenitors (Prog); Blue, tdTom⁺ macrophages (Mφ); Green, tdTom⁺ endothelial cells (EC). Repeated measures ANOVA. Multiple comparison test: **p*<0.05 and †*p*<0.01 vs EC.

(e) Immunofluorescence microscopy of sections of hindlimb muscle at day 14 that had received injections of MatrigelTM control (top), E9.5 YS CFU-M progenitors (middle) or Aortic (Ao) CFU-M progenitors (bottom). Counter-staining with antibody to laminin (red) for myocytes and DAPI (blue) for nuclei. Graphs show capillary and arteriolar density. N=4-10 mice/gp, three sections per mouse. One-way ANOVA. †*p*<0.01 vs control. Scale bar, 100 µm. See **Appendix 6** for bigger images.

(f) Primary transfer of GFP⁺ YS CFU-M progenitors after hindlimb ischaemia surgery with two-week follow-up.

(g) Laser Doppler perfusion images on days 0 and 14 after ischaemia in mice receiving cell-free control (above) and YS progenitors (below). Graph shows results. N=4 for Prog, n=6 for control. Mixed effects two-way ANOVA: *p*=0.0004 for time; *p*=0.08 for group; *p*=0.02 for time x group. Multiple comparison test: †*p*<0.01 for prog vs control.

(h) Flow cytometry plots and graph show fate of GFP⁺ YS progenitors in recipient hindlimb muscle. N=4 mice. Green histogram, Sample; Grey, FMO control. Repeated measures ANOVA. Multiple comparison test: †*p*<0.01 and ‡*p*<0.001 vs prog.

Data summarised as mean±SD.

Experimental Design	MatrigelTM <i>In vitro</i> N=3-6	Aortic Ring <i>Ex vivo</i> N=2	Hindlimb <i>In vivo</i> N=3-6
Surface marker	CD45⁺CD11b⁺F4/80⁺ Macrophages		
CD206 ⁺	75.8±23.9%	85.1±20.6%	68.4±17.0%
CX ₃ CR1 ⁺	74.5±16.3%	90.9±13.4%	91.0±8.7%
MHCII ⁺	5.8±2.5%	2.9±0.0%	19.4±7.2%
LYVE-1 ⁺	45.0±21.4%	83.9±0.8%	61.2±16.5%
CCR2 ⁺	5.5±4.1%	N/A	27.6±1.9%
	CD45⁻CD31⁺ and/or CD45⁻CDH5⁺ Endothelial cells		
VEGFR2 ⁺	40.0±13.3%	23.6±0.8%	77.9±19.4%
TIE2 ⁺	80.4±3.9%	N/A	N/A
LYVE-1 ⁺	10.7±2.9%	18.7±3.1%	16.4±5.0%

Table 4-1. Sub-lineage fate of aortic EndoMac progenitors in different experimental models.

Expression of select surface markers on macrophages and endothelial cells produced by adult aortic progenitors in MatrigelTM cord-forming assay, aortic ring assay and after intramuscular injection in hindlimb ischaemia model. N-values relate to number of different experiments in which these markers were assessed. N/A, Not assessed.

Results summarised as mean±SD.

Experimental Design	<i>Cx3cr1</i> ^{CreER-YFP} x <i>Rosa</i> ^{tdTom} Aortic Rings N=6	<i>Csf1r</i> ^{MerCreMer} x <i>Rosa</i> ^{mT/mG} Aortic Rings N=3
tdTom ⁺ or GFP ⁺	20.6±8.1%	19.8±5.2%
Surface marker	tdTom ⁺ or GFP ⁺ CD45 ⁺ CD11b ⁺ F4/80 ⁺ Macrophages	
CD206 ⁺	90.9±7.2%	94.8±4.7%
CX ₃ CR1 ⁺	97.9±3.6%	91.6±8.1%
MHCII ⁺	5.6±0.9%	3.2±0.2%
LYVE-1 ⁺	59.5±33.9%	37.5±10.7%
	tdTom ⁺ or GFP ⁺ CD45 ⁺ CD31 ⁺ and/or CD45 ⁺ CDH5 ⁺ Endothelial cells	
VEGFR2 ⁺	76.2±5.8%	71.4±5.8%
LYVE-1 ⁺	15.2±5.9%	21.1±3.2%

Table 4-2. Sub-lineage fate of YS-derived cells in aortic ring assays.

Expression of select surface markers on YS-derived macrophages and endothelial cells formed by Day 7 in aortic ring assays from E9.5 tamoxifen-induced *Cx3cr1*^{CreER-YFP} x *Rosa*^{tdTom} and E8.5 tamoxifen-induced *Csf1r*^{MerCreMer} x *Rosa*^{mT/mG} mice. Results shown for tdTom⁺ and GFP⁺ labelling are absolute expression (i.e. not normalised to brain microglia).

Results summarised as mean±SD.

4.3.6 Regulatory effects of angiotensin II on aortic EndoMac progenitors

The renin-angiotensin system, and specifically angiotensin II (AngII), play key regulatory roles in myelopoiesis (572, 573) and vascular inflammation (571, 574). When pluripotent stem cells undergo haemato-endothelial differentiation *in vitro*, they transition through bipotent haemangioblasts that form colonies and express ACE (CD143) and the receptors for AngII, ATR1 and ATR2 (564). We found that ACE was expressed on EndoMac progenitors from aortic CFU-M in culture ($86.9 \pm 6.7\%$, $n=5$), fresh aortic digests ($85.9 \pm 11.0\%$, $n=5$) and CFU-M from YS ($99.8 \pm 0.05\%$, $n=3$) (**Figure 4-12a-c**). Aortic progenitors also expressed ATR1 ($87.4 \pm 1.2\%$, $n=3$) and ATR2 ($84.9 \pm 13.5\%$, $n=3$) at surface protein (**Figure 4-12a**) and mRNA level (**Figure 4-12d**). AngII induced concentration-dependent increases in 1° CFU-M yield from aortic cells (**Figure 4-12e**) and at 100 nM stimulated CFU-M to self-renew for four passages (**Figure 4-12f**). Treatment with inhibitors of ACE (enalapril), ATR1 (losartan) and ATR2 (PD123,319) showed that inhibition of ATR1 and ATR2 reduced aortic CFU-M yield (**Figure 4-12g**). To study the effect of AngII on differentiation, we injected 10^4 GFP⁺ aortic progenitors into the peritoneum of C57BL/6 mice and assessed their fate by flow cytometry 72 h later (**Figure 4-12h-j**). Daily *i.p.* injections of AngII promoted expansion of progenitor-derived macrophages by 14.5-fold and 3.4-fold compared to PBS and M-CSF, respectively (**Figure 4-12j-k**). In keeping with these different effects on EndoMac progenitors, qPCR showed that AngII upregulated mRNA levels of genes involved in cell division (*Ccna1*, *Ccnb1*, *Ccnd2*), self-renewal (*Myc*, *Nanog*, *Klf4*) (575, 576), myelopoiesis (*Klf4*, *Irf8*) (577), M2-like macrophage polarisation (*Arg1*) and endothelial specification and angiogenesis (*Cdh5*, *Cd248*) (**Figure 4-12l-p**).

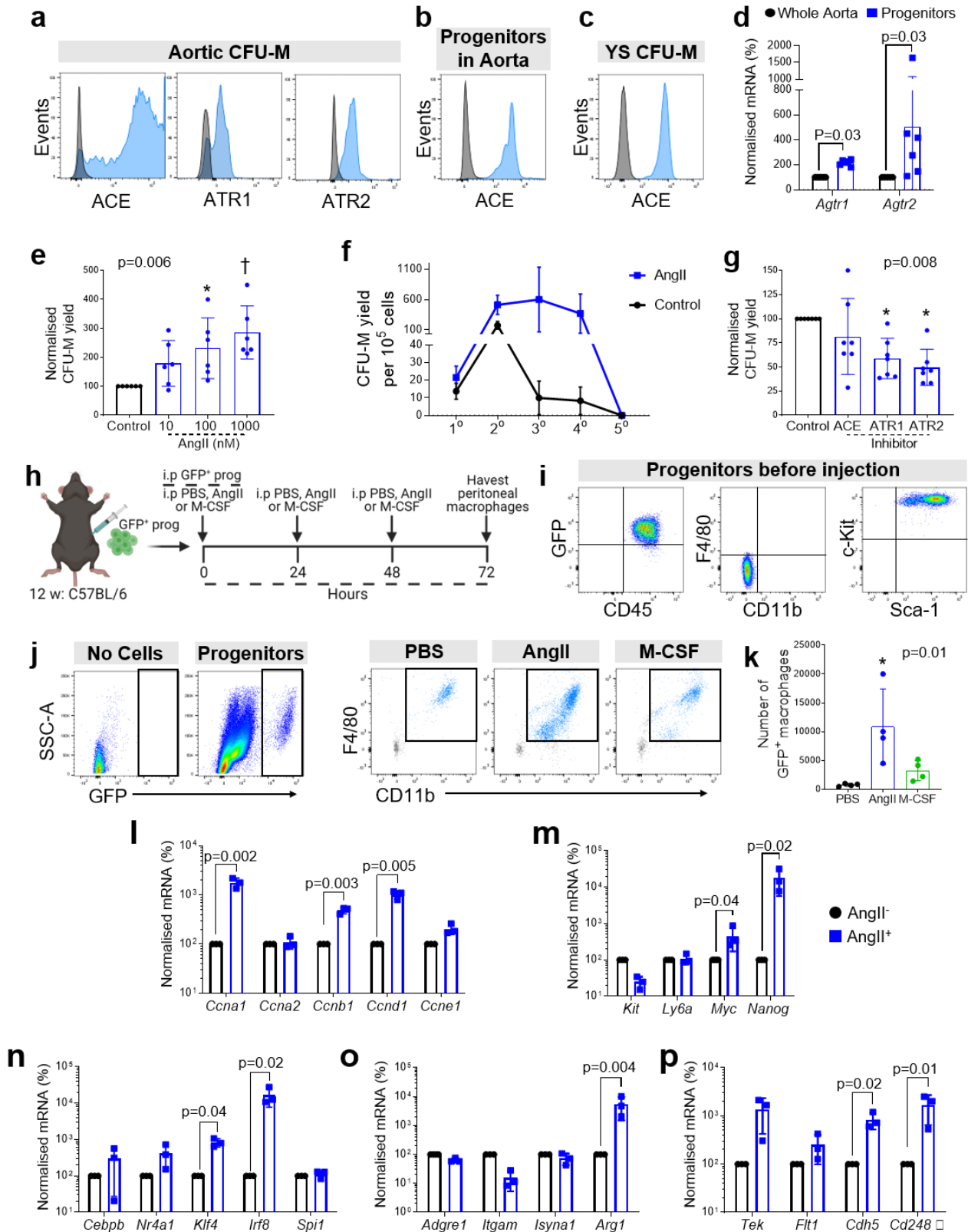


Figure 4-12. Regulatory effects of angiotensin II on aortic EndoMac progenitors.

(a) Surface expression of ACE, ATR1 and ATR2 on progenitors from aortic CFU-M. N=3 12 w C57BL/6 mice.

- (b)** ACE expression on progenitors in C57BL/6 aortic digests. N=3 mice.
- (c)** ACE expression on progenitors from E9.5 YS CFU-M. N=3 mice.
- (d)** Normalised mRNA expression of *Agtr1* and *Agtr2* in progenitors from aortic CFU-M relative to same donor aortic digests. N=6 12 w C57BL/6 mice. Wilcoxon signed rank tests.
- (e)** Aortic CFU-M yield with different concentrations of AngII, normalised to no AngII control. N=6 12 w C57BL/6 mice. Friedman test. Multiple comparison tests: * $p < 0.05$, † $p < 0.01$ vs control.
- (f)** Aortic CFU-M yield across serial passages in the presence or absence of 100 nM AngII. N=3 12 w C57BL/6 mice.
- (g)** Aortic CFU-M yield in the presence of inhibitors to ACE (Enalapril), AT1R (Losartan) and AT2R (PD123,319) normalised to control. N=3 12 w C57BL/6 mice. Friedman test. Multiple comparison tests: * $p < 0.05$ vs control.
- (h)** Peritoneal transfer assay.
- (i)** Immunophenotype of GFP⁺ aortic progenitors before intraperitoneal injection.
- (j)** Flow cytometry of peritoneal gavage shows macrophage fate of GFP⁺ aortic progenitors after 72 h of daily injections of PBS, AngII or M-CSF.
- (k)** Number of macrophages produced by progenitors under different conditions. N=4 mice/gp. One-way ANOVA. Multiple comparisons test: * $p < 0.05$ vs PBS.
- (l-p)** mRNA expression for select genes in aortic progenitors after treatment with AngII, normalised to no AngII control. Genes related to (L) cell cycle; (M) progenitor/stem cell biology and self-renewal; (N) myelopoiesis; (O) macrophages; (P) endothelial biology and angiogenesis. mRNA transcripts were first normalised to *β-actin*. Paired *t*-tests. N=3-4 12 w C57BL/6 mice.

Data summarised as mean±SD.

4.3.7 YS-derived EndoMac progenitors expand early in AngII-induced inflammation

AngII-induced vascular inflammation is characterised by expansion of adventitial macrophages (578), adventitial fibrosis (524) and in some mouse strains, development of aortic aneurysms (579). A recent study reported that after 10 d of exposure to AngII, adventitial macrophages expanded due to both recruitment of BM-derived cells and local proliferation of embryonic-derived macrophages (571). As AngII induced progenitor expansion and differentiation to macrophages within 72 h in our peritoneal transfer assay, we focused on its early effects on aortic progenitors and macrophages after systemic infusion by osmotic pump. In *Flt3^{Cre}* x *Rosa^{mT/mG}* mice, 48 h of AngII caused increases in both *Flt3-Cre⁺* and *Flt3-Cre⁻* macrophages compared to PBS control (2.9 and 5.8-fold, respectively) (**Figure 4-13a,b**), with more *Flt3-Cre⁺* and *Flt3-Cre⁻* macrophages in S-phase of cycle (both 2.9-fold vs PBS) (**Figure 4-13c**). As a potential contributor to the expansion of non-BM derived macrophages, AngII also induced expansion and proliferation of *Flt3-Cre⁻* progenitors, with fold comparisons compared to PBS: 3.0x for aortic CFU-M yield (**Figure 4-13d**); 8.5x for progenitor number assessed by flow cytometry (**Figure 4-7e**); and 10.5x for progenitors in S-phase (**Figure 4-13f**).

We next used adult *Cx3cr1^{CreER-YFP}* x *Rosa^{tdTom}* mice that had been administered TAM at E9.5. AngII or PBS were infused by osmotic pump for 12 h, 48 h or 168 h (7 d) (**Figure 4-13g**). AngII increased the numbers of *tdTom⁺* cells in aorta at all three time-points compared to PBS (fold comparisons by flow cytometry: 1.7x at 12 h, 1.8x at 48 h, 2.9x at 168 h) (**Figure 4-13h**). This was supported by confocal microscopy analysis of sections of descending aorta at 48 h (**Figure 4-13i, 4-14a**). As measured by CFU-M yield, the AngII-induced expansion of EndoMac progenitors was highest at 12 h (6.1-fold vs PBS), still evident at 48 h (3.3-fold) but no longer at 168 h (1.3-fold) (**Figure 4-13j**). We also used a strategy of BrdU pulse-chase in these mice to test for an ancestral relationship between YS-derived progenitors, macrophages and endothelium in aorta under AngII-induced inflammation. BrdU was injected 11 h after the start of AngII or PBS, with aortas studied at the same three time-points of infusion

as above (**Figure 4-13g**). Progenitors were by far the most proliferative YS-derived population in both PBS and AngII groups after 12 h of treatment (**Figure 4-13k**). As the number of BrdU⁺ tdTom⁺ cells remained relatively constant over the study duration (**Figure 4-13l-m**), we analysed how BrdU labelling was distributed across different YS-derived populations over time. Whereas it was highest in progenitors at 12 h and diminished thereafter (**Figure 4-13n**), it progressively increased in tdTom⁺ macrophages and endothelial cells in AngII aortas, peaking in both at 168 h (**Figure 4-13o,p**). A similar pattern of redistribution of BrdU labelling was seen among GFP⁺ YS-derived cells over 14 d of pulse-chase in steady state aortas from E8.5 TAM-induced 12 w *Csf1r*^{MerCreMer} x *Rosa*^{mT/mG} mice (**Figure 4-14b-i**). Together with our earlier results, these findings support a model whereby YS-derived progenitors undergo very early proliferative expansion in response to AngII, followed by differentiation into macrophages and endothelium.

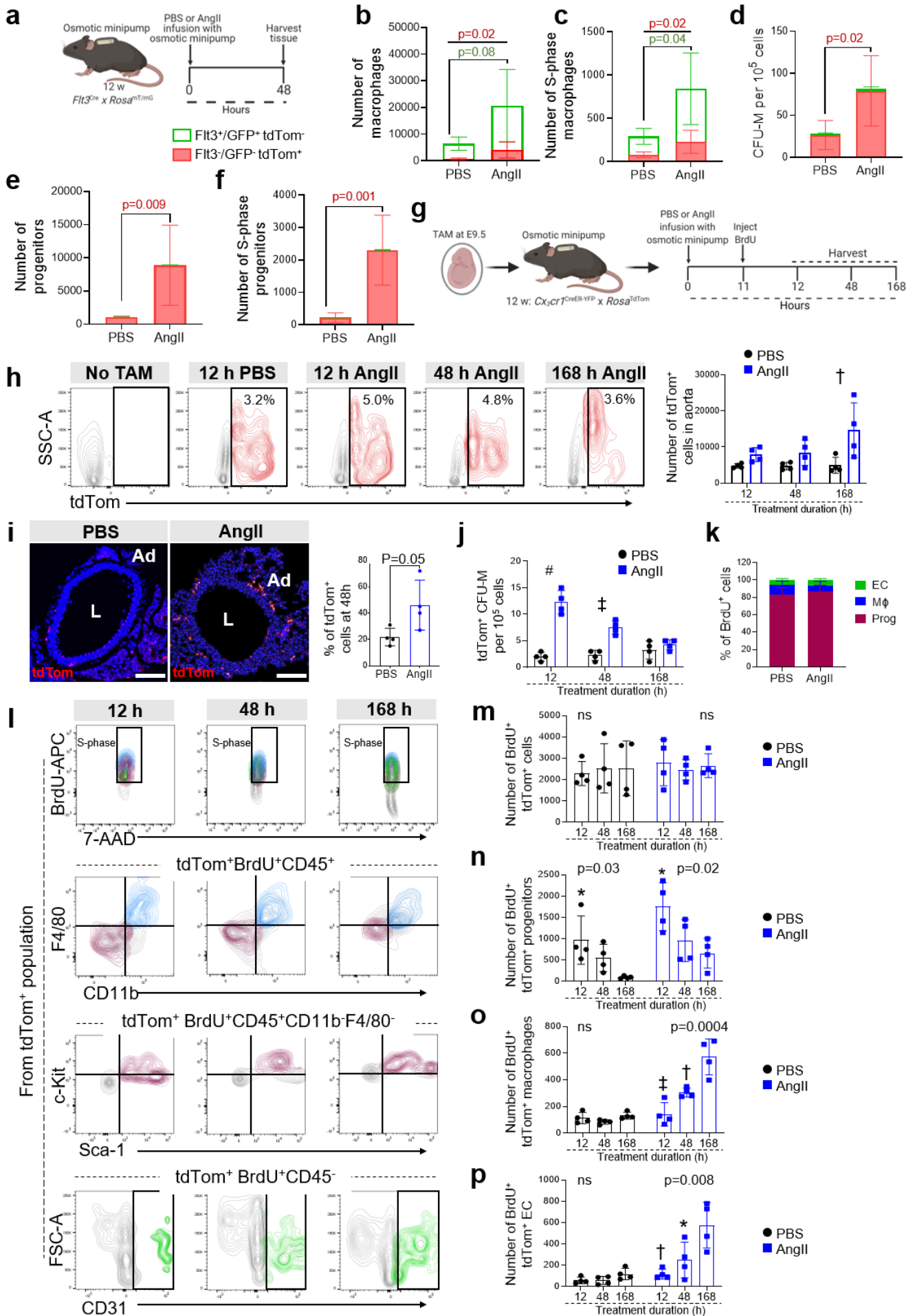


Figure 4-13. YS-derived EndoMac progenitors proliferate early during angiotensin II-induced vascular inflammation.

(a) PBS or AngII infusion in *Flt3^{Cre}* x *Rosa^{mT/mG}* mice for 48 h.

(b-f) Graphs compare aortas from PBS and AngII groups for the numbers of *Flt3⁺* and *Flt3⁻*: **(b)** macrophages, **(c)** S-phase macrophages, **(d)** CFU-M yield, **(e)** progenitors and **(f)** S-phase progenitors (n=6 mice/gp). Flow cytometry used for **(b)**, **(c)**, **(e)** and **(f)**. Unpaired *t*-tests. Red p-values, *Flt3⁻* comparisons; Green p-values, *Flt3⁺*.

(g) PBS or AngII infusion in E9.5 TAM-induced *Cx3cr1^{CreER-YFP}* x *Rosa^{tdTom}* mice with BrdU pulse-chase.

(h) Flow cytometry of *tdTom⁺* labelling in aortic digests. No TAM Cre control also shown. Percentages are mean *tdTom⁺*. N=4 mice. Graph shows number of *tdTom⁺* cells in aortas. Two-way ANOVA: Interaction p=0.17, time p=0.002, group p=0.14. Group comparison: †p<0.01.

(i) Confocal microscopy of descending aorta after 48h of PBS or AngII. Graph shows % of *tdTom⁺* cells in adventitia (Ad). N=4, three sections each. Unpaired *t*-test. L, Lumen. Nuclei stained with DAPI. Scale bar, 50 µm.

(j) *tdTom⁺* aortic CFU-M yield (n=4 mice/gp). Two-way ANOVA: Interaction p<0.0001, time p=0.0005, group p<0.0001. Group comparisons: #p<0.0001, ‡p<0.001.

(k) Percentages of *BrdU⁺* cells that were progenitors (Prog), macrophages (Mφ) or endothelial cells (EC) after 12 h of PBS or AngII and 1 h after BrdU. N=4 mice/gp.

(l) Flow cytometry of aortic digests after different durations of AngII, gated from *tdTom⁺* cells. Maroon, Prog; Blue, Mφ; Green, EC.

(m-p) Numbers of *BrdU⁺* *tdTom⁺* **(m)** cells, **(n)** progenitors, **(o)** macrophages and **(p)** EC following BrdU pulse-chase for different durations of PBS or AngII infusion. N=4 mice/gp. p-values are from one-way ANOVA for each treatment. ns, not significant. Multiple test comparisons: *p<0.05, †p<0.01, ‡p<0.001 vs 168 h.

Data summarised as mean±SD.

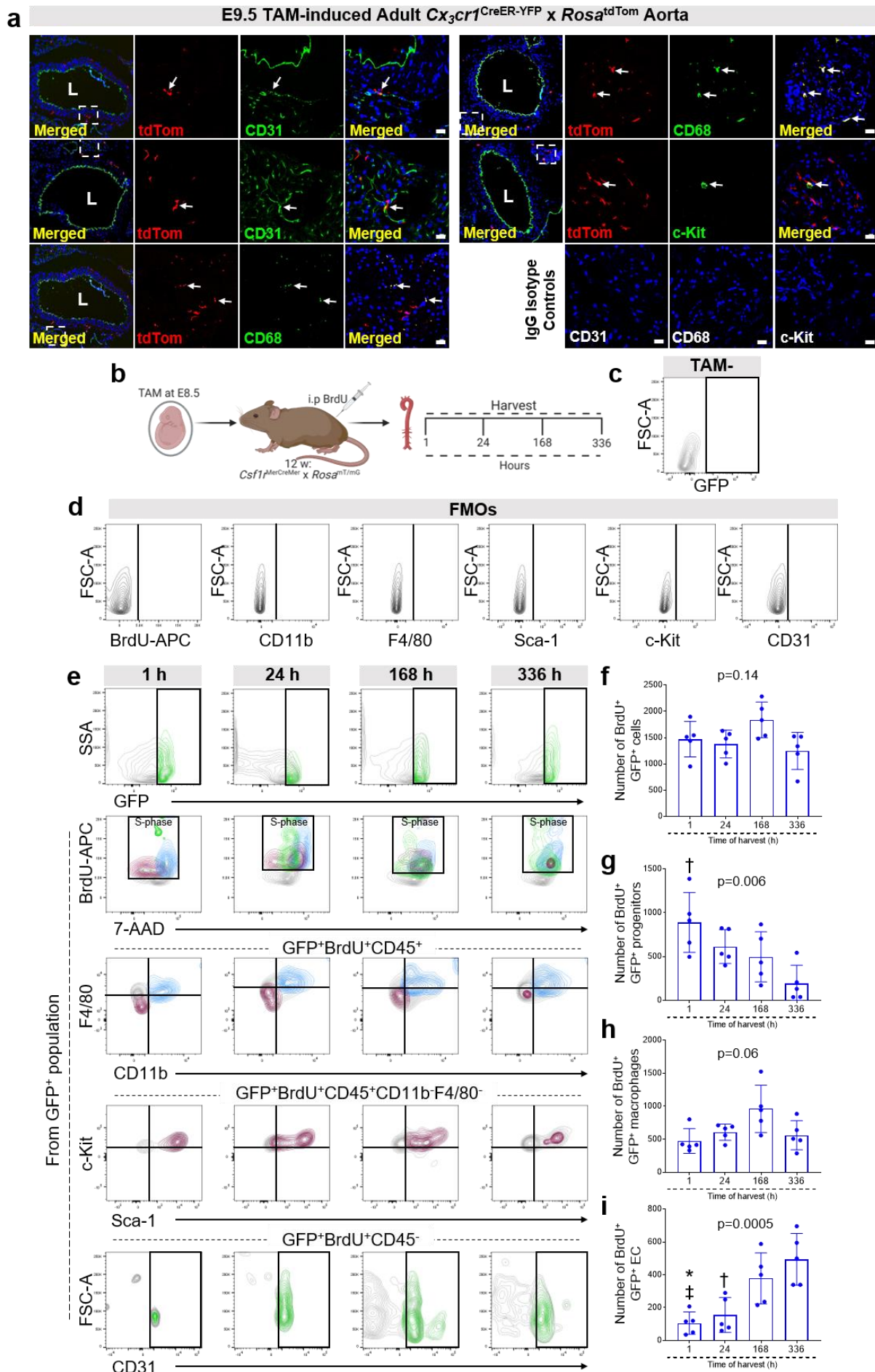


Figure 4-14. BrdU pulse-chase supports differentiation of YS-derived progenitors into macrophages and endothelial cells in healthy aorta.

(a) Confocal microscopy images of sections of aorta from adult *Cx3cr1*^{CreER-YFP} x *Rosa*^{tdTom} mice that were induced with TAM at E9.5 and treated with AngII infusion for 48 h. Lower power images are merged from staining for CD31 (green), tdTom (red) and DAPI (blue). Dotted boxes relate to adjacent magnified images that show tdTom⁺ cells in adventitia co-staining for CD31, CD68 or c-Kit (arrows). L, Lumen. Scale bar, 10 μ m. See **Appendix 7** for bigger images.

(b) BrdU pulse-chase in 12 w *Csf1r*^{MerCreMer} x *Rosa*^{mT/mG} mice that were induced with TAM at E8.5.

(c) Flow cytometry for no TAM Cre control.

(d) Fluorescence-minus-one (FMO) controls.

(e) Flow cytometry of aortic digests at different time-points after BrdU injection. Maroon, Prog; Blue, M ϕ ; Green, EC.

(f-i) Numbers of BrdU⁺ GFP⁺ **(f)** cells, **(g)** progenitors, **(h)** macrophages and **(i)** EC at different time-points after BrdU pulse. N=5 mice/gp. p-values are from Kruskal-Wallis in **(f)** and **(h)** and one-way ANOVA in **(g)** and **(i)**. Multiple test comparisons: *p<0.05 vs 168 h; †p<0.01 and ‡p<0.001 vs 336 h. Data summarised as mean $\pm$ SD.

4.4 Discussion

The intimate association between the haematopoietic and endothelial lineages begins early in development. Converging evidence indicates that YS EMPs give rise to both macrophages and endothelial cells in adult mouse tissues (132, 555). In this study we identify and characterise undifferentiated EndoMac progenitors in murine aorta, heart and lung that also originate from a YS source. These progenitors are more abundant at birth, at which time they have enhanced clonal and self-renewal capacity and are maintained independently of definitive haematopoiesis. As shown by differentiation, adoptive transfer and BrdU pulse-chase studies, they are bipotent for macrophage and endothelial lineages and possess vasculogenic capacity. In association with expression of the haemangioblastic markers, ACE, AT1R and AT2R, we demonstrate a pathophysiologically relevant regulatory role for AngII, which rapidly stimulates their clonogenic, self-renewal and differentiation properties.

Most mature cell types rely on the self-renewal of stem cells and proliferation and differentiation of transient amplifying progenitors for their homeostatic turnover and recovery after tissue insult. Although there has been conjecture about how embryonically derived macrophages are maintained after birth, prevailing opinion favours their ability to self-renew without losing functional or differentiated status (178). This was initially based on experiments showing self-renewal of macrophages in which MafB and c-Maf were genetically knocked out (580). Subsequent studies have provided contrasting data either supporting the long-lived nature of tissue-resident macrophages, especially microglia (581), their stochastic turnover (553, 582), repopulation by clonal expansion (583, 584) or differentiation from local progenitors (585). The mechanistic basis for macrophage self-renewal has been linked to downregulation of MafB/cMaf, which occurs constitutively in some macrophage populations (e.g. alveolar macrophages) or can be induced (e.g. by M-CSF). This in turn activates a self-renewal gene network, centred around *Myc*, *Klf2* and *Klf4* (576). Evidence that embryonically derived macrophages undergo *bona fide* self-renewal in aorta and heart is somewhat circumstantial and includes the persistent labelling of some macrophage subtypes after postnatal induction of fate-mapping in *Cx3cr1^{CreER}* and

Csf1r^{MerCreMer} reporter mice (168, 569). However, these results are also compatible with the turnover and differentiation of previously unrecognised EndoMac progenitors, which express CX₃CR1 and CSF1R.

As shown here, growth of CFU-M from adult tissues is not due to clonal proliferation of monocytes or macrophages, but rather YS-derived progenitors. The finding that CFU-M undergo self-renewal from single cell origins paved the way for us to study their composition and uncover a unique LIN⁻CD45^{+/Lo}CD11b⁻F4/80⁻Sca-1⁺c-Kit⁺CX₃CR1⁺CSF1R⁺ fingerprint, that identifies progenitors in both digested tissue and culture-derived CFU-M. This surface phenotype distinguishes EndoMac progenitors from BM HSCs and their lineage-committed progenitors, such as macrophage/dendritic cell progenitors and common monocyte progenitors, which lack Sca-1 but express Flt3/CD135 and Ly6C, respectively (586, 587). As a relatively high percentage of progenitors are actively dividing even in steady state, it is not surprising that their ability to self-renew is finite and diminishes with aging, along with their clonogenic and angiogenic capacity. Combined with a lack of circulatory renewal from BM HSCs, this helps explain why tissue progenitor numbers decrease postnatally at a faster rate than their less proliferative, durable endothelial and macrophage progeny. However, we also demonstrate that their proliferation and self-renewal can be amplified by stimulatory cues, such as elicited by AngII.

As in previous studies (588), we found negligible contribution from YS to the adult HSC pool. As revealed by embryonic profiling across different gestational ages and timed induction of fate-mapping, EndoMac progenitors emerge in YS around E7.5-E8.5 and expand there until E10.5, by which time they have migrated intra-embryonically to AGM, heart and other tissues. This aligns with prior findings for multipotent EMPs and CX₃CR1⁺ pre-macrophages (560). Throughout our differentiation assays, EndoMac progenitors adopted the fast-track pathway of macrophage differentiation characteristic of early YS EMPs, producing macrophages without passing through a monocyte intermediate stage. This separates them from late EMPs that colonise foetal liver where they produce monocytes and other lineage-committed progenitors (561). This was reflected here by mixed growth of different CFU types from E12.5-15.5 liver, as distinct from selective CFU-M growth from AGM and heart at the same ages.

Whereas the multipotency of YS EMPs includes primitive erythroid cells, megakaryocytes, mast cells and neutrophils (30, 558, 589), we found no evidence that postnatal EndoMac progenitors produce these lineages (not shown). Their differentiation capacity for macrophages and endothelium is therefore positioned between that of EMPs (30, 132) and pre-macrophages in YS (559). Consistent with this, they have an intermediate surface marker phenotype that retains expression of the early EMP markers, c-Kit, CSF1R, CD93, CD16/32 and CD45 (Lo) but not CD41, while acquiring the pre-macrophage marker, CX₃CR1. By comparison, pre-macrophages are otherwise characterised as CD45⁺c-Kit⁻CD11b^{Lo} (559).

The classical origin of embryonic vascular endothelial cells is the differentiation of mesoderm-derived angioblasts around E7.0 YS in mice (590). Subsequently, endothelial cells undergo local proliferation during tissue angiogenesis, with evidence for clonal expansion in adult tissues, such as heart and muscle under ischaemic insult (591). Other identified sources of postnatal endothelial renewal include circulating endothelial progenitor cells (516) and tissue-resident endovascular progenitors (229). Plein *et al.* recently reported that endothelial cells in YS and some embryonic and adult tissues also derive from YS EMPs (132), although this was not reproduced by another study that used alternative lineage mapping techniques (563). In tracking the fate of early YS CX₃CR1⁺ and CSF1R⁺ progenitors, we examined both their macrophage and endothelial progeny through the lens of EndoMac progenitors. While endothelial cells were the most abundant lineage labelled in heart at each of the three ages studied, E15.5 aorta contained a predominance of YS-derived progenitors, that were replaced after birth by increasing numbers of macrophages and endothelial cells. In addition to the self-renewal of YS-derived macrophages and endothelium, another feasible explanation for this finding is the postnatal transformation of progenitors into these two distinct lineages. We consistently observed this to occur across different *in vitro*, *in vivo* and BrdU pulse-chase studies, which also highlighted the highly proliferative nature of the progenitor population. Although our results suggest a differentiation bias toward the endothelial lineage in the settings of post-ischaemia repair and *in vitro* angiogenesis, these progenitors can also rapidly generate macrophages, as seen after peritoneal injection. Importantly, they

appear predisposed to forming macrophages that are LYVE-1⁺MHCII^{-Lo}CCR2⁻, consistent with the identity of YS-derived macrophages that reside in heart and adventitia (168, 569, 571).

The century-old notion of the haemangioblast, a bipotent progenitor for endothelial and haematopoietic cells in development (542), has been supported by *in vitro* evidence of a common pathway of haemato-endothelial differentiation from pluripotent stem cells (564, 565, 592). Given their macrophage-restricted haematopoietic potential and lack of expression of the canonical haemangioblast marker, Brachyury, we do not propose that EndoMac progenitors are *bona fide* haemangioblasts. However, their expression of other haemangioblast markers, namely ACE and both AngII receptor subtypes (564), focused our attention on AngII as a regulator of their properties within adult vasculature. AngII stimulated the proliferative, clonogenic, self-renewal and macrophage-forming capacity of aortic EndoMac progenitors, and upregulated relevant genes, such as *Nanog*, *Myc*, *Irf8* and *Arg1*. Together with our results from short-term AngII infusion in *Flt3*^{Cre} and *Cx3cr1*^{CreER} mice, this indicates that these progenitors are primed to provide an immediate proliferative response to AngII, that helps to feed the expansion of YS-derived adventitial macrophages and endothelial cells. As shown here and in another recent study (571), this is complemented by recruitment and proliferation of BM-derived macrophages, which also contribute to AngII-induced adventitial inflammation.

4.5 Conclusion

In conclusion, our discovery of EndoMac progenitors adds to the recognised fate of YS EMPs in embryonic and postnatal tissues. Our findings demonstrate that these YS-derived progenitors provide a local source of macrophage and endothelial renewal that can be rapidly switched on during the early phases of tissue inflammation and neovascularisation postnatally.

Chapter 5

Yolk sac-derived Endothelial-Macrophage progenitor cells in murine skeletal muscle contribute to neovascularisation and perfusion recovery after hindlimb ischaemia.

5.1 Introduction

Skeletal muscle (SkM) is a plastic tissue with significant capacity to regenerate and adapt following severe damage, that may occur due to trauma, ischaemia or various forms of myopathy and muscular dystrophy. Peripheral artery disease causing ischaemia can result in lower limb amputation, and is a major cause of morbidity and health economic burden worldwide (593). Ischaemia and resulting SkM damage trigger a complex and coordinated response, involving different cell populations that interact to mediate progression from a pro-inflammatory to anti-inflammatory state, in parallel with neovascularisation, fibrosis and ultimately muscle regeneration and repair (499, 500, 594-597). Among these interactions, macrophages and endothelial cells share a strong, bidirectional relationship which is essential for efficient vascular remodelling and recovery of the ischaemic tissue (486, 598).

Macrophages are the major infiltrating immune population in ischaemic SkM, where they play various important functions, that include: 1) secreting pro-inflammatory cytokines and chemokines to promote early leukocyte infiltration; 2) phagocytosis and removal of necrotic cell debris; 3) release of matrix metalloproteinases for extracellular matrix remodelling; 4) promoting endothelial cell proliferation, migration and angiogenesis by producing pro-angiogenic factors and guiding the sprouting of new blood vessels through cell-to-cell contact; and 5) supporting activation and differentiation of muscle stem cells to regenerate myocytes (486, 598). This functional pleiotropy reflects the vast heterogeneity and plasticity that is more broadly characteristic of tissue-resident macrophages and has been attributed to their diverse ontogeny and the local regulatory effects of different tissue and disease environments (123, 554, 599-601).

Traditional views of macrophage infiltration in ischaemic SkM emphasised the early and transient recruitment of inflammatory monocytes (CCR2⁺Ly6C^{Hi}CX₃CR1^{Lo} subtype in mice) from blood, which arise from definitive haematopoiesis in bone marrow (BM) (602, 603). Monocytes differentiate into macrophages, which proliferate and mature in a process that has been shown in mice to involve downregulation of Ly6C and upregulation of CX₃CR1, CD11c and MHC class II (MHCII) over time

(602). Notably, this is regulated by instructive cues expressed by local vascular endothelial cells, such as delta like canonical Notch ligand 1 (Dll1). Previous studies have also suggested that macrophages undergo polarisation switches from the classically activated, pro-inflammatory M1-like state to the alternatively activated M2-like state as tissue healing progresses, although this historical classification system is oversimplified and outdated (461, 486, 492).

It is now established that in many murine tissues, resident macrophages are also embryonically derived from yolk sac (YS) erythromyeloid progenitor cells (EMPs), that emerge in two distinct waves, primitive and definitive haemopoiesis, from embryonic day 7 (E7) and E8.5, respectively (2, 93, 109). Early EMPs contribute to primitive haemopoiesis, express c-Kit and colony stimulating factor 1 receptor (CSF1R) and differentiate into CX₃CR1⁺ pre-macrophages and mature macrophages. This wave sees the emergence of microglia in brain (1, 93, 123). In contrast, definitive haemopoiesis sees the appearance of late EMPs which are c-myb⁺ and migrate to foetal liver, where they form foetal monocytes that seed macrophages in different embryonic tissues, except for brain (2). Embryonically derived macrophages persist into postnatal life and are maintained independently of monocyte recruitment and BM haematopoiesis, through what is widely regarded to be proliferative self-renewal (162, 166, 194, 604). A recent study of macrophage diversity in steady state mouse SkM identified two main subsets, distinguished by surface expression of LYVE-1 and MHCII (172). In addition to finding transcriptomic differences between these subpopulations, it also demonstrated different ontogenies, whereby LYVE-1^{Lo}MHCII^{Hi} macrophages predominantly arose from definitive haematopoietic stem cells (HSCs) and circulating monocytes, while LYVE-1^{Hi}MHCII^{Lo} macrophages had mixed origins from HSCs and early and late YS EMPs.

Aided by paracrine and structural support from macrophages, endothelial cells proliferate, migrate, and coalesce to form vascular labyrinths in ischaemic SkM, that restore oxygen and nutrient delivery to the regenerating tissue (220, 365). Single-cell sequencing data have uncovered that tissue-resident endothelial cells are also heterogeneous (605-607), which may be a product of both different microenvironments and different developmental origins. Lineage-tracing using multi-colour reporter

mice has recently shown that endothelial cells mostly undergo clonal expansion to generate new vessels in response to ischaemia in heart and SkM, with limited contribution from circulating BM-derived progenitors (597, 605, 606). Aligned with this, other studies have identified locally resident hierarchies of endovascular progenitor or stem cells that occupy the walls of blood vessels and are highly proliferative (226, 229, 608, 609).

A shared ancestral link between haematopoietic cells and endothelium has long been postulated based on the presence of haemogenic endothelium in embryonic YS and aorta (136, 548) and the emergence of bipotent haemangioblasts during primitive vascular development and haematopoiesis (57, 610, 611). Interestingly, Plein *et al.* have reported that YS EMPs are also a source of endothelial cells that persist in the vasculature of postnatal mouse brain, heart and liver, although this has not yet been described for SkM (132). We recently discovered a YS-derived population of undifferentiated endothelial-macrophage (EndoMac) progenitors in mouse aorta, heart and lung (Chapter 4). These bipotent cells display a unique LIN⁻CD45^{+/Lo}CD11b⁻F4/80⁻Sca-1⁺c-Kit⁺CX₃CR1⁺CSF1R⁺ surface marker phenotype, are highly proliferative even in steady state, and have capacity for clonal expansion, self-renewal and vasculogenesis, which can be further augmented under certain stimulatory cues. Here we establish the presence of YS-derived, locally maintained EndoMac progenitors in SkM of mouse hindlimb and demonstrate their proclivity for rapid expansion and differentiation into macrophages and endothelial-lined blood vessels after acute ischaemia.

5.2 Methods in brief (also see Chapter 2)

5.2.1 Overview

Breeding pairs of C57BL/6 (C57BL/6J), *Cx3cr1*^{GFP/+} (B6.129P-Cx3cr1^{tm1Litt}/J), *Flt3*^{Cre} (Tg.Flt3^{cre}/ERT2), *Rosa*^{mT/mG}(Gt(ROSA)26Sor^{tm4}(ACTB-tdTomato,-EGFP)Luo/J), *Csf1r*^{Mer-iCre-Mer} (FVB-Tg(Csf1r-cre/Esr1*)1Jwp/J), *Cx3cr1*^{YFP-creER} (B6.129P2(Cg)-Cx3cr1^{tm2.1}(cre/ERT2)^{Litt}/WganJ), *UBI-GFP* (C57BL/6-Tg(UBC-GFP)30Scha/J) mouse strains were acquired from The Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed in the animal care facilities at SAHMRI, and were maintained on standard chow diet. Skeletal muscle (SkM) from the quadriceps and gastrocnemius of these mice was either prepared into tissue sections for immunofluorescence staining and confocal microscopy, or enzymatically digested into single cell suspensions for use in different experiments, including methylcellulose colony forming assay, MatrigelTM-based angiogenesis and multi-colour flow cytometry. Several experiments also made use of the murine model of hindlimb ischaemia (511) in combination with the *Flt3*^{Cre} x *Rosa*^{mT/mG} or tamoxifen (TAM)-inducible *Csf1r*^{MerCreMer} x *Rosa*^{mT/mG} fate-mapping strains or with adoptive transfer of GFP⁺ SkM EndoMac progenitors, to study their proliferative response and fate in the setting of peripheral ischaemia. All animal experiments and care were performed in accordance with the standards stated in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Academy of Sciences, Bethesda, MD, USA) and in accordance with the National Health and Medical Research Council of Australia research guidelines and Australian Code for the Care and Use of Animals for Scientific Purposes. Ethics approval was obtained from the SAHMRI animal ethics committee (ID SAM117, SAM155, SAM308 and SAM432.19).

5.2.2 Statistical analysis

Where relevant, data analysis was performed blinded to study group. Statistical analyses were performed using Prism (GraphPad version 9). Data sets were tested for normality of distribution by Shapiro-Wilk test. Statistical comparisons were performed with parametric or non-parametric unpaired or paired two-sample *t*-tests or ANOVA (with post-test multiple comparisons), as specified. Results are expressed as mean $\pm$ standard deviation of multiple experiments. In all cases, statistical significance was established at two-tailed $p < 0.05$. The statistical parameters and the number of mice used per experiment are found in the figure legends.

5.3 Results

5.3.1 Skeletal muscle contains CX₃CR1⁺c-Kit⁺ CFU-M progenitors that are capable of self-renewal

Single cell suspensions were prepared from digests of quadriceps and gastrocnemius SkM from 8-12 week-old (w) C57BL/6J mice and cultured in methylcellulose for 14 days (d) to study haematopoietic colony formation. As described previously for mouse aorta (262), SkM cells generated the full spectrum of haematopoietic CFUs but showed marked predilection for CFU-M (>95%) (**Figure 5-1a**). Primary (1°) SkM CFU-M yield was comparable to that of donor-matched aortic cells (29.8±14.9 vs 23.5±8.9 colonies/10⁵ cells, n=12) (**Figure 5-1b**), with the majority being small colonies containing 30-100 cells (**Figure 5-1c**).

In keeping with the properties of CFU-M progenitors in other tissues (Chapters 4), we next confirmed the ability of SkM CFU-M to undergo self-renewal in secondary (2°) methylcellulose cultures. First, we replated the entire cellular content of 1° methylcellulose cultures and re-cultured for another 14 d. 2° CFU-M yield was ~6-fold that of 1° CFU-M yield (**Figure 5-1d**). To examine colony self-renewal further, we isolated individual CFU-M from 1° cultures under microscopic guidance and disaggregated each colony into single cell suspensions that were seeded in different wells. Importantly, SkM CFU-M were observed to renew from single cell origins (**Figure 5-1e**), with 81.1% of 1° CFU-M giving rise to at least one 2° colony and a yield of 1 new colony formed for every 15.2±9.5 cells plated (3 donor mice).

This finding led us to study the immunophenotypic identity of self-renewing CFU-M progenitors. As reported for aorta, heart, lung and embryonic YS in Chapter 4, CFU-M were enriched with LIN⁻CD45⁺CD11b⁻F4/80⁻Sca-1⁺c-Kit⁺CX₃CR1⁺ progenitors, which accounted for ~90% of their cellular composition (n=6 mice, 2 independent experiments) (**Figure 5-1f**). Although SkM CFU-M progenitors

also displayed moderate expression of CSF1R, the receptor for macrophage colony-stimulating factor (M-CSF), they were negative or lowly expressing for various other macrophage (CD206, CD86, CCR2, MerTK, MHCII, CD64, Ly6C, LYVE-1) (**Figure 5-1h**) and endothelial (CDH5, VEGFR2, LYVE-1, TIE2) surface markers, except for CD31, which is also known to be expressed on myeloid progenitors in both embryonic YS and postnatal BM (612-616) (**Figure 5-1i**).

Flow cytometry analysis identified that LIN⁻CD45^{+/Lo}CD11b⁻F4/80⁻c-Kit⁺Sca-1⁺CX₃CR1⁺ progenitor cells made up 0.4±0.3% of all viable cells in fresh digests of 12 w SkM or 3816±2862 cells/g (n=12 mice) (**Figure 5-1g**). Notably, ~30% of progenitor cells in steady state muscle were found to occupy the proliferating S or G2/M phases of cycle, as determined by bromodeoxyuridine (BrdU) uptake (**Figure 5-1j**).

We corroborated the presence of CX₃CR1⁺ (GFP⁺) c-Kit⁺ progenitor cells in sections of gastrocnemius muscle from adult *Cx3cr1*^{GFP/+} mice using fluorescence confocal microscopy (**Figure 5-1k**). Flow cytometry revealed that in these mice, progenitors accounted for 24.1±4.7% of CX₃CR1⁺ (GFP⁺) cells in SkM digests, while LIN⁻CD45⁺CD11b⁺F4/80⁺ macrophages made up another 46.7±7.6% (n=6 mice) (**Figure 5-1l**). Having previously established that CX₃CR1 helps to regulate the clonal expansion of CFU-M progenitors in aorta (Chapter 4), we compared CFU-M yield and progenitor numbers from gastrocnemius in *Cx3cr1*^{GFP/+} mice and their homozygote *Cx3cr1*^{GFP/GFP} littermates that lack the functional *Cx3cr1* allele. *Cx3cr1*^{GFP/GFP} SkM displayed a trend for lower 1° CFU-M yield (21.7±8.1 versus 37.3±21.4 per 10⁵ cells, p=0.07, n=5 mice) (**Figure 5-1m**), with a non-significant difference also seen in progenitor prevalence as determined by flow cytometry (**Figure 5-1n**). Collectively, these results show that adult mouse SkM contains CFU-M progenitor cells that are capable of self-renewal with similar surface marker expression as YS-derived EndoMac progenitors that we have identified in other tissues.

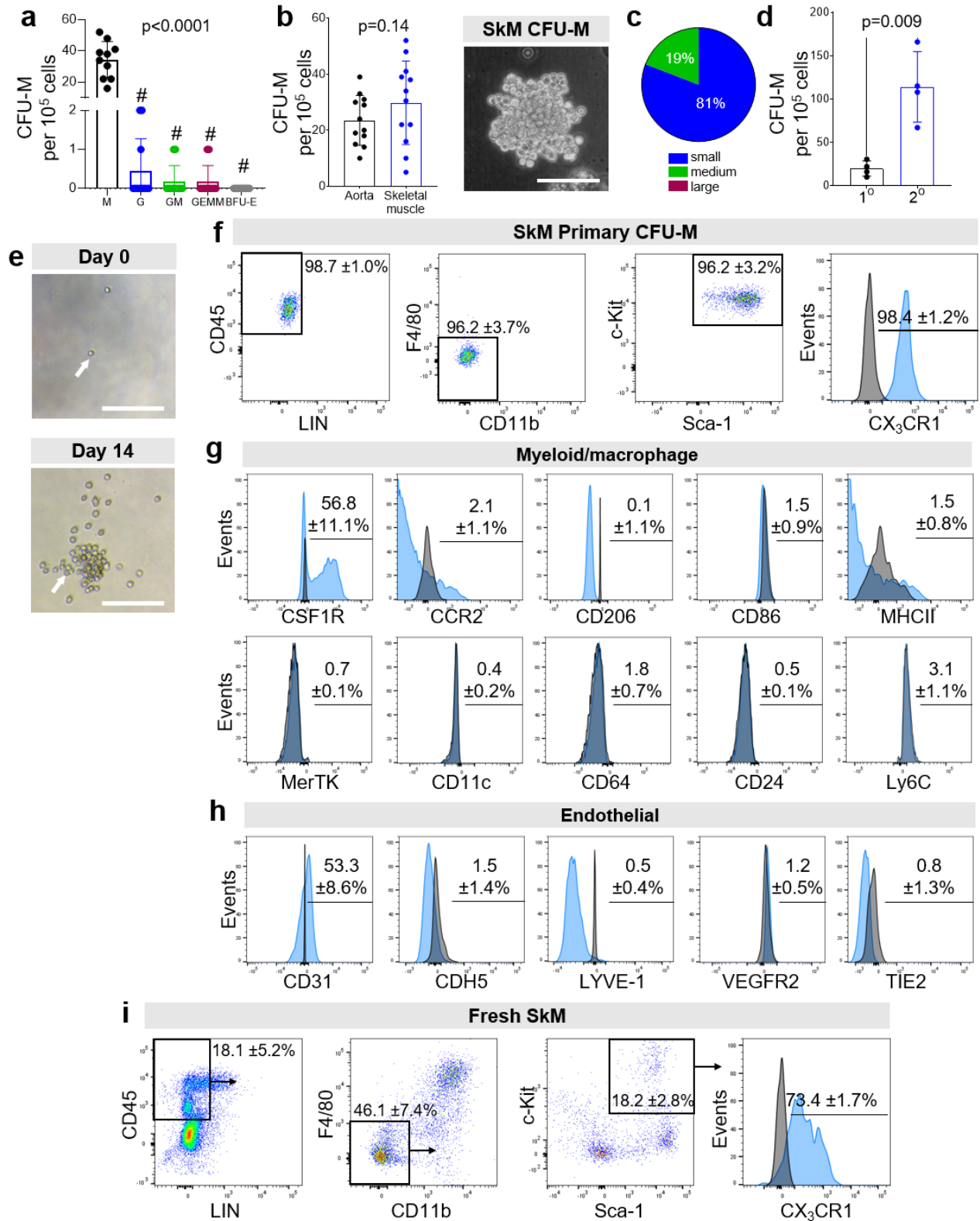


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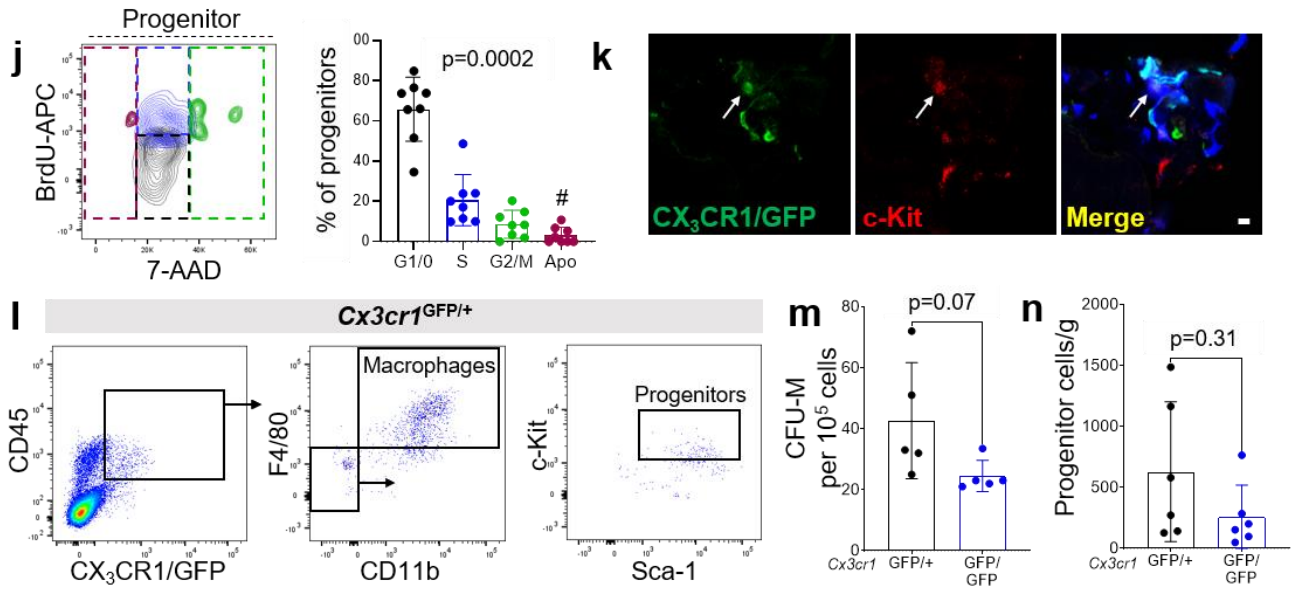


Figure 5-1. Skeletal muscle contains CD45⁺c-Kit⁺CX₃CR1⁺ CFU-M progenitors capable of self-renewal.

(a) Frequency of different CFU types from C57BL/6 skeletal muscle (SkM) cells. N=5 mice. M, Macrophage; GM, Granulocyte-macrophage; GEMM, Granulocyte-erythrocyte-monocyte-megakaryocyte; G, Granulocyte; BFU-E, Burst-forming units-erythroid. Kruskal-Wallis tests. Multiple test comparisons: #p<0.0001 for comparison to CFU-M.

(b) Comparison of 1° CFU-M from donor-matched aorta and SkM digests. N=12 mice. Paired *t*-test. Example of a medium-sized (100-1000 cells) CFU-M from 12 w C57BL/6 SkM cells. Scale bar, 100 μm.

(c) Pie chart shows breakdown of CFU-M by colony size. N=5 mice.

(d) Donor-matched primary (1°) and secondary (2°) CFU-M yield from adult C57BL/6 SkM. N=4 mice. Paired *t*-test.

(e) Self-renewal of 2° colony from single cell obtained from 1° CFU-M. Scale bar, 100 μm.

(f) Flow cytometry of cells from 1° SkM CFU-M. N=3 mice. Blue histogram, Sample. Dotted, FMO control.

(g-h) Flow cytometry histograms depict expression of different **(g)** myeloid/macrophage and **(h)** endothelial markers on surface of cells from 1° SkM CFU-M (blue histogram). Grey histograms, FMO controls. Representative of n=3 12 w C57BL/6 mice.

(i) Flow cytometry plots of SkM digest show progenitor population. N=4 mice.

(j) Flow cytometry contour plot for bromodeoxyuridine (BrdU) versus 7-aminoactinomycin (7-AAD) gated from SkM progenitors, shows cells in G1/0 (black), S (blue), G2/M (green) and apoptotic (maroon) phases. Percentages of progenitors in each phase from 12 w C57BL/6 SkM. N=8 mice. One-way ANOVA. Multiple comparison tests: #p<0.0001 for comparison to G1/0.

(k) Confocal microscopy of SkM from 12 w *Cx3cr1*^{GFP/+} mouse shows CX₃CR1⁺c-Kit⁺ progenitors. Scale bar, 10 µm. See **Appendix 8** for bigger images.

(l) Flow cytometry gating strategy used to quantify CD45^{+/Lo}CD11b⁺F4/80⁺Sca-1⁺c-Kit⁺CX₃CR1⁺ progenitor cells and CD45⁺CD11b⁺F4/80⁺ CX₃CR1⁺ macrophages in gastrocnemius SkM of 12 w *Cx3cr1*^{GFP/+} mice.

(m-n) Frequency of **(m)** CFU-M and **(n)** progenitors from SkM of 12 w *Cx3cr1*^{GFP/+} and *Cx3cr1*^{GFP/GFP} mice. N=7-10 mice. Unpaired *t*-tests.

Data summarised as mean±SD.

5.3.2 Skeletal muscle CFU-M progenitors arise from embryonic YS and are not derived from BM-derived haematopoiesis

We next interrogated the origins of CFU-M progenitors in adult mouse SkM. Because definitive HSCs transiently up-regulate growth factor fms-like tyrosine kinase 3 (*Flt3*) as they differentiate (570), we used *Flt3*^{Cre} x *Rosa*^{mT/mG} mice to determine whether SkM progenitors arise from a BM (definitive HSC) source, in which they would be *Flt3*-Cre⁺ and therefore GFP⁺. As expected, >90% of haematopoietic CFUs, including CFU-M, from BM and spleen were GFP⁺tdTom⁻ (*Flt3*-Cre⁺). In contrast, the vast majority of SkM CFU-M were GFP⁻tdTom⁺ (*Flt3*-Cre⁻), as shown both by fluorescence microscopy (**Figure 5-2a**) and flow cytometry (**Figure 5-2b**). CD45⁺CD11b⁺F4/80⁻ blood monocytes demonstrated ~98.5% GFP⁻tdTom⁻ expression (**Figure 5-2c**), which represents near complete Cre-Lox recombination efficiency in this fate-mapping system. By comparison, Lin⁻CD45^{+/Lo}CD11b⁻F4/80⁻Sca-1⁺c-Kit⁺ progenitors from 12 w gastrocnemius/quadriceps were ~94% GFP⁻tdTom⁺ (n=4 mice) (**Figure 5-2d**). These data indicate that SkM CFU-M progenitors are independent of *Flt3*⁺ definitive haematopoiesis, as occurs in postnatal BM.

Together with our discovery that extra-embryonic YS seeds CFU-M progenitors in aorta and other murine tissues, the results from *Flt3* Cre-Lox fate-mapping prompted us to study whether SkM progenitors are also embryonically derived. Postnatal age-profiling revealed that progenitors in gastrocnemius/quadriceps were most abundant in newly born (P1) mice and progressively reduced in number in adulthood (12 w) and advanced age (52 w) (**Figure 5-2e,f**). Similarly, P1 CFU-M comprised higher proportions of medium and large-sized colonies, suggesting greater capacity for clonal expansion and/or self-renewal, while these larger colonies were very infrequent from 52 w SkM (**Figure 5-2g**). These findings were suggestive of prenatal seeding, which was supported by the presence of c-Kit⁺CX₃CR1⁺ progenitor cells in sections of hindlimb from *Cx3cr1*^{GFP/+} mice at E15.5 gestation (**Figure 5-2h**).

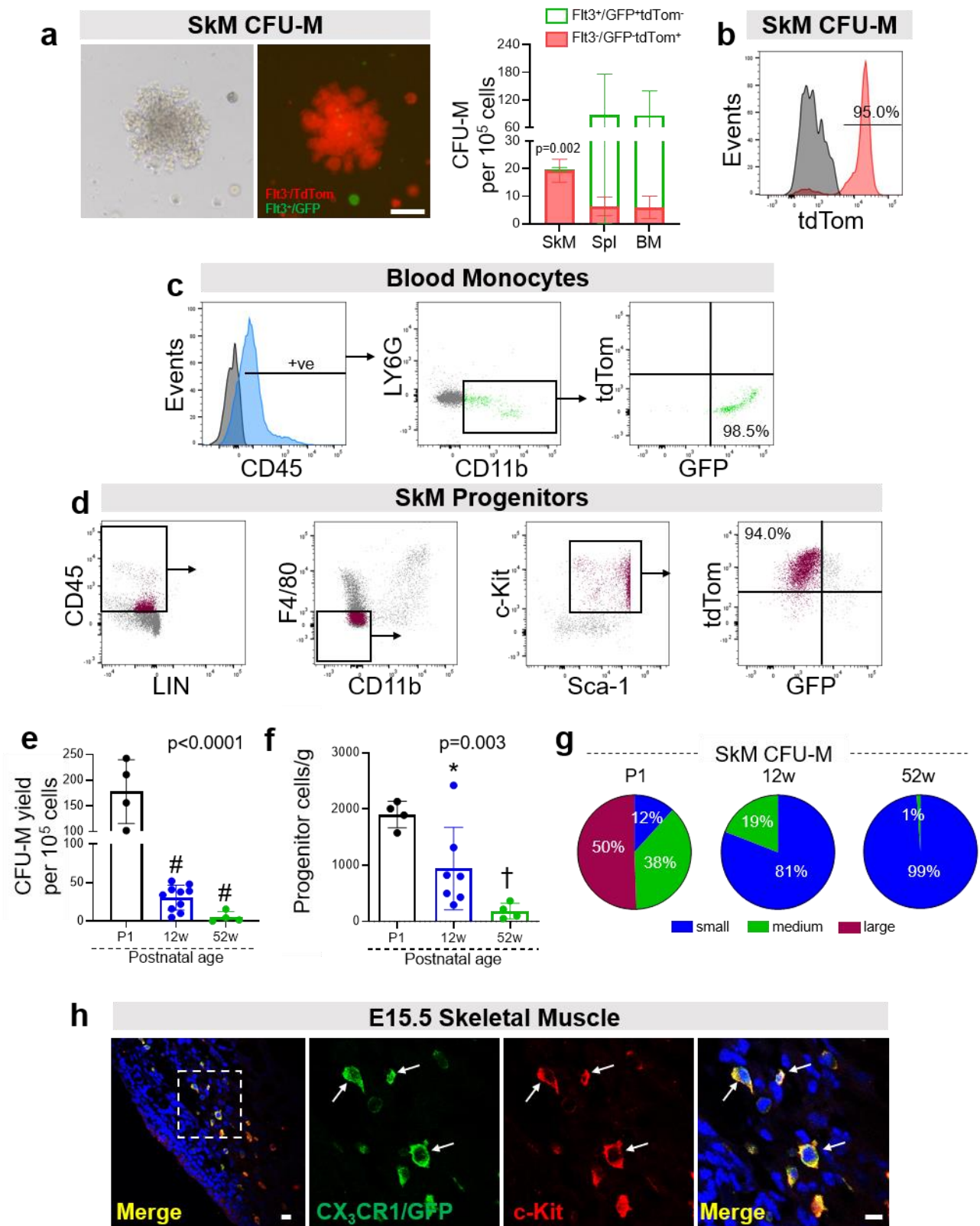


Figure 5-2. SkM CFU-M progenitors do not arise from definitive haematopoiesis.

(a) Light and fluorescence microscopy of SkM CFU-M from adult *Flt3^{Cre}* x *Rosa^{mT/mG}*, mouse. Graph of Flt3⁺ and Flt3⁻ CFU-M yield from different tissues. N=5 mice. Spl, Spleen. BM, Bone marrow. Paired *t*-tests for SkM. Scale bar, 100 μ m.

(b) Representative tdTom expression of cells in CFU-M from *Flt3^{Cre}* x *Rosa^{mT/mG}* SkM.

(c-d) Flow cytometry showing **(c)** GFP (*Flt3-Cre⁺*) expression of blood CD45⁺CD11b⁺LY6G⁻ monocytes (green) and **(d)** tdTom (*Flt3-Cre⁻*) expression of SkM progenitors (maroon) from 12 w *Flt3^{Cre}* x *Rosa^{mT/mG}* mice. Percentages represent mean of n=4 mice.

(e-f) Age comparisons of **(e)** 1^o CFU-M yield and **(f)** number of progenitors for C57BL/6 SkM. N=3-6 mice. One-way ANOVA for both. Multiple comparison tests: * *p*<0.05; †*p*<0.01 and #*p*<0.0001 for comparison to P1.

(g) Pie charts show classification of CFU-M based on colony size for SkM at different postnatal ages. Percentages represent mean of n=3-7 mice.

(h) Confocal microscopy of CX₃CR1⁺c-Kit⁺ progenitor cells (arrows) in lower hindlimb muscle at E15.5 from *Cx3cr1^{GFP/+}* embryos. Scale bar, 10 μ m.

Data summarised as mean $\pm$ SD.

Wang *et al.* recently reported that a subset of resident macrophages in postnatal SkM originates from a CSF1R⁺ YS source (172). We therefore used two complementary 4-hydroxytamoxifen (TAM)-induced lineage-tracing systems to elucidate whether SkM CFU-M progenitors are also YS-derived. TAM was administered *in utero* to *Csf1r*^{MerCreMer} $\times$ *Rosa*^{mT/mG} mice at E8.5 to induce expression of GFP labelling in YS CSF1R⁺ cells, including primitive macrophages, EMPs, and their progeny (132, 554) (**Figure 5-3a**). Tissues were harvested postnatally at 12 w, at which time 54.1% of brain microglia were labelled GFP⁺ (n=4 mice). As microglia exclusively arise from YS, the observed number of GFP⁺ cells indicate incomplete efficiency of TAM-induced Cre-Lox recombination. Induction at E8.5 also labelled 44.9% of progenitors, 16.3% of macrophages and 6.7% of endothelial cells (up to 11.0% for CDH5⁺ endothelial cells) in hindlimb SkM, with negligible GFP expression observed in blood monocytes by flow cytometry (**Figure 5-3b-d**). After normalising for microglia, this meant that 83% of SkM progenitors were YS-derived. In support of this, normalised labelling was 100% for CFU-M from SkM, compared to <4% for BM colonies, as assessed by fluorescence microscopy (**Figure 5-3e**). Flow cytometry also revealed that progenitors accounted for 11.7 $\pm$ 8.5% of GFP⁺ cells in 12 w SkM digests, while 15.2 $\pm$ 1.9% were macrophages and 72.7 $\pm$ 6.3% were endothelial cells (**Figure 5-3f,g**). Similar results were obtained from *Cx3cr1*^{CreER-YFP} $\times$ *Rosa*^{tdTom} mice that had been administered TAM at E9.5 to induce tdTom labelling in *Cx3cr1*⁺ YS cells and their progeny (**Figure 5-4**). Therefore in addition to resident macrophages (172), our data show for the first time that YS CSF1R⁺ CX₃CR1⁺ progenitors also give rise to CFU-M progenitors and endothelial cells in postnatal SkM, consistent with our observations for other tissues.

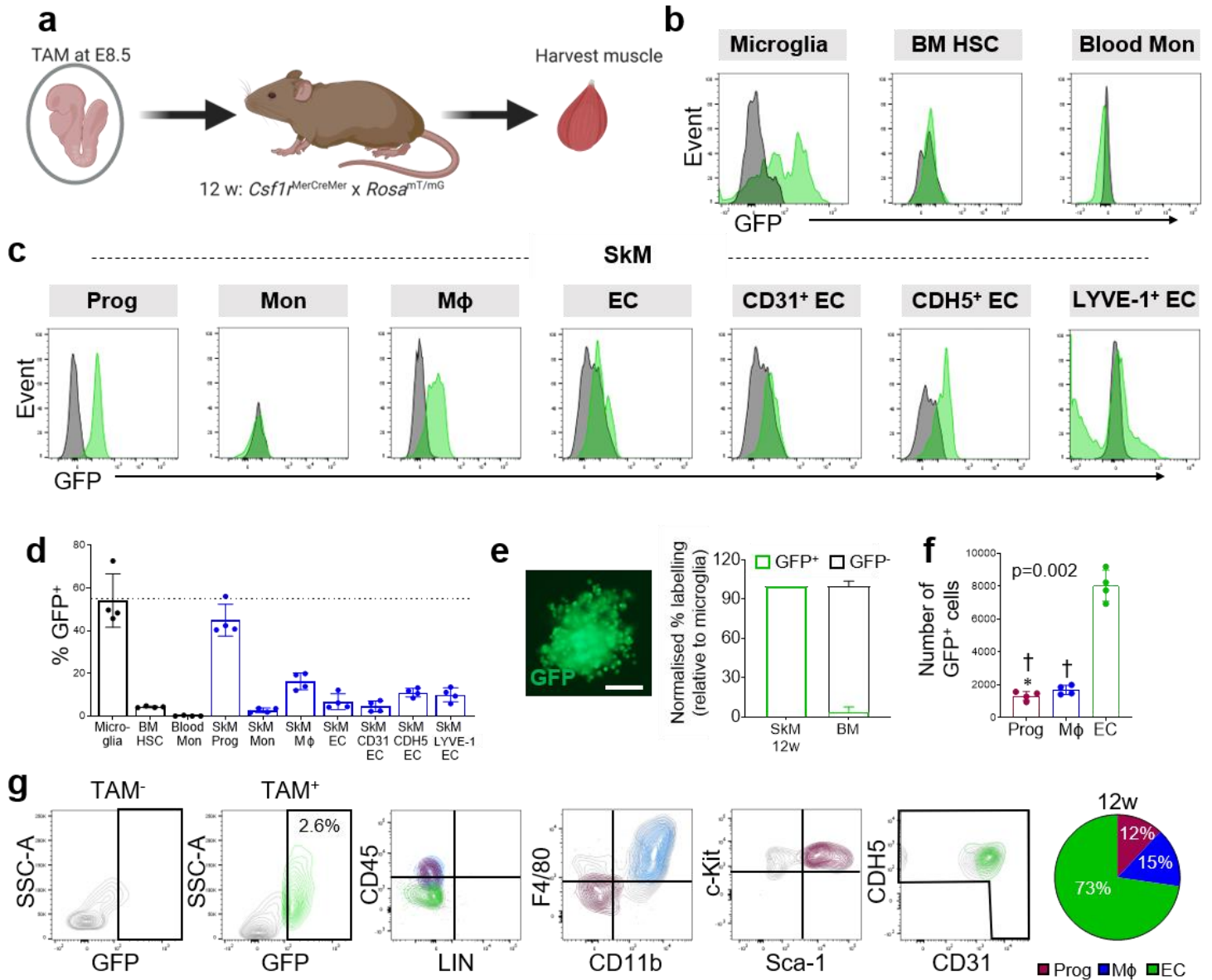


Figure 5-3. SkM progenitors arise from an E8.5 YS CSF1R⁺ source.

(a-g) Pregnant *Csf1r*^{MerCreMer} x *Rosa*^{mT/mG} mice were induced with TAM at E8.5, with offspring analysed at 12 w. **(a)** Experimental design.

(b-c) Representative histograms shows percentage of GFP expression in different cell types and **(d)** summarised percentage in graph. Green histogram, Sample; Grey, No TAM Cre control. Mon, monocytes; HSC, haemopoietic stem cells; Prog, Progenitors; Mφ, Macrophages; EC, Endothelial cells.

(e) GFP⁺ CFU-M from 12 w SkM. Graph shows GFP⁺ vs GFP⁻ status of CFU-M from 12 w SkM and BM, with results normalised to CNS microglia. N=4 mice. Scale bar, 100 μm.

(f) Numbers of GFP⁺ progenitors (prog), macrophages (M ϕ) and endothelial cells (EC) in 12 w SkM. N=4 mice. One-way ANOVA. Multiple comparisons test: †p<0.05 for comparison to EC.

(g) Flow cytometry examples of GFP⁺ progenitors (maroon), macrophages (blue) and endothelial cells (green) in 12 w SkM. Pie charts show lineage breakdown of GFP⁺ cells. N=4 mice.

Data summarised as mean $\pm$ SD.

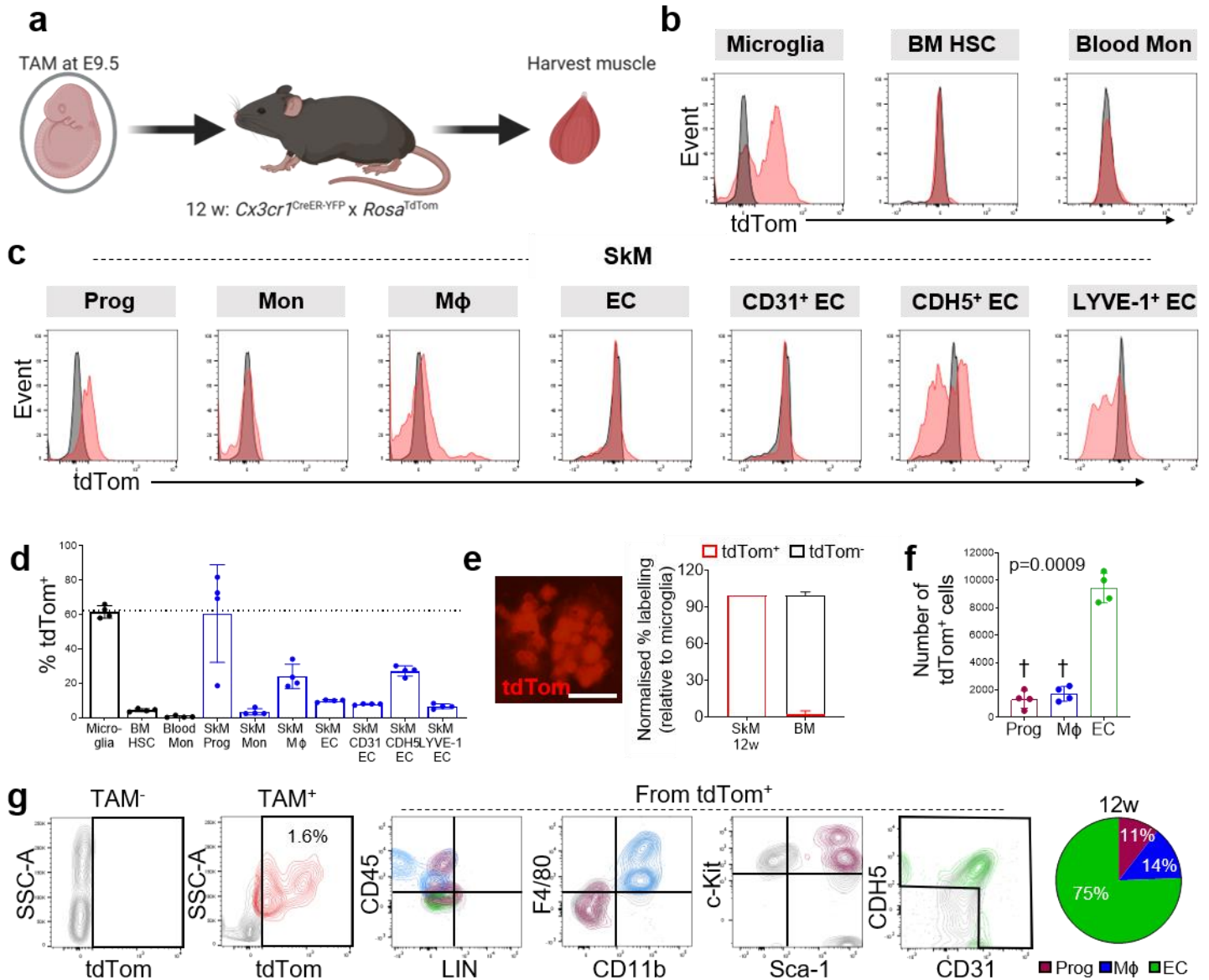


Figure 5-4. SkM progenitors arise from an E9.5 YS CX₃CR1⁺ source.

(a-g) Pregnant *Cx3cr1*^{CreER-YFP} × *Rosa*^{tdTom} mice were induced with TAM at E9.5, with offspring analysed at 12 w. **(a)** Experimental design.

(b-c) Representative histograms shows percentage of tdTom expression in different cell types and **(d)** summarised percentage in graph. Red histogram, Sample; Grey, No TAM Cre control. Mon, monocytes; HSC, haemopoietic stem cells; Prog, Progenitors; Mφ, Macrophages; EC, Endothelial cells.

(e) tdTom⁺ CFU-M from 12 w SkM. Graph shows tdTom⁺ vs tdTom⁻ status of CFU-M from 12 w SkM and BM, with results normalised to CNS microglia. N=4 mice. Scale bar, 100 μm.

(f) Numbers of tdTom⁺ progenitors (prog), macrophages (M ϕ) and endothelial cells (EC) in 12 w SkM. N=4 mice. One-way ANOVA. Multiple comparisons test: †p<0.05 for comparison to EC.

(g) Flow cytometry examples of tdTom⁺ progenitors (maroon), macrophages (blue) and endothelial cells (green) in 12 w SkM. Pie charts show lineage breakdown of GFP⁺ cells. N=4 mice.

Data summarised as mean $\pm$ SD.

5.3.3 YS-derived SkM progenitors proliferate and expand rapidly in response to ischaemia.

Interacting closely together, macrophages and endothelial cells are central players in the inflammatory and neovascularisation responses that occur in SkM in response to ischaemia (486, 598, 617-619). We therefore examined the kinetics of these two populations following surgical hindlimb ischaemia in parallel with resident SkM progenitors. 8-12 w *Flt3^{Cre}* x *Rosa^{mT/mG}* mice were subjected to permanent ligation of their left iliac artery and distal vessels to induce peripheral ischaemia, with tissues harvested at 3 d and 14 d after surgery (246) (**Figure 5-5a**). Circulating monocytes were entirely GFP⁺ and increased in frequency by two-fold at both time-points after ischaemia compared to uninjured mice (**Figure 5-5b,c**). CFU-M yield was ~2.5-fold higher from ischaemic gastrocnemius/quadriceps SkM compared to the contralateral non-ischaemic limb at both 3 d and 14 d (**Figure 5-5d**). These colonies were predominantly GFP⁺tdTom⁺ (*Flt3-Cre⁻*) and therefore not BM-derived and were of larger size than those from non-ischaemic SkM (**Figure 5-5e**). Flow cytometry also showed that ischaemia resulted in a 3-fold increase in GFP⁺tdTom⁺ progenitors in SkM at 3 d, with numbers returning toward levels seen in non-ischaemic SkM by 14 d (**Figure 5-5f-h**).

Interestingly, ~30% of CD11b⁺F4/80⁺ macrophages in non-ischaemic SkM were GFP⁺tdTom⁺ (*Flt3-Cre⁻*), which is in keeping with results from another recent study (172). This proportion increased to 36% at 14 d post-ischaemia (**Figure 5-5f, i**). Increases in absolute numbers were seen for both GFP⁺tdTom⁺ and GFP⁺tdTom⁻ macrophages in ischaemic SkM at both time-points, with fold comparisons versus non-ischaemic SkM: 5.4x at 3 d and 4.0x at 14 d for GFP⁺tdTom⁺ macrophages, and 5.3x at 3 d and 3.1x at 14 d for GFP⁺tdTom⁻ macrophages (**Figure 5-5j**). By comparison, 70-90% of endothelial cells were GFP⁺tdTom⁺ (*Flt3-Cre⁻*) and these showed a more striking increase in number at 14 d after ischaemia (**Figure 5-5k, l**).

To elucidate the proliferative status of progenitors and macrophages in SkM after hindlimb ischaemia, we also administered BrdU in these experiments and analysed cell cycle activity by flow cytometry. In keeping with their early expansion after ischaemic surgery, we detected higher proportions and numbers of progenitors in S-phase at day 3 of ischaemia than at day 14 or in non-ischaemic SkM (**Figure 5-6a-d**). Meanwhile, increases occurred in both the proportions and absolute numbers of GFP⁺tdTom⁻ and GFP⁺tdTom⁺ macrophages that were in S-phase at both early and late time-points in ischaemic SkM (**Figure 5-6c-e**).

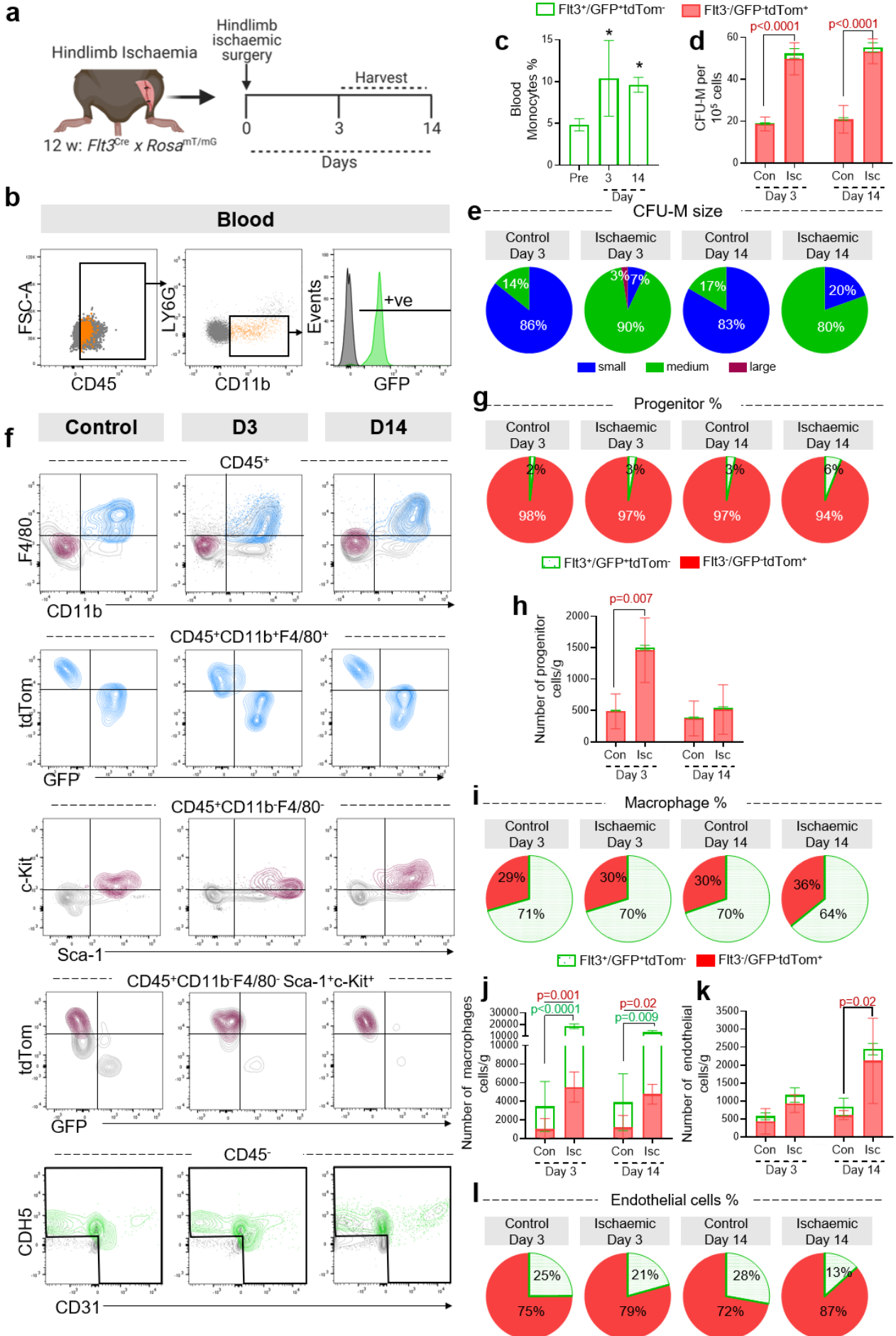


Figure 5-5. Expansion of resident progenitors, macrophages and endothelial cells and BM-derived macrophages in hindlimb muscle in response to ischaemia.

- (a) Schematic shows the induction of hindlimb ischaemia in *Flt3^{Cre}* x *Rosa^{mT/mG}* mice, with tissues harvested at 3 d and 14 d after surgery.
- (b) Flow cytometry shows GFP (*Flt3-Cre⁺*) expression of blood monocytes.
- (c) Graph summarises percentages of blood monocyte by *Flt3-Cre* status from *Flt3^{Cre}* x *Rosa^{mT/mG}* mice before and after hindlimb ischaemia. N=4-6 mice/gp. One-way ANOVA. Multiple comparison: * $p < 0.05$ for comparison to control.
- (d) CFU-M yield and *Flt3-Cre* status from non-ischaemic (Con) and ischaemic (Isc) SkM. Paired t test. Red P-values, *Flt3-Cre⁻* comparisons; Green P-values, *Flt3-Cre⁺*.
- (e) Pie charts show classification of CFU-M based on colony size from non-ischaemic and ischaemic SkM. Percentages represent mean of n=6 mice/gp.
- (f) Flow cytometry profiling of cells in gastrocnemius SkM at different time-points after ischaemic surgery.
- (g) Percentages of progenitors that express GFP and tdTom from *Flt3^{Cre}* x *Rosa^{mT/mG}*, from non-ischaemic and ischaemic SkM. Percentages represent mean of n=6 mice/gp.
- (h) Graphical quantification of numbers of *Flt3-Cre⁺* (green) and *Flt3-Cre⁻* (red) progenitors in non-ischaemic and ischaemic SkM. N=6 mice/gp. Paired t-tests. Red p-values, *Flt3-Cre⁻* comparisons; Green p-values, *Flt3-Cre⁺*.
- (i) GFP and tdTom expression of macrophages. Percentages represent mean of n=6 mice.
- (j-k) Graphical quantification of numbers of *Flt3-Cre⁺* (green) and *Flt3-Cre⁻* (red) (j) macrophages and (k) endothelial cells in non-ischaemic and ischaemic SkM. N=6 mice/gp. Paired t-tests. Red p-values, *Flt3-Cre⁻* comparisons; Green p-values, *Flt3-Cre⁺*.
- (l) GFP and tdTom expression of endothelial cells. Percentages represent mean of n=6 mice.
- Data summarised as mean $\pm$ SD.

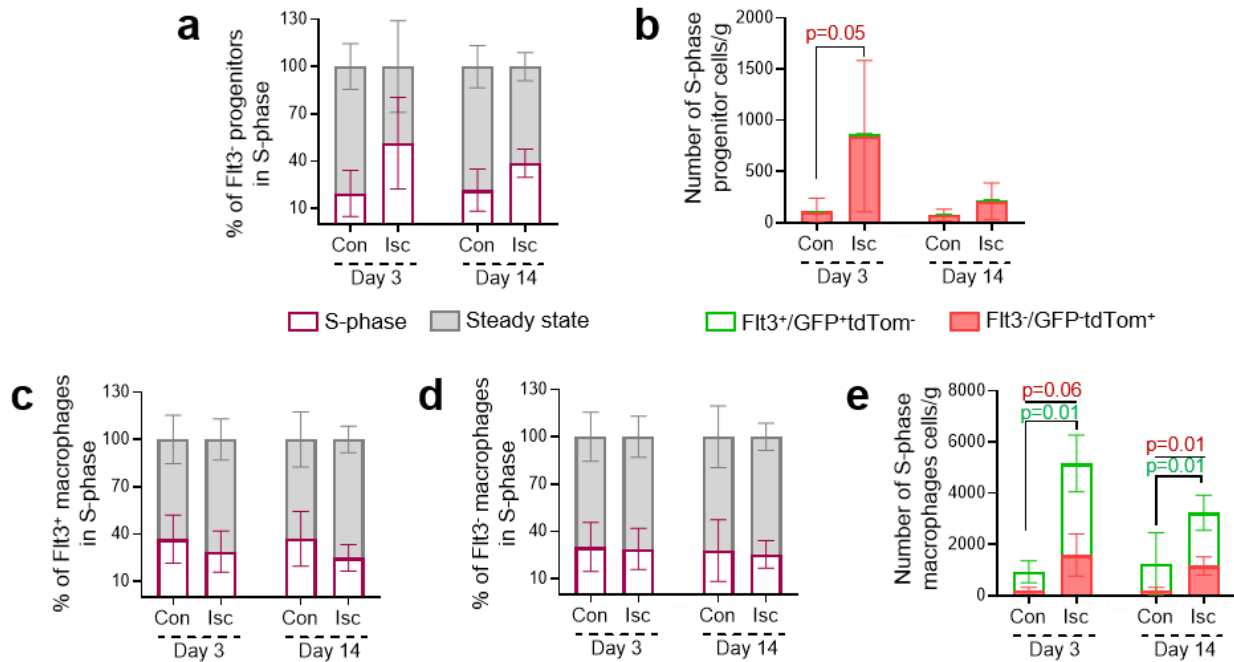


Figure 5-6. Increased proliferation of resident progenitors and macrophages and BM-derived macrophages in ischaemic hindlimb muscle.

(a) Percentages of progenitors in S-phase in non-ischaemic (Con) and ischaemic (Isc) SkM. N=6 mice/gp.

(b) Graphical quantification of numbers of Flt3-Cre⁺ (green) and Flt3-Cre⁻ (red) S-phase progenitors in non-ischaemic and ischaemic SkM. N=6 mice/gp. Paired *t*-tests. Red p-values, Flt3⁻ comparisons.

(c-d) Percentages of **(c)** Flt3-Cre⁺ and **(d)** Flt3-Cre⁻ macrophages in S-phase in non-ischaemic and ischaemic SkM. N=6 mice/gp.

(e) Graphical quantification of numbers of Flt3⁺ (green) and Flt3⁻ (red) S-phase macrophages in non-ischaemic and ischaemic SkM. N=6 mice/gp. Paired *t*-tests. Red p-values, Flt3⁻ comparisons; Green p-values, Flt3⁺.

Data summarised as mean±SD.

Because these results show that non-BM derived progenitors and macrophages expand in SkM following ischaemia, we also performed ischaemic surgeries in TAM-induced 12 w *Csf1r^{MerCreMer}* x *Rosa^{mT/mG}* mice to determine whether these cells are ultimately derived from YS (**Figure 5-7a**). In these studies, we also harvested tissue at 1 d post-surgery to uncover which cell population(s) proliferate and expand earliest. In keeping with our observations from *Flt3^{Cre}* x *Rosa^{mT/mG}* ischaemic mice, we found a significant increase in CFU-M yield in ischaemic limbs at each time-point studied (**Figure 5-7c**). Flow cytometry showed sustained increases in the overall number of YS-derived GFP⁺ cells in SkM after ischaemia (fold comparisons versus non-ischaemic SkM: 5.8x at 1 d, 5.2x at 3 d, 7.1x at 14 d) (**Figure 5-7d, e**). Remarkably, GFP⁺ progenitors expanded by 35-fold in ischaemic SkM at 1 d post-surgery, with their numbers diminishing but still higher than in control limbs at 3 d (7.3-fold) and 14 d (3.1-fold) (**Figure 5-7f**). In contrast, the differences between ischaemic and non-ischaemic limbs were most pronounced for the number of GFP⁺ macrophages at 3 d (14-fold) (**Figure 5-7g**) and for GFP⁺ endothelial cells at 14 d (5-fold) (**Figure 5-7h**). After normalisation of GFP expression to microglia, 80-100% of progenitors and 28-38% were determined to be YS EMP-derived at all timepoints after ischaemia (**Figure 5-7i-j**). Meanwhile, the contributions of EMPs to endothelial cells increased from 10-20% in control limbs to 32% in 3 d ischaemic limbs and 56% in 7 d ischaemic limbs (**Figure 5-7k**). As further proof that YS-derived cells participate in the response to ischaemia in SkM, we also detected robust GFP⁺CD31⁺ structures, resembling vascular networks in day 14 hindlimbs, with GFP⁺c-Kit⁺ progenitors and GFP⁺CD68⁺ macrophages in proximity (**Figure 5-7l**).

We next considered whether the early expansion of YS-derived progenitors may directly contribute to the later accumulation of YS-derived macrophages and endothelial cells in ischaemic SkM. We performed BrdU pulse-chase experiments, in which BrdU was administered by intraperitoneal injection at a fixed time of 23 h after hindlimb ischaemic surgery in E8.5 TAM-induced 12 w *Csf1r*^{MerCreMer} x *Rosa*^{mT/mG} mice. Ischaemic and non-ischaemic limbs were then harvested at four different time-points relative to ischaemic surgery: 24 h, 72 h, 168 h (7 d) and 336 h (14 d) (**Figure 5-8a, b**). At 24 h, the total number of BrdU⁺ GFP⁺ cells were more than 18-fold higher in ischaemic than non-ischaemic SkM, highlighting a profound, early proliferative response to ischaemia. Notably, progenitors represented the most proliferative YS-derived population in both limbs at this time, accounting for 81% and 65% of BrdU⁺ cells in non-ischaemic and ischaemic SkM, respectively (**Figure 5-8c**). As the overall number of BrdU⁺ GFP⁺ cells did not significantly change over time (**Figure 5-8d**), we examined the re-distribution of BrdU labelling across the different GFP⁺ populations to probe for a possible ancestral relationship between progenitors and the other two YS-derived cell types. BrdU⁺ GFP⁺ progenitors were most abundant at 24 h after surgery in both limbs, before disappearing completely by 168 h in non-ischaemic SkM and progressively diminishing to less than a third of their number by 336 h in ischaemic SkM (**Figure 5-8e**). In contrast, BrdU⁺ GFP⁺ macrophages peaked at 72 h in non-ischaemic and 168 h in ischaemic limbs (**Figure 5-8f**), while BrdU⁺ GFP⁺ endothelial cells peaked in both limbs at 336 h (**Figure 5-8g**). In addition, the median fluorescence intensity (MFI) of the overall BrdU⁺ signal among YS-derived cells also diminished over time (**Figure 5-8b,h**) and was brightest in progenitors and macrophages at the earliest time-point and dimmest in endothelial cells at study end (**Figure 5-8i-l**). Together, these results align to demonstrate that hindlimb ischaemia induces an early proliferative response and accumulation of YS-derived SkM progenitors. The subsequent re-distribution and dimming of BrdU label suggest that they then undergo differentiation into macrophages and endothelial cells, which would contribute to the expansion of both of these YS-derived lineages.

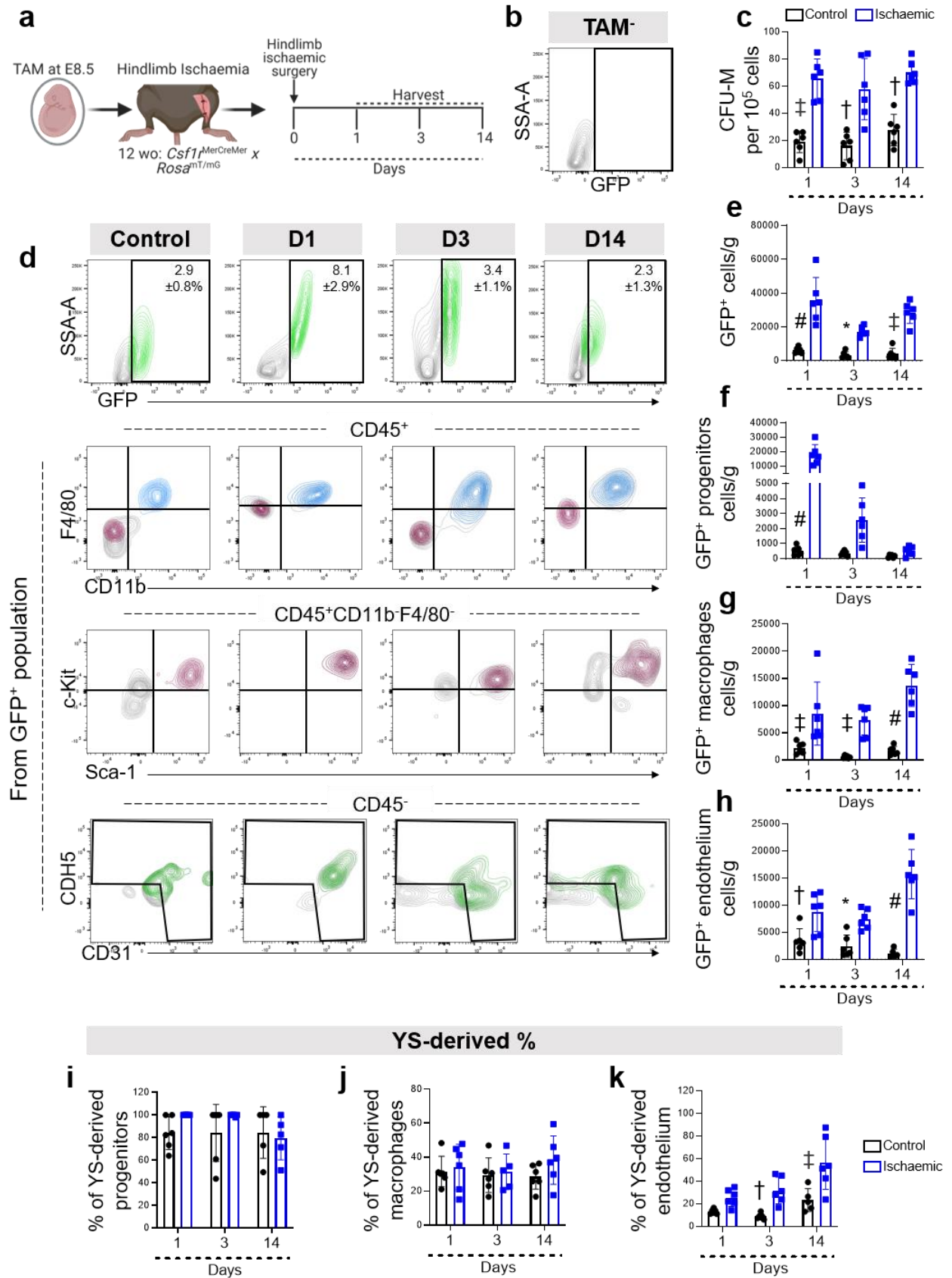


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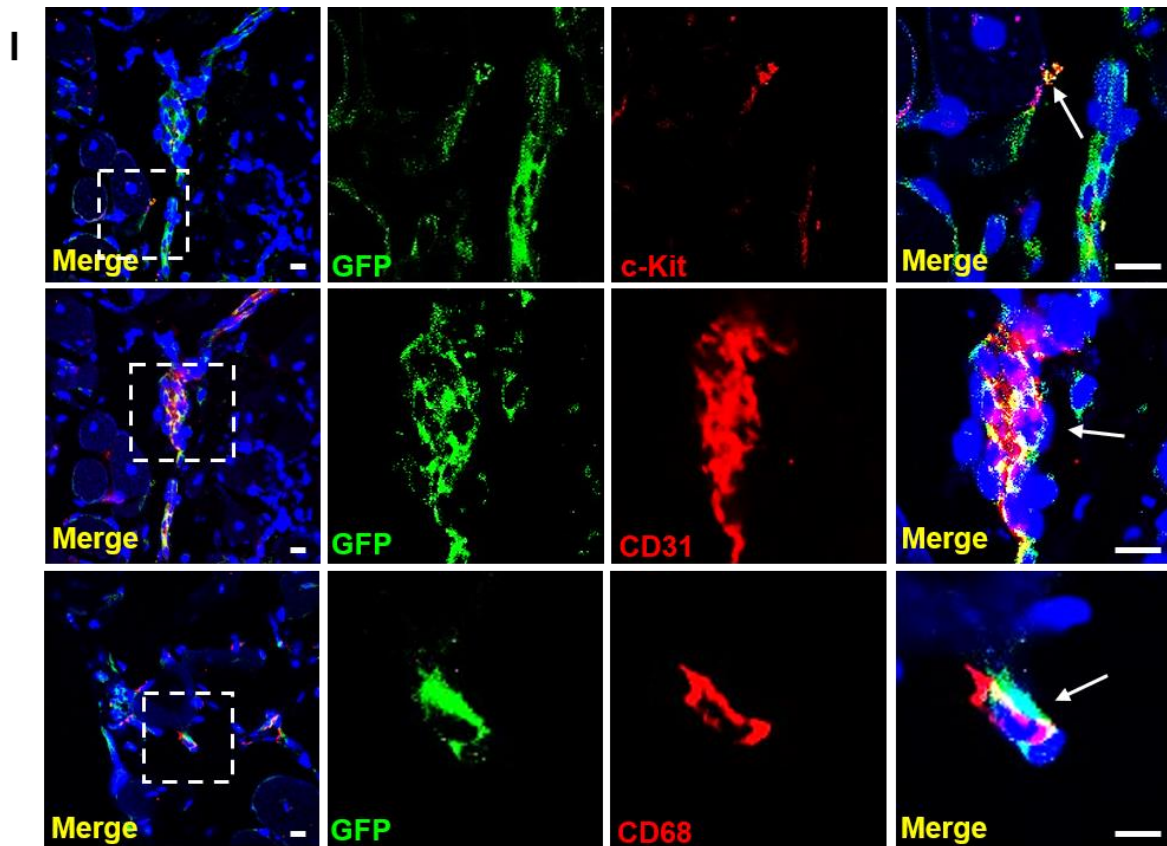


Figure 5-7. YS-derived CFU-M progenitors expand early after induction of ischaemia in SkM.

(a) Schematic shows the induction of hindlimb ischaemia in E8.5 TAM-induced adult *Csf1r*^{MerCreMer} $\times$ *Rosa*^{mT/mG}, with tissues harvested at 1 d, 3 d, and 14 d after surgery.

(b) No tamoxifen (TAM) Cre control also shown.

(c) CFU-M yield from non-ischaemic (black) and ischaemic (blue) SkM. N=6 mice/gp. Mixed effects two-way ANOVA: $p=0.04$ for time; $p=0.0001$ for group; $p=0.9$ for time $\times$ group. $\dagger p<0.01$ and $\ddagger p<0.001$.

(d) Flow cytometry profiling of YS-derived GFP⁺ cells in gastrocnemius SkM at different time-points after ischaemic surgery.

(e) Number of GFP⁺ cells (not normalised to microglia) per gram of SkM at different time-points. N=6 mice/gp. Mixed effects two-way ANOVA: $p=0.003$ for time; $p<0.0001$ for group; $p=0.03$ for time $\times$ group. $*p<0.05$, $\dagger p<0.01$ and $\#p<0.0001$.

(f-h) Graphical quantification of numbers of GFP⁺ **(e)** progenitors, **(f)** macrophages and **(g)** endothelial cells in non-ischaemic and ischaemic SkM. N=6 mice/gp. Mixed effects two-way ANOVA: *p<0.05, †p<0.01, ‡p<0.001 and #p<0.0001.

(i-k) Graphical quantification of normalised GFP expression in **(i)** progenitors, **(j)** macrophages and **(k)** endothelial cells in non-ischaemic and ischaemic SkM. Normalised to microglia. N=6 mice/gp. Mixed effects two-way ANOVA: †p<0.01 and ‡p<0.001. Scale bar, 10 µm.

(l) Co-expression of GFP with c-Kit, CD31 and CD68 (arrows) in gastrocnemius 14 d after ischaemic surgery.

Data summarised as mean±SD.

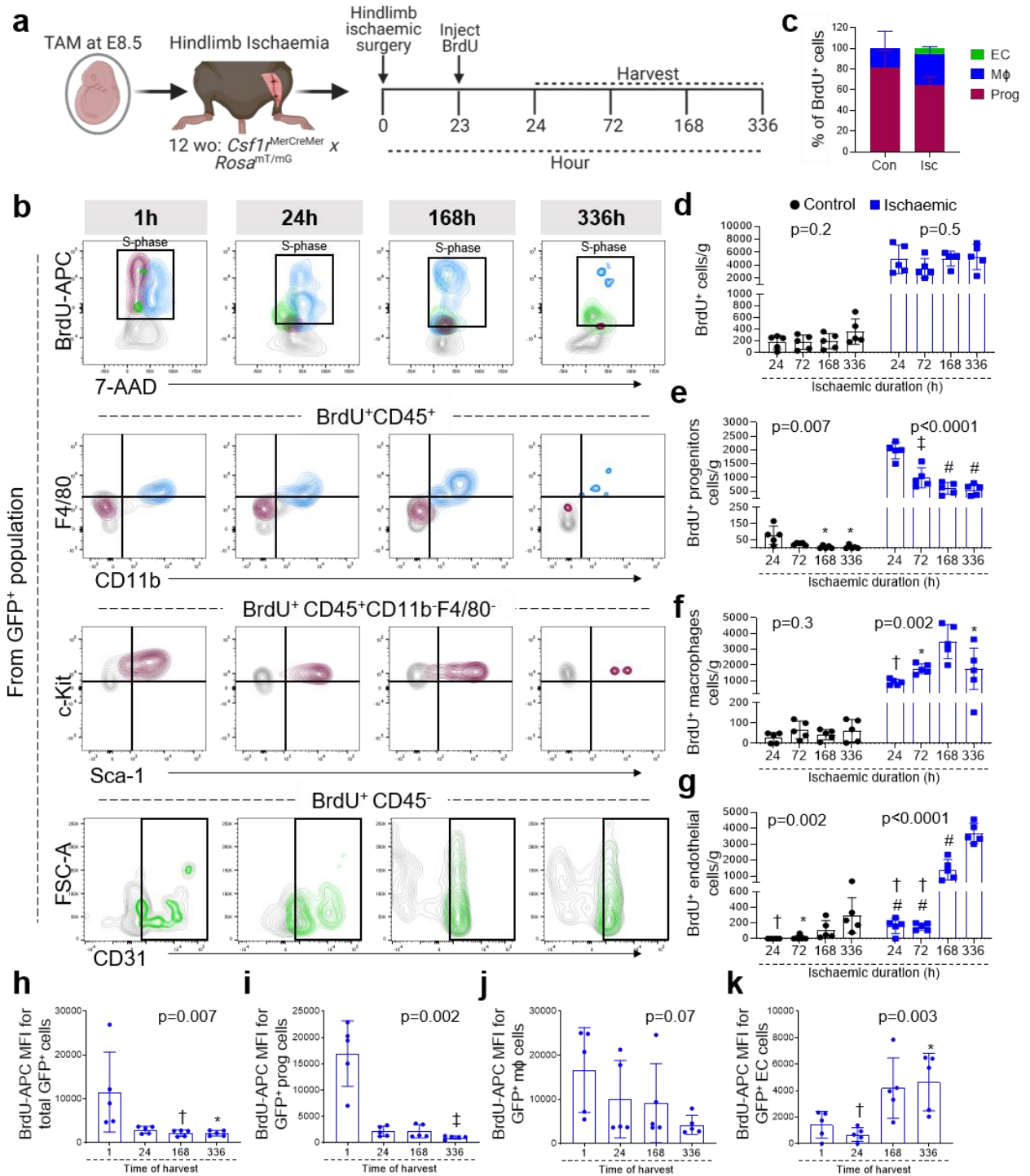


Figure 5-8. BrdU pulse-chase reveals early proliferation of YS-derived progenitors in ischaemic muscle followed by differentiation into macrophages and endothelial cells.

(a) BrdU pulse-chase in hindlimb ischaemic 12 w *Csfl^{MerCreMer} x Rosa^{mT/mG}* mice that were induced with TAM at E8.5.

(b) Flow cytometry of SkM digests at different time-points after BrdU injection. Maroon, Prog; Blue, M ϕ ; Green, Endothelial cells.

(c) Percentages of BrdU⁺ cells that were progenitors (Prog), macrophages (M ϕ) or endothelial cells (EC) after 24 h of ischaemia (isc) or control (con). N=5 mice/gp.

(d-g) Numbers of BrdU⁺ GFP⁺ **(d)** cells, **(e)** progenitors, **(f)** macrophages and **(g)** endothelial cells at different time-points after BrdU pulse. N=5 mice/gp. P-values are from Kruskal-Wallis in **(e)** and **(g)** and one-way ANOVA in **(d)** and **(f)**.

(h-k) MFI of BrdU in GFP⁺ **(h)** cells, **(i)** progenitors, **(j)** macrophages and **(k)** endothelial cells at different time-points after BrdU pulse in ischaemic limbs. N=5 mice/gp. P-values are from Kruskal-Wallis in **(h)**, **(i)** and **(j)** and one-way ANOVA in **(k)**.

Data summarised as mean $\pm$ SD.

5.3.4 SkM CFU-M progenitors have bipotent plasticity for macrophages and endothelial cells

In the previous chapters, we established the bipotent endothelial-macrophage plasticity and vasculogenic capacity of YS-derived CFU-M progenitors in postnatal aorta. Together with the results from BrdU pulse-chase for ischaemic SkM, this led us to directly examine the differentiation potential and vasculogenic capacity of SkM CFU-M progenitors. Progenitor cells isolated from 12 w SkM CFU-M were cultured in MatrigelTM for 7 d in the presence of endothelial cell growth media, enriched with angiogenic growth factors, including vascular endothelial growth factor (VEGF). By comparison to donor-matched aortic progenitors, SkM progenitors displayed greater angiogenic cord-forming capacity and produced more mature, interconnected networks comprising branching cords similar to those seen with endothelial cultures (**Figure 5-9a**). Flow cytometry revealed that these networks formed by SkM progenitors mostly contained newly formed endothelial cells, with some macrophages but very few remaining progenitor cells (**Figure 5-9b**).

We next performed adoptive transfer experiments to directly study the fate of SkM progenitors in their native environment under ischaemic insult. 12 w C57BL/6 mice underwent hindlimb ischaemia surgery and then immediately received injections of cell-free MatrigelTM (control) or SkM CFU-M progenitors prepared from ubiquitously GFP donor mice ($\sim 1.5 \times 10^4$ cells) into their quadriceps and gastrocnemius (**Figure 5-9c**). Laser Doppler imaging demonstrated that perfusion recovery was doubled by day 14 in the ischaemic limb of mice that received SkM progenitors compared to control (**Figure 5-9d**). Progenitors also resulted in a significant increase in CD31⁺ capillary density and trend toward increased CD31⁺SMA⁺ arteriolar density in sections of treated SkM (**Figure 5-9e**). Confocal microscopy showed that donor SkM progenitors produced lumen-containing GFP⁺CD31⁺ tubular structures, resembling new blood vessels, with nearby GFP⁺CD68⁺ macrophages (**Figure 5-9f**). Finally, flow cytometry analysis of day 14 SkM digests also confirmed that GFP⁺ progenitors had largely transformed into endothelial cells and macrophages, with $\sim 10\%$ retaining their progenitor phenotype. (**Figure 5-9g**).

Given these *in vivo* results, we also tried to simulate the ischaemic environment *in vitro* by repeating methylcellulose and MatrigelTM cultures under hypoxia. SkM CFU-M yield was doubled in hypoxic conditions (**Figure 5-9h**), while cord-forming capacity of SkM progenitors was also enhanced by 1.6-fold (**Figure 5-9i**). Interestingly, the latter was associated with a selective increase in production of endothelial cells (also reflected by increased cord length) in the presence of hypoxia, without altering the number of newly formed macrophages (or cord branch number).

These *in vitro* and *in vivo* data confirm the identity of SkM CFU-M progenitors as vasculogenic, bipotent EndoMac progenitors and demonstrate that hypoxia augments their clonogenic and angiogenic properties while also favouring their endothelial differentiation. This equips these YS-derived cells with the ability to expand and promote neovascularisation and perfusion recovery after ischaemic insult.

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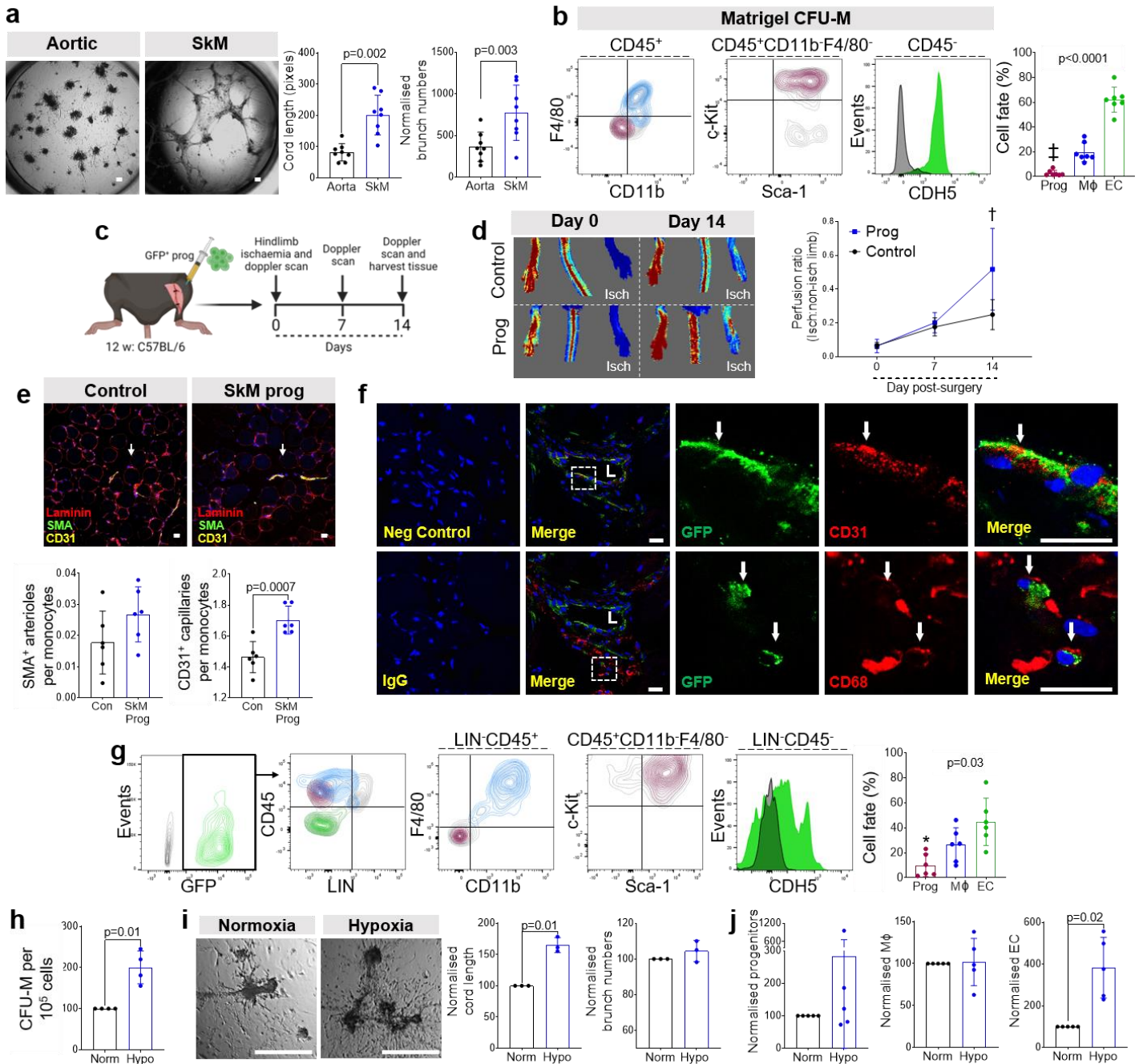


Figure 5-9. SkM CFU-M progenitors form endothelial cells and macrophages and have vasculogenic capacity.

(a) Light microscopy images of cord networks formed at 7 d from 12 w C57BL/6 aortic and SkM CFU-M progenitors plated in Matrigel™. Graphs display comparisons of cord length and branch number. N=8 mice. Paired *t*-test. Scale bar, 100 μ m.

- (b)** Flow cytometry plots and graph show fate of CFU-M derived progenitors in MatrigelTM. N=4 mice. Green histogram, Sample; Grey, FMO control. Friedman test. Multiple comparison tests: ‡p<0.001 for comparison to EC.
- (c)** Primary transfer of GFP⁺ SkM progenitors after hindlimb ischaemia surgery with two-week follow-up.
- (d)** Laser doppler perfusion images on day 0 and day 14 after ischaemia in mice receiving cell-free control (above) and progenitors (below). Graph shows results. N=6 mice/gp. Mixed effects two-way ANOVA: p<0.0001 for time; p=0.02 for group; p=0.01 for time x group. Multiple comparison test: † p<0.01 for prog vs control.
- (e)** Immunofluorescence microscopy of sections of gastrocnemius at day 14 that had received injections of MatrigelTM control or SkM progenitors. Counter-staining with antibody to laminin (red) for myocytes and DAPI (blue) for nuclei. Graphs show capillary and arteriolar density (n=6 mice/gp, three sections per mouse). Unpaired *t*-test. Scale bar, 100 µm. See **Appendix 9** for bigger images.
- (f)** Confocal microscopy of ischaemic gastrocnemius shows neovessels lined by GFP⁺CD31⁺ endothelial cells (top) with nearby GFP⁺CD68⁺ macrophages (bottom). L, Lumen. Scale bar, 20 µm.
- (g)** Flow cytometry plots and graph show fate of GFP⁺ donor cells in recipient gastrocnemius. N=6 mice. Maroon, Progenitors (Prog); Blue, Macrophages (Mφ); Green, Endothelial cells (EC). Green histogram, Sample; Grey, FMO control. Repeated measures ANOVA. Multiple comparison test: * p<0.05 vs EC. See **Appendix 10** for bigger images.
- (h)** SkM CFU-M yield under normoxic and hypoxic conditions. N=5 mice/gp. Paired *t*-test.
- (i)** Light microscopy images of cord networks formed at 7 d from SkM progenitors plated in MatrigelTM exposed to normoxic (left) or hypoxic (right) conditions, and summary of the total cord length and number of branch points for the networks formed. N=3 mice/gp. Paired *t*-test. Scale bar, 100 µm.
- (j)** Summary of progenitors (left), macrophages (middle) and endothelial cells (right) as normalised cell composition of these networks exposed to normoxic or hypoxic conditions. N=6 mice/gp. Paired *t*-test. Data summarised as mean±SD.

5.3.5 YS-derived EndoMac progenitors are predisposed to forming LYVE-1^{Hi}MHCII^{Lo} macrophages in ischaemic SkM

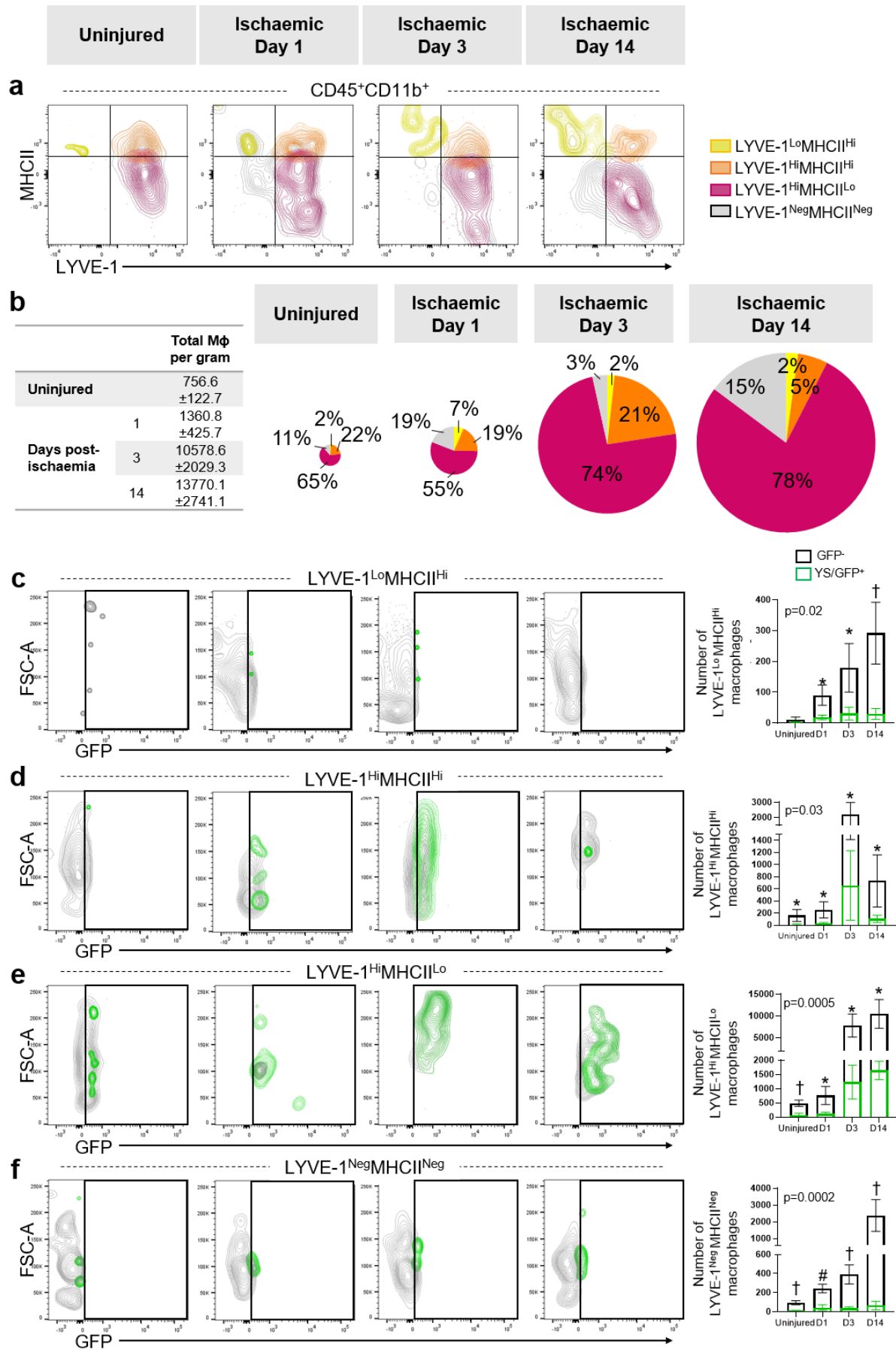
Wang *et al.* recently reported that resident macrophages in steady state SkM express the markers, CD64, MerTK, CD163 and CD206 and comprise two distinct subsets that can be distinguished by their surface expression of LYVE-1 and MHCII: LYVE-1^{Lo}MHCII^{Hi} and LYVE-1^{Hi}MHCII^{Lo} (172). In addition to finding transcriptomic differences between these subpopulations, they also demonstrated different ontogenies, whereby LYVE-1^{Lo}MHCII^{Hi} macrophages predominantly arose from definitive HSCs (e.g. in BM) and circulating monocytes, while LYVE-1^{Hi}MHCII^{Lo} macrophages had mixed HSC and non-HSC origins. We therefore focused our attention on these macrophage subtypes in SkM during recovery from ischaemia and re-interrogated their origins through the new lens of YS-derived SkM EndoMac progenitors.

Using E8.5 TAM-induced 12 w *Csf1r*^{MerCreMer} $\times$ *Rosa*^{mT/mG} mice, we first determined that steady state SkM contained mostly LYVE-1^{Hi}MHCII^{Lo} macrophages, of which 30.7 $\pm$ 11.6% were YS-derived after normalisation for GFP⁺ microglia labelling (**Figure 5-10a**). Unlike Wang's study, we observed a second, smaller LYVE-1^{Hi}MHCII^{Hi} subpopulation, of which <3% was YS-derived, with very few macrophages displaying the previously described LYVE-1^{Lo}MHCII^{Hi} surface phenotype. Following ischaemia, we observed a rapid and progressive expansion of total macrophage number between day 1 and 3, with a further, more modest increase out to day 14 (**Figure 5-10b**). This involved expansion of each macrophage subpopulation, albeit with different kinetics, including the emergence of a fourth LYVE-1^{Neg}MHCII^{Neg} subset that was virtually absent in non-ischaemic SkM (**Figure 5-10a-f**). LYVE-1^{Lo}MHCII^{Hi} macrophages remained the most dominant subtype and increased sequentially with each time-point studied (1.6-fold at day 1, 16-fold at day 3 and 22-fold at day 14 compared to non-ischaemic SkM). Notably the YS-derived proportion of these cells remained relatively constant throughout (~15% GFP⁺, and when normalised for microglial labelling, ~30% YS-derived) (**Figure 5-10c**). By comparison, LYVE-1^{Hi}MHCII^{Hi} macrophages peaked at day 3 of ischaemia (~14-fold compared to non-

ischaemic) aided by an 80-fold expansion of GFP⁺ LYVE-1^{Hi}MHCII^{Hi} cells, before decreasing in number at day 14 (**Figure 5-10d**). The much smaller LYVE-1^{Lo}MHCII^{Hi} and LYVE-1^{Neg}MHCII^{Neg} populations both peaked at day 14 with minimal contribution from the YS source (**Figure 5-10e, f**).

To verify these results, we also studied the kinetics of these macrophage subtypes in SkM of *Flt3*^{Cre} x *Rosa*^{mT/mG} mice at days 3 and 14 after hindlimb ischaemia. This showed similar expansions of predominantly GFP⁺tdTom⁺ (*Flt3*-Cre⁻) LYVE-1^{Hi}MHCII^{Lo} macrophages peaking at day 14. Meanwhile, the late increase seen in LYVE-1^{Lo}MHCII^{Hi} macrophages predominantly involved GFP⁺tdTom⁻ (*Flt3*-Cre⁺) cells, indicating their dependence on definitive BM haematopoiesis. Finally, resident *Flt3*-Cre⁻ macrophages contributed to ~15% of LYVE-1^{Hi}MHCII^{Hi} macrophages when their number peaked at day 3 post-ischaemia, before becoming negligible again at day 14 (**Appendix 11**).

These results highlight a specific predilection for YS to give rise to resident LYVE-1^{Hi}MHCII^{Lo} macrophages in both steady state and ischaemic SkM. Further analyses of the fate of SkM EndoMac progenitors during *in vitro* MatrigelTM angiogenesis assays revealed that ~73% of the macrophages formed were LYVE-1^{Hi}MHCII^{Lo} while ~16% were LYVE-1^{Hi}MHCII^{Hi} (**Figure 5-11a**). A similar result was obtained when GFP⁺ progenitors were injected into ischaemic hindlimbs, with the LYVE-1^{Hi}MHCII^{Lo} subset making up ~82% of all donor-derived macrophages in day 14 SkM (**Figure 5-11b**). In combination, these results support the mixed ontogeny of macrophages in steady state and ischaemic SkM and moreover, indicate that resident EndoMac progenitors are a source of the YS-derived LYVE-1^{Hi}MHCII^{Lo} subpopulation that expands substantially and progressively after ischaemia is induced.


 Figure 5-10. LYVE-1^{Hi} MHCII^{Lo} and LYVE-1^{Hi} MHCII^{Hi} arise from a YS-derived origin.

(a) Flow cytometry analysis of MHCII and LYVE-1 expression in gastrocnemius digests of E8.5 TAM-induced *Csf1r*^{MerCreMer} $\times$ *Rosa*^{mT/mG} mice at different timepoints after hindlimb ischaemia.

(b) Absolute density of macrophages per gram of SkM in non-ischaemic and ischaemic hindlimbs at different timepoints after surgery. Pie charts drawn to scale, compare the breakdown of macrophage subsets from these limbs. N=5 mice. LYVE-1^{Lo}MHCII^{Hi} (yellow) LYVE-1^{Hi}MHCII^{Hi} (orange), LYVE-1^{Hi}MHCII^{Lo} (dark red) and LYVE-1^{Neg}MHCII^{Neg} (grey).

(c-f) Flow cytometry contour plots and adjacent graphs show the number and GFP expression status of

(c) LYVE-1^{Lo}MHCII^{Hi}, **(d)** LYVE-1^{Hi}MHCII^{Hi}, **(e)** LYVE-1^{Hi}MHCII^{Lo} and **(f)** LYVE-1^{Neg}MHCII^{Neg} macrophage subpopulations ischaemic muscle non-ischaemic and ischaemic SkM at 1 d, 3 d and 14 d after ischaemic surgery. N=5 mice. Paired *t*-tests GFP⁺ vs GFP⁻: **p*<0.05; †*p*<0.01; ‡*p*<0.001 and #*p*<0.0001.

Data summarised as mean±SD.

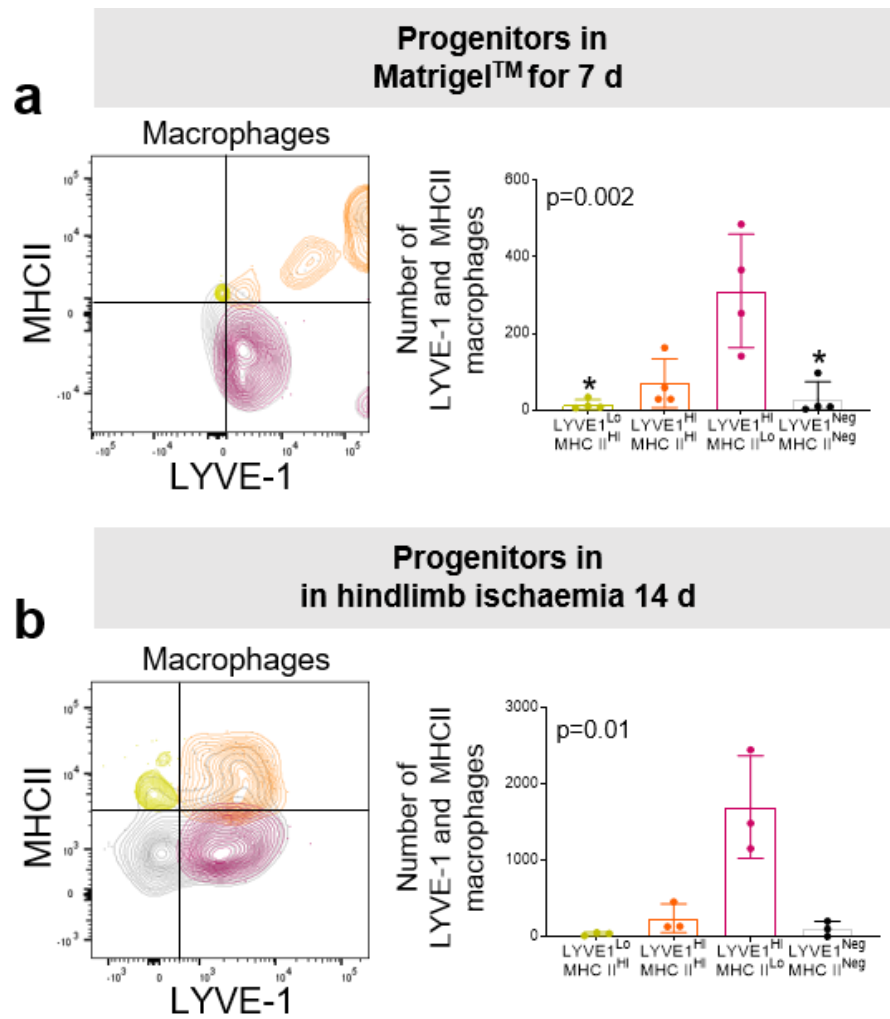


Figure 5-11. SkM EndoMac progenitors preferentially give rise to LYVE-1^{Hi}MHCII^{Lo} macrophages.

(a) Flow cytometry profiling of MHCII and LYVE-1 expression on macrophages formed from adult SkM progenitors after 7 d in Matrigel™ *in vitro*. N=4 mice. Friedman test. Multiple test comparisons: * $p<0.05$ for comparison to LYVE-1^{Hi}MHCII^{Lo}.

(b) Flow cytometry contour plot of MHCII and LYVE-1 expression on macrophages formed from GFP⁺ SkM progenitors 14 d after intramuscular transfer into ischaemic hindlimb. N=3 mice. Friedman test. LYVE-1^{Lo}MHCII^{Hi} (yellow) LYVE-1^{Hi}MHCII^{Hi} (orange) and LYVE-1^{Hi}MHCII^{Lo} (dark red).

Data summarised as mean±SD.

5.4 Discussion

Intimate interactions between macrophages and endothelial cells are integral to the coordinated inflammatory and neovascularisation responses that are required to minimise tissue damage after ischaemia (598). Over the past few years, historical views about the developmental origins of both cell types in steady state and ischaemic SkM have been challenged. Growing evidence now places importance on their local maintenance and proliferation (172, 365, 597), in addition to, or even more so than their renewal from circulatory precursors (209, 486, 620-622). As shown for other tissues, the extra-embryonic YS has emerged as a common source of both macrophages and endothelial cells that persist into adult life (93, 132). Having newly identified YS-derived, bipotent EndoMac progenitors in postnatal murine aorta, heart and lung, this chapter establishes their presence in steady state quadriceps and gastrocnemius SkM. Moreover, we highlight their striking proclivity to proliferate and expand in the first 24 h after ischaemic insult, before producing macrophages, predominantly of the MHCII^{Lo}LYVE-1^{Hi} resident subtype, and endothelial-lined neovessels that facilitate perfusion recovery. Finally, by also examining macrophages and endothelial cells together in each of our fate-mapping experiments, our study sheds important light on the relative contributions of definitive HSCs and YS EMPs to these respective lineages in both non-ischaemic and ischaemic SkM.

Although extensively studied for other tissues (2, 93, 101, 162, 164, 166, 171, 194, 404, 623), information on the origins, phenotypic and transcriptomic heterogeneity of macrophages in SkM has only recently become available (172). Using constitutional Cre-Lox fate-mapping under the Flt3 promoter crossed to a YFP reporter, Wang *et al.* found that only 35-45% of macrophages in limb and diaphragm muscle arose from a definitive haematopoietic source at 4 w of age, with this proportion increasing modestly to around 45-55% by 12 w (172). In contrast, in the same study results from sublethal irradiation and BM transplant indicated a greater contribution of around 80-90% by BM HSCs 12 w post-transplantation, with HSCs preferentially giving rise to MHCII^{Hi}LYVE-1^{Lo} macrophages via CCR2⁺ circulating monocytes. Notably, this macrophage subset was relatively absent from SkM at the time of birth but made up about a quarter of the total resident population at 4 w of postnatal life.

Interestingly, as a result of TAM-induced lineage mapping in *Csf1r^{CreER}* mice, the authors estimated that YS only contributed to 5% of total resident macrophages in 4 w quadriceps and 12.8% in diaphragm, with these amounts increasing to 48.6% and 34.8% for the MHCII^{Lo}LYVE-1^{Hi} subset, respectively. Given that *Flt3^{Cre}* driven labelling does not distinguish between early YS EMP and late EMP/foetal monocyte sources, they inferred that foetal monocytes of non-HSC origin make a greater contribution to *Flt3-Cre⁺* macrophages in 4 w SkM than early EMPs (172). This is in line with some (2, 122, 171), but not all previous studies (93) for other tissue macrophages. Using the same *Flt3^{Cre}* promoter system crossed with a *Rosa^{mT/mG}* reporter, we achieved near 100% recombination efficiency as reflected by GFP⁺ labelling of blood monocytes and observed that 30% of macrophages in steady state 12 w hindlimb SkM were from non-HSC ancestry. In contrast to Wang's study (172), the results of our TAM-induced *Csf1r^{MerCreMer}* and *Cx3cr1^{CreER}* fate-mapping suggest that all of these *Flt3-Cre⁺* macrophages can be accounted for as originating from E8.5 CSF1R⁺ and E9.5 CX₃CR1⁺ sources, with the majority of these belonging to the dominant MHCII^{Lo}LYVE-1^{Hi} pool.

Until now, studies have widely concluded that embryonically derived tissue macrophages achieve local maintenance through proliferative self-renewal, without undergoing phenotypic or functional dedifferentiation (172, 178, 194, 624). In most tissues described, there appears to be a lack of direct evidence showing that this actually occurs *in vivo*. However, the ability for mature macrophages to self-renew has been demonstrated for alveolar macrophages and has been linked to certain lineage-specific epigenetic and transcription factor regulators (604, 625, 626). In addition to showing that SkM macrophages from both HSC and YS origins actively proliferate, especially after ischaemia, our study has also uncovered EndoMac progenitors as another source for the postnatal maintenance and expansion of YS-derived macrophages in SkM. As in aorta, these progenitors can be defined by their ability to form CFU-M that can clonally self-renew *in vitro*. Both in culture and fresh SkM digests, they have a distinct LIN⁻CD45^{+/Lo}CD11b⁻F4/80⁻Sca-1⁺c-Kit⁺ immunophenotype, without expressing mature myeloid or endothelial surface markers, other than CX₃CR1, CSF1R and CD31, which importantly are also found on YS EMPs and/or pre-macrophage precursors (1, 33, 93, 123). They are seeded prenatally, such that they are relatively abundant at birth, at which time they have heightened proliferative capacity,

as evident by larger colony sizes. Given that a considerable percentage of these progenitors are actively cycling even under normal conditions, it follows that their capacity for self-renewal is limited. This was evident by complete disappearance of BrdU-labelled YS-derived progenitors in steady state adult SkM 7 d after pulse-chase. In the absence of ongoing replacement from BM HSCs, the pool of SkM resident EndoMac progenitors therefore diminishes as a function of postnatal ageing. Similar depletions have been described for other tissue-resident stem cell populations, including muscle stem cells, which undergo age-related loss of repression of senescence pathways (627) and clonal drift (628).

After culture in MatrigelTM and transfer into ischaemic hindlimb, adult EndoMac progenitors mostly transformed into endothelial cells albeit with robust macrophage formation also. Notably, only ~3% and ~10% of the progenitor-derived total population retained progenitor status under these respective conditions, as defined by surface marker expression. This suggests that the progenitors undergo asymmetrical division and differentiation. Hypoxia and ischaemia particularly stimulate their clonogenic, proliferative and endothelial-forming vasculogenic capacity, with evidence for increased self-renewal also. Ischaemic surgery induced a >20-fold expansion in BrdU⁺ progenitors after just 24 h. Although this number subsequently declined, with progressive re-distribution of the BrdU label to YS-derived macrophages and endothelial cells, we also observed retention of a considerable number of BrdU⁺ progenitors after 14 d with diminished fluorescence intensity for the BrdU signal. This implies that there was ongoing production of new progenitors from the original actively cycling pool, in parallel with *de novo* formation of macrophages and endothelial cells. In the absence of multi-colour clonal tracing studies, we cannot yet determine whether this occurred solely by asymmetrical division or whether some progenitor clones were induced to undergo symmetrical division by ischaemia.

In certain tissues, endothelial cells are known to arise from different developmental origins, possess heterogeneous phenotypic and molecular properties and acquire a considerable degree of functional specialisation (629). Although relatively little is known about the heterogeneity and ontogeny of SkM endothelial cells, studies have implicated roles for both circulating, BM-derived and local, non-BM derived progenitors (630, 631). Here, we show that the majority of endothelial cells in SkM are from

non-HSC (Flt3-Cre⁻) sources and these markedly expand in number post-ischaemia. This is in keeping with other studies that have highlighted the contributions of locally-derived endothelial progenitors (228, 620, 632-634) and clonal expansion of local endothelial cells (597, 635, 636) to ischaemia-induced neovascularisation. Importantly, our embryonic fate-mapping experiments reveal that ~12% of endothelial cells in SkM are derived from CSF1R⁺ YS EMPs, and this increases to >50% 14 d after ischaemia. Moreover, as seen for aorta, lung and heart in Chapter 4, endothelial cells and not macrophages account for the majority of YS-derived cells in adult muscle. This complements the recent finding that YS EMPs are a source of endothelial-lined blood vessels in embryonic and postnatal mouse brain, heart, lung and liver (132). In that study embryonic liver endothelial cells of YS EMP origin were observed by single-cell sequencing to significantly express *Oit3*, *Mrc1* (*Cd206*), *Stab2* and *Lyve1* compared to other non-EMP endothelial cells. In addition to producing macrophages, especially of the resident MHCII^{Lo}LYVE-1^{Hi} subtype, our data strongly implicate that EndoMac progenitors are a renewable and robust source of these YS-derived endothelial cells, especially in the ischaemic setting. Although not a major focus here, we observed that YS EMPs and EndoMac progenitors also give rise to LYVE-1⁺ endothelial cells, and this is consistent with recent evidence revealing the YS origins of lymphatic endothelium in the heart (637).

The precise mechanisms governing the proliferative, self-renewal and differentiation properties of EndoMac progenitors in muscle under different pathophysiological conditions now require careful evaluation. This includes the molecular, transcriptional and epigenetic factors that predispose their expansion and formation of endothelial cells under hypoxic and ischaemic conditions. As discussed above, the results from BrdU pulse-chase indicate that progenitors undergo an enormous proliferative burst, before producing macrophages and then endothelial cells. While we have found no evidence that their formation of macrophages involves a monocyte stage, it is not yet clear whether they transition through a hierarchy of other intermediate states of differentiation and at what point this diverges into the endothelial and macrophage lineages and their respective subtypes. Finally, it is important to note that in the ischaemic milieu, our adoptive transfer experiment shows that the engraftment of EndoMac progenitors is associated with vasculogenesis and favourable restoration of tissue perfusion. Pending

the demonstration of analogous progenitors in human muscle, this has possible therapeutic implications for critical limb ischaemia, which still carries an unacceptably high mortality and morbidity burden (638). This could take the form of direct stem cell transplantation or the use of pharmacological or other modulatory treatments that upregulate their salutary properties.

5.5 Conclusion

Like postnatal aorta, heart and lung, mouse skeletal muscle contains clonogenic, self-renewing EndoMac progenitor cells of early YS origin, that have high proliferative capacity and are maintained after birth without circulatory input from BM HSCs. In the setting of ischaemia, these bipotent progenitors contribute directly to the expansion of local macrophages and endothelial cells and in turn the inflammatory and neovascularisation responses that are essential to promote muscle recovery.

Chapter 6

Divergent roles of yolk sac-derived
Endothelial-Macrophage progenitor cells
in skin wound healing and melanoma
growth.

6.1 Introduction

In Chapter 4 and 5, we uncovered the existence of a unique YS-derived progenitor population that gives rise to both macrophages and endothelial cells in postnatal murine aorta, heart, lung and muscle. Given the widespread distribution of tissue-resident macrophages of embryonic origin (93, 101, 122, 162), our discovery prompts consideration of whether these endothelial-macrophage (EndoMac) progenitors also exist in other tissues in which macrophages and endothelial cells interact closely to maintain steady state homeostasis and mount inflammatory and neovascular responses to pathological insult. This especially holds true in the skin during the well-orchestrated process of wound healing (424, 639, 640). Wound healing involves four broad stages: 1) haemostasis, 2) inflammation, 3) proliferation, and 4) resolution/remodelling (412). If these steps are not fully controlled, these processes may lead to maladaptive inflammation and abnormal neovessel growth, which in turn can increase the severity of tissue injury and contribute to development of chronic skin diseases, potentially even malignancy (413-415, 432, 460, 641, 642). In both the healing response to cutaneous wound injury and the pathogenesis of skin cancers (e.g. melanoma), the transition from one stage to the next depends on the activation, maturation and differentiation of various cell populations that include macrophages and endothelial cells (413-415, 432, 460, 641, 643).

Macrophages and endothelial cells play central, intricately linked roles, particularly during formation of new blood vessels via angiogenesis (7, 10). During skin wound healing, angiogenesis forms as outgrowths from the endothelium of existing blood vessels, which occurs through the coordinated involvement of endothelial cell migration, proliferation and cord formation, which are supported structurally and topically by local and recruited immune cells, including macrophages (424, 639, 640). Endothelial cells in skin have been shown to originate either from proliferative renewal of resident endothelial cells (644-646), differentiation of local progenitor cells (647), or the recruitment and differentiation of circulating bone marrow (BM)-derived endothelial progenitor cells (EPCs) (206, 214, 229, 230, 645-660). Some of these local ancestral populations, such as CD31^{Lo}VEGFR2^{Lo} endovascular progenitors, have also been found to initiate early vasculogenesis during the establishment of

melanoma, thereby promoting tumour enlargement and metastasis (230). Melanoma vascularisation and growth have also been correlated to elevated numbers of tumour-associated macrophages (TAM ϕ) in the tumour microenvironment, highlighting the importance of macrophages in supporting pathological, as well as physiological neovascularisation (661).

In addition to the ancestral diversity of endothelial cells, it is now well recognised that skin macrophages also comprise mixed composition of embryonic and monocyte-derived macrophages (101, 162, 164, 404). Several studies have demonstrated that epidermal Langerhans cells and subsets of dermal macrophages arise from extra-embryonic yolk sac (YS) erythromyeloid progenitors (EMPs) in mice (92, 109, 122). EMPs arise as early as embryonic day 7 (E7.0) and peak in number in YS around E8.5-9.5 when they begin to enter the bloodstream to seed other organs (2, 93, 109). An early wave of EMPs, which express c-Kit and colony-stimulating factor receptor (CSF1R), gives rise to CX₃CR1⁺ pre-macrophage precursors and mature tissue macrophages, without passing through a monocyte intermediate stage (2, 98, 99, 109, 175). A second wave of EMPs colonises foetal liver and thereafter other tissues via foetal liver monocytes (2). Although previous studies have provided conflicting insights as to whether early or late EMPs are the original source of embryonically derived dermal macrophages and Langerhans cells (98, 165, 175, 176, 195, 662), both have been shown to be maintained after birth independently of input from definitive BM haematopoietic stem cells (HSCs) or circulating monocytes (93, 109, 121, 122, 164, 175, 197, 663).

It is also unclear whether macrophages of different origins in skin function differently under homeostatic and diseased conditions. Tamoutounour *et al.* elucidated the composition, ontogeny and functional specialisation of dermal CD11b⁺CD11c⁺ dendritic cells and five different subclasses of dermal CD11b⁺ monocytes (denoted “P1”), monocyte-dendritic cells (“P2” and “P3”) and macrophages (“P4” and “P5”) in healthy murine skin (404). They inferred a precursor-progeny relationship between extravasated Ly6C^{Hi} blood monocytes and the P1 → P2 → P3 subsets, which expressed *Il10*-related transcripts consistent with an anti-inflammatory role and could stimulate naive T cells. In contrast, P4 and P5 macrophages appeared to arise from mixed sources. In steady state, a majority were maintained

independently of CCR2 and Flt3 and aligned by transcriptomic analysis with embryonically derived macrophages. However, in BM irradiation and parabiotic chimaeras, some were found to originate from adult circulating monocytes in a CCR2-dependent manner (404). Whereas P4 and P5 dermal macrophages were enriched with phagocytic properties, they were unable to stimulate T cells or migrate to draining cutaneous lymph nodes. How these different dermal myeloid subtypes relate to traditional views of sequential “M1” (pro-inflammatory) and then “M2” (anti-inflammatory) macrophage involvement in skin wound healing (424, 664-666) and tumour progression (451, 667-669) remains uncertain.

Current consensus attributes the local maintenance and turnover of embryonically derived skin-resident macrophages to a largely unproven capacity to undergo proliferative self-renewal without loss of functional differentiation (164). However, an alternative explanation is that postnatal skin also contains a niche of progenitors that can support macrophage renewal under steady state and mediate their expansion in disease. The previous chapters reported the existence of YS-derived, self-renewing, tissue-resident progenitor cells that can differentiate into both endothelial cells and macrophages in postnatal mouse aorta, heart, lung and skeletal muscle. Here, we investigated for analogous EndoMac progenitors in skin and examined for its contribution to vasculogenic and inflammatory responses to wound injury and melanoma growth.

6.2 Methods in brief (also see Chapter 2)

6.2.1 Overview

Breeding pairs of C57BL/6 (C57BL/6J), *Cx3cr1*^{GFP/+} (B6.129P-Cx3cr1^{tm1Litt}/J), *Flt3*^{Cre} (Tg.*Flt3*^{cre}/ERT2), *Rosa*^{mT/mG} (Gt(ROSA)26Sor^{tm4}(ACTB-tdTomato,-EGFP)Luo/J), *Csf1r*^{Mer-iCre-Mer} (FVB-Tg(*Csf1r*-cre/*Esr1**)1Jwp/J), *Cx3cr1*^{YFP-creER} (B6.129P2(Cg)-Cx3cr1^{tm2.1}(cre/ERT2)Litt/WganJ) and *UBI-GFP* (C57BL/6-Tg(UBC-GFP)30Scha/J) mouse strains were acquired from The Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed in the animal care facility at SAHMRI, and were maintained on standard chow diet. Skin from these mice comprising epidermis and dermis, but not subcutaneous fat, was prepared into tissue sections for immunofluorescence staining and confocal microscopy, or enzymatically digested into single cell suspensions for use in different experiments, including methylcellulose colony forming unit (CFU) and MatrigelTM-based angiogenesis assays and multi-colour flow cytometry. Several experiments also made use of validated murine wound healing (512) and B16 melanoma cell cancer models (670) in combination with the *Flt3*^{Cre} x *Rosa*^{mT/mG} or tamoxifen (TAM)-inducible *Csf1r*^{MerCreMer} x *Rosa*^{mT/mG} fate-mapping strains or with adoptive transfer of GFP⁺ Skin EndoMac progenitors. All animal experiments and care were performed following the standards stated in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Academy of Sciences, Bethesda, MD, USA) and in accordance with the National Health and Medical Research Council of Australia research guidelines and Australian Code for the Care and Use of Animals for Scientific Purposes. Ethics approval was obtained from the SAHMRI animal ethics committee (ID SAM117, SAM155, SAM308 and SAM432.19).

6.2.2 Statistical analysis

Where relevant, analysis was performed blinded to study group. Statistical comparisons were performed using Prism (GraphPad version 9). Data sets were tested for normality of distribution by Shapiro-Wilk test. Statistical comparisons were performed with parametric or non-parametric unpaired or paired two-sample *t*-tests or ANOVA (with post-test multiple comparisons), as specified. Results are expressed as mean $\pm$ standard deviation of multiple experiments. In all cases, statistical significance was established at two-tailed $p < 0.05$. The statistical parameters and the number of mice used per experiment are found in the figure legends.

6.3 Results

6.3.1 Self-renewing, clonogenic skin CFU-M progenitors express CX₃CR1 and CSF1R

Skin digests from 8-12 w C57BL/6 mice generated the full range of haematopoietic CFU subtypes in methylcellulose but showed predilection for colony forming units-macrophage (CFU-M), which comprised almost 90% of all colonies formed (**Figure 6-1a**). As we had previously discovered and comprehensively characterised postnatal aortic CFU-M progenitors (Chapters 3 and 4), we compared skin and aortic CFU-M yield from donor-matched mice and found ten-fold higher yield from skin (**Figure 6-1b**). Therefore, among the different non-haematopoietic tissues studied so far (aorta, heart, lung, muscle, skin), skin is the most enriched with CFU-M progenitors. Skin CFU-M ranged in size, with a greater proportion meeting our criteria for medium size (100-1000 cells) than from aorta ($p=0.04$, **Figure 6-1c**).

Numerous studies have inferred that resident macrophages from embryonic origin can self-renew postnatally, including those in skin (165). We therefore investigated the ability of skin CFU-M to self-renew *in vitro* through two different assays. Firstly, the whole cellular content from primary (1°) CFU assays were re-plated for another 14 days (d) in methylcellulose. CFU-M yield from these secondary (2°) cultures was enriched to seven-fold that of 1° culture (**Figure 6-1d**). Secondly, individually isolated 1° CFU-M were dissociated into single cell preparations, which were re-plated so that the content of one colony occupied one well of a 96-well plate. This firstly showed that 2° CFU-M arose from single cell origins (**Figure 6-1e**). 92.1% of 1° colonies gave rise to at least one new 2° colony, with a self-renewal efficiency of 1 colony per 3.7 ± 0.7 cells plated. By comparison, we had previously determined that the self-renewal capacity of age-matched aortic CFU-M was 1 CFU-M per 14.8 ± 16.3 cells (Chapter 4).

Having established the clonal self-renewal capacity of adult skin CFU-M, we next used flow cytometry to study the cellular composition of 1° CFU-M. Like aorta, heart, lung and muscle (Chapters 4 and 5),

CFU-M from adult skin were enriched with progenitor cells that were negative for mature lineage (LIN), monocyte-macrophage (CD11b, F4/80) and endothelial (CD144/CDH5) markers, but positive for the pan-leukocyte antigen, CD45, the haematopoietic progenitor/stem cell markers, Sca-1 and c-Kit, as well as CX₃CR1 and CSF1R, which are expressed on YS pre-macrophages and EMPs, respectively and tissue-resident macrophages (2, 93, 121, 168, 552, 554) (**Figure 6-1f** and **Appendix 12**). We identified a matching LIN⁻CD45^{+/Lo}CD11b⁻F4/80⁻Sca-1⁺c-Kit⁺CX₃CR1⁺CSF1R⁺ population in fresh digests of 12 w C57BL/6 skin with a prevalence of 0.6±0.5% of all viable skin cells (n=12 mice) (**Figure 6-1g**). Importantly, this is within the same order of magnitude as 1° CFU-M yield from skin (~200 CFU-M/10⁵ cells or 0.2%). Analysis of bromodeoxyuridine (BrdU) uptake 24 h after intraperitoneal (*i.p.*) injection demonstrated that this was an actively proliferating population, with 13.3±4.6% progenitors in the S-phase of cell cycle (**Figure 6-1h**).

As skin CFU-M progenitors expressed CX₃CR1, we also studied their presence in *Cx3cr1*^{GFP/+} reporter mice, where green fluorescence protein (GFP) is expressed in association with the *Cx3cr1* allele (155). CX₃CR1 (GFP) was expressed in 5.4±2.0% of viable skin cells (n=6), as quantified by flow cytometry. FACS immunoselection showed that CFU-M forming capacity was enriched from CX₃CR1^{Bright} cells and completely absent from the CX₃CR1^{Neg} fraction (**Figure 6-1i-k**). Notably, 34.4±7.4% of CD45⁺ CX₃CR1⁺ (GFP⁺) cells in skin were progenitors, with another 50.2±10.8% consisting of CD11b⁺F4/80⁺ macrophages (n=6) (**Figure 6-1i**). As further evidence that CX₃CR1 is an important marker of CFU-M progenitors, we also compared *Cx3cr1*^{GFP/+} mice with their *Cx3cr1*^{GFP/GFP} littermates, which lack the functional *Cx3cr1* allele. Both the CFU assay and flow cytometry revealed marked depletion of the progenitor population from *Cx3cr1*^{GFP/GFP} skin (**Figure 6-1l, m**).

Together, these results uncover the existence of self-renewing, clonogenic progenitors in adult mouse skin, that express the same surface markers as EndoMac progenitors in other tissues. These include both CX₃CR1 and CSF1R, which are recognised markers of embryonic YS myelopoiesis.

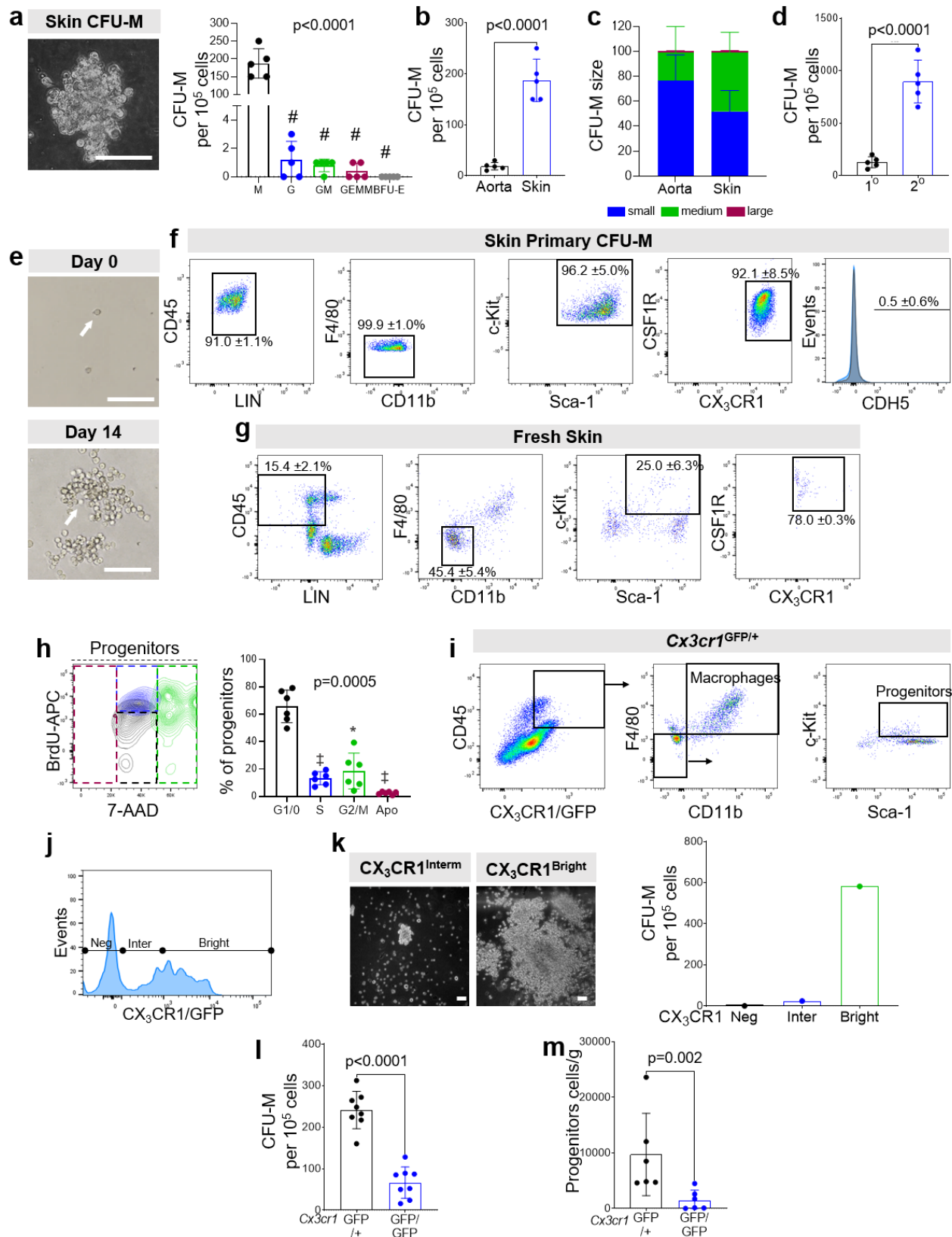


Figure 6-1. Skin contains $c\text{-Kit}^+ \text{CX}_3\text{CR1}^+ \text{CSF1R}^+$ CFU-M progenitors capable of self-renewal.

- (a)** Light microscopy image of CFU-M grown from 12 w C57BL/6 skin digest after 14 d in methylcellulose. Adjacent graph shows frequencies of different CFU types from C57BL/6 skin cells. M, Macrophage; G, Granulocyte; GM, Granulocyte-macrophage; GEMM, Granulocyte-erythrocyte-monocyte-megakaryocyte; BFU-E, Burst-forming units-erythroid. N=5 mice. Kruskal-Wallis test. Multiple test comparisons: # $p < 0.0001$ for comparison to CFU-M. Scale bar, 100 μm .
- (b)** Comparison of primary (1°) CFU-M yield from same donor aorta and skin digests. N=5 mice. Paired *t*-test.
- (c)** Breakdown of small, medium and large CFU-M from same donor 12 w C57BL/6 aorta and skin cells. N=5 mice. Paired *t*-test comparing aortic vs skin medium colonies, $p = 0.04$.
- (d)** Donor-matched 1° and 2° CFU-M yield from 12 w C57BL/6 skin. N=5 mice. Paired *t*-test.
- (e)** Self-renewal of 2° CFU-M from single cell (arrows) obtained from 1° CFU-M. Scale bar, 100 μm .
- (f-g)** Flow cytometry of cells from **(f)** 1° skin CFU-M (n=3) and **(g)** freshly digested skin cells revealing progenitor population (n=4 mice).
- (h)** Flow cytometry of bromodeoxyuridine (BrdU) versus 7-aminoactinomycin (7-AAD) gated from progenitors in skin digest. Dotted boxes represent G1/0 (black), S (blue), G2/M (green) and apoptotic (maroon) phases. Graph shows percentage of progenitors in each phase. N=6 mice. One-way ANOVA. Multiple comparison tests: * $p < 0.05$ and ‡ $p < 0.001$ for comparison to G1/0.
- (i)** Flow cytometry shows presence of $\text{CD11b}^+\text{F4/80}^+\text{Sca-1}^+\text{c-Kit}^+$ progenitors and $\text{CD11b}^+\text{F4/80}^+$ macrophages among $\text{CD45}^+\text{CX}_3\text{CR1}^+$ cells in skin of 12 w $\text{Cx3cr1}^{\text{GFP}/+}$ mice. Representative of N=6 mice.
- (j)** Histogram used for FACS isolation of $\text{CX}_3\text{CR1}^{\text{Neg}}$, $\text{CX}_3\text{CR1}^{\text{Interm}}$ and $\text{CX}_3\text{CR1}^{\text{Bright}}$ populations from 12 w $\text{Cx3cr1}^{\text{GFP}/+}$ skin.
- (k)** Graph shows 1° CFU-M yield from each sorted population (one experiment using skin from six pooled mice). Scale bar, 100 μm .
- (l-m)** Frequency of **(l)** 1° CFU-M and **(m)** progenitors from skin of 12 w $\text{Cx3cr1}^{\text{GFP}/+}$ and $\text{Cx3cr1}^{\text{GFP}/\text{GFP}}$ mice. N=6-8 mice. Unpaired *t*-tests.

Data summarised as mean $\pm$ SD.

6.3.2 Skin CFU-M progenitors arise from embryonic YS and are not derived from BM-derived haematopoiesis

We next investigated the ontogeny of these progenitor cells and their relationship to definitive and YS haematopoiesis. Definitive HSCs transiently upregulate growth factor fms-like tyrosine kinase 3 (Flt3) as they differentiate (671). We therefore used the well-established *Flt3^{Cre}* x *Rosa^{mT/mG}* fate-mapping model to distinguish cells that have arisen from definitive HSCs which express GFP (*Flt3-Cre⁺*), from all other cells which are GFP⁻ and fluoresce with tdTomato (tdTom) instead (*Flt3-Cre⁻*) (671). As expected, >90% of haematopoietic colonies grown from BM or spleen were GFP⁺tdTom⁻ (**Figure 6-2a**). In contrast, the vast majority of skin CFU-M were GFP⁺tdTom⁺, as shown by both fluorescence microscopy (**Figure 6-2a**), and flow cytometry profiling of the progenitor cells contained within 1° CFU-M (**Figure 6-2b**). Furthermore, whereas blood monocytes were ~98.5% GFP⁺ consistent with their HSC origins (**Figure 6-2c**), LIN⁻CD45^{+/Lo}CD11b⁻F4/80⁻Sca-1⁺c-Kit⁺ progenitor cells in freshly digested skin were ~95% GFP⁺tdTom⁺ (n=4) (**Figure 6-2d**). These results indicate that skin CFU-M progenitors are independent of BM haematopoiesis and their circulating monocyte progeny.

We also used *Flt3^{Cre}* x *Rosa^{mT/mG}* mice to quantify the proportions of skin macrophages, endothelial cells and their respective subtypes that originate from a definitive Flt3⁺ source. 64.1±8.3% of LIN⁻CD45⁺CD11b⁺F4/80⁺ macrophages and 43.5±16.3% of LIN⁻CD45⁻CD31⁺ and/or LIN⁻CD45⁻CDH5⁺ endothelial cells were Flt3-Cre⁺ (GFP⁺), indicating that both lineages have mixed origins in adult skin (**Figure 6-2b**). CD11b⁺CD45⁺ myeloid subsets were further analysed based on the flow cytometric gating strategy described by Tamoutounour *et al.* (404) (**Figure 6-2e**). In steady state skin, we found the following percentage breakdown of CD11b⁺CD45⁺ subpopulations with Flt3-Cre⁺ status provided in the adjacent brackets: P1 14.3% of total (62.4% Flt3-Cre⁺), P2 2.2% (93.4%), P3 14.8% (92.3%), P4 32.8% (73.7%), P5 0.7% (96.1%) and CD11c⁺ dendritic cells 38.5% (81.4%) (**Figure 6-2f, g**). However, it is important to recognise that these data apply to digests of whole skin, in which we did not separate dermis from epidermis.

For other tissues (e.g. muscle, heart, aortic adventitia), macrophages have also been classified based on their expression of major histocompatibility complex-II (MHCII) and LYVE-1 (172, 194, 624). In most of these studies, resident macrophages of mixed embryonic and definitive haematopoietic origin have been identified as MHCII^{Lo}LYVE-1⁺, whereas those renewed by definitive HSCs and circulating monocytes are MHCII^{Hi}LYVE-1⁻. Here we determined that skin myeloid cells, defined as LIN⁻CD45⁺CD11b⁺ can also be subclassified using these phenotypes, with 55.5% MHCII^{Lo}LYVE-1⁺ (67.0% Flt3-Cre⁺), 36.8% MHCII^{Hi}LYVE-1^{Lo} (98.4% Flt3-Cre⁺) and 4.2% MHCII^{Hi}LYVE-1^{Hi} (99.3% Flt3-Cre⁺) (**Figure 6-2h**). Finally, with respect to endothelium, we observed that a higher proportion of CD45⁻CD31⁺ cells (86.3±4.1%, n=4) were Flt3-Cre⁺ compared to CD45⁻CDH5⁺ (36.8±3.3%, n=4; p<0.0001 for Flt3-Cre⁺ LIN⁻CD45⁻CD31⁺ vs LIN⁻CD45⁻CDH5⁺ cells) endothelial cells, while definitive haematopoiesis contributed to only a small minority of LYVE-1⁺ lymphatic endothelial cells (**Figure 6-2i**).

Finally, we also performed experiments in *Flt3*^{Cre} x *Rosa*^{mT/mG} mice in which we injected clodronate liposomes by *i.p.* injection to deplete blood monocytes (**Figure 6-2i, k**). This led to an expected striking reduction in Flt3-Cre⁺ macrophages in skin, without significantly altering the pool of Flt3-Cre⁻ macrophages (**Figure 6-2l**) or progenitors (**Figure 6-2m, n**). This further consolidates the evidence that skin CFU-M progenitors are independent of definitive BM haematopoiesis and circulating monocytes.

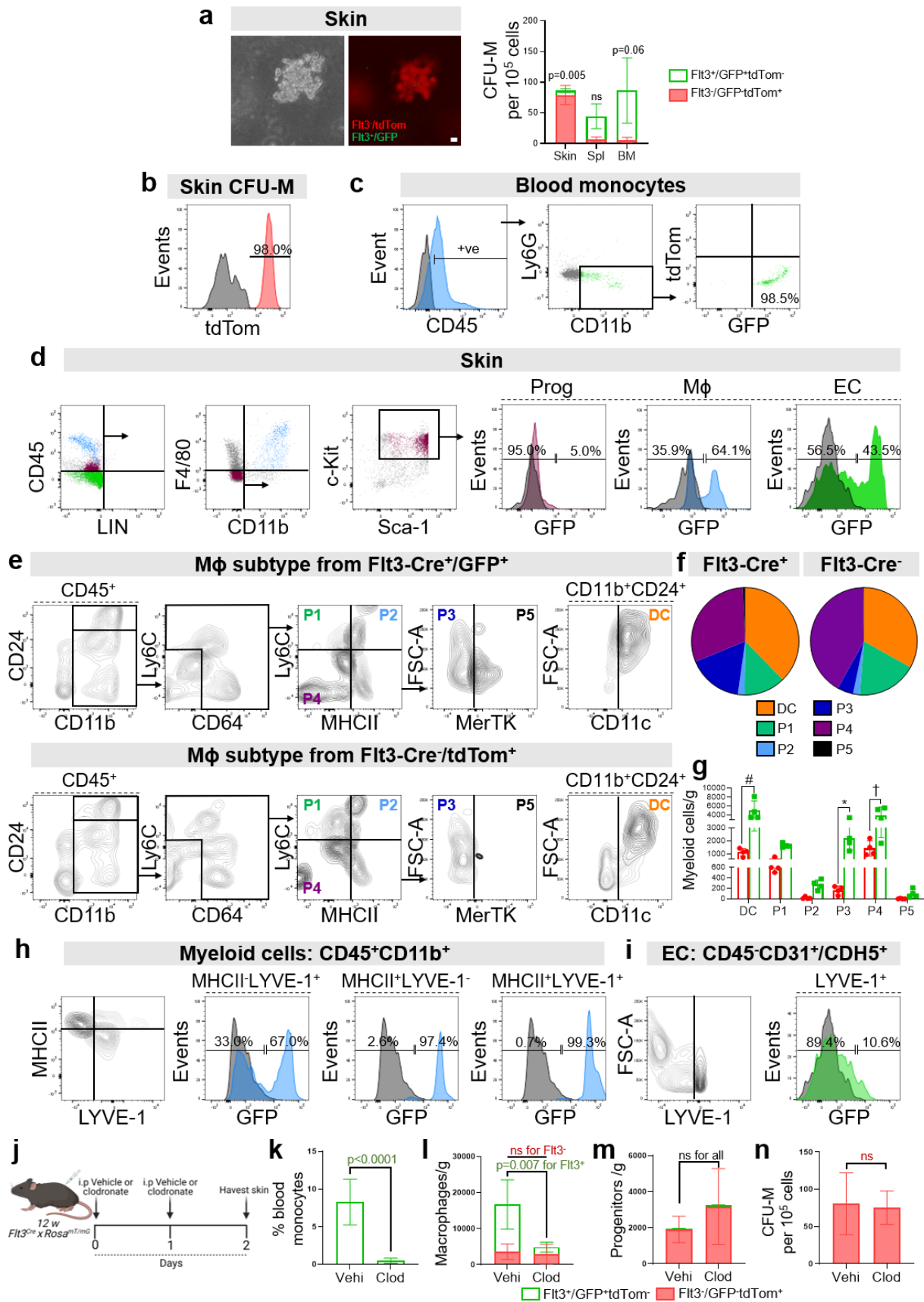


Figure 6-2. Skin CFU-M progenitors are not derived from BM-derived haematopoiesis.

- (a) Light and fluorescence microscopy of CFU-M from skin of adult *Flt3^{Cre}* x *Rosa^{mT/mG}* mice. Graph of *Flt3-Cre⁺* and *Flt3-Cre⁻* CFU-M yield from different tissues. N=5 mice. Spl, Spleen. BM, Bone marrow. Paired *t*-tests for each tissue. Scale bar, 20 μ m.
- (b) Flow cytometry for tdTom expression of cells in skin CFU-M from *Flt3^{Cre}* x *Rosa^{mT/mG}* mice. N=3 mice.
- (c) Flow cytometry for tdTom versus GFP expression of blood CD45⁺CD11b⁺LY6G⁻ monocytes from *Flt3^{Cre}* x *Rosa^{mT/mG}* mice.
- (d) Flow cytometry for GFP⁺ (*Flt3-Cre⁺*) status of progenitors (maroon), macrophages (M ϕ , blue) and endothelial cells (EC, green) in skin of *Flt3^{Cre}* x *Rosa^{mT/mG}* mice. Percentages represent mean of n=4 mice.
- (e) Flow cytometry gating strategy used to assess different myeloid cell populations in skin digests from adult *Flt3^{Cre}* x *Rosa^{mT/mG}* mice, according to classification system by Tamoutounour *et al.* (404)
- (f) Pie chart represents the breakdown of *Flt3-Cre⁺* and *Flt3-Cre⁻* myeloid cells in skin according to this classification system. N=6 mice.
- (g) Graph shows quantification of *Flt3-Cre⁺* and *Flt3-Cre⁻* myeloid cell subtypes in adult skin. N=4 mice. Mixed effects two-way ANOVA: $p < 0.0001$ for myeloid subgroup; $p < 0.0001$ for cell origin; $p = 0.0008$ for subgroup x origin. Multiple comparison: * $p < 0.05$, † $p < 0.01$ and # $p < 0.0001$ for *Flt3⁺* vs *Flt3⁻*
- (h). Flow cytometry profiling of MHCII and LYVE-1 expression on CD45⁺CD11b⁺F4/80⁺ macrophages in adult *Flt3^{Cre}* x *Rosa^{mT/mG}* skin and their *Flt3-Cre⁺* (GFP⁺) status. N=4 mice. Blue histogram, Sample; Grey, FMO control.
- (i) LYVE-1 expression on endothelial cells with adjacent histogram showing *Flt3-Cre⁺* (GFP⁺) status. N=4 mice. Green histogram, Sample; Grey, FMO control.
- (j) Schematic for clodronate (Clod) or vehicle (Vehi) liposomal treatment of *Flt3^{Cre}* x *Rosa^{mT/mG}* mice.
- (k-n) Graphs compare Vehi and Clod-treated mice for the presence of *Flt3-Cre⁺* and *Flt3-Cre⁻* (k) blood monocytes, (l) skin macrophages, (m) skin progenitors and (n) skin CFU-M yield. N=4-5 mice. Unpaired *t*-tests. Red *p*-values, *Flt3-Cre⁻* comparisons; green *p*-values, *Flt3-Cre⁺*.

Data summarised as mean or mean $\pm$ SD.

We proceeded to narrow down the origin of skin CFU-M progenitors by studying their presence across four different pre- and postnatal ages in C57BL/6 mice: E15.5, postnatal day 1 (P1), 12 w and 52 w. CFU-M yield was highest from embryonic and P1 skin and decreased with postnatal ageing (**Figure 6-3a**). Embryonic and P1 CFU-M also formed more quickly in methylcellulose (not shown) and were larger in size, as reflected by more medium and large colonies (E15.5: 510.2±95.2 medium and 78.6±52.3 large CFU-M/10⁵ cells; P1: 159.9±48.2 medium and 2.9±1.2 large; 12 w: 13.1±4.4 medium and 0.2±0.3 large; 52 w: 2.1±0.9 medium and 0 large; n=4/age) (**Figure 6-3b**). Age-related declines were also found for the prevalence of progenitors in skin as measured by flow cytometry (**Figure 6-3c**) and their clonal self-renewal efficiency, which was defined by the number of progenitors from 1° CFU-M needed to form a 2° CFU-M (**Figure 6-3d**).

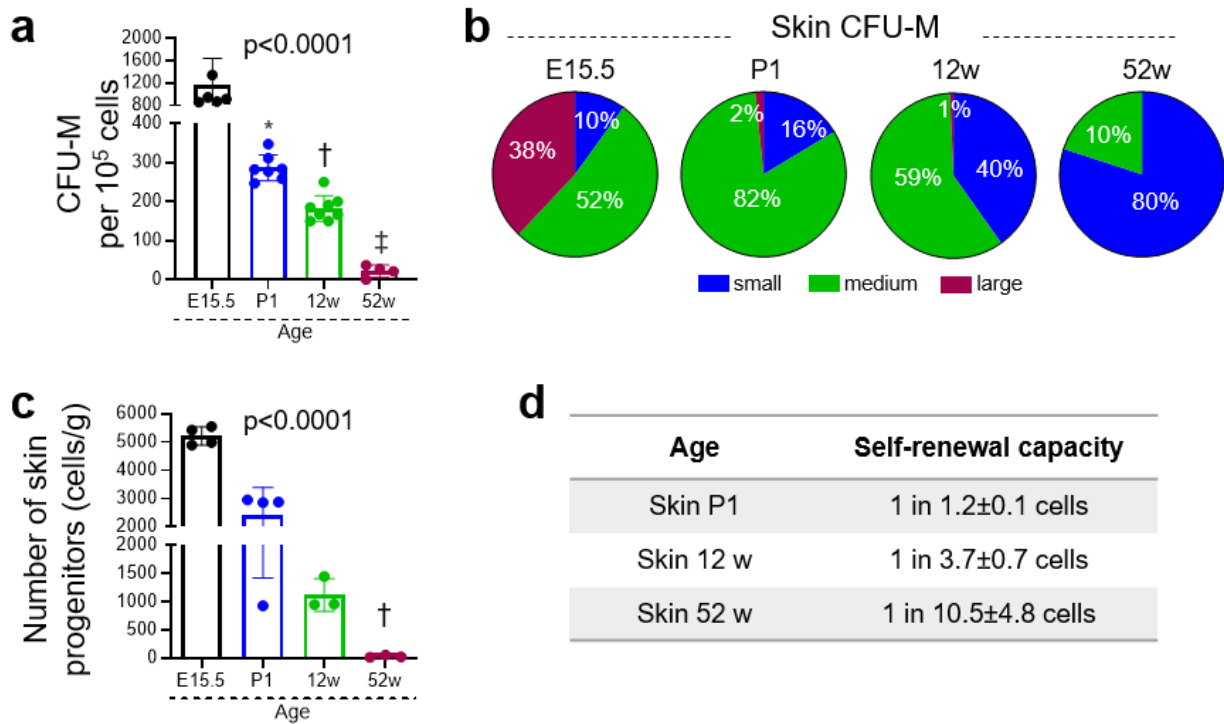


Figure 6-3. Age-related differences in the prevalence, clonal and self-renewal capacity of CFU-M progenitors.

(a) Age comparisons of CFU-M yield for C57BL/6 skin. N=3-8 mice/age. Kruskal-Wallis. Multiple comparison tests: * $p < 0.05$ for P1 vs 52 w; † $p < 0.01$ and ‡ $p < 0.001$ for comparison to E15.5.

(b) Pie charts show classification of skin CFU-M based on colony size at different ages. N=3-6 for each.

(c) Age comparisons for the number of progenitors per gram (g) of C57BL/6 skin. N=3-4 mice/age. Kruskal-Wallis. Multiple comparison tests: † $p < 0.01$ for comparison to E15.5.

(d) CFU-M self-renewal capacity from skin at different ages, expressed as the number (mean $\pm$ SD) of 1° CFU-M progenitors required to generate one 2° CFU-M. N=3-5 mice/age.

As skin CFU-M progenitors are independent from definitive haematopoiesis and most abundant at E15.5 and P1, we next studied whether they derive from extra-embryonic YS, which was found to be the source of EndoMac progenitors in aorta, heart, lung and muscle (Chapters 4 and 5). YS EMPs give rise to CX₃CR1⁺ pre-macrophages and macrophages from E8.5, which then begin to colonise the embryo starting at E9.5 (1, 101, 123). Firstly, we generated *Cx3cr1*^{CreER-YFP} x *Rosa*^{tdTom} mice, which we injected at E8.5 or E9.5 with tamoxifen (TAM) to pulse label *Cx3cr1*⁺ cells and their progeny, and tracked these cells in skin at E15.5, P1 and 12 w. Brain microglia, which originate entirely from YS myelopoiesis (98), were used as a positive control. There was limited tdTom labelling of microglia (13.4±5.7%, n=3) after induction at E8.5, with negligible tdTom expression in skin (data not shown). However, induction at E9.5 robustly labelled microglia, as well as skin progenitors, macrophages and endothelial cells in 12 w *Cx3cr1*^{CreER-YFP} x *Rosa*^{tdTom} mice, but very few blood monocytes (**Figure 6-4a-c**). After normalising for tdTom labelling of microglia, we determined that ~74% of progenitors, ~32% of macrophages and ~4% of endothelial cells in 12 w skin were derived from an E9.5-induced CX₃CR1⁺ source, although the normalised proportion of labelled endothelial cells increased to ~28% when considering only the CDH5⁺ subset. Progenitors, macrophages and endothelium were also labelled with tdTom at E15.5 and P1. Notably, the numbers of tdTom⁺ macrophages and endothelial cells in skin increased markedly after birth, countered by a modest decrease in the number of tdTom⁺ progenitors (**Figure 6-4d**). This was associated with corresponding age-related changes to the breakdown of tdTom⁺ cells as depicted in **Figure 6-4e**. Finally, we observed 100% normalised tdTom⁺ labelling of skin CFU-M by fluorescence microscopy from each age studied (E15.5, P1 and 12 w), whereas colonies from E15.5 foetal liver, P1 and 12 w BM, were predominantly tdTom⁻ (**Figure 6-4f**).

We also performed complementary TAM-inducible *Csf1r*^{MerCreMer} x *Rosa*^{mT/mG} lineage-tracing to investigate whether skin CFU-M progenitors arise specifically from early CSF1R⁺ YS EMPs. We injected TAM into pregnant dams at E8.5 and analysed for GFP expression in skin cells from 12 w offspring. Brain microglia was again used as a positive control. Induction at E8.5 labelled microglia, skin progenitors, macrophages and endothelial cells in adult *Csf1r*^{MerCreMer} x *Rosa*^{mT/mG} mice, with negligible labelling of monocytes in blood (**Figure 6-5a-c**). In this experiment, after normalisation of

GFP expression to microglia, 100% of progenitors were determined to be YS EMP-derived, along with ~32% of macrophages and ~25% of all endothelial cells. Overall, flow cytometry revealed that progenitors accounted for 14% of GFP⁺ cells in 12 w skin, matching our *Cx3cr1*^{CreER} mapping results at the same age, with macrophages and endothelial cells making up 29% and 57%, respectively (n=6 mice) (**Figure 6-5d, e**). Normalised GFP labelling was 100% for skin CFU-M as opposed to BM CFU-M which were GFP⁻ (**Figure 6-5f**). Importantly, there were no GFP⁺ skin CFU-M in the absence of TAM induction of Cre-Lox recombination in *Csf1r*^{MerCreMer} x *Rosa*^{mT/mG} littermate controls (**Figure 6-5f**).

Finally, FACS was used to isolate GFP⁺ YS EMP-derived progenitors (CD45^{+/Lo}CD11b⁻F4/80⁻Sca-1⁺c-Kit⁺), macrophages (CD45⁺CD11b⁺F4/80⁺) and endothelial cells (CD45⁻CD31⁺) from skin digests of E8.5 TAM-induced, 12 w *Csf1r*^{MerCreMer} x *Rosa*^{mT/mG} mice. Wright-Giemsa staining showed that progenitors had similar morphology to their counterparts in aorta (see Chapter 4) and lacked the surface membrane protrusions and intracellular vacuolations seen in macrophages (**Figure 6-5g**). Quantitatively, they had similarly sized nuclei to macrophages but smaller overall cell areas, resulting in significantly higher nuclear:cell ratio (**Figure 6-5g**). Crucially, CFU-M were only produced by sorted progenitors, with neither YS-derived macrophages nor endothelial cells displaying any capacity for colony formation in methylcellulose (**Figure 6-5h**).

Overall, these results indicate that CFU-M progenitor cells are derived from YS EMPs and are seeded prenatally in skin. Although they persist postnatally, their prevalence diminishes as mice age without replenishment from definitive HSCs. Interestingly, this is countered by an increased abundance of both YS-derived macrophages and endothelial cells in adult skin, prompting consideration of whether the progenitor pool contributes to expansion and maintenance of these mature lineages after birth.

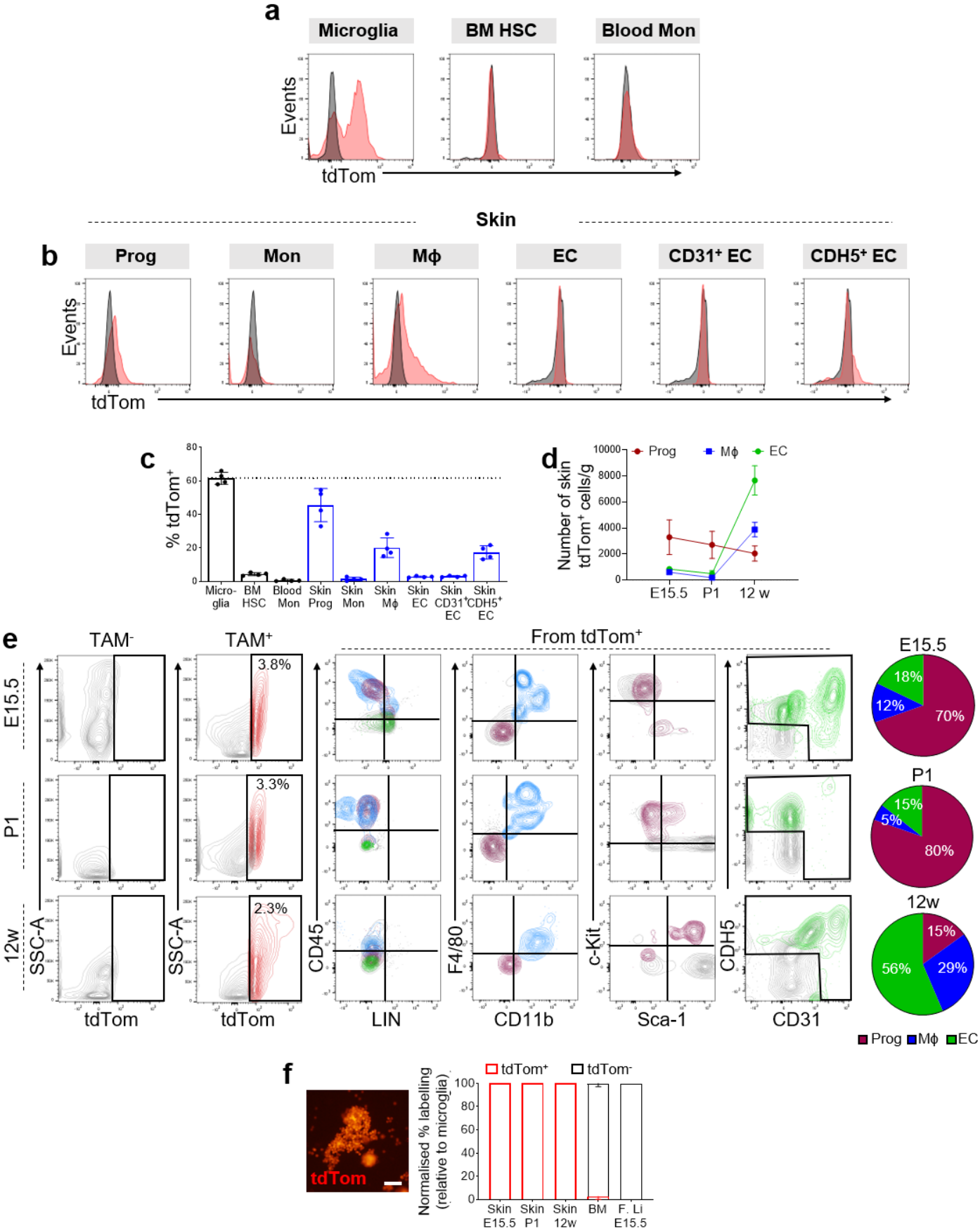


Figure 6-4. *Cx3cr1*⁺ YS cells give rise to progenitors, macrophages and endothelial cells that survive into adulthood.

(a-f) E9.5 tamoxifen (TAM)-induced labelling of *Cx3cr1*^{CreER-YFP} x *Rosa*^{tdTom} mice, with analysis at E15.5, P1 or 12 w.

(a-b) Representative histograms show tdTom expression in different cell populations at 12 w of age. Mon, Monocytes; HSC, Haematopoietic stem cells; Prog, Progenitors; Mφ, Macrophages; EC, Endothelial cells. Red histogram, Sample; Grey, No TAM Cre control.

(c) Graph summarises the % tdTom⁺ expression in these populations. Dotted line corresponds to mean tdTom⁺ expression in microglia, which were used as a positive control. N=4 mice.

(d) Numbers of tdTom⁺ progenitors (prog), macrophages (Mφ) and endothelial cells (EC) in skin at different ages (n=3 for E15.5 and P1 and n=4 for 12 w). Mixed effects two-way ANOVA: p<0.0001 for time; p=ns for cell type; p=0.0001 for time x group.

(e) Flow cytometry plots show tdTom⁺ progenitors (maroon), Mφ (blue) and EC (green) in skin at different ages. Pie charts show lineage breakdown of tdTom⁺ cells at these ages. N=3-4/age.

(f) tdTom⁺ CFU-M from 12 w skin. Graph shows tdTom⁺ vs tdTom⁻ status of CFU-M from different tissues and ages, with results normalised to labelling of brain microglia. N=4/age. BM, Bone marrow. F. Li, Foetal liver. Scale bar, 50 μm.

Data summarised as mean±SD.

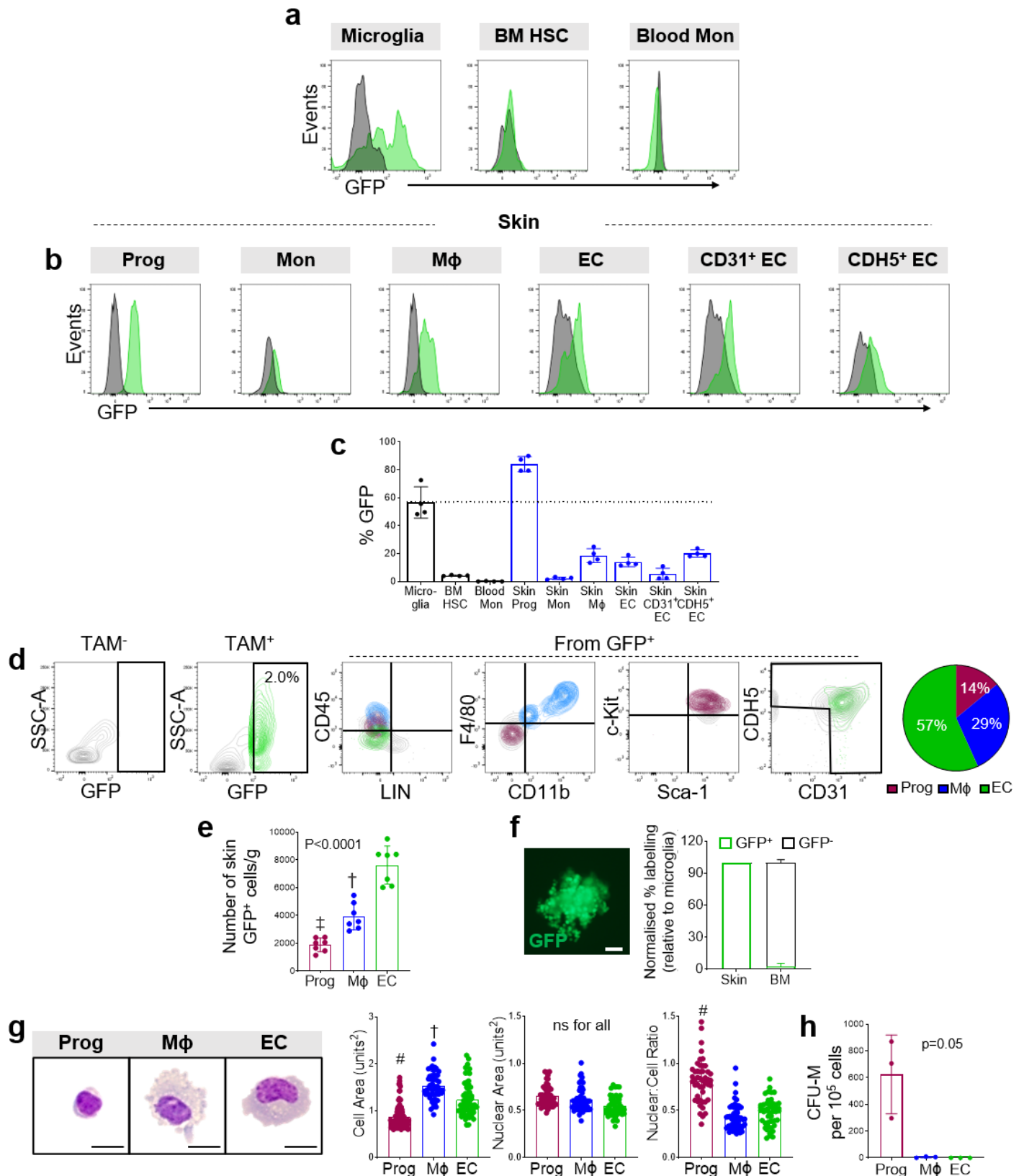


Figure 6-5. *Csflr*⁺ YS EMPs give rise to progenitors, macrophages and endothelial cells that survive into adulthood.

(a-h) *Csflr*^{MerCreMer} *x* *Rosa*^{mT/mG} mice were induced with TAM at E8.5 and analysed at 12 w.

(a-b) Representative histograms show GFP expression in different cell populations. Mon, Monocytes; HSC, Haematopoietic stem cells; Prog, Progenitors; M ϕ , Macrophages; EC, Endothelial cells. Green histogram, Sample; Grey, No TAM Cre control.

(c) Graph summarises the % GFP⁺ expression in these populations. Dotted line corresponds to mean GFP⁺ expression in microglia, which were used as a positive control. N=4 mice.

(d) Flow cytometry plots show GFP⁺ progenitors (maroon), M ϕ (blue) and EC (green) in skin. Pie chart shows lineage breakdown of GFP⁺ cells. N=6 mice. No TAM control also shown.

(e) Graph shows numbers of GFP⁺ progenitors, M ϕ and EC in 12 w skin. N=6 mice. Repeated measures ANOVA. Multiple test comparisons: †p<0.01 and ‡p<0.001 vs EC.

(f) GFP⁺ CFU-M from 12 w skin. Graph shows GFP⁺ vs GFP⁻ status of CFU-M from skin and BM, with results normalised to microglia. N=4 mice. Scale bar, 50 μ m.

(g) Wright-Giemsa staining of sorted GFP⁺ populations, with results for total cell area, nuclear area and nuclear:cell area ratio. N=47-51 per cell type. Kruskal-Wallis tests. Multiple test comparisons: #p<0.0001 vs M ϕ and EC; †p<0.01 vs EC. Scale bar, 100 μ m.

(h) CFU-M yield from GFP⁺ progenitors, M ϕ and EC (three experiments, six skin each). Friedman test. Data summarised as mean $\pm$ SD.

5.3.3 YS-derived progenitors expand during wound healing

Both macrophages and endothelial cells have been shown to expand during the wound healing process (418, 660). We therefore investigated the kinetics of YS-derived progenitors in response to skin wound injury, in parallel with these other two cell types. Skin wounds were created on the dorsum of 12 w *Csf1r*^{MerCreMer} x *Rosa*^{mT/mG} mice that had been induced with TAM at E8.5 (**Figure 6-6a**) (512). Wounds were harvested 1, 3 and 7 d after surgery, and digested to analyse their cellular composition through flow cytometry. Uninjured skin was obtained from sites remote to the wounds of the same mice, while skin from non-induced *Csf1r*^{MerCreMer} x *Rosa*^{mT/mG} mice were used as GFP negative controls (**Figure 6-6b, c**). We found significant expansion of GFP⁺ YS EMP-derived cells in wounds at all three time-points compared to uninjured skin (**Figure 6-6d**). As a comparison to uninjured skin, the number of GFP⁺ progenitors were at least two-fold higher at each time-point, although their absolute number decreased as wound healing progressed, from 3898±1292 cells/g at day 1 to 1840±999 cells/g at day 7 (**Figure 6-6e**). A similar trend was observed for CFU-M yield, whereby fold-comparisons for wounds compared to uninjured skin were 3.5x higher at 1 d, 5x at 3 d and 1.8x at 7 d (**Figure 6-6f**). Conversely, we observed increasing numbers of YS-derived macrophages and endothelial cells in wounds over time, with GFP⁺ endothelial cells especially peaking at day 7 (**Figure 6-6g, h**). These results could be explained either by proliferative self-renewal of these cells in their mature form or by their differentiation from the progenitors that had expanded earlier. After normalisation of GFP expression to microglia, 96-100% of progenitors were determined to be YS EMP-derived at all timepoints after wound injury. Meanwhile, the contributions of EMPs to macrophages and endothelial cells increased from 33% and 15% in uninjured skin, respectively, to 46% and 47% in 3 d wounds and 61% and 50% in 7 d wounds (**Figure 6-6i-k**). Confocal microscopy also confirmed the presence of YS-derived GFP⁺ endothelial cells (CD31⁺) and macrophages (CD68⁺) in healed wounds (day 7 after surgery) (**Figure 6-6l**). Taken together, the response to cutaneous wound injury involves early expansion of resident EMP-derived progenitors, along with progressive accumulation of EMP-derived macrophages and endothelial cells as the wound site closes.

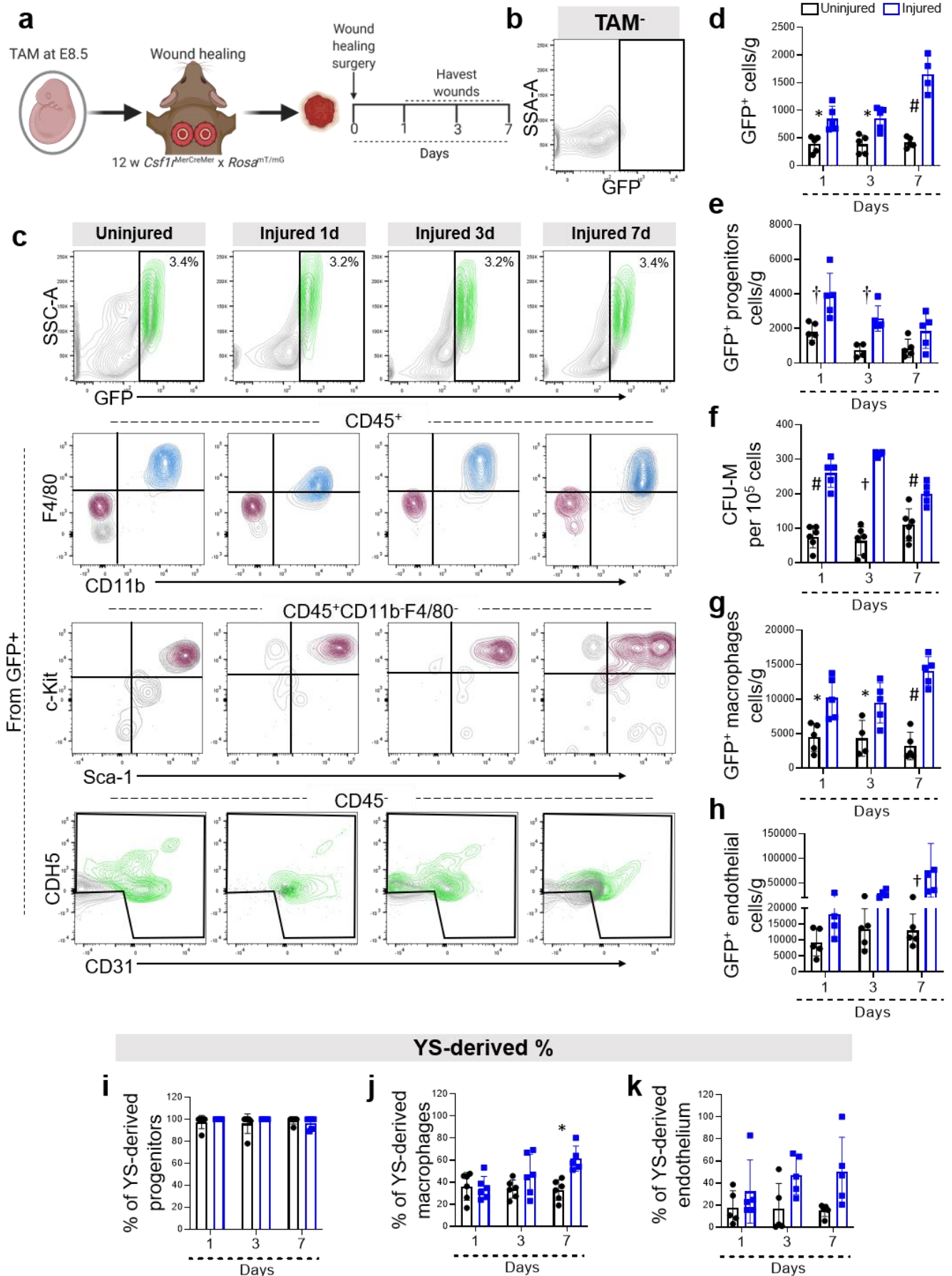


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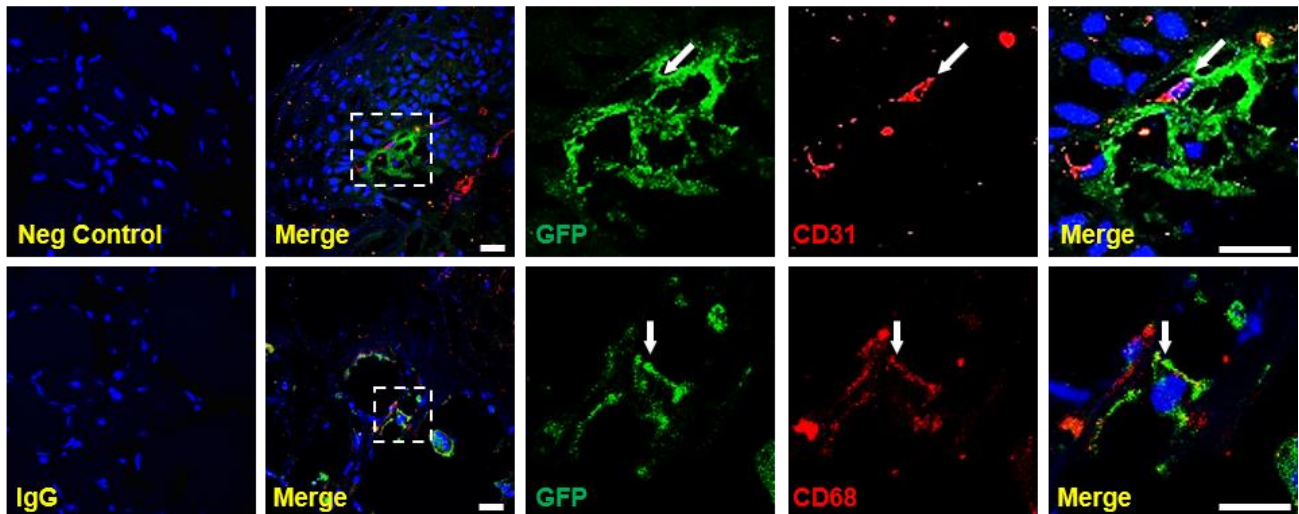


Figure 6-6. YS-derived CFU-M progenitors expand early during wound healing.

(a) Schematic showing wound sites on dorsal surface of 12 w *Csf1r^{MerCreMer} x Rosa^{mT/mG}* mice which had been induced with TAM at E8.5. Wounds were harvested 1 d, 3 d and 7 d after injury.

(b) Flow cytometry for GFP expression in a no TAM Cre control.

(c) Flow cytometry profiling of YS-derived GFP⁺ cells in skin at different time-points after wound healing surgery.

(d-h) Graphical quantifications of **(d)** all GFP⁺ and GFP⁺ **(e)** progenitors, **(f)** CFU-M yield, **(g)** macrophages and **(h)** endothelial cells at different time-points after wound injury with comparison to uninjured skin. N=5 mice/gp. Two-way ANOVA results: **(d)** p=0.0009 for time; p=0.002 for group; p=0.002 for time x group; **(e)** p=0.001 for time; p<0.0001 for group; p=0.3 for time x group; **(f)** p=0.2 for time; p<0.0001 for group; p=0.0005 for time x group; **(g)** p=0.3 for time; p=0.002 for group; p=0.07 for time x group; **(h)** p=0.06 for time; p=0.02 for group; p=0.07 for time x group; Multiple comparison used at each time-point: *p<0.05, †p<0.01 and #p<0.0001 for uninjured vs wounded skin.

(i-k) Graphs show % GFP expression normalised to microglia for **(i)** progenitors, **(j)** macrophages and **(k)** endothelial cells at different time-points after wound injury with comparison to uninjured skin. N=5 mice/gp. Two-way ANOVA results: **(i)** p=0.8 for time; p=0.3 for group; p=0.4 for time x group; **(j)** p=0.1 for time; p=0.03 for group; p=0.02 for time x group; **(k)** p=0.5 for time; p=0.004 for group; p=0.5 for time x group; Multiple comparison tests: *p<0.05 for uninjured vs wounded skin.

(I) Confocal microscopy images showing GFP⁺ CD31⁺ and CD68⁺ cells (arrows) in skin 7 d after wound injury. No antibody and IgG isotype control staining also shown. Scale bar, 20 μ m.

Data summarised as mean $\pm$ SD.

6.3.4 Skin CFU-M progenitors differentiate into macrophages and endothelial cells *in vitro*.

Our findings thus far led us to investigate whether skin CFU-M progenitors differentiate into macrophages and endothelial cells to help account for the local expansion of these YS-derived lineages that occurs in steady state skin after birth (**Figure 6-4, 6-5**) and in response to wound injury (**Figure 6-6**). Purity-checked progenitors were isolated from 12 w skin CFU-M and cultured in Matrigel™ for 7 d with endothelial growth media (EGM-10, CC-4542, Lonza, Switzerland). As per aortic and muscle EndoMac progenitors in Chapters 4 and 5, skin progenitors formed complex, interconnected networks of branching cords (**Figure 6-7a**), which by flow cytometry were shown to contain a predominance of newly formed endothelial cells, along with smaller numbers of macrophages and progenitors (**Figure 6-7b**). The macrophages that were produced comprised both the MHCII^{Hi}LYVE-1^{Hi} and MHCII^{Lo}LYVE-1^{Hi} phenotypes (**Figure 6-7b**), and also expressed CX₃CR1, CSF1R and high levels of the M2-like mannose receptor, CD206, with fewer cells expressing the M1-like co-stimulatory molecule, CD86 (**Table 6-1**). Meanwhile, the endothelial progeny no longer expressed c-Kit but retained Sca-1, which is known to be found on some endothelial cells (672, 673) (**Figure 6-7b**). Approximately a quarter of endothelial cells also were positive for the lymphatic marker, LYVE-1 (**Figure 6-7b, Table 6-1**). It is important to highlight here that neither macrophage colony-stimulating factor (M-CSF) nor vascular endothelial growth factor C (VEGF-C) were added to the endothelial growth media used in this assay. Interestingly, a small percentage of the cells produced in Matrigel™ co-expressed CD45, F4/80 and c-Kit but neither CD11b nor Sca-1 (**Figure 6-7b**). To the best of our knowledge, this LIN⁻CD45⁺CD11b⁻F4/80⁺c-Kit⁺Sca-1⁻ immunophenotype does not conform to any previously recognised cell population. This population also expressed CX₃CR1, CSF1R, LYVE-1, but not CD206, CD86 or MHCII. Given that it straddles the surface marker expression of CFU-M progenitors and macrophages, we considered that it may represent a transitional state of progenitor cell differentiation and henceforth refer to this subpopulation as “intermediate” throughout the rest of this chapter. Overall, the results of this assay indicate that like their aortic and muscle counterparts, skin CFU-M progenitors are at least bipotent for endothelial cells and macrophages *in vitro*.

Angiogenic cord-forming capacity, as measured by cumulative cord length and the number of branch-points was highest for CFU-M progenitors from P1 skin and lower from 12 and 52 w mice (**Figure 6-7c**). To establish the importance of macrophage progeny in the cord-forming capacity of skin progenitors, experiments were also performed with the addition of liposomal clodronate to the MatrigelTM every second day to deplete macrophages. While this did not significantly affect the total length of cords formed, it did result in reduction of cord branching (**Figure 6-7d,e**), consistent with the known ability of macrophages to support endothelial tip cell anastomosis (7).

Immediately after wound injury, the wound microenvironment becomes hypoxic (674). Hypoxia is a well-known regulator of both embryonic and adult stem cell biology (675). We therefore studied the effects of hypoxia on adult skin progenitors in culture and observed a 1.5-fold increase in 1° CFU-M yield in methylcellulose compared to normoxic conditions (**Figure 6-7f**), along with a trend toward enhanced cord-formation in MatrigelTM (**Figure 6-7g**). The latter result was accompanied by a significant increase in endothelial cell, but not macrophage formation under these conditions (**Figure 6-7h-j**).

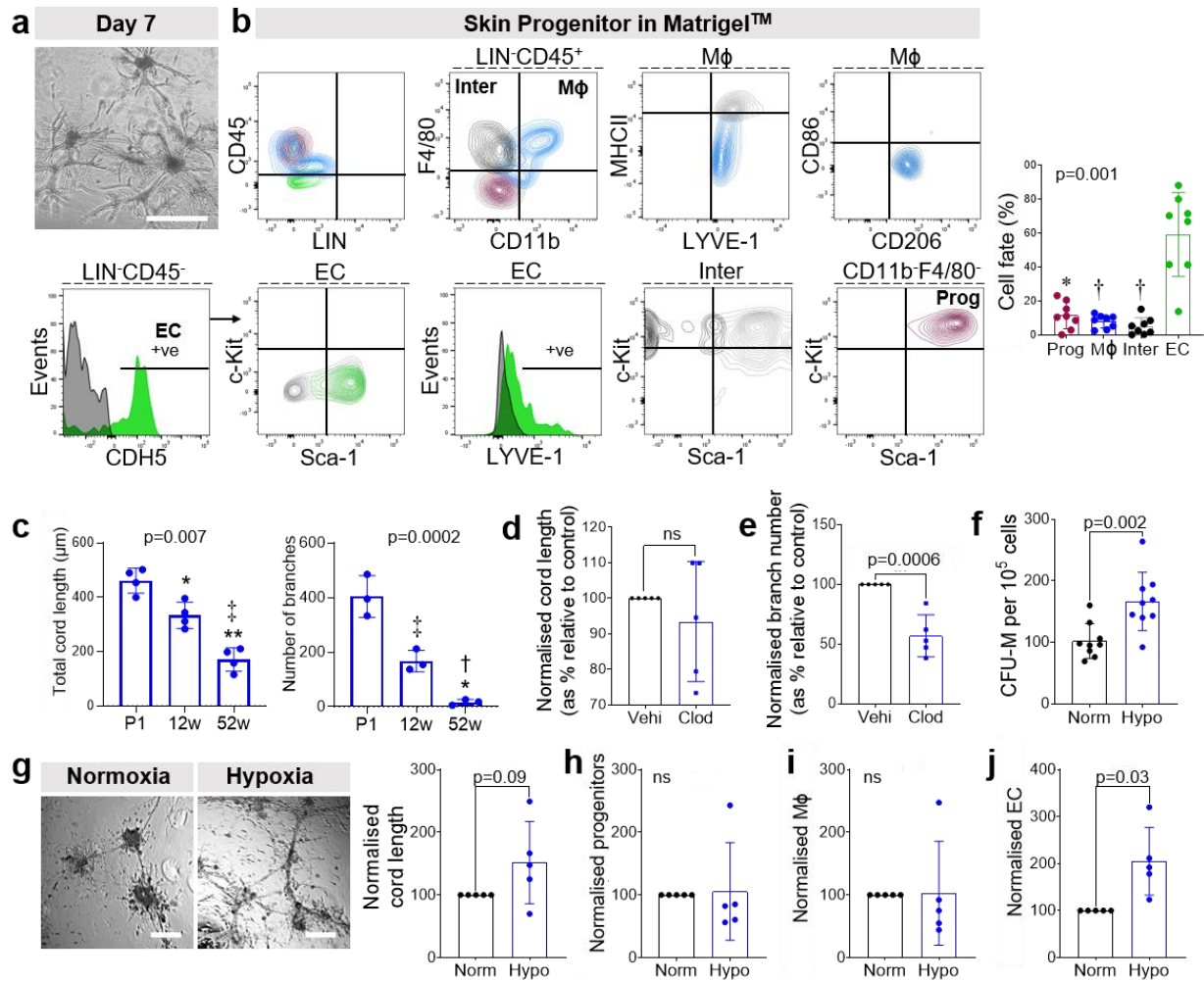


Figure 6-7. Skin CFU-M progenitors have endothelial and myeloid plasticity *in vitro*.

(a) Light microscopy images of cord networks formed after 7 d from 12 w C57BL/6 skin CFU-M progenitors plated in Matrigel™. Scale bar. 100 μ m.

(b) Flow cytometry plots and graph show fate of CFU-M derived progenitors in Matrigel™. N=8 mice. Maroon, Progenitors (Prog); Blue, Macrophages (M ϕ); Black, Intermediate population (Inter); Green, Endothelial cells (EC). Green histogram, Sample; Grey, FMO control. One-way ANOVA. Multiple comparison tests: * $p < 0.05$ and † $p < 0.01$ for comparison to EC.

(c) Total cord length and number of branches produced in Matrigel™ by skin progenitors from different aged C57BL/6 mice. N=3-4/age. One-way ANOVA. Multiple test comparisons: * $p < 0.05$, † $p < 0.01$ and ‡ $p < 0.001$ for comparison to P1; ** $p < 0.05$ for comparison to 12 w.

(d-e) Normalised data for **(d)** total cord length and **(e)** number of branch points after skin progenitors were cultured in Matrigel™ for 7 d in the presence of clodronate (Clod) relative to control vehicle (Vehi) liposomes. N=5 mice. Paired *t*-test. ns, not significant.

(f) Skin CFU-M yield under normoxic and hypoxic conditions. N=9 mice. Paired *t*-test.

(g) Light microscopy images of cord networks formed after 7 d from 12 w C57BL/6 skin CFU-M progenitors plated in MatrigelTM under normoxic or hypoxic conditions with summary of the total cord length normalised to normoxia. N=5 mice. Paired *t*-test. Scale bar, 100 μ m.

(h-j). Normalised number of **(h)** progenitor, **(i)** macrophage and **(j)** endothelial cells in MatrigelTM networks exposed to hypoxic relative to normoxic conditions. N=5 mice. Paired *t*-tests.

Data summarised as mean $\pm$ SD.

Matrigel™ In vitro N=3	
Surface marker	CD45⁺CD11b⁺F4/80⁺ Macrophages
CD206 ⁺	81.3±19.8%
CD86 ⁺	14.2±2.1%
CX ₃ CR1 ⁺	79.1±20.1%
MHCII ⁺	7.5±3.9%
LYVE-1 ⁺	91.2±8.1%
CSF1R ⁺	67.8±4.8%
	CD45⁺CD31⁺ and/or CD45⁺CDH5⁺ Endothelial cells
VEGFR2 ⁺	13.0±3.0%
LYVE-1 ⁺	25.1±3.3%
	CD45⁺CD11b⁺F4/80⁺ Intermediate
CD206 ⁺	2.1±2.5%
CD86 ⁺	1.5±3.4%
CX ₃ CR1 ⁺	71.3±10.9%
MHCII ⁺	1.8±0.6%
LYVE-1 ⁺	90.0±1.0%
CSF1R ⁺	88.5±5.8%

Table 6-1. Sub-lineage fate of skin EndoMac progenitors in Matrigel™.

Expression of select surface markers on macrophages, endothelial cells and the “intermediate” population produced by adult skin progenitors in the Matrigel™ cord-forming assay. N=3 mice. Results are summarised as mean±SD.

We next examined further whether YS-derived cells are involved in skin neovascularisation. We induced *ex vivo* vascular sprouting by culturing dissected pieces of mouse skin in MatrigelTM from both E9.5 TAM-induced adult *Cx3cr1*^{CreER-YFP} x *Rosa*^{tdTom} and E8.5 TAM-induced adult *Csf1r*^{MerCreMer} x *Rosa*^{mT/mG} reporter mice (**Figure 6-8a,f**). TdTom⁺ and GFP⁺ vascular-like outgrowths formed from these cultures over 7 d, respectively (**Figure 6-8b,g**). Flow cytometry also showed that by comparison to freshly digested skin, these skin sprouts had a much higher content of tdTom⁺ (**Figure 6-8d**) or GFP⁺ cells (**Figure 6-8i**), respectively. These YS-derived cells predominantly consisted of endothelial cells and macrophages, with <5-10% being progenitors (**Figure 6-8e, j**). Additionally, we found that the labelled macrophages in both experiments were mostly positive for CX₃CR1 and MHCII, with some expression of CD206 and LYVE-1 (**Table 6-2**). These skin sprouting assays therefore revealed expansion of YS-derived cells compared to steady state skin, although progenitors were sparser than expected. This was perhaps not surprising given that the previous results in this chapter and Chapters 4 and 5 show diminution of the progenitor pool after their differentiation into macrophages and endothelium.

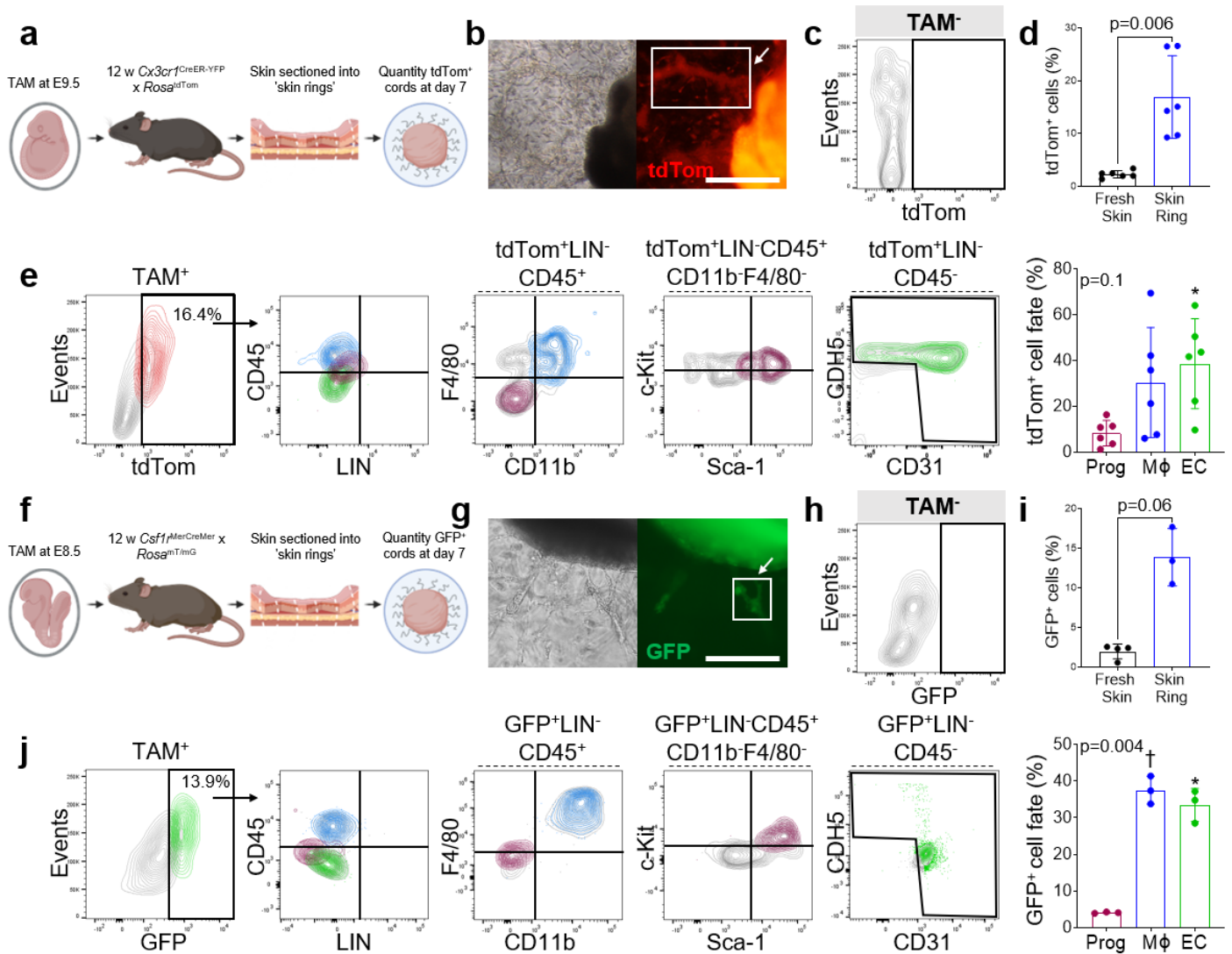


Figure 6-8. YS-derived cells are involved in skin neovascularisation *ex vivo*.

(a) Schematic for skin ring assay performed using skin from E9.5 TAM-induced adult *Cx3cr1*^{CreER-YFP} x *Rosa*^{tdTom} mice to label YS-derived cells as tdTom⁺.

(b) Light and fluorescence microscopy of skin ring shows tdTom⁺ sprouting. Scale bar, 100 µm.

(c) Flow cytometry of skin ring sprouting assay from 12 w *Cx3cr1*^{CreER-YFP} x *Rosa*^{tdTom} mouse without TAM-induction (i.e. negative control for tdTom expression).

(d) Graph compares % tdTom⁺ expression (not normalised to microglia) for day 7 skin ring assays with fresh digests of whole skin. N=4-6 mice/gp. Unpaired *t*-test.

(e) Flow cytometry of skin ring sprouting assay from 12 w *Cx3cr1*^{CreER-YFP} x *Rosa*^{tdTom} mouse with TAM-induction at E9.5. Graph shows lineage fate of YS-derived tdTom⁺ cells. N=6 mice. Maroon,

tdTom⁺ progenitors (Prog); Blue, tdTom⁺ macrophages (M ϕ); Green, tdTom⁺ endothelial cells (EC).

Repeated measures ANOVA. Multiple comparison test: * $p < 0.05$ vs prog.

(f) Schematic for skin ring assay performed using skin from E8.5 tamoxifen-induced adult *Csf1r*^{MerCreMer} x *Rosa*^{mT/mG} mice to label YS-derived cells GFP⁺.

(g) Light and fluorescence microscopy of skin ring shows GFP⁺ sprouting. Scale bar, 100 μ m.

(h) Flow cytometry of skin ring sprouting assay from 12 w *Csf1r*^{MerCreMer} x *Rosa*^{mT/mG} mouse without TAM-induction.

(i) Graph compares % GFP⁺ expression (not normalised to microglia) in day 7 skin ring assays with fresh digests of whole skin. N=3-4 mice/gp. Unpaired *t*-test.

(j) Flow cytometry of skin ring sprouting assay from 12 w *Csf1r*^{MerCreMer} x *Rosa*^{mT/mG} mouse with TAM-induction at E8.5. Graph shows lineage fate of YS-derived GFP⁺ cells. N=3 mice. Repeated measures ANOVA. Multiple comparison test: * $p < 0.05$ and † $p < 0.01$ vs prog.

Data summarised as mean $\pm$ SD.

Experimental Design	<i>Cx3cr1</i> ^{CreER-YFP} x <i>Rosa</i> ^{tdTom} Skin Rings N=6	<i>Csf1r</i> ^{MerCreMer} x <i>Rosa</i> ^{mT/mG} Skin Rings N=3
tdTom ⁺ or GFP ⁺	16.4±7.8%	13.9±3.6%
Surface marker	tdTom ⁺ or GFP ⁺ CD45 ⁺ CD11b ⁺ F4/80 ⁺ Macrophages	
CD206 ⁺	50.5±0.5%	74.8±17.2%
CX ₃ CR1 ⁺	88.7±5.7%	81.4±18.4%
MHCII ⁺	99.9±0.1%	100±0%
LYVE-1 ⁺	35.2±12.2%	59.2±37.1%
	tdTom ⁺ or GFP ⁺ CD45 ⁺ CD31 ⁺ and/or CD45 ⁺ CDH5 ⁺ Endothelial cells	
VEGFR2 ⁺	37.4±17.9%	54.3±28.4%
LYVE-1 ⁺	30.7±17.2%	25.9±7.4%

Table 6-2. Sub-lineage fate of YS-derived cells in skin ring assays.

Expression of select surface markers on YS-derived macrophages and endothelial cells formed by Day 7 in skin ring assays from E9.5 tamoxifen-induced *Cx3cr1*^{CreER-YFP} x *Rosa*^{tdTom} and E8.5 tamoxifen-induced *Csf1r*^{MerCreMer} x *Rosa*^{mT/mG} mice. Results shown for tdTom⁺ and GFP⁺ labelling are absolute expression (i.e. not normalised to brain microglia). Results are summarised as mean±SD.

6.3.5 Skin CFU-M progenitors accelerate wound closure *in vivo*

We next performed adoptive transfer experiments to examine the differentiation potential and vasculogenic capacity of progenitors and their effect on wound healing *in vivo*. Purity-checked progenitors were used from 1° skin CFU-M prepared from 8-12 w ubiquitous GFP donor mice. They were injected at a dose of 1.5×10^4 cells in 50 μ L Matrigel™ into the perimeter of one of the wounds created on the dorsum of 12 w C57BL/6 mice, with 50 μ L of Matrigel™ injected as cell-free control into the other wound (**Figure 6-9a**). Cell transfer resulted in accelerated wound closure (**Figure 6-9b, c**), however there was no significant difference seen in wound perfusion, as measured by laser doppler imaging (**Figure 6-9d**). Immunostaining identified GFP⁺ CD31⁺ and CD68⁺ cells in wounds after 10 d (**Figure 6-9e**). Differentiation of donor GFP⁺ progenitors into endothelial cells predominantly, but also macrophages and the “intermediate” subpopulation was further shown by flow cytometry analysis of wound digests, with <10% of engrafted GFP⁺ cells retaining the progenitor phenotype (**Figure 6-9f**). 86% of new GFP⁺ CD11b⁺F4/80⁺ macrophages were MHCII^{Lo}LYVE-1^{Hi}, with high co-expression of CD206, CX₃CR1 and CSF1R but much lower expression of CD86. The intermediate subpopulation also co-expressed CX₃CR1, CSF1R and LYVE-1, with low expression of CD86 and MHCII, while LYVE-1 was expressed on ~60% of GFP⁺ endothelium, suggesting lymphatic endothelial differentiation (**Figure 6-9f, Table 6-3**). These results establish that skin CFU-M progenitors also have at least endothelial-macrophage bipotential *in vivo*, seemingly with the ability to form lymphatic endothelium, although this requires more definitive investigation. Importantly, these properties are associated with favourable acceleration of wound closure.

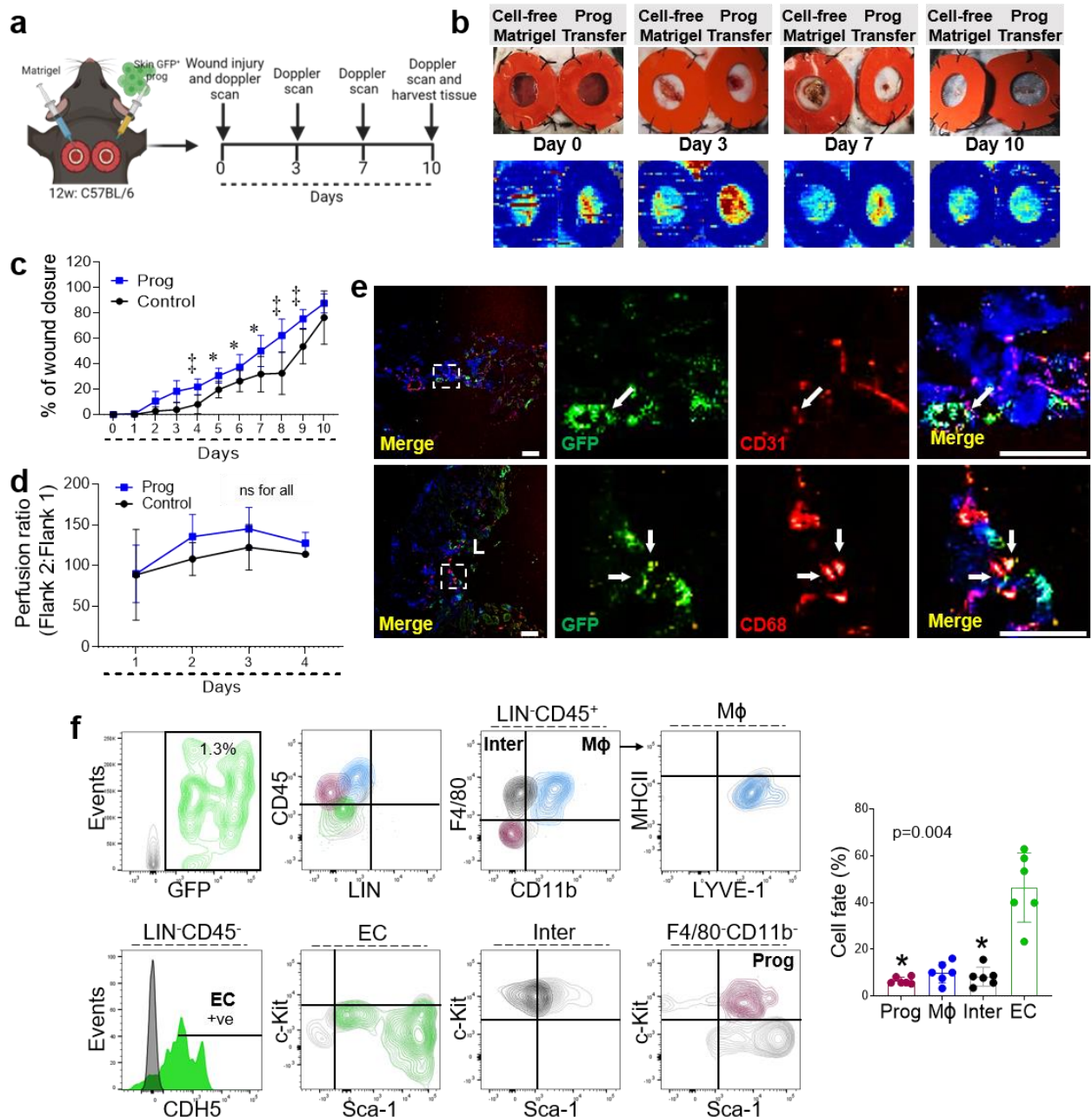


Figure 6-9. Skin CFU-M progenitors have endothelial-macrophage bipotency and facilitate wound closure *in vivo*.

(a) Schematic for adoptive transfer study.

(b) Photographs of wounds imaged on days 0, 3, 7 and 10 (above) and representative laser Doppler perfusion images of wounds at different time-points (below).

(c) Graph shows the percentage of wound closure achieved in the cell transfer and control wounds over time. N=6 mice. Mixed effects two-way ANOVA: $p < 0.0001$ for time; $p = 0.02$ for group; $p = 0.03$ for time x group. Multiple comparison: * $p < 0.05$ and ‡ $p < 0.001$ control vs prog.

(d) Summary of doppler perfusion results at early time-points. N=6 mice. Unpaired *t*-tests. ns, not significant. Mixed effects two-way ANOVA: *p*=ns for time; *p*=ns for group; *p*=ns for time x group. Multiple comparison, with no significant differences found.

(e) Confocal microscopy images show GFP⁺CD31⁺ and GFP⁺CD68⁺ cells in wounds at day 10. Scale bar, 50 μ m.

(f) Flow cytometry plots and graph show fate of GFP⁺ donor cells in recipient wounds. Maroon, Progenitors (Prog); Blue, Macrophages (M ϕ); Green, Endothelial cells (EC); Black, Intermediate population (Inter). Green histogram, Sample; Grey, FMO control. N=6 mice. Repeated measures ANOVA. Multiple comparison test: **p*<0.01 vs EC.

Data summarised as mean $\pm$ SD.

Wound Transfer <i>In vivo</i> N=3	
Surface marker	CD45 ⁺ CD11b ⁺ F4/80 ⁺ Macrophages
CD206 ⁺	96.8±2.4%
CD86 ⁺	12.1±6.9%
CX ₃ CR1 ⁺	89.4±8.0%
MHCII ⁺	14.7±2.8%
LYVE-1 ⁺	95.8±2.3%
CSF1R ⁺	92.5±4.1%
	CD45 ⁺ CD31 ⁺ and/or CD45 ⁺ CDH5 ⁺ Endothelial cells
VEGFR2 ⁺	96.9±3.4%
LYVE-1 ⁺	59.9±12.7%
	CD45 ⁺ CD11b ⁺ F4/80 ⁺ Intermediate
CD206 ⁺	1.1±3.9%
CD86 ⁺	0.9±2.3%
CX ₃ CR1 ⁺	81.4±9.8%
MHCII ⁺	7.6±2.2%
LYVE-1 ⁺	96.0±1.9%
CSF1R ⁺	94.6±5.9%

Table 6-3. Sub-lineage fate of skin EndoMac progenitors in wound healing model.

Expression of select surface markers on macrophages and endothelial cells produced by adult skin progenitors after injection into the wound healing model. N=3-6 mice. Results are summarised as mean±SD.

6.3.6 YS-derived skin EndoMac progenitors proliferate and expand in melanoma

TAM ϕ s and tumour vascularisation are important determinants of cancer growth (676). Having established that EndoMac progenitors enhance wound healing, we also studied their fate in a model of melanoma (670). First, we injected 5×10^5 B16-F10 murine melanoma cells (provided by Prof Claudine Bonder, University of South Australia) under the dorsal skin of 12 w *Flt3^{Cre}* x *Rosa^{mT/mG}* mice, which then received *i.p.* clodronate or vehicle control liposomes for 12 d (**Figure 6-10a**). Clodronate had the expected effect of severely depleting circulating monocytes (**Figure 6-10b**). Although this was associated with a non-significant trend for reduced GFP⁺ (Flt3-Cre⁺) CD68⁺ macrophages in sections of tumour, it also resulted in a compensatory increase in tdTom⁺ (Flt3-Cre⁻) macrophages, so that overall macrophage density was preserved (**Figure 6-10c**). It followed that clodronate had no significant effect on tumour size (**Figure 6-10d**) or weight (**Figure 6-10e**).

Given the lack of effect of monocyte depletion, we next studied the contribution of YS EMP-derived resident cells to melanoma growth. B16-F10 cells were injected into the skin of E8.5 TAM-induced 12 w *Csf1r^{MerCreMer}* x *Rosa^{mT/mG}* mice. Tumours were harvested at 7 d, which was the earliest time that they had formed a palpable lump, and at 14 d when they were more established (**Figure 6-11a**). Tumours contained an overall increased density of GFP⁺ EMP-derived cells at both time-points compared to normal skin (**Figure 6-11b, c**). Of these, progenitors only showed significant expansion at day 7 (1.8-fold compared to normal), while macrophages were significantly increased at both days 7 (4.3-fold) and 14 (2.8-fold), and endothelial cells only at day 14 (1.6-fold) (**Figure 6-11d-g**). After normalisation of GFP expression to microglia, ~92% of progenitors and ~12% of endothelial cells were determined to be YS EMP-derived in both early and late tumours. Meanwhile, the contribution of YS EMPs to macrophages increased from ~30% in control skin to 47% in day 7 tumours and then back down to 29% at day 14 (**Figure 6-6h-j**). Over 60% of GFP⁺ progenitors were in the proliferating S-phase of cycle in tumours at both time-points, compared to <20% in unaffected skin, as assessed 24 h after BrdU administration (**Figure 6-11k, l**). However, the absolute number of proliferating GFP⁺ progenitors was

highest at day 7 (**Figure 6-11m**). These data reveal for the first time that YS-derived resident cells expand during melanoma formation. Within the delayed time window studied here (days 7 and 14), this was most striking for macrophages. Although the tumour milieu appears to have a sustained pro-mitogenic effect on EndoMac progenitors, their expansion occurs in early-stage tumorigenesis, and it is possible that this would have been even more apparent had we studied the first few days after melanoma seeding.

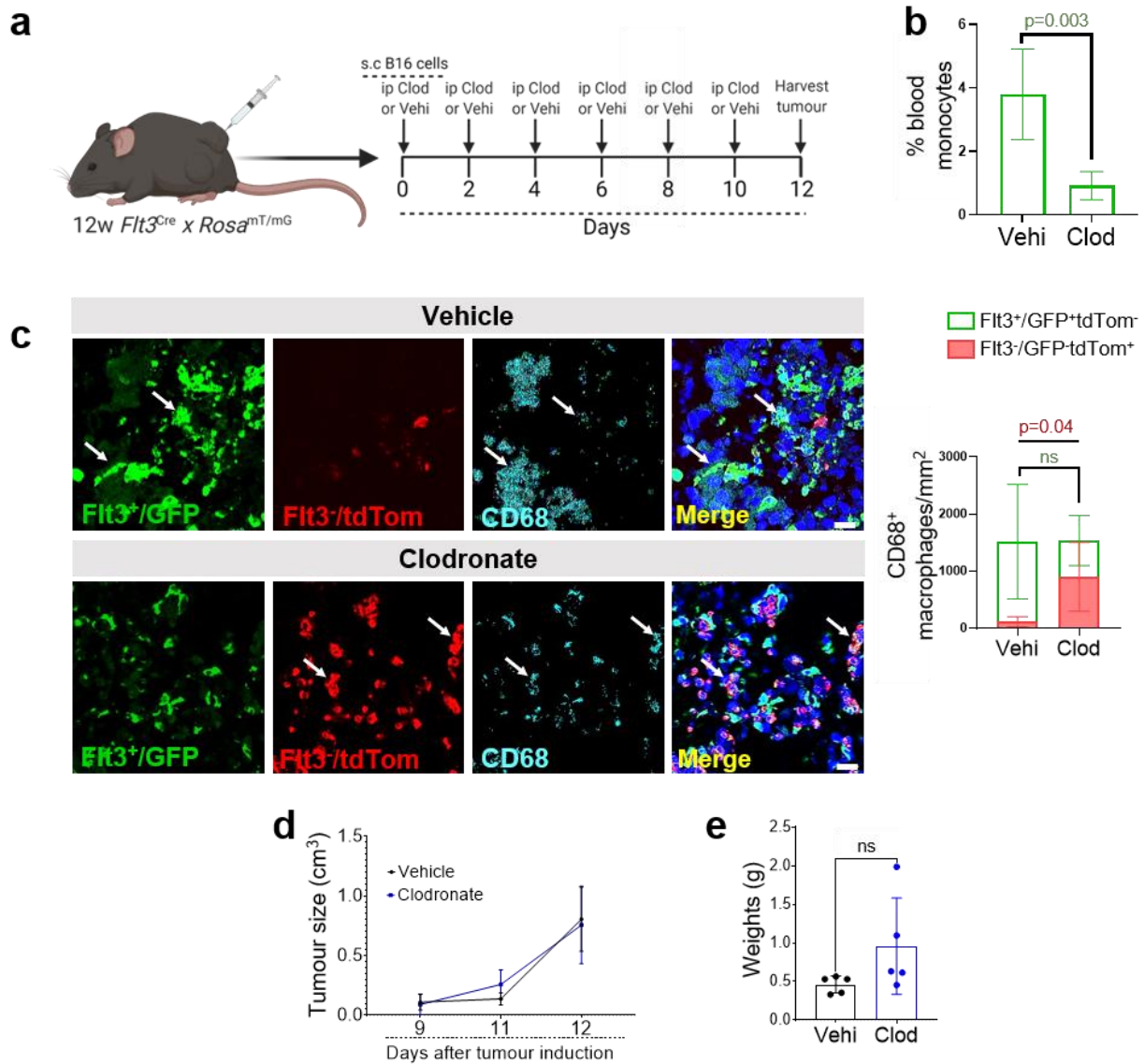


Figure 6-10. Resident macrophages support tumour cell growth with or without the presence of recruited monocytes/macrophages.

(a) Schematic showing experimental design of injected B16-F10 melanoma cells to induce tumours in 12 w *Flt3^{Cre} x Rosa^{mT/mG}* mice which also received either vehicle (Vehi) or clodronate (Clod) injections.

(b) Graph compares the two groups for *Flt3*-Cre⁺ (GFP⁺) blood monocytes after 12 d. N=5 mice/gp. Unpaired *t*-test. Note, *Flt3*-Cre⁻ monocytes were negligible and therefore are not shown.

(c) Immunostaining of day 12 tumours showing GFP⁺ and tdTom⁺ CD68⁺ cells in vehicle or clodronate treated *Flt3^{Cre} x Rosa^{mT/mG}* mice. N=5 mice/gp. Graph compares the number of *Flt3*-Cre⁻ and *Flt3*-Cre⁺ CD68⁺ macrophages per mm². 5 sections from each of 5 mice per group. Unpaired *t*-tests. Red p-value, *Flt3*-Cre⁻ comparison. Green p-value *Flt3*-Cre⁺. Scale bar, 20 μ m.

(d) Tumour size from *Flt3^{Cre}* x *Rosa^{mT/mG}* receiving vehicle (black) or clodronate (blue) injections. N=5 mice/gp. Mixed effects two-way ANOVA: $p < 0.0001$ for time; $p = \text{ns}$ for group; $p = \text{ns}$ for time x group. Multiple comparison, with no significant differences found.

(e) Weights of day 12 tumours harvested from *Flt3^{Cre}* x *Rosa^{mT/mG}* that had received vehicle or clodronate injection. N=5 mice/gp. Unpaired *t*-test. ns, not significant.

Data summarised as mean $\pm$ SD.

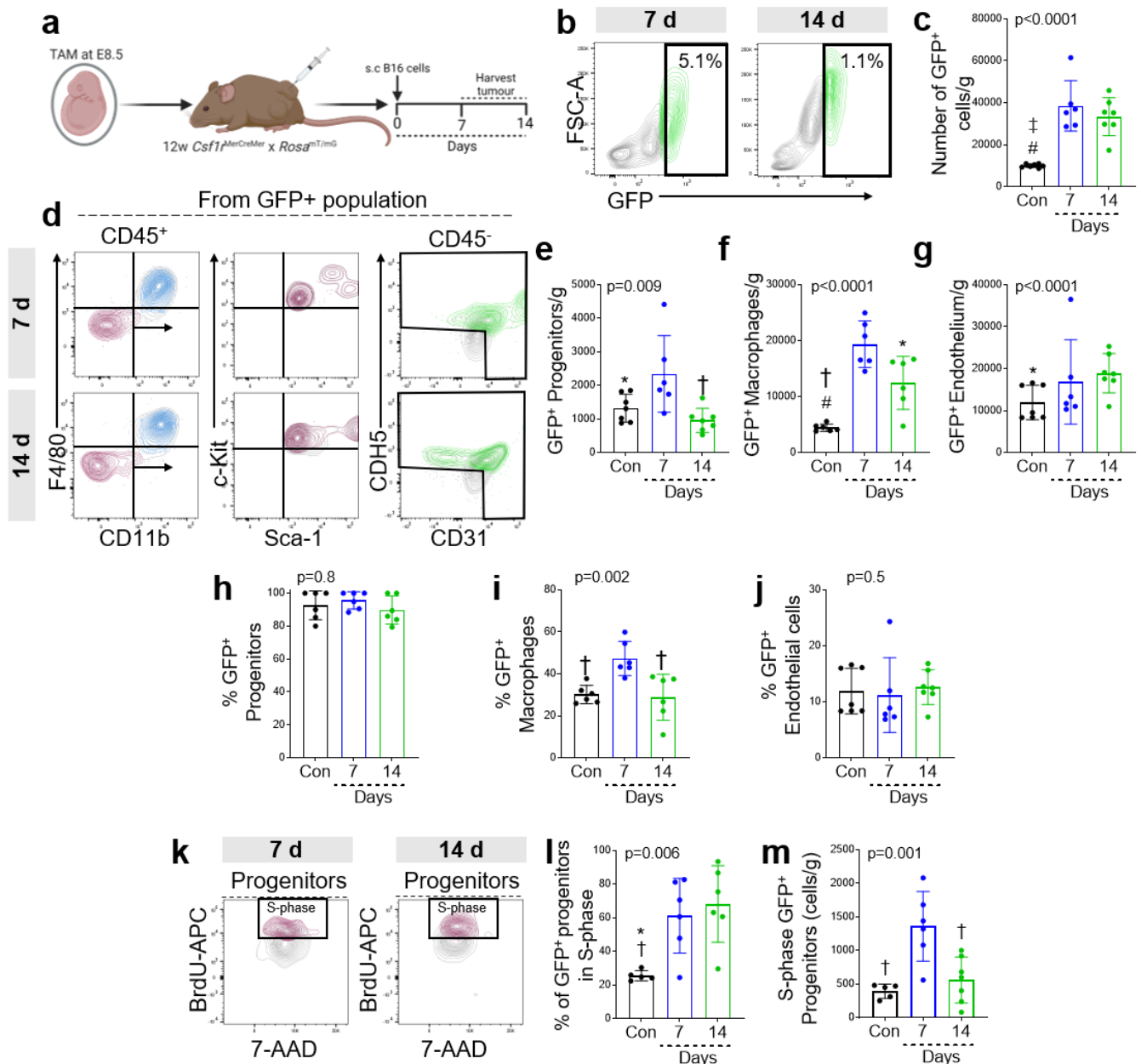


Figure 6-11. YS-derived cells, including EndoMac progenitors, expand during melanoma development.

(a) Schematic showing experimental design of injected B16-F10 melanoma cells to induce tumours in 12 w *Csf1^{MerCreMer}* x *Rosa^{mT/mG}* mice which had been induced with TAM at E8.5. Tumours were harvested at 7 d (early tumour) and 14 d (late tumour).

(b) GFP⁺ expression in tumours. Mean % from N=6-7 mice/gp.

(c) Number of YS-derived GFP⁺ cells in healthy (con) skin and early and late tumours. N=6-7 mice/gp. One-way ANOVA. Multiple comparison: ‡p<0.001 versus 14 d tumours, and #p<0.0001 versus 7 d.

(d) Flow cytometry of GFP⁺ progenitors (maroon), Mφ (blue) and EC (green) in 7 d and 14 d tumours.

(e-g) Graphical quantification of numbers of GFP⁺ **(e)** progenitors, **(f)** macrophages and **(g)** endothelial cells in healthy (con) skin and early and late tumours. N=6-7 mice/gp. **(e, f)** One-way ANOVA and **(g)** Kruskal-Wallis test. Multiple comparison test: * $p < 0.05$, † $p < 0.01$, and # $p < 0.0001$ versus 7 d tumours.

(h-j) Graphical quantification of GFP expression normalised to microglia for **(h)** progenitors, **(i)** macrophages and **(j)** endothelial cells in healthy (con) skin and early and late tumours. N=6-7 mice/gp.

(h, i) One-way ANOVA and **(j)** Kruskal-Wallis test. Multiple comparison test: † $p < 0.01$ versus 7 d tumours.

(k) Flow cytometry for BrdU uptake versus 7-aminoactinomycin (7-AAD) staining gated from GFP⁺ progenitors in tumours at days 7 and 14, showing cells in S-phase.

(l, m) Graphs show **(l)** percentage and **(m)** numbers per gram of GFP⁺ progenitors that were in S-phase of active cell cycle in healthy skin and early and late tumours. N=5-7 mice/gp. One-way ANOVA. Multiple comparison test: * $P < 0.05$ for 14 d tumour and † $p < 0.01$ for 7 d tumours.

Data summarised as mean±SD.

6.3.7 EndoMac progenitors promote melanoma growth and favour macrophage formation in tumours

The finding that EndoMac progenitors proliferate and expand in melanoma led us to also examine their effect on tumour growth. Progenitors were isolated from skin CFU-M grown from adult ubiquitous GFP mice. After confirming their purity, 1.5×10^4 GFP⁺ progenitors were co-injected with 5×10^5 B16-F10 cells into the skin of one flank of C57BL/6 recipients, with the other flank receiving cancer cells only (**Figure 6-12a**). The addition of progenitors resulted in increased tumour size and weight compared to control (**Figure 6-12b, c**). In each tumour, donor GFP⁺ cells formed CD31⁺ vascular-like structures, with clusters of GFP⁺CD68⁺ macrophages also found within the tumour (**Figure 6-12d**). Tumours were also dissociated and stained for flow cytometry. Although this identified GFP⁺ macrophages, progenitors and endothelial cells in decreasing order of prevalence, the dominant donor-derived population of cells expressed the “intermediate” surface phenotype described above (**Figure 6-12e**). Notably, the majority of GFP⁺ CD11b⁺F4/80⁺ macrophages were of the MHCII^{Lo}LYVE-1^{Hi} subtype and co-expressed CX₃CR1, CSF1R and CD206, with fewer CD86⁺ cells (**Figure 6-12e, Table 6-4**). Meanwhile, the intermediate population also expressed CX₃CR1, CSF1R and LYVE-1, with little expression of CD206, CD86 and MHCII. As observed in the wound transfer study, ~60% of progenitor-derived endothelial cells were positive for LYVE-1, suggesting lymphatic differentiation (**Table 6-4**).

These results suggest that in the tumour microenvironment, skin EndoMac progenitors favour macrophage differentiation, as distinct from their predilection for endothelial cells in the wound injury model. To investigate this further, we performed *in vitro* MatrigelTM assays, in which skin progenitors were cultured in the presence of supernatant from melanoma cells. Compared to the same concentration of control media, melanoma supernatant surprisingly resulted in almost complete abrogation of cord formation, so that day 7 cultures only contained discrete cell clumps with little sprouting (**Figure 6-13a**). Flow cytometry analysis of the content of these wells showed that this was not due to reductions in either cell viability (**Figure 6-13b**) or residual progenitor numbers (**Figure 6-13c**), but rather trends

toward less endothelial formation (**Figure 6-13d**) and more macrophages (**Figure 6-13e**), consistent with our *in vivo* results.

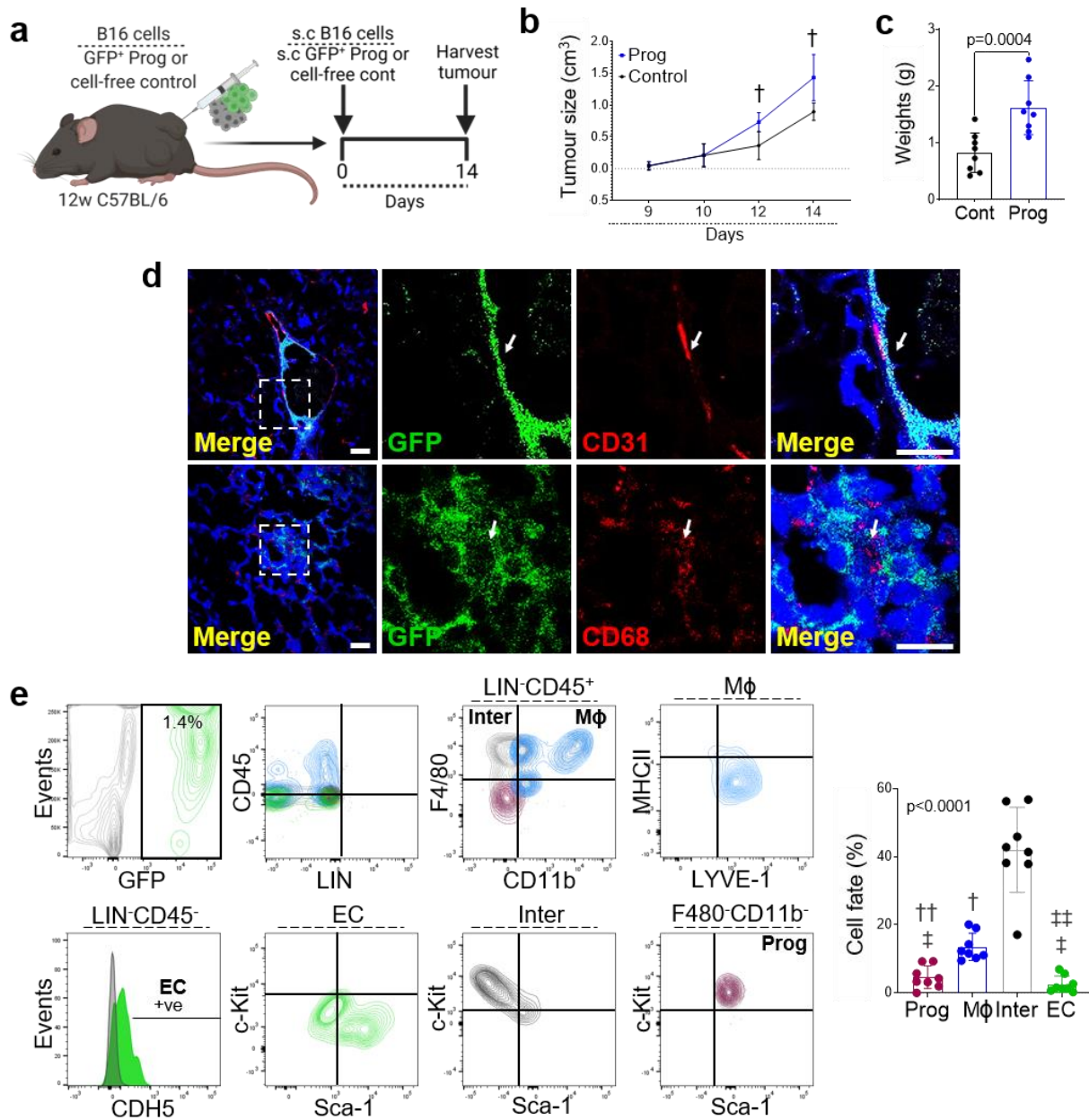


Figure 6-12. EndoMac progenitors promote melanoma growth.

(a) Schematic showing experimental design of co-injecting GFP⁺ skin progenitors (prog) or MatrigelTM cell-free control (cont) with B16-F10 melanoma cells to induce tumours in 12 w C57BL/6 mice.

(b) Tumour size from C57BL/6 mice receiving MatrigelTM only (Control) or skin progenitors (Prog). N=8 mice. Mixed effects two-way ANOVA: $p < 0.0001$ for time; $p = 0.001$ for group; $p = 0.0002$ for time x group. Multiple comparison test: $\dagger p < 0.01$ for prog vs control.

(c) Weights of tumours from the progenitor and control groups. N=8 mice. Unpaired *t*-test.

(d) Confocal microscopy showing GFP⁺ CD31⁺ and CD68⁺ co-staining in tumours after 14 d. Scale bar, 20 μ m.

(e) Flow cytometry and accompanying graph show the fate of GFP⁺ progenitors in melanoma tumours after 14 d. N=8 mice. Maroon, Progenitors (Prog); Blue, Macrophages (M ϕ); Green, Endothelial cells (EC); Black, Intermediate population (Inter). Green histogram, Sample; Grey, FMO control. Repeated measures ANOVA. Multiple comparison test: †p<0.01 and ‡ p<0.001 versus Inter; ††p<0.01 and ‡‡ p<0.001 versus M ϕ .

Data summarised as mean $\pm$ SD.

Melanoma Transfer <i>In vivo</i> N=6	
Surface marker	CD45⁺CD11b⁺F4/80⁺ Macrophages
CD206 ⁺	74.9±15.5%
CD86 ⁺	21.2±10.9%
CX ₃ CR1 ⁺	99.4±0.9%
MHCII ⁺	21.0±4.2%
LYVE-1 ⁺	89.1±3.5%
CSF1R ⁺	66.2±18.1%
CD45⁺CD31⁺ and/or CD45⁺CDH5⁺ Endothelial cells	
VEGFR2 ⁺	96.9±3.4%
LYVE-1 ⁺	58.5±12.6%
CD45⁺CD11b⁺F4/80⁺ Intermediate	
CD206 ⁺	1.7±3.2%
CD86 ⁺	1.9±3.1%
CX ₃ CR1 ⁺	62.5±9.9%
MHCII ⁺	3.5±4.3%
LYVE-1 ⁺	88.9±4.1%
CSF1R ⁺	95.6±3.9%

Table 6-4. Surface marker expression on macrophage and endothelial populations produced by EndoMac progenitors in the melanoma model.

Shown are the mean±SD values for percent expression of different surface markers expressed by donor-derived macrophages (defined as LIN⁻CD45⁺CD11b⁺F4/80⁺), endothelial cells (LIN⁻CD45⁺CD31⁺ and/or CDH5⁺) or intermediate cells (CD45⁺CD11b⁺F4/80⁺) 14 d after co-injection of GFP⁺ EndoMac progenitors with melanoma cells.

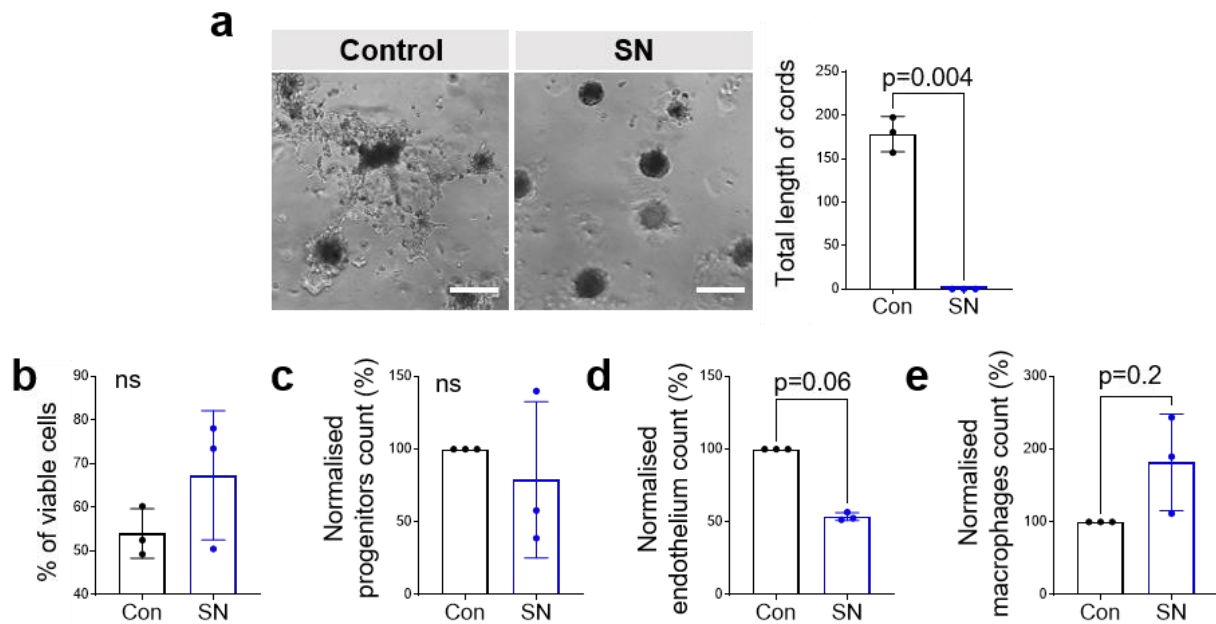


Figure 6-13. Melanoma supernatant eliminates angiogenic cord formation from skin EndoMac progenitors in Matrigel™.

(a) Light microscopy images of day 7 Matrigel™ cultures in the presence of 50% control media (control) and 50% melanoma supernatant (SN). Graph shows total cord length. N=3 mice. Paired *t*-test. Scale bar, 100 μ m.

(b-e) Graphs show comparisons by flow cytometry for **(b)** viable cells and numbers of **(c)** progenitors, **(d)** endothelium and **(e)** macrophages expressed as % relative to control media. N=3 mice. Paired *t*-tests. ns, not significant.

Data summarised as mean $\pm$ SD.

6.3.8 Elucidation of the heterogeneity of macrophages derived from YS EMPs and EndoMac progenitors in wound healing and melanoma

Finally, we also performed a series of analyses around macrophage heterogeneity in the different wound healing and melanoma experiments, using the classification system described by Tamoutounour *et al.* (404). The gating strategy for this is shown in **Figure 6-2**. Briefly, CD11b⁺CD24^{Lo/-} cells were used to gate Ly6C⁺ and/or CD64⁺ cells. These cells were further classified based on a Ly6C versus MHCII plot into Ly6C^{Hi}MHCII⁻ (P1), Ly6C^{Hi-to-Int}MHCII⁺ (P2), and Ly6C^{Lo}MHCII^{Neg} (P4) subpopulations. The remaining Ly6C⁻MHCII⁺ compartment was then classified into two further subsets: MerTK⁻ (P3) and MerTK⁺ (P5). Finally, dendritic cells (DCs) were defined as CD11b⁺CD24⁺CD11c⁺ (**Figure 6-2**). Among these subtypes, Tamoutounour *et al.* concluded that the P1 and P2 cells are more pro-inflammatory, P4 and P5 more anti-inflammatory macrophages and P3 intermediate between the two (404). In E8.5 TAM-induced 12 w *Csf1r*^{MerCreMer} x *Rosa*^{mT/mG} steady state skin, we found that the most abundant subpopulations overall were DC, P4, P3 and P1 in decreasing order, with very few P2 and P5 cells (**Figure 6-14a**). Among GFP⁺ YS EMP-derived cells, the order of prevalence from highest to lowest was P4, DC, P1 and P3 (**Figure 6-14b**). As such, YS EMPs made the biggest contribution to P1 and P4 cells (**Figure 6-14c**), which aligned well with our earlier data for *Flt3*-Cre⁻ macrophages in the *Flt3*^{Cre} x *Rosa*^{mT/mG} mice (**Figure 6-2g**).

After wound injury, the overall number of DCs expanded by day 1 and fell back towards the steady state level by day 7 (**Figure 6-14d**). This pattern was accentuated within the YS-derived pool (6.5x at 1 d, 8.6x at 3 d and 2.9x at 7 d compared to steady state), which contributed to ~40% of DCs throughout wound healing (**Figure 6-14e, f**). Meanwhile, the overall population of P1 cells progressively increased, peaking at 7 d (4.6x compared to steady state) (**Figure 6-14g**). This was supported by an early and sustained contribution from YS EMPs (**Figure 6-14h, i**). Remarkably, the P2 compartment increased by more than forty-fold at 3 d after wound injury from very low numbers at steady state and at 1 d (**Figure 6-14j**), with minimal contribution from YS (**Figure 6-14k, l**). Neither the total nor GFP⁺ P3 populations changed significantly as a result of wound injury (**Figure 6-14m-o**). P4 macrophages

showed elevated expansion from day 1 to day 7, with the same trend seen in EMP-derived macrophages (**Figures 6-14p-r**). Finally, the P5 population remained of negligible size throughout (**Figures 6-14s-u**). Therefore tissue-resident EMP-derived cells make the biggest contribution to DCs (days 1-3), pro-inflammatory P1 macrophages (days 1-7) and anti-inflammatory P4 macrophages (day 7) during wound healing. In keeping with this, we also found that EndoMac progenitors mostly formed P1, P4 macrophages and DCs (decreasing order) in both MatrigelTM *in vitro* (**Figure 6-14v, w**) and at day 10 after injection into wounds (**Figure 6-14x, y**).

In melanomas growing in E8.5 TAM-induced 12 w *Csf1r*^{MerCreMer} $\times$ *Rosa*^{mT/mG} mice, we found that the total and GFP⁺ DC populations were both expanded by approximately four-fold in day 7 tumours compared to normal skin, before becoming relatively depleted at day 14 (**Figure 6-15a-c**). P1 cells increased to a similar extent at both time-points (**Figure 6-15d**), with a bigger contribution from the EMP-derived pool at day 7 (**Figure 6-15e, f**). The P2 and P3 compartments remained relatively small and unchanged overall, despite a significant increase in EMP-derived P2 macrophages (**Figure 6-15g-l**). The anti-inflammatory P4 subset expanded progressively with melanoma growth, becoming the largest population at day 14 (**Figure 6-15m**), with robust input from GFP⁺ EMP-derived cells (**Figure 6-15n, o**). A similar kinetic profile was observed for P5 macrophages, albeit on a much smaller scale (**Figure 6-15p-r**). Consistent with these findings, injection of GFP⁺ EndoMac progenitor cells into the melanoma model predominantly formed macrophages of the P1 and P4 subtypes at day 14 when tumours were harvested (**Figure 6-15s, t**).

The immunophenotypic heterogeneity of skin macrophages therefore depends on pathophysiological context, as well as ontogeny. The sub-lineage fate of CSF1R⁺ YS EMPs predominantly consists of DCs, P1 and P4 cells to varying degrees for different stages of wound healing and melanoma formation. Importantly, this overlaps well with the macrophage subtypes that are produced by EndoMac progenitors under the same conditions. This provides another layer of corroborative evidence that these ancestral cells contribute to the local renewal and expansion of EMP-derived macrophages in both healthy and diseased adult skin.

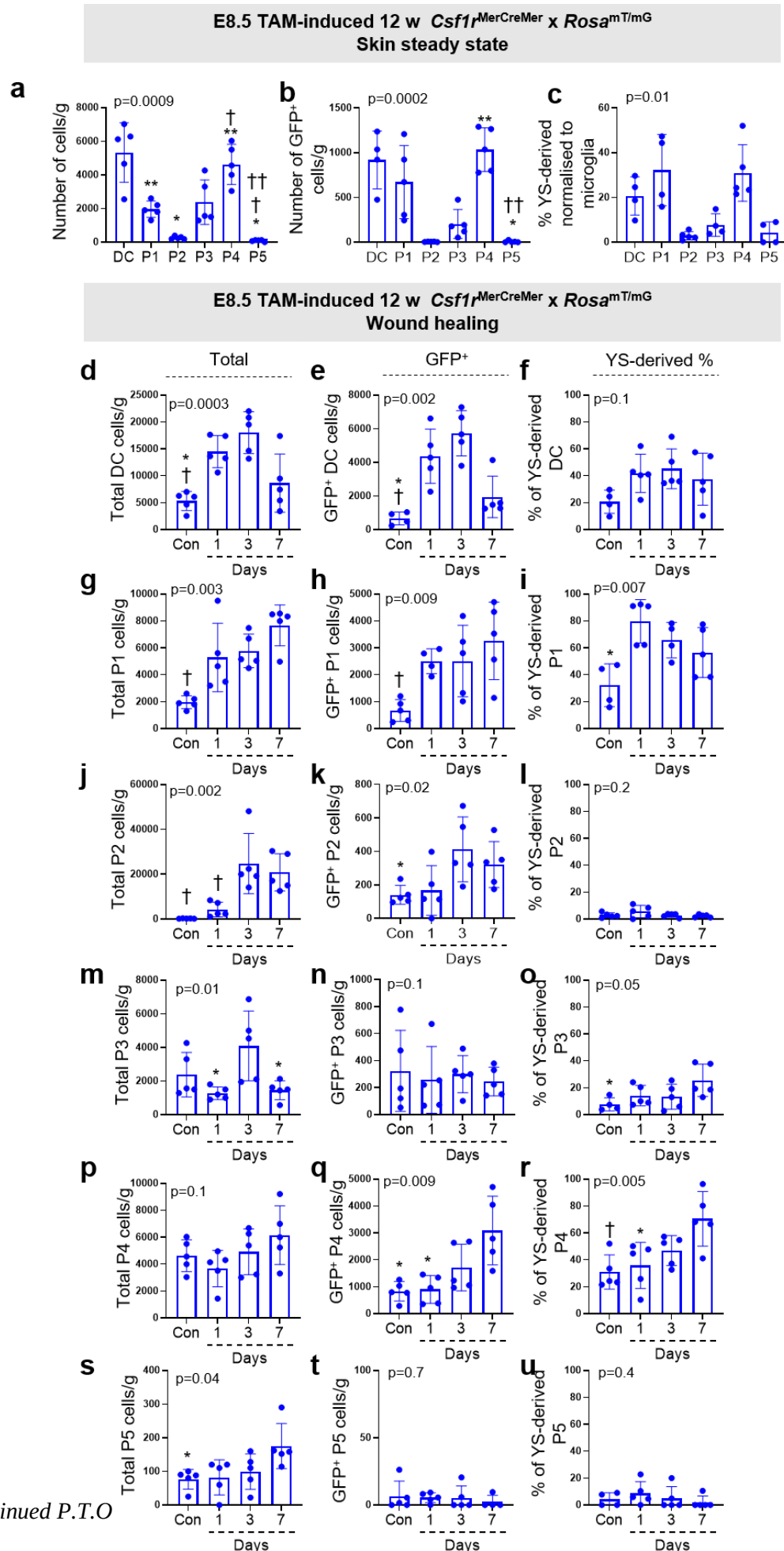


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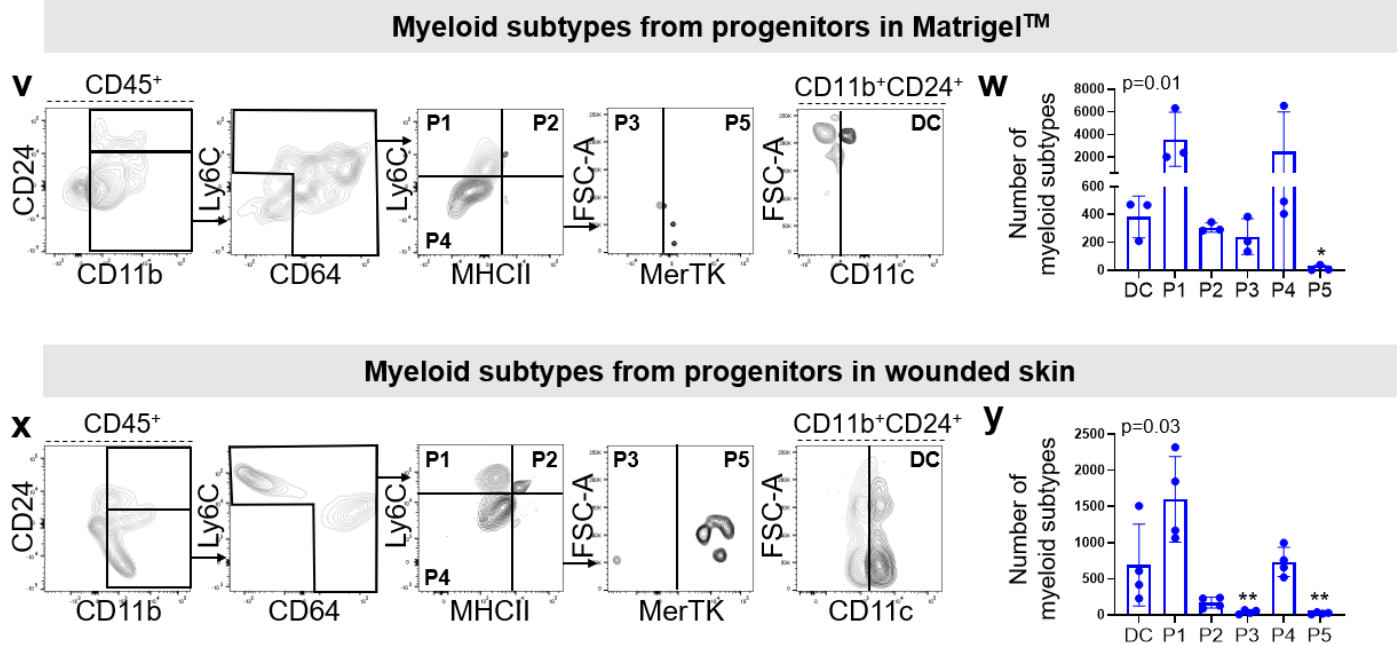


Figure 6-14. Myeloid subtypes formed in healing wounds.

(a-c) Graphical quantification of **(a)** absolute number, **(b)** number of YS EMP-derived (GFP⁺) and **(c)** normalised percentage (%) of YS EMP-derived myeloid subtypes in steady state skin from E8.5 TAM-induced 12 w *Csf1r*^{MerCreMer} x *Rosa*^{mT/mG} mice. N=5 mice/gp. Repeated measures ANOVA. Multiple comparison test: **p*<0.05 versus DC; ***p*<0.05 versus P2, †*p*<0.01 versus P1; ††*p*<0.01 versus P4.

(d-f) Graphs compare the **(d)** absolute number, **(e)** number of YS EMP-derived (GFP⁺) and **(f)** normalised percentage of YS EMP-derived cells between steady state (con) skin and wounds at different time-points for DCs. N=5 mice/gp. **(d, f)** One-way ANOVA and **(e)** Kruskal-Wallis test. Multiple comparison test: **p*<0.05 for comparison to day 1 and †*p*<0.01 for comparison to day 3.

(g-i) Graphs compare the **(g)** absolute number, **(h)** number of GFP⁺ and **(i)** normalised percentage of YS EMP-derived cells between steady state (con) skin and wounds at different time-points for P1 cells. N=5 mice/gp. **(h)** One-way ANOVA and **(g, i)** Kruskal-Wallis test. Multiple comparison test: **p*<0.05 for comparison to day 1 and †*p*<0.01 for comparison to day 7.

(j-l) Graphs compare the **(j)** absolute number, **(k)** number of GFP⁺ and **(l)** normalised percentage of YS EMP-derived cells between steady state (con) skin and wounds at different time-points for P2 cells. N=5 mice/gp. **(k, l)** One-way ANOVA and **(j)** Kruskal-Wallis test. Multiple comparison test: **p*<0.05 for comparison to day 3 and †*p*<0.01 for comparison to day 3 and day 7.

(m-o) Graphs compare the **(m)** absolute number, **(n)** number of GFP⁺ and **(o)** normalised percentage of YS EMP-derived cells between steady state (con) skin and wounds at different time-points for P3 cells. N=5 mice/gp. **(m, n, o)** One-way ANOVA. Multiple comparison test: In **(m)** *p<0.05 for comparison to day 3; in **(o)** *p<0.05 for comparison to day 7.

(p-r) Graphs compare the **(p)** absolute number, **(q)** number of GFP⁺ and **(r)** normalised percentage of YS EMP-derived cells between steady state (con) skin and wounds at different time-points for P4 cells. N=5 mice/gp. **(p, r)** One-way ANOVA and **(q)** Kruskal-Wallis. Multiple comparison test: *p<0.05 and †p<0.01 for comparison to day 7.

(s-u) Graphs compare the **(s)** absolute number, **(t)** number of GFP⁺ and **(u)** normalised percentage of YS EMP-derived cells between steady state (con) skin and wounds at different time-points for P5 cells. N=5 mice/gp. **(s, t, u)** Kruskal-Wallis test. Multiple comparison test: *p<0.05 for comparison to day 7.

(v-w) Myeloid subtypes produced by skin CFU-M progenitors after 7 d of culture in Matrigel™. N=3 mice. Repeated measures ANOVA. Multiple comparison test: *p<0.05 versus P1.

(x-y) Myeloid subtypes produced by skin CFU-M progenitors after adoptive transfer into wounds *in vivo*. N=4 mice. Repeated measures ANOVA. Multiple comparison test: *p<0.05 versus P1; **p<0.05 versus P4.

Data summarised as mean±SD.

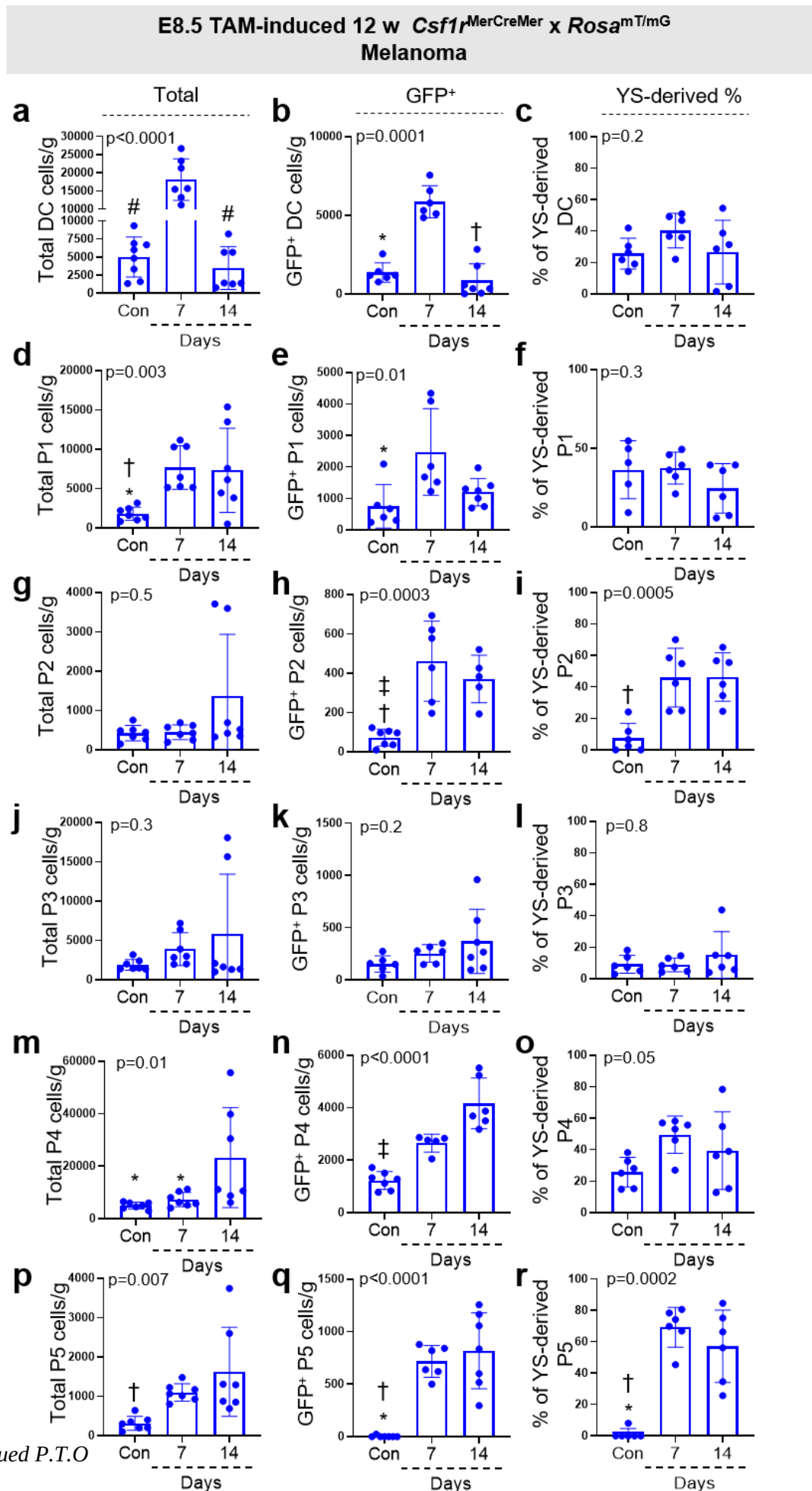


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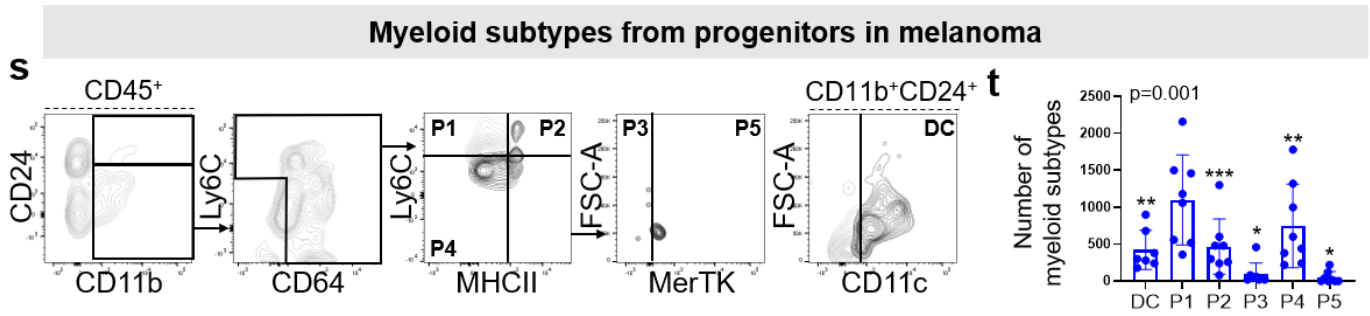


Figure 6-15. Myeloid subtypes in early and late tumours.

(a-c) Graphs compare the **(a)** absolute number, **(b)** number of YS EMP-derived (GFP⁺) and **(c)** normalised percentage of YS EMP-derived cells between steady state (con) skin and tumours at different time-points for DCs. N=7-8 mice/gp. **(a, c)** One-way ANOVA and **(b)** Kruskal-Wallis test. Multiple comparison test: * $p < 0.05$, † $p < 0.01$ and # $p < 0.0001$ for comparison to day 7.

(d-f) Graphs compare the **(d)** absolute number, **(e)** number of YS EMP-derived (GFP⁺) and **(f)** normalised percentage of YS EMP-derived cells between steady state (con) skin and tumours at different time-points for P1. N=7-8 mice/gp. **(f)** One-way ANOVA and **(d, e)** Kruskal-Wallis. Multiple comparison test: **(d)** * $p < 0.05$ and † $p < 0.01$ for comparison to day 7 and 14; **(e)** * $p < 0.05$ for test comparison to day 7.

(g-i) Graphs compare the **(g)** absolute number, **(h)** number of YS EMP-derived (GFP⁺) and **(i)** normalised percentage of YS EMP-derived cells between steady state (con) skin and tumours at different time-points for P2. N=7-8 mice/gp. **(h, i)** One-way ANOVA and **(g)** Kruskal-Wallis test. Multiple comparison test: † $p < 0.01$ for comparison to day 14 and ‡ $p < 0.001$ for comparison to day 7.

(j-l) Graphs compare the **(j)** absolute number, **(k)** number of YS EMP-derived (GFP⁺) and **(l)** normalised percentage of YS EMP-derived cells between steady state (con) skin and tumours at different time-points for P3. N=7-8 mice/gp. **(k)** One-way ANOVA and **(j, l)** Kruskal-Wallis test. Multiple comparison test: not significant for all.

(m-o) Graphs compare the **(m)** absolute number, **(n)** number of YS EMP-derived (GFP⁺) and **(o)** normalised percentage of YS EMP-derived cells between steady state (con) skin and tumours at different time-points for P4. N=7-8 mice/gp. **(m)** One-way ANOVA and **(n, o)** Kruskal-Wallis test. Multiple comparison test: * $p < 0.05$, † $p < 0.01$ and ‡ $p < 0.001$ for comparison to day 14.

(p-r) Graphs compare the **(p)** absolute number, **(q)** number of YS EMP-derived (GFP⁺) and **(r)** normalised percentage of YS EMP-derived cells between steady state (con) skin and tumours at different time-points for P5. N=7-8 mice/gp. **(p)** One-way ANOVA and **(q, r)** Kruskal-Wallis. Multiple comparison test: *p<0.05 for comparison to day 7; †p<0.01 for comparison to day 14.

(s-t) Myeloid subtypes produced by skin CFU-Ms after 14 d in tumours. **(t)** Graphical quantification. N=8 mice. Repeated measures ANOVA. Multiple comparison test: *p<0.05 versus P1; **p<0.05 versus P5; ***p<0.05 versus P3.

6.4 Discussion

Accurate regulation of events in wound healing is essential; failure to initiate key steps at the right time delays healing leading to development of chronic wounds, while aberrant initiation of wound healing processes may produce cell behaviours that promote development of skin cancers (413). Although knowledge of both skin wound healing and tumorigenesis is well established, treatments have not advanced sufficiently in recent years to address the considerable burden they cause to individual patients and the healthcare system (677, 678). Because of the biological parallels between them, lessons learned from mechanistic research into wound healing may provide us with insights into the mechanisms that are involved in skin tumorigenesis and *vice versa*. Having already discovered bipotent YS-derived EndoMac progenitors in postnatal mouse aorta, heart, lung and muscle, we embarked on this study to investigate for the existence of similar progenitors in skin, with the expectation that this resident ancestral population would be involved in both wound healing and melanoma.

Our findings show that skin does in fact contain progenitors, that share the same early CX₃CR1⁺ and CSF1R⁺ YS ontogeny and similar phenotypic, clonogenic, self-renewal and differentiation properties as EndoMac progenitors in aortic adventitia and muscle. Using a combination of methylcellulose-based CFU assays and flow cytometry, we have established that these progenitors are seeded in skin during embryonic development, as evident by their abundant presence at E15.5 and immediately after birth. Thereafter, they are maintained independently of definitive haematopoiesis. Despite high proliferative activity even in steady state, their prevalence diminishes with postnatal ageing and this occurs in parallel with declining clonogenic and self-renewal capacity. Intriguingly, between birth and adulthood their decreasing numbers appear to be replaced by the accumulation of differentiated YS-derived macrophages and endothelial cells. Our results indicate that this may be explained, at least in part, by the differentiation of progenitors into both of these daughter lineages postnatally. From a pathophysiological perspective, we show that progenitors locally expand within the first day after wound injury and this milieu predisposes their transformation into endothelial cells, which is associated with favourable acceleration of wound closure. In contrast, in melanoma their proliferative expansion

is accompanied by predilection for producing macrophages and in this setting, they maladaptively promote tumour growth.

As in other tissues (121, 164, 663), the source of macrophage renewal and expansion in skin has been a topic of ongoing debate. Epidermal Langerhans cells, which are a form of non-lymphoid DCs, were among the first tissue-resident myeloid cells in postnatal skin found to originate embryonically (93, 101, 121, 122, 175, 663). Previous studies have variably shown that Langerhans cells arise from early and late EMPs, as well as foetal liver HSCs, leaving the relative contributions of these sources uncertain. For example, using inducible c-Kit-Cre lineage-tracing, Sheng *et al.* reported that foetal liver HSCs contribute to ~80% of postnatal Langerhans cells (175), whereas Gomez-Perdiguero *et al.* determined that 30% are from early YS EMPs. Dermal macrophages are particularly heterogeneous and their different subtypes have also been linked to diverse ontogeny, based on results from *Flt3l*^{-/-} and *Ccr2*^{-/-} knockout-mice in steady state and inflamed skin (404). Notably, by using E9.5 TAM-induced *Cx3cr1*^{creERT2} x *Rosa*^{YFP} mice, Kolter *et al.* found that only ~7% of dermal macrophages originate from YS primitive macrophage precursors or early EMPs, while ~23.5% of dermal macrophages originate from late EMPs (165). Although we did not distinguish between dermis and epidermis in the current study, our own fate-mapping results indicate that ~35% of adult skin macrophages, defined as CD45⁺CD11b⁺F4/80⁺, arise independently of definitive Flt3⁺ haematopoiesis, and almost all of these can be accounted for from E8.5 CSF1R⁺ and/or E9.5 CX₃CR1⁺ origins. To date, the expert consensus has interpreted that these embryonically derived macrophages are maintained postnatally through a remarkable and sustained capacity for local proliferation and self-renewal, without losing their functional and differentiated status (177). However, another explanation that has been put forth recently for the durability of embryonically seeded skin macrophages, is that they remain in a stable, quiescent state, with only a 2% rate of turnover in the first two months of postnatal life, compared to 50% turnover for the rest of the dermal macrophage pool (165).

Among different clonal tracing studies, Ghigo *et al.* used multi-colour lineage mapping to show that expansion of epidermal Langerhans cells does not occur stochastically, but rather from local, clonal

proliferative units composed of dividing parent cells and their terminally differentiated daughter cells (180). Other groups have also reported clonal increases in macrophages (679) and fibroblasts (680) after wound injury, along with unpublished results from a thesis, which interestingly showed numerous clonal patches of unidentified F4/80⁻ cells (681). In keeping with this, we have identified a macrophage-specific clonogenic capacity of skin, that cannot be attributed to clonal division of monocytes or macrophages. This was demonstrated by our own FACS isolation experiments here, as well as previous research that shows that these mature myeloid cells do not produce CFU-M unless genetically modified to do so (178). Rather, our results show that these colonies are formed from a highly proliferative, undifferentiated progenitor population, that originates from E8.5 YS EMPs and expresses a LIN⁻CD45^{+/-Lo}CD11b⁻F4/80⁻Sca-1⁺c-Kit⁺CX₃CR1⁺CSF1R⁺ surface marker phenotype, that distinguishes it from HSCs and other lineage-committed myeloid and endothelial progenitors (6, 229, 230, 246, 262, 263, 631, 633, 660, 682). Importantly, these CFU-M forming progenitors can renew themselves clonally at the single cell level. However, in both *in vitro* and adoptive transfer studies, we observed that relatively few undifferentiated progenitors remain after undergoing transformation into their macrophage and endothelial progeny. This suggests that they undergo asymmetric division during differentiation and maturation and have only restricted ability to self-renew.

Interestingly, this Chapter found that the microenvironment in which skin EndoMac progenitors were exposed to affected their differentiation outcome. After culture in MatrigelTM and transfer into wounds, progenitors mostly transformed into endothelial cells. Further analyses revealed that hypoxia, which is present in early wounds, stimulated their clonogenic, proliferative and endothelial-forming, vasculogenic capacity (674). In keeping with this, wound injury induced a 2-fold expansion in YS-derived progenitors within the first 24 h, while accumulation of YS-derived macrophages and endothelial cells occurred later. In fact, TAM-induction of E8.5 CSF1R⁺ YS EMPs revealed that ~35% of macrophages and ~16% of endothelial cells in steady state adult skin are EMP-derived, and this increases to ~60% and ~50%, respectively, after 14 d of wound healing. This aligns with previous studies that have demonstrated the contributions of resident macrophages (424, 639, 683, 684) and endothelial progenitors (644-646) to the wound healing process. Importantly, progenitor cells

accelerated wound closure, suggesting that they play an adaptive role in wound repair. It follows that depletion of EndoMac progenitors, as found here to be the case in *Cx3cr1*^{GFP/GFP} skin, would be expected to delay wound closure. Indeed, these CX₃CR1-deficient mice have been shown by other groups to have a decreased rate of wound closure compared to their heterozygous *Cx3cr1*^{GFP/+} littermates, which have a single functioning *Cx3cr1* allele (685-687). Recently, we have found the same in association with a reduction in the number of progenitor cells (**Appendix 13**), macrophages and mature endothelial-lined capillaries in these *Cx3cr1*^{GFP/GFP} wounds (data not shown).

In contrast to wound injury, the melanoma microenvironment primed the progenitors to form macrophages *in vivo*, with consistent results shown for the *in vitro* MatrigelTM assay in the presence of conditioned media from melanoma cells. As well as favouring the macrophage phenotype, transferred donor progenitors accelerated tumour initiation and growth; however, we are yet to study whether metastasis or the vascularisation of these tumours were affected. Fate-mapping showed expansion of both YS EMP-derived progenitors and macrophages by 1.8- and 4.3-fold, respectively, 7 days after melanoma seeding compared to unaffected skin, with both populations then declining in number by 14 days. By comparison, the expansion of EMP-derived endothelial cells was more modest but their numbers remained constant between days 7 and 14. Based on the kinetics of EndoMac progenitor proliferation in other models, we speculate that the magnitude of progenitor expansion may be greater at earlier time-points during melanoma development, which could not be assessed reliably here due to methodological limitations

In the tumour microenvironment, TAM ϕ help to promote tumour growth, invasion and metastasis. However, there is very little known about the ontogeny of TAM ϕ in melanoma, partly due to the lack of specific surface markers to discriminate them from other macrophage subtypes (446, 452). Previous fate-mapping studies have shown that TAM ϕ consist of both YS EMP and BM HSC-derived macrophages in mouse models of brain (688, 689), breast (690), pancreatic (453), lung (454) and hepatocellular cancers (691), as well as colon adenoma (669). To the best of our knowledge, this study is the first to show that mixed developmental origins also apply to TAM ϕ in melanoma. Moreover, we

have shown that expansion of locally resident macrophages can compensate for depletion of monocyte-derived macrophages, such that tumour size is not affected. This is associated and perhaps at least partly mediated by early expansion and then differentiation of the YS-derived EndoMac progenitor pool. Interestingly, another study of lung cancer revealed that resident macrophages contributed to tumour growth, while accumulation of BM-derived macrophages led to tumour dissemination (454). This may explain why tumour size was not reduced by clodronate-depletion of monocytes, although to definitively confirm this we would also need to study tumour cell density. TAM ϕ have also been associated with enhanced angiogenesis to assist tumour growth and spread (692). Depletion of YS EMP-derived macrophages in murine hearts has been associated with a reduction in angiogenesis (693), but little is known yet about the contribution of TAM ϕ from different origins to tumour vascularisation. Further evaluation of the vascular density and integrity of monocyte-depleted melanomas will help us to understand the respective contributions of local and BM-derived TAM ϕ to tumour angiogenesis.

As for other tissues, macrophage heterogeneity in skin can no longer be simplified into the historical M1 versus M2 classification system. Using a unique flow cytometry-based system, Tamoutounour *et al.* reported in 2013 that steady state dermis has an abundance of DCs, P5, P3 and P1 cells (404), while other groups have demonstrated that MHCII⁺LYVE-1⁺ macrophages are the dominant subtype in adult mouse skin (175, 197, 694, 695). Our results are mostly consistent with both sets of findings, although we observed that Ly6C^{Lo}MHCII⁻ P4 macrophages were much more prevalent than their Ly6C⁻MHCII⁺MerTK⁺ P5 counterparts, with YS EMPs contributing to ~4% of these. In wounded TAM-induced *Csf1r*^{MerCreMer} fate-mapping mice, the dominant YS-derived myeloid population switched from the inflammatory, P1 subset in the first 24 h after wound injury to the more anti-inflammatory P4 population at later time-points, when wound closure was almost complete. This supports other studies that have reported macrophage phenotype based on traditional M1 and M2-related markers (423, 424, 683, 696). According to the “M1/M2 paradigm”, mainly based on *in vitro* polarisation experiments, pro-inflammatory M1 macrophages display anti-tumour characteristics, whereas M2-macrophages are generally considered pro-tumorigenic (451, 667, 668). Both populations can co-exist in tumours, although M2-like MHCII^{Lo} macrophages (which overlap with the P1 and P4 subtypes) are claimed to

be enriched in hypoxic regions of tumour and M1-like MHCII^{Hi} macrophages (which include P2, P3 and P5) in normoxic areas. As reported for other cancers (451, 667-669), we observed a progressive predominance of YS-derived P4 and MHCII^{Lo} macrophages during melanoma development, which are notionally pro-tumoural. This raises important questions about cancer therapies targeting specific macrophage subsets and suggests that better outcomes may be achieved by selectively depleting resident, embryonically derived macrophages, while preserving the phagocytic activities of recruited, monocyte-derived macrophages.

Consistently, our differentiation and adoptive transfer experiments indicated that skin EndoMac progenitors do not form macrophages by traversing through a monocyte stage. Instead, we observed formation of a new “intermediate” population that expressed CD45, F4/80, c-Kit, CX₃CR1, CSF1R and LYVE-1 but not CD11b, Sca-1 or MHCII, and was especially prominent in the melanoma model. Although further evaluation is now required to confirm the transcriptional and functional properties of these cells, their expression of c-Kit suggests that they may still be in an immature, albeit macrophage-committed state. Interestingly, this surface phenotype resembles CD11b^{Lo}F4/80^{Hi}CX₃CR1^{Hi} Langerhans cell precursors, which were previously identified as a YS-derived ancestral population in E17.5 embryonic dermis (122). In contrast, we also found that progenitor-derived endothelial cells mostly maintained Sca-1 expression, which was suggested in a recent study to associate with higher proliferative capacity than for Sca-1⁻ endothelial cells in adult mouse femoral artery (682). Unbiased subcluster analysis of our flow cytometry data in parallel with single cell RNA-sequencing should help to clarify the hierarchy by which YS-derived EndoMac progenitors differentiate into macrophages, endothelial cells and their subtypes, and potentially uncover any new intermediate populations. Identification of the key genes, signalling pathways and growth factors that drive the expansion and differentiation of EndoMac progenitors during wound healing and melanoma growth will also be important for developing therapies that can selectively promote (in wounds) or dampen (in melanomas) them. These are likely to be both disease- and time-specific.

An important limitation of the current study is that all experiments were performed without separating skin into its dermal and epidermal layers. However, another member of our group has recently shown that EndoMac progenitors are indeed present in both layers. Other objectives for future research are to investigate for analogous progenitor cells in human skin, especially with a long-term view to cell transplantation or reprogramming to help treat resistant burns, ulcers or chronic wounds, as occur in patients with diabetes and peripheral vascular disease.

6.5 Conclusion

This Chapter has established the presence of YS-derived EndoMac progenitor cells in postnatal mouse skin. The fate and function of these bipotent progenitors are influenced by the local disease microenvironment. Although they favour the endothelial lineage and promote healing after wound injury, they are predisposed to produce macrophages in the setting of melanoma, where they maladaptively enhance tumour growth.

Chapter 7

General discussion

7.1 General overview and key findings

Embryonic development involves a highly coordinated series of events that need to be faithfully replicated. Deep knowledge and understanding of the normal developmental processes essential for proper organogenesis are fundamental, not only to advance “basic” biology and research, but also to provide insights into disease mechanisms and advance clinical science. Developmental biology and stem cell biology are at the root of regenerative medicine, a field in rapid emergence partly due to progress in technology and engineering. However, the challenge to design new strategies that can detect, prevent, and cure diseases must be pushed forward first by our understanding of normal biological systems.

New blood vessel formation is central to normal organ development and many pathophysiological processes and is closely intertwined with inflammation. Macrophages accumulate at sites of neovascularisation and support endothelial cells that line new blood vessels. It follows that a better understanding of how the intricate relationships between macrophages and endothelial cells come about may open up new therapeutic opportunities to target major disease processes. One important clue may lie in the ontogenies of both cell lineages, which increasingly appear to overlap during early prenatal life. Converging lines of evidence indicate that tissue-resident macrophages and endothelial cells share a common source from EMPs in extra-embryonic YS. Current expert consensus contends that YS-derived macrophages and endothelial cells are seeded in tissues prenatally and are maintained after birth by local self-renewal in their mature, differentiated state. As an alternative explanation for this, this thesis identified for the first time the existence of undifferentiated, bipotent EndoMac progenitor cells, seeded from embryonic development, in postnatal murine tissues.

In Chapter 3, we first built on the earlier finding that murine arterial adventitia contains Sca-1⁺CD45⁺ macrophage progenitor cells, that are able to selectively generate CFU-M in methylcellulose (262, 263). In addition to forming CFU-M and macrophages, we made the surprising observation that aortic Sca-1⁺CD45⁺ cells are also vasculogenic and produce endothelial-lined neovessels after *in vivo* transfer

(246). As the adventitial Sca-1⁺CD45⁺ compartment is heterogeneous in its cellular composition, we were not able to claim that they were bipotent progenitors for endothelial and myeloid lineages at a single cell level. However, we were prompted to study the true nature of aortic CFU-M more precisely. The pivotal finding in Chapter 4 that these colonies undergo self-renewal from single cell origins enabled us to comprehensively phenotype their progenitor cell content. We were therefore able to extend their surface marker characterisation beyond the imprecise Sca-1⁺CD45⁺ phenotype described previously (19, 20), to a unique fingerprint based on at least eight markers: LIN⁻CD45^{+/Lo}CD11b⁻F4/80⁻Sca-1⁺c-Kit⁺CX₃CR1⁺CSF1R⁺. This immunophenotype has not been described before and crucially identifies progenitors in both freshly digested tissues and CFU-M grown from a range of embryonic and postnatal mouse tissues: aorta, heart, lung and YS (Chapter 4), skeletal muscle (Chapter 5), and skin (Chapter 6).

By profiling the tissue distribution and abundance of CFU-M progenitors pre- and postnatally and performing complementary fate-mapping with *Flt3*, *Cx3cr1* and *Csf1r* promoters, we have established that they originate from YS EMPs, are seeded in embryonic tissues around E10.5 and are then maintained independently of definitive haematopoiesis, albeit in declining numbers after birth. In keeping with the established literature (2, 30, 93, 108), we have found that YS CSF1R⁺ and CX₃CR1⁺ progenitors do not contribute to the adult HSC pool and that YS-derived EndoMac progenitors do not circulate in peripheral blood. In addition, our results support the recent (132, 545), but disputed finding (133), that YS EMPs are also a source of mature endothelial cells and demonstrate that this applies not only to heart and lung (132), but also aorta, muscle and skin. Moreover, our studies indicate that in each of these tissues at steady state, the fate of YS EMPs surprisingly favours the endothelial lineage over macrophages.

Using a combination of *in vitro*, *ex vivo* and *in vivo* differentiation studies, along with lineage mapping and BrdU pulse-chase, we have now clearly determined that CFU-M progenitors are bipotent for macrophages and endothelial cells at a clonal level and they possess striking vasculogenic capacity. Confirmation that YS-derived EndoMac progenitors are present in different postnatal tissues also led

us to interrogate their expansion and fate under different pathophysiological conditions, that are underpinned by inflammatory and neovascularisation responses. By performing CFU assays and flow cytometry analysis in parallel, we observed that progenitors undergo robust expansion in different models of disease. Notably, their numbers increased by: 1) ~3-fold in atherosclerotic aorta (Chapter 3); 2) between 2 and 6-fold in AngII-induced aortic inflammation (Chapter 4); 3) between 2.5 and 7-fold in muscle after hindlimb ischaemia (Chapter 5); and 4) ~2-fold in skin after wound injury and induction of melanoma (Chapter 6). Our BrdU pulse-chase studies in Chapters 4 and 5 indicated that this accumulation of progenitors begins with a very early proliferative response, that is followed by their differentiation into macrophages and endothelial cells, which would promote local inflammation and neovascularisation after tissue insult or disease. Furthermore, our results suggest that in different settings, these progenitors either play adaptive roles to promote tissue repair (e.g. after injury or ischaemia), or maladaptively help drive disease pathogenesis, such as in atherosclerosis and tumorigenesis.

Taken together, our findings represent a major breakthrough in extending what was already known about the long-term fate of YS EMPs. Although YS-derived EndoMac progenitors are clearly not the dominant source of macrophages and endothelial cells in postnatal tissues, their existence does provide a new model that helps to explain and bring together several key observations from the existing literature:

- a) Long-held speculation around the existence of bipotent haemato-endothelial progenitors in postnatal tissues, which until now has never been definitively shown *in vivo*.
- b) Independent observations from different fate-mapping studies that YS EMPs give rise to both tissue-resident macrophages and endothelial cells.
- c) Apparent self-renewal of YS-derived macrophages in their mature state postnatally and clonal expansion of endothelial cells without BM or circulatory input.
- d) Interdependence of macrophages and endothelial cells in developmental and pathophysiological neovascularisation.

7.2 Bridging existing knowledge gaps

Previous studies have used TAM-induced E8.5 *Csf1r*-Cre and E9.5 *Cx3cr1*-Cre fate-mapping to show the YS origins of postnatal tissue-resident macrophages (2, 30, 93, 99) and osteoclasts (134), while E8.5 *Csf1r*-Cre fate-mapping has also shown that YS EMPs contribute to prenatal erythrocytes (135), postnatal mast cells (137), natural killer (NK) cells (697) and vascular and lymphatic endothelial cells (132, 698). Here, we identified that both fate-mapping approaches label EndoMac progenitors along with tissue-resident macrophages and endothelial cells. Importantly, our findings support a shared ancestral relationship that links these three YS-derived populations in their various tissue niches postnatally, without ongoing renewal from definitive BM HSCs or circulating progenitors (**Figure 7-1**). Numerous threads of evidence show the intertwined association between endothelium and haematopoietic cells, especially macrophages, in embryonic development (20, 42, 110, 127, 699), postnatal tissue homeostasis (4, 7, 10, 15, 16) and disease (342, 347, 362, 364, 700). The notion that this may be explained during early embryogenesis by a common haemangioblast progenitor was first introduced almost a century ago, borne out of the intimate relationship between developmental vasculogenesis and primitive haematopoiesis in extra-embryonic YS (542). Although *in vitro* studies using embryonic stem cells and induced pluripotent stem cells have shown a common pathway of haemato-endothelial differentiation (564, 592, 701, 702), demonstration of the *in vivo* existence of haemangioblasts has proven notoriously difficult, especially after birth (52, 53).

7.2.1 Why does defining the origins of macrophages and endothelial cells matter?

Defining the origins of macrophages and endothelial cells is not only a matter of intellectual interest, but it also has implications for developing new treatment paradigms for different pathophysiological conditions. Previous studies have indicated that tissue macrophages and endothelial cells from different origins have important transcriptional and functional differences and therefore respond to different regulatory cues, including soluble growth factors and cytokines (88, 132, 166, 167, 169, 172, 194). This is analogous to the heterogeneity of vascular smooth muscle cells (SMC), which have been shown to originate from three distinct embryonic sources (703, 704), along with postnatal resident stem/progenitor cells (235, 705) and circulating progenitors from BM (703-705). The diverse ontogeny of vascular SMC is reflected in their anatomical distribution. The embryonic SMC colonising the ascending aorta are derived partly from the secondary heart field and partly from neural crest (245, 706), whereas SMC residing in the remaining aortic arch are neural crest-derived (707), and those populating the descending aorta originate from somatic mesoderm (708). Studies have shown that these different sources of SMC influence their gene-expression profiles and responses to pathological states (709) and this in turn might explain why different regions of the vasculature react differently when exposed to the same stimulus. Examples of this include the regional susceptibility of arteries to calcification (710, 711), aneurysm formation (712), and atherosclerosis (235, 713).

Although this thesis did not specifically study the distribution of EndoMac progenitors in different regions of the aorta or other arteries, previous studies by my supervisor (262, 263) suggested heterogeneity in their abundance, whereby CFU-M yield was more prevalent in thoracic and abdominal aortas than carotid, femoral and renal arteries. Furthermore, preliminary experiments by our group using human internal mammary artery (IMA) and saphenous vein (SV) specimens have revealed much lower CFU-M recovery from IMA. This may have implications for adventitial remodelling and atherogenesis in these vessels after coronary artery bypass grafting. Further experiments are now required to elucidate

whether tissue- and region-specific differences in the presence and function of EndoMac progenitors have implications in disease processes.

7.2.2 Origins of macrophages and endothelial cells

The origin of endothelial cells prenatally and postnatally is complex. Apart from the earliest vessels arising in the YS and embryo, blood vessels were thought to subsequently develop through sprouting angiogenesis from the proliferation and migration of established endothelial cells. In recent years, a growing body of literature has suggested that distinct populations of progenitor cells also contribute to blood vessel growth throughout embryonic, as well as postnatal life. Endothelial progenitors have been described in BM (208), blood (206) and in solid, non-haematopoietic tissues, including aorta (214, 220-223, 226-228, 231), heart (714), and skin (229, 230, 715, 716). Recent studies have also indicated that YS EMPs contribute endothelial cells to the blood and lymphatic vessels of some embryonic and adult organs (132, 545). Here, we were able to quantify the contributions of both YS progenitors and definitive Flt3⁺ HSCs to endothelial cells in different mouse tissues under different conditions. In healthy adult aorta, lung and skin, we found that up to 55%, 40% and 45% of endothelial cells, respectively, were derived from a definitive haematopoietic source based on *Flt3*-Cre fate-mapping. In contrast, this contribution was only ~30% in heart and ~25% in skeletal muscle. Furthermore, embryonic fate-mapping indicated that ~12% of endothelial cells in aorta, heart and muscle were derived from CSF1R⁺ YS EMPs, compared to ~16% in skin and ~8% in lung, with supportive results also provided by complementary *Cx3cr1*-Cre lineage-tracing. Although not a primary focus here, our data also suggest that the contribution of YS progenitors was highest for endothelial cells expressing CDH5 (VE-cadherin, CD144), as seen for muscle (Chapter 5) and skin (Chapter 6). In both of these tissues, we also found preliminary evidence to support the YS origins of LYVE-1⁺ lymphatic endothelial cells, and importantly observed progressive expansion of the YS-derived, tissue-resident endothelial pool in different disease states.

Similarly, across the tissues studied here, we also verified that YS EMPs are an important source of resident macrophages, especially of the LYVE-1⁺MHCII^{-Lo} subtype. Based on flow cytometry, we quantified this embryonic contribution to be ~25% of all CD11b⁺F4/80⁺ macrophages in steady state adult aorta, ~18% in heart, ~34% in lung, ~30% in muscle and ~35% in skin. Interestingly, for aorta and skin, our age profiling studies also showed a marked increase in the YS-derived macrophage compartment between birth and adulthood. Although our fate mapping systems did not allow us to definitively distinguish between early and late EMPs, the weight of our data supports an early CSF1R⁺ EMP origin for each of these tissue-resident macrophage populations, in keeping with studies by Gomez Perdiguero *et al.* (101, 121, 551, 555). This differs from other data, which have concluded that most embryonically derived macrophages in liver (2, 175), kidney (2, 175), lung (2, 175), gut (2, 175), skin (2, 175, 197) and muscle (172) come from late CSF1R^{Lo} EMPs and/or c-Kit⁺ definitive HSCs via foetal liver monocytes.

In demonstrating that EndoMac progenitors are a highly proliferative and self-renewable source of locally maintained macrophages and endothelium postnatally, it is important to emphasise that our results do not necessarily contradict the current dogma that embryonically derived macrophages also undergo self-renewal in their differentiated state. However, with the exception of a few studies (164, 177), we believe that most of the published data used to support this notion is at best circumstantial (194, 624, 717). Evidence for the self-renewal of certain subtypes of macrophages in aorta, heart, lung, muscle and skin has been based on their lack of reconstitution from BM-derived monocytes (e.g. in parabiosis and irradiation transfer studies), their proclivity for proliferative expansion after insult and their persistent reporter labelling after postnatal induction of fate-mapping in *Cx3cr1*^{CreER} and *Csf1r*^{MerCreMer} mice (168, 569, 633). However, these results are also compatible with macrophage renewal from CSF1R⁺CX₃CR1⁺ EndoMac progenitors.

Our different studies highlight that the selective growth of CFU-M from “non-haematopoietic” tissues is not due to proliferating monocytes or macrophages, but rather undifferentiated progenitors. Crucially, we have provided direct evidence that these progenitors can renew themselves clonally *in vitro* without

altering their immunophenotype, while also showing that after *in vitro* and *in vivo* differentiation a proportion of progenitors still retain their undifferentiated status. This indicates that they are endowed with properties that are necessary for self-maintenance and amplification, while also being able to transform phenotypically into mature daughter cells. The enriched presence of EndoMac progenitors in neonatal tissues is consistent with prenatal seeding. Meanwhile, their diminishing prevalence with increasing postnatal age suggests that their capacity for self-renewal is limited and that they may be vulnerable to age-related senescence, which is known to affect other tissue stem/progenitor cells (718). This would progressively exhaust their reserves, both through increased apoptosis (which was not studied here) and loss of their proliferative and self-renewal properties. The latter was supported by our findings that CFU-M from older tissues were smaller in size and less capable of generating secondary colonies when re-plated in methylcellulose and vascular-like cords in Matrigel™. Overall, we believe that it is feasible that the local maintenance and replenishment of YS-derived tissue macrophages occur because of the proliferative self-renewal of both undifferentiated progenitors and differentiated macrophages, with these respective sources making different contributions depending on age, tissue type and stage of disease.

7.2.3 Clonal capacity of macrophages and endothelial cells

An important limitation to our studies so far, is that we have not yet investigated for clonal expansion of EndoMac progenitors or their progeny *in vivo*, by performing lineage-tracing with multi-colour reporter (e.g. Confetti or Rainbow) mice. These studies would also help to elucidate whether EndoMac progenitors undergo asymmetrical or symmetrical division during steady state and in response to disease. Over recent months, another PhD candidate in our group has collected tissues from PBS and AngII-treated *Csf1r*^{MerCreMer} x *Rosa*^{Rainbow/+} mice. Some of these mice were induced with TAM at E8.5 to specifically study clonal accumulation of all YS EMP-derived cells, while others were induced with TAM postnatally to focus on the clonal expansion and fate of locally-resident CSF1R⁺ cells, including EndoMac progenitors. Results are still pending.

7.2.4 Determining the identity of tissue-resident EndoMac progenitors

Throughout our studies, we have considered whether tissue-resident EndoMac progenitors may simply overlap with other stem/progenitor cell populations that have already been described. In particular, we have focused on their similarities and differences with the following five cell types: 1) haemangioblasts (40, 42, 46, 50, 55, 110, 719, 720); 2) primitive macrophage precursors in YS (96, 98, 99, 101); 3) early and late YS EMPs (2, 93, 108, 109, 131); 4) embryonic endothelial progenitor cells (eEPC) (224); and 5) mesoangioblasts (199) (**Table 7-1**).

Like haemangioblasts, EndoMac progenitors first emerge in YS between E7.0-E7.5 as shown by the low yield of CFU-M at this gestational age in Chapter 4. They can self-renew and differentiate into endothelial and haematopoietic cells at a clonal level, although the latter is restricted to macrophages and possibly dendritic cells, at least from adult tissue sources. Notably, they express ACE (CD143), AT1R and AT2R, which have been identified as haemangioblast markers in previous *in vitro* studies (46). However, EndoMac progenitors do not express other canonical haemangioblast markers, namely VEGFR2 (Flk1) or Brachyury, at either surface protein or mRNA level (data not shown).

As discussed in Chapter 4, EndoMac progenitors possess some but not all of the characteristics of early YS EMPs, including shared expression of several surface markers [e.g. CD93 (AA4.1), c-Kit, CSF1R] and bipotency for the endothelial and macrophage lineages, although EMPs also produce other primitive haematopoietic cells (e.g. erythrocytes) (93, 101, 121, 123, 131, 132). Furthermore, tissues which have been shown to contain EMP-derived macrophages (e.g. aortic adventitia, heart, lung, muscle, skin) (92, 93, 123, 166, 172, 637, 721, 722), were also shown here to harbour EndoMac progenitors, beginning prenatally. Meanwhile, several threads of evidence suggest that they are distinct from late EMPs which are *Myb*-dependent and have limited self-renewal capacity after E16.5 (2, 114). In particular, EndoMac progenitors display low transcript levels for *Myb* (data not shown), do not produce CD11b⁺F4/80^{-Lo} monocytes or other circulating progeny and minimally colonise foetal liver.

Another possibility is that EndoMac progenitors are simply a postnatal residue of primitive macrophage precursors that have been identified in YS between E7.5 and E9.5 and have been characterised as $CD45^{Lo/-}c\text{-Kit}^+CX_3CR1^+CSF1R^+$. These precursors also exclusively give rise to CFU-M when cultured in colony-forming haematopoietic media and generate primitive macrophages in YS, as well as brain microglia (96, 98, 99). Although we have identified EndoMac progenitors in embryonic brain beginning at E10.5, they mostly disappear from this site immediately after birth (data not shown). We have also considered the overlap between EndoMac progenitors and eEPC that are characterised as $TIE2^+VEGFR2^{Lo/-}c\text{-Kit}^+Sca-1^+$ and found in YS at E7.5 (225). These progenitors also form small spherical clusters in culture, which give rise to endothelial cell networks, but do not seem to have haematopoietic potential. Furthermore, EndoMac progenitors are negative for TIE2 and VEGFR2 in their undifferentiated state and lose surface expression of c-Kit upon endothelial differentiation.

Finally, it is worth comparing the characteristics of EndoMac progenitors with embryonic mesoangioblasts, which have multipotent, clonogenic capacity for macrophage, endothelial and mesenchymal lineages (202, 723). Mesoangioblast colonies formed in semisolid culture medium contain cells that are $PDGFR\alpha^+PDGFR\beta^+CD90^+CD31^{Lo}CD34^+CD43^-CD73^-$ (724-727). Similarly, the progenitors contained within CFU-M from aorta and other solid tissues, also express CD90, CD31, and CD34, but neither of the PDGF receptors, PDGFR α (CD140a) or PDGFR β (CD140b) (**Figure 4-2, Figure 7-2**). Curiously, we have occasionally observed spontaneous contractile activity of both CFU-M in methylcellulose and progenitor-derived cords in MatrigelTM, which has prompted us to speculate that EndoMac progenitors may also have mesenchymal or muscular plasticity. As reported in Chapter 3, $Sca-1^+CD45^+$ aortic cells were unexpectedly able to generate $Sca-1^+CD45^-$ progeny *in vitro*. More recently, we have found that pure populations of postnatal EndoMac progenitors also give rise to $CD45^-CD31^-$ cells that are strongly positive for PDGFR α and CD90 both in MatrigelTM assays and after adoptive transfer *in vivo* (data not shown). Our lineage-tracing studies have also consistently revealed the presence of non-haematopoietic, non-endothelial cells derived from $CSFR1^+$ and CX_3CR1^+ YS progenitors that express mesenchymal/fibroblast markers. Given what is known about endothelial-to-mesenchymal transformation and the fibroblastic fate of other tissue-resident endovascular progenitors

(230, 231), we believe that future studies should investigate whether YS EMPs and EndoMac progenitors also share this potential.

Taken together, our current knowledge of EndoMac progenitors suggests that they are distinct from other previously defined embryonic and postnatal cell types. Our working model is that they are a self-renewing derivative of early YS EMPs, that are at the very least bipotent and possibly multipotent (**Figure 7-1**). Much more work is now needed to unravel their place in the hierarchy of macrophage and endothelial ontogeny. Based on our available data, we propose that they are immediately ancestral to CX₃CR1⁺ pre-macrophage precursors in embryonic life, and may transition to CD11b⁺F4/80^{Hi} macrophages via intermediate Sca-1⁺c-Kit⁺CD11b⁺F4/80⁺ precursors postnatally. Similarly, their differentiation toward CD45⁺CD31⁺ and/or CDH5⁺ endothelial cells may also involve a proliferative Sca-1⁺ transitional state. The next steps to investigate these theories are to culture these progenitors under different lineage-specific inductive conditions and investigate for cluster and subcluster diversity of their progeny through unbiased analysis of our flow cytometry data and single cell RNA-sequencing.

	EndoMac progenitors	Haem-angioblasts	Primitive Mφ precursors	Haemogenic Endothelium	Early EMPs	Late EMPs	eEPC	Mesoangioblast	References
Surface Phenotype	Lin ⁻ CD45 ^{+/Lo} CD11b ⁻ F4/80 ⁻ Sca-1 ⁺ c-Kit ⁺ CX ₃ CR1 ⁺ CSF1R ⁺	VEGFR2 ⁺ Bry ⁺ Tal1 ⁺	CD41 ^{Lo} c-Kit ^{- or +} CX ₃ CR1 ⁺ CSF1R ⁺	TIE2 ⁺ CD31 ⁺ CDH5 ⁺	CD45 ^{Lo} CD41 ⁺ c-Kit ⁺ CSF1R ⁺ CD16/32 ⁺ CD93 (AA4.1) ^{Lo}	CD45 ^{Lo} c-Kit ⁺ CSF1R ^{Lo} CD16/32 ⁺ CD93 (AA4.1) ⁻	CD34 ⁺ VEGFR2 ⁺ TIE2 ⁺ c-Kit ⁺ Sca-1 ⁺	VEGFR2 ⁺ Bry ⁻	(38, 41, 51, 74-77, 89, 98, 116-118, 224, 225, 246, 262, 263, 728)
Time of origin (murine)	~E7.5-E8.5	~E7.5	~E7.5-E8.5	~E8.25	~E7.5-E8.5	~E9.5	~E7.5	~E8.5	(61, 89, 98, 116-118, 728)
Origin	YS	YS	YS	YS and AGM	YS	YS then migrate to foetal liver	YS	YS	(61, 89, 98, 116-118, 728)
Differentiate into?	Resident Mφ; EC; Intermediate Mφ; Other	EC; Erythrocytes; Primitive Mφ precursors	Primitive YS Mφs; Microglia	EC; EMPs; HSC; T and B cell progenitors	EC; Resident Mφ; Osteoclasts; Megakaryocytes; Mast cells; NK cells	Foetal monocytes; Resident Mφ; Erythrocytes; Megakaryocytes; Mast cells; NK cells	EC	SMC; Skeletal muscle; Mφ; EC; Cardiac muscle	(59, 60, 62-64, 91, 98, 100, 728).
Haematopoiesis	Prim	Prim	Prim	Def	Prim/Def	Def	N/A	N/A	(25, 35, 89)
Dependent on	<i>Myc</i> <i>Cx3cr1</i>	<i>Myc</i>	<i>Myc</i> <i>Tgfb2</i>	<i>Myb</i> <i>Runx1</i>	<i>Myc</i> <i>Runx1</i>	<i>Myb</i> <i>Runx1</i>	N/A	N/A	(2, 79, 80, 101, 125)
Flt3⁻ or Flt3⁺	Flt3 ⁻	Flt3 ⁻	Flt3 ⁻	Flt3 ⁻	Flt3 ⁻ and Flt3 ⁺	Flt3 ⁺	Flt3 ⁻	Flt3 ⁻	(728)
Self-renew properties	Self-renew	N/A	Self-renew	N/A	Self-renew	Limited self-renewal	N/A	N/A	(89, 114, 200, 728).
CFU	CFU-M	BL-CFC	CFU-Ms	N/A	CFU-M; G; GEMM	CFU-M; G; GEMM; E	MAC and ECFC	N/A	(41, 225, 728)
Tissues seeded	Aorta; Heart; Lung; Skin; Muscle	N/A	Brain; Epidermal skin	YS; AGM	Aorta; Heart; Lung; Dermis and epidermal skin; Muscle; Gut; Liver; Adipose tissue	Lung; Dermis and epidermal Skin; Muscle; Gut; Liver	N/A	N/A	(2, 89, 91-93, 100, 101, 108, 131, 132, 135-139, 728).

Table 7-1. Properties of YS-derived EndoMac progenitors compared to other progenitor cells of interest.

AGM, Aorta-gonad-mesonephro; BL-CFU, Blast colony forming cells; Bry, Brachyury; CFU-M, Colony forming unit macrophage; Def, Definitive haemopoiesis; E, Embryonic day; EC, Endothelial cell; ECFC, Endothelial colony-forming cells; EMP, Erythromyeloid progenitors; Mφ, Macrophage; MAC, Myeloid angiogenic cells; N/A, Not available; Prim, Primitive haemopoiesis; SMC, Smooth muscle cell; YS, Yolk Sac.

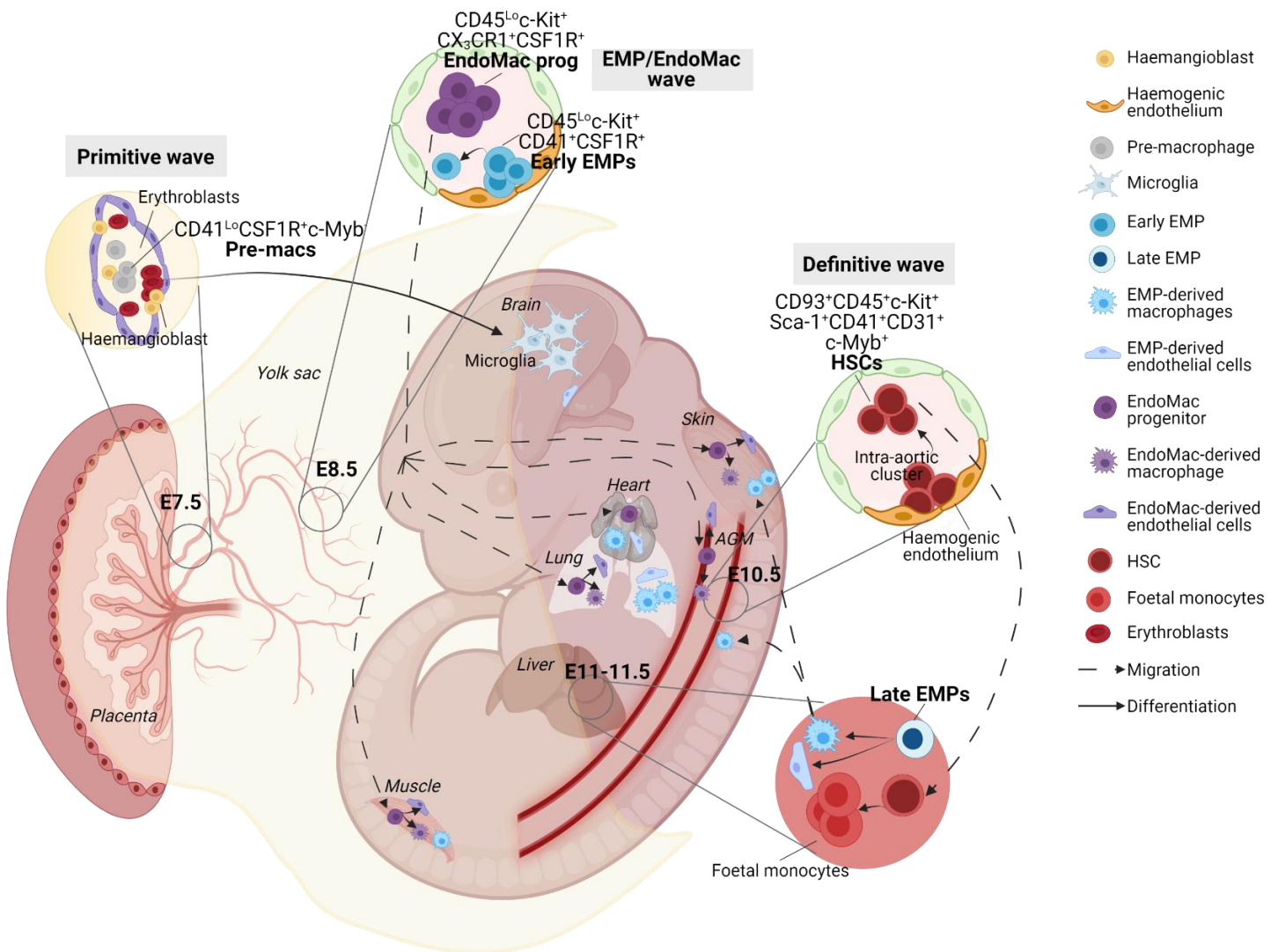


Figure 7-1. A new paradigm that proposes that YS-derived EndoMac progenitors seed different tissues during embryonic development and these persist after birth.

AGM, Aorta-gonad-mesonephro; CD31, Platelet endothelial cell adhesion molecule; CD41, Integrin alpha-IIb; CD45, Protein tyrosine phosphatase, receptor type, C; CD93, Cluster of Differentiation 93; c-Kit, Proto-oncogene c-KIT; c-Myb, MYB proto-oncogene; CSF1R, colony stimulating factor 1 receptor; CX₃CR1, CX3C chemokine receptor 1; E, Embryonic day; EMP, Erythromyeloid progenitors; EndoMac prog, Endothelial-macrophage progenitors; HSC, Haematopoietic stem cell.

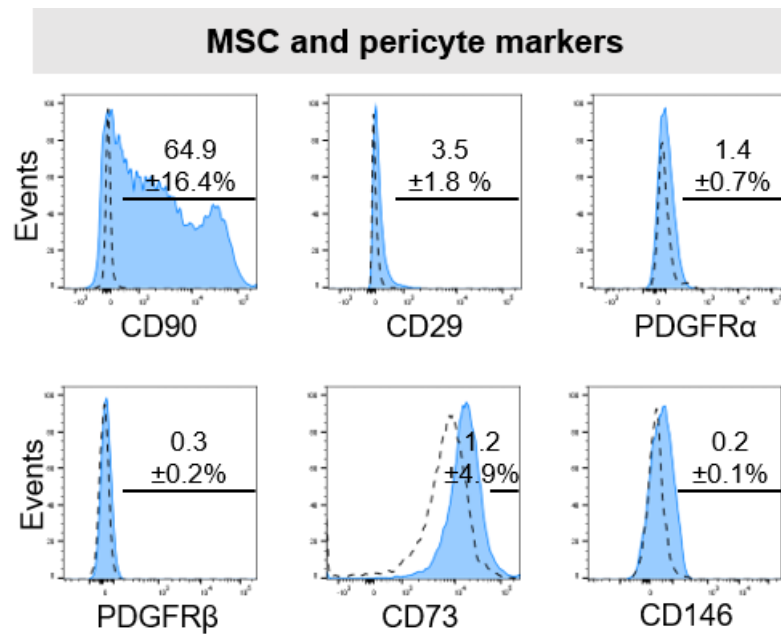


Figure 7-2. Expression of mesoangioblast, mesenchymal stem cell (MSC) and pericyte surface markers on progenitors contained within 1° aortic CFU-M.

Flow cytometry histograms depict expression of different types of surface markers on cells from primary (1°) aortic CFU-M (blue histogram). Dotted histogram, FMO control. Representative of n=3 12 w C57BL/6 mice. Data summarised as mean±SD.

7.2.5 The influence of different tissue niches on EndoMac progenitors

Microenvironmental signals tightly regulate the development and homeostasis of all tissues. It has been shown that macrophages strongly adapt to their tissue niches after prolonged exposure (729, 730). Therefore, even BM-derived, tissue-resident macrophages eventually acquire some capacity for self-maintenance, along with tissue-specific transcriptomic and functional properties, similar to their EMP-derived counterparts (731, 732). It follows, that EndoMac progenitors also display a degree of tissue specification, as summarised in **Table 7-2**.

One such example is that EndoMac progenitors from heart only formed clusters after 7 d in MatrigelTM, with minimal sprouting or inter-connected cords (**Figure 7-3**). Flow cytometry revealed that these clusters contained CD11b⁺F4/80⁺ macrophages and cells of the “intermediate” pre-macrophage phenotype, but very few endothelial cells. This result resembled the fate of skin progenitors when they were cultured with the conditioned media from melanoma cells. Similarly, although not shown in Chapter 4, AngII-induced expansion of EndoMac progenitors occurred much later in heart than aorta, and this was associated with a smaller accumulation of YS-derived endothelial cells compared to macrophages. Meanwhile, AngII had little effect on any of the YS-derived populations in the skin or muscle of these mice (data not shown). Clearly, different tissues and pathophysiological insults expose EndoMac progenitors to different regulatory cues, including cytokines, growth factors, transcriptional and epigenetic modifications. This will need to be studied by techniques like RNA- and ATAC-sequencing, which in turn should help define key signalling pathways that are age-, tissue- or disease-specific. Further comparative analyses could also address whether EndoMac progenitors exhibit gender or strain/species-related differences in their properties.

Experiment		YS E9.5	12 w Aorta	12 w Heart	12 w Lung	12 w Skin	12 w SkM
Stem cell marker identification	Flow cytometer	Lin ⁻ CD45 ^{+/Lo} CD11b ⁻ F4/80 ⁻ c-Kit ⁺ CX ₃ CR1 ⁺ CSF1R ⁺ *	Lin ⁻ CD45 ^{+/Lo} CD11b ⁻ F4/80 ⁻ Sca-1 ⁺ c-Kit ⁺ CX ₃ CR1 ⁺ CSF1R ⁺	Lin ⁻ CD45 ^{+/Lo} CD11b ⁻ F4/80 ⁻ Sca-1 ⁺ c-Kit ⁺ CX ₃ CR1 ⁺ CSF1R ⁺	Lin ⁻ CD45 ^{+/Lo} CD11b ⁻ F4/80 ⁻ Sca-1 ⁺ c-Kit ⁺ CX ₃ CR1 ⁺ CSF1R ⁺	Lin ⁻ CD45 ^{+/Lo} CD11b ⁻ F4/80 ⁻ Sca-1 ⁺ c-Kit ⁺ CX ₃ CR1 ⁺ CSF1R ⁺	Lin ⁻ CD45 ^{+/Lo} CD11b ⁻ F4/80 ⁻ Sca-1 ⁺ c-Kit ⁺ CX ₃ CR1 ⁺ CSF1R ⁺
	Average number of CFU-M per 10 ⁵ cells	~361	~28	~22	~77	~187	~30
Clonogenic, proliferative and self-renewal capacity	Size breakdown of Primary CFU-M [†]	Small: ~12% Medium: ~26% Large: ~62%	Small: ~72% Medium: ~27% Large: ~1%	Small: ~40% Medium: ~60% Large: ~0%	Small: ~62% Medium: ~36% Large: ~2%	Small: ~52% Medium: ~47% Large: ~1%	Small: ~81% Medium: ~19% Large: ~0%
	Self-renewal capacity	1 in ~1 cells	1 in ~14.8 cells	N/A	N/A	1 in ~3.7 cells	1 in ~15.2 cells
	% in S-phase <i>in vivo</i> ‡	N/A	~21%	~11%#	~15%#	~13%	~21%
	Matrigel <i>in vitro</i>	EC > Mφ > Prog	EC > Mφ > Prog	Inter > Mφ > EC > Prog	N/A	EC > Prog > Mφ > Inter	EC > Mφ > Prog
Multi-lineage differentiation**	Lineage-tracing <i>in vivo</i>	N/A	EC > Mφ > Prog	Mφ > EC > Prog	EC > Mφ > Prog	EC > Mφ > Prog	EC > Mφ > Prog
	Adoptive Transfer <i>in vivo</i>	EC > Mφ > Prog	EC > Mφ > Prog	N/A	N/A	Wound healing: EC > Mφ > Inter > Prog Melanoma: Inter > Mφ > Prog > EC	EC > Mφ > Prog
Regulators	-	N/A	AngII, M-CSF, CX ₃ CL1, Hypoxia [#]	AngII [#] , M-CSF [#]	AngII [#] , M-CSF [#]	Hypoxia	Hypoxia

Table 7-2. Comparison of EndoMac progenitors from different mouse tissue sources.

Data sets represent the approximate mean from multiple donor mice.

AngII, Angiotensin II; BrdU, Bromodeoxyuridine; EC, Endothelial cells; Inter, Intermediate population; M-CSF, Macrophage Colony Stimulating Factor; Mφ, Macrophages; N/A, Not available; Prog, Progenitors.

*YS E9.5 EndoMac progenitors express Sca-1 during culture *in vitro* but not *in vivo*; † Colony size classified as small (~30-100 cells), medium (~100-1000 cells) and large (>1000 cells); ‡ S-phase was analysed by flow cytometry from 12 w *Cx3cr1*^{GFP/+} mice receiving BrdU 24 h before sacrifice; **Multi-lineage differentiation data are displayed in order of abundance of cell progeny; # Data not shown in this thesis.

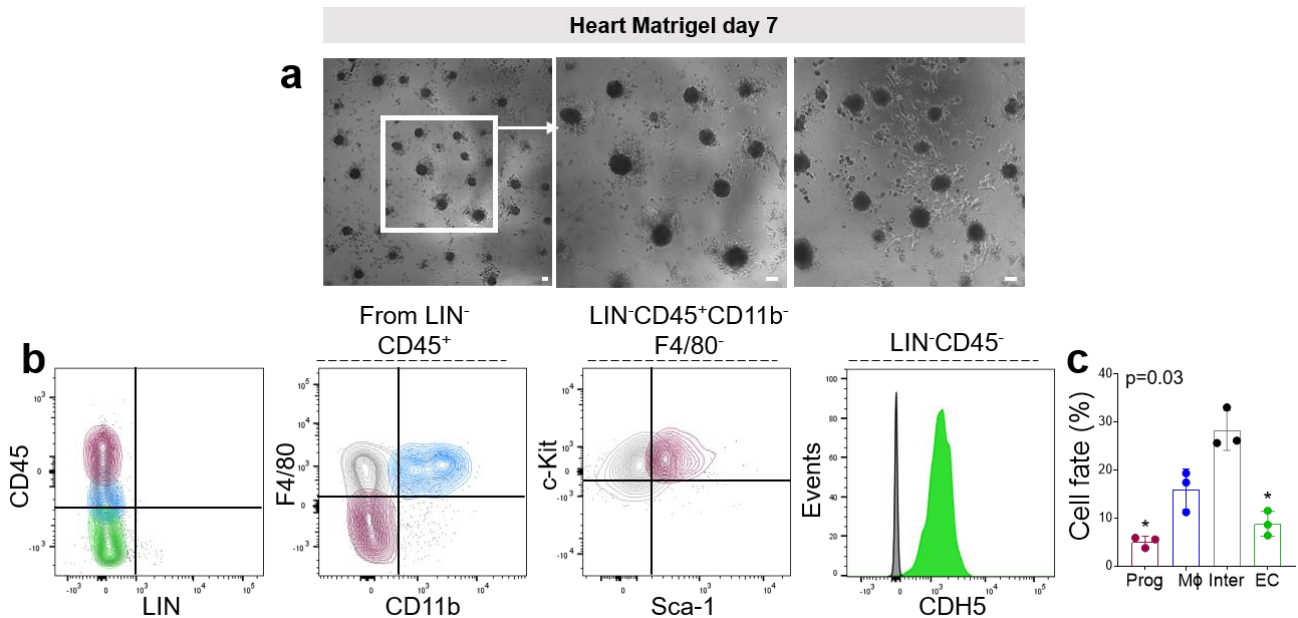


Figure 7-3. *In vitro* fate of heart EndoMac progenitors in Matrigel™.

EndoMac progenitors were isolated and purity-checked from CFU-M grown from 12 w C57BL/6 heart digests, and then cultured in Matrigel™ in the presence of endothelial growth media.

(a) Light microscopy images were taken at day 7 of culture in Matrigel™. White box indicates magnified area. Scale bar, 100 μ m.

(b-c) Flow cytometry plots and **(c)** adjacent graph display the cellular composition of these cultures. N=3 mice. Repeated measures ANOVA. Multiple comparison test: *p<0.05 vs Inter. Maroon, Progenitors (Prog); Blue, Macrophages (Mφ); Black, Intermediate population (Inter); Green, endothelial cells (EC). Green histogram, Sample; Grey, FMO control.

7.3 Other areas of ongoing investigation

7.3.1 Adventitial EndoMac progenitors as an “outside-in” driver of atherosclerosis

Although often viewed simplistically as the build-up of cholesterol plaques in the inner layer of blood vessels, the pathology of atherosclerosis is in fact transmural (233). In addition to “inside-out” recruitment of lipoproteins and inflammatory cells from luminal blood to plaque, increasing evidence also points to an “outside-in” pathway of atherosclerosis, mediated by changes that occur in the outer vessel layer, or adventitia (342, 348). Importantly, these changes involve the adventitial expansion of both macrophages and endothelial-lined *vasa vasorum*, that infiltrate and destabilise plaque (233). Understanding these adventitial changes remains an important step to developing new treatment paradigms for atherosclerosis.

Based on our results for adventitial Sca-1⁺CD45⁺ cells in Chapter 3, we hypothesise that the proliferation and differentiation of YS-derived EndoMac progenitors may account for the rapid expansion of adventitial macrophages and *vasa vasorum*, which begins early in atherosclerosis and then continues to drive growth and destabilisation of plaque from the outside of the vessel in (**Figure 7-4**). So far, I have performed two pilot experiments to begin to test this. Firstly, we have crossed *Flt3*^{Cre} x *Rosa*^{mT/mG} mice onto the atherogenic *Apoe*^{-/-} background. *Flt3*^{Cre} x *Rosa*^{mT/mG} x *Apoe*^{-/-} mice were then fed chow or HCD from 8 w of age, for either 4, 12 or 24 w to induce early, established or advanced atherosclerosis in their aortas. Preliminary analysis of flow cytometry and CFU-M assays showed up to a six-fold expansion of aortic progenitors after only 4 w of HCD (**Figure 7-5a-e**). CFU-M from these aortas were also larger and displayed a greater capacity for self-renewal (**Figure 7-5f-h**). Furthermore, this experiment has also demonstrated that macrophages in atherosclerotic aortas come from mixed *Flt3*-Cre⁺ and *Flt3*-Cre⁻ sources. The number of GFP⁺ BM-derived macrophages in aorta increased by 2.2-fold from 4 w to 12 w of HCD, while there was also a 1.9-fold increase in tdTom⁺ non-BM derived macrophages over the same period (**Figure 7-5i**). Fluorescence microscopy also revealed the presence

of both tdTom⁺ and GFP⁺ CD68⁺ macrophages in atherosclerotic plaques after 12 w HCD (**Figure 7-5j**).

Among various possible explanations, we speculate that the early expansion of adventitial EndoMac progenitors may contribute to the accumulation of tdTom⁺ (Flt3-Cre⁻) macrophages in arteries with established plaques (160, 266, 297). To examine the fate of these progenitors in atherosclerosis, I have also performed transplant studies in which I injected aortic EndoMac progenitors from ubiquitous GFP donor mice into the carotid adventitia of recipient *Apoe*^{-/-} mice, that had undergone tandem stenosis surgery to induce unstable, rupture-prone plaques (733) (**Figure 7-6a**). A control group received injections of cell-free MatrigelTM for comparison. Recipient mice were fed HCD for 6 w before and 6 w after surgery, followed by tissue harvests. Although analysis of the effect of progenitor transfer on carotid plaque burden and stability is ongoing, my initial results show engraftment of donor-derived GFP⁺ cells in the injected carotids of all recipients, with ~75% localised to adventitia and ~25% to plaque (n=5 mice) (**Figure 7-6b,c**). Using a combination of confocal microscopy and flow cytometry, I have also already confirmed the transformation of donor progenitors into both macrophages and endothelial cells, with few undifferentiated progenitors remaining 6 w after transfer (**Figure 7-6b,d**).

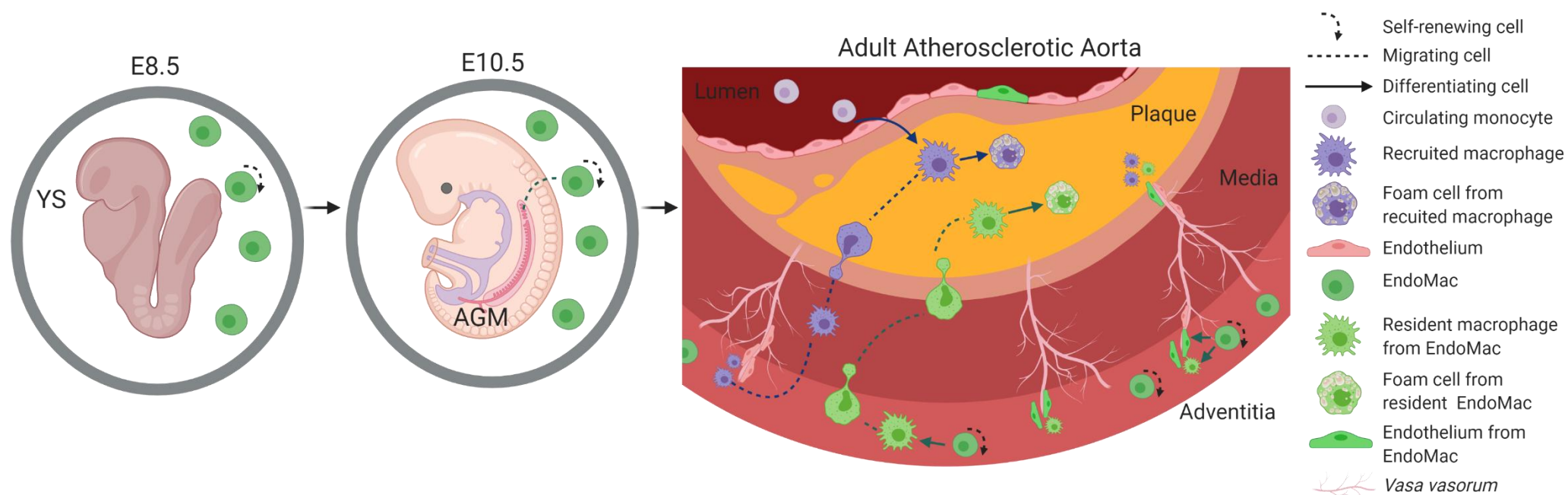


Figure 7-4. Proposed schematic of YS-progenitors contributing to atherosclerotic plaque and *vasa vasorum* formation.

AGM, Aorta-gonad-mesonephros; E, Embryonic day; EndoMac, Endothelial-macrophage progenitors; YS, Yolk sac.

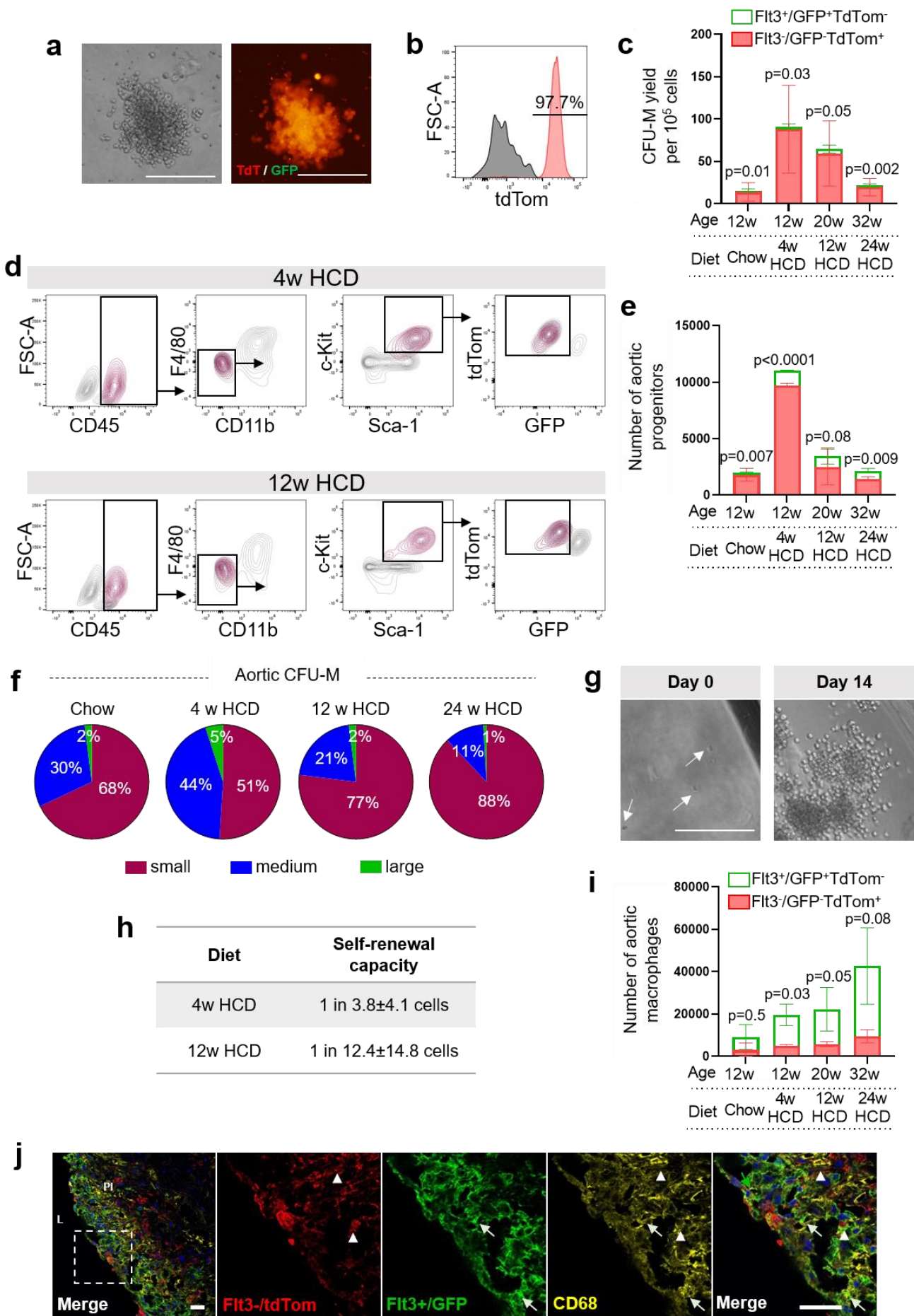


Figure 7-5. Expansion of EndoMac progenitors in atherosclerotic aorta.

- (a)** Light and fluorescence microscopy of aortic CFU-M from *Flt3^{Cre}* x *Rosa^{mT/mG}* x *Apoe^{-/-}* mice fed HCD for 4 w.
- (b)** Flow cytometry histogram confirms tdTom expression of cells contained in CFU-M from 4 w HCD *Flt3^{Cre}* x *Rosa^{mT/mG}* x *Apoe^{-/-}* aorta.
- (c)** Graph compares CFU-M yield and their *Flt3-Cre⁻* versus *Flt3-Cre⁺* status from the aortas of *Flt3^{Cre}* x *Rosa^{mT/mG}* x *Apoe^{-/-}* mice after different durations of chow or HCD. N=3-6 mice/gp. Mixed effects two-way ANOVA: $p < 0.0001$ for time; $p < 0.0001$ for group; $p < 0.0001$ for time x group. p-values from multiple comparison.
- (d)** Representative flow cytometry plots show presence of progenitors (maroon) in aortas from *Flt3^{Cre}* x *Rosa^{mT/mG}* x *Apoe^{-/-}* mice after 4 and 12 w of HCD, and their tdTom versus GFP fluorescence.
- (e)** Graphical quantification of numbers of *Flt3-Cre⁺* (green) and *Flt3-Cre⁻* (red) progenitors in aortas from *Flt3^{Cre}* x *Rosa^{mT/mG}* x *Apoe^{-/-}* mice maintained on chow or HCD for different durations. N=3-6 mice/gp. Mixed effects two-way ANOVA: $p = 0.02$ for time; $p < 0.0001$ for group; $p = 0.003$ for time x group. p-values from multiple comparison.
- (f)** Pie charts show classification of CFU-M based on colony size for *Flt3^{Cre}* x *Rosa^{mT/mG}* x *Apoe^{-/-}* mice maintained on chow or HCD for different durations. N=3-6 mice/gp.
- (g)** Light microscopy images showing self-renewal of secondary CFU-M from single cell origins. Donor aorta shown was from a *Flt3^{Cre}* x *Rosa^{mT/mG}* x *Apoe^{-/-}* mice fed HCD for 4 w.
- (h)** Self-renewal capacity of CFU-M progenitors from aortas of *Flt3^{Cre}* x *Rosa^{mT/mG}* x *Apoe^{-/-}* mice after 4 and 12 w of HCD, as represented by the number of cells in a 1° CFU-M needed to generate one 2° CFU-M. N=3-6 mice/gp.
- (i)** Graphical quantification of the numbers of *Flt3-Cre⁺* and *Flt3-Cre⁻* CD11b⁺F4/80⁺ macrophages in aortic digests from *Flt3^{Cre}* x *Rosa^{mT/mG}* x *Apoe^{-/-}* fed chow or HCD for different durations. N=3-6 mice/gp. Mixed effects two-way ANOVA: $p = 0.02$ for time; $p = 0.005$ for group; $p = 0.2$ for time x group. p-values from multiple comparison.

(j) Confocal microscopy showing GFP⁺ (Flt3-Cre⁺) (arrow) and tdTom⁺ (Flt3-Cre⁻) (arrowheads) CD68⁺ cells in a cross-section of aortic sinus plaque from a *Flt3*^{Cre} x *Rosa*^{mT/mG} x *Apoe*^{-/-} mouse after 12 w of HCD. Scale bar, 50 μ m.

Data are represented as mean or mean $\pm$ SD of replicate experiments.

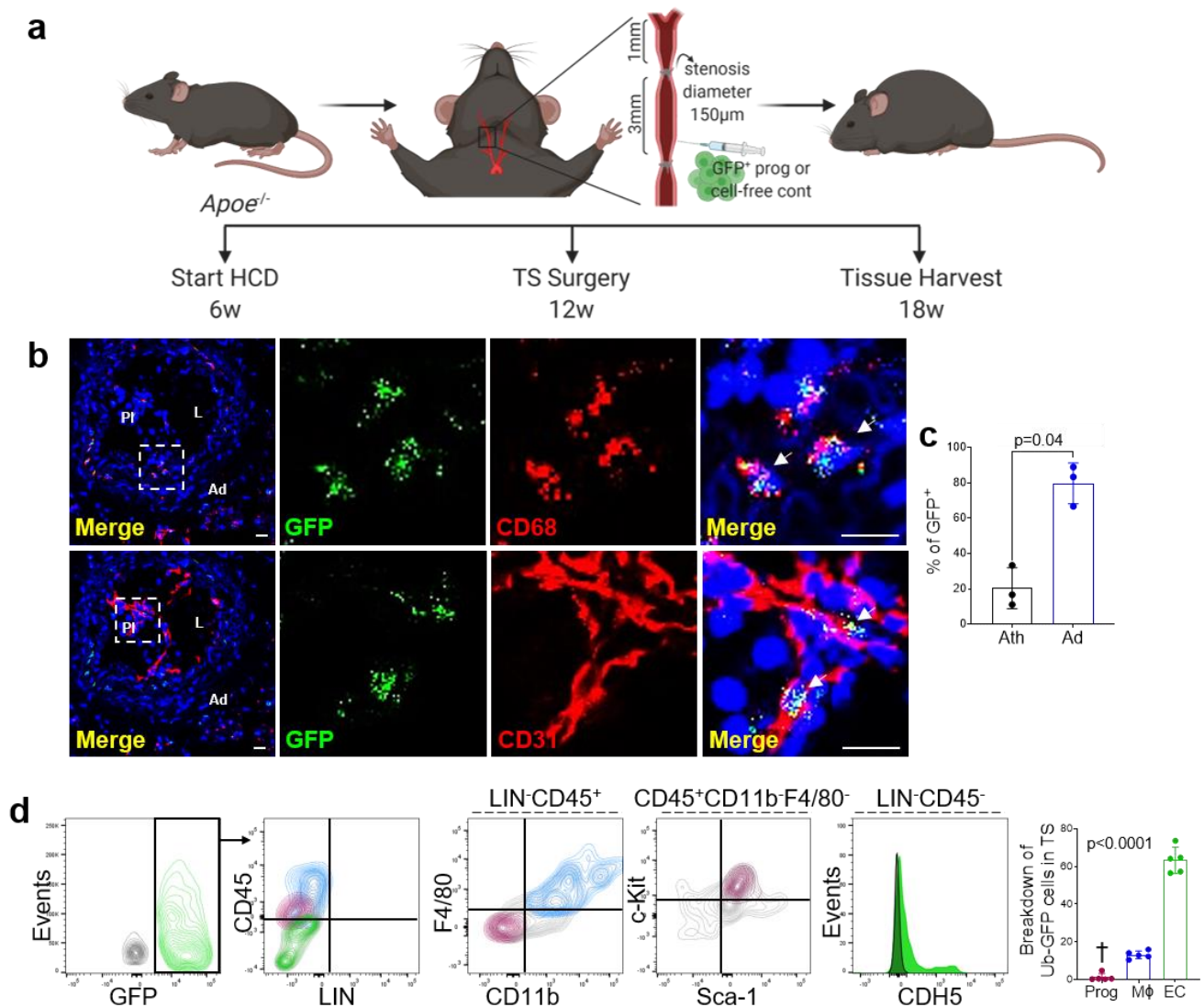


Figure 7-6. The fate of aortic EndoMac progenitors in atherosclerotic plaque.

(a) Experimental design: *Apoe^{-/-}* mice were commenced on HCD at 6 w of age and then 6 w later underwent surgery to create a tandem stenosis (150 µm outer diameter) around the right common carotid artery, with the distal point 1 mm from the carotid artery bifurcation and proximal point 3 mm from the distal stenosis (733). Aortic CFU-M progenitors from 12 w GFP donor mice (1.5×10^4 cells in 60 µL MatrigelTM) were injected into the adventitia of the right carotid artery. A group of mice received MatrigelTM only as a cell-free control. After surgery, mice continued HCD for a further 6 w until sacrifice and tissue harvest.

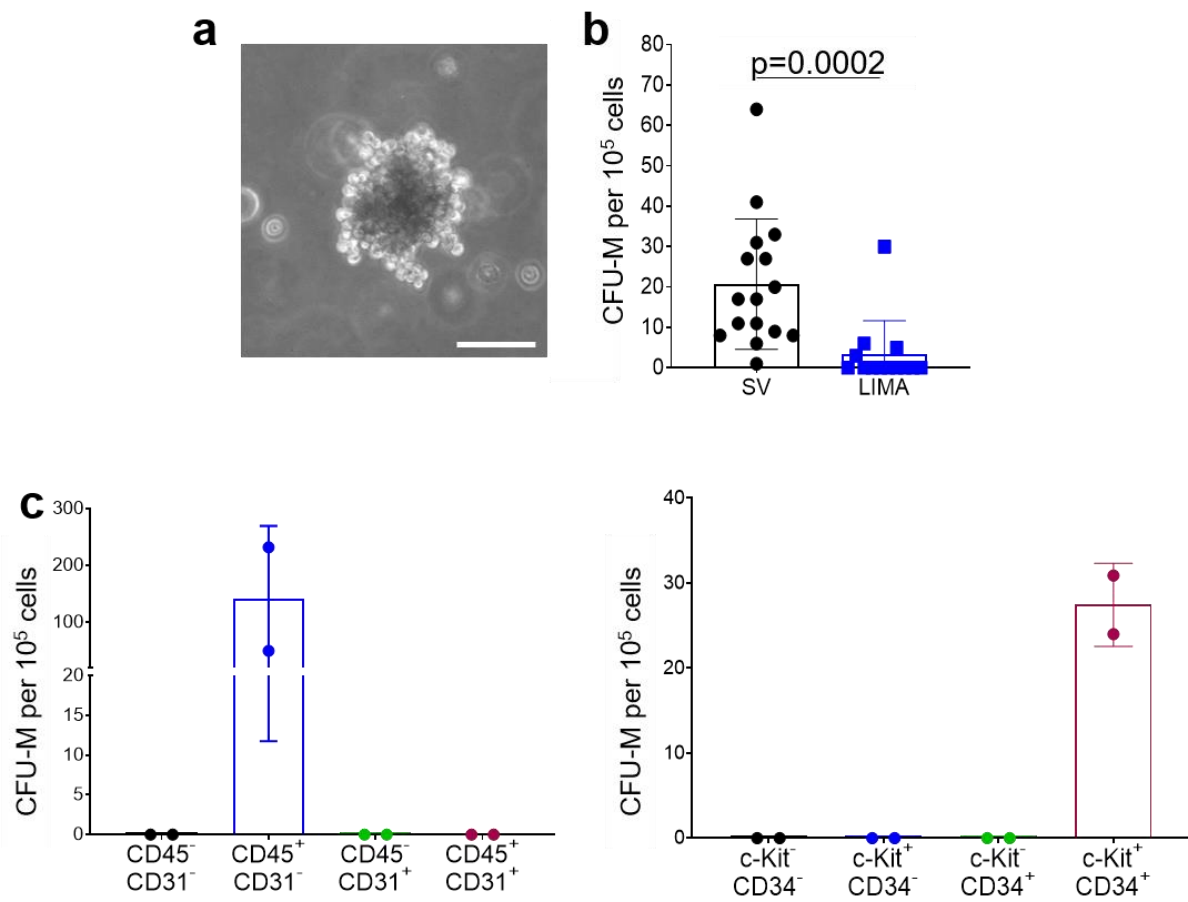
(b) Confocal microscopy showing GFP⁺ donor-derived cells in carotid plaque that co-express CD68 or CD31. Scale bar, 50 µm.

(c) Graph compares distribution of GFP⁺ cells in adventitia versus plaque of recipient carotid arteries (5 sections examined from each of 3 mice). Paired *t*-test.

(d) Flow cytometry profiling of carotid digests 6 w after tandem stenosis surgery and progenitor cell transfer. Graph shows fate of progenitors. N=5 mice. Friedman test. Multiple comparisons test: † $p < 0.01$ compared to EC. Maroon, Progenitors (Prog); Blue, Macrophages (M ϕ); Green, Endothelial cells. Green histogram, Sample; Grey, FMO control.

7.3.2 Discovery of EndoMac progenitors in human tissue

Moving forward, a major translational objective is to investigate analogous progenitor cells in human tissues. Recently, I co-supervised with A/Prof Psaltis an Honours student, Dr Aaron Long, who identified CFU-M forming progenitors in human blood vessels. Segments of SV and IMA were obtained at the time of coronary artery bypass grafting and vessel digests cultured in methylcellulose. As per mouse aorta, these human vessels selectively produced CFU-M after 14 d of culture. However, we observed important vessel-specific differences, such that colonies were substantially more prevalent and larger from SV than IMA (**Figure 7-7**). FACS sorting revealed that these CFU-M arose from a CD45⁺CD31⁻c-Kit⁺CD34⁺ population (n=2 experiments, **Figure 7-7**), which to the best of our knowledge has not previously been described. Another key finding was that the presence of diabetes significantly reduced the prevalence of human CFU-M progenitors, along with their angiogenic capacity in MatrigelTM (**Figure 7-8**). This is of particular interest as diabetic arteries are known to form fewer and less mature *vasa vasorum* (734). Importantly, these results were also corroborated by showing that mouse aortic EndoMac progenitors are also severely depleted in the presence of streptozotocin-induced diabetes, with downregulation of their proliferative, self-renewal and vasculogenic properties.



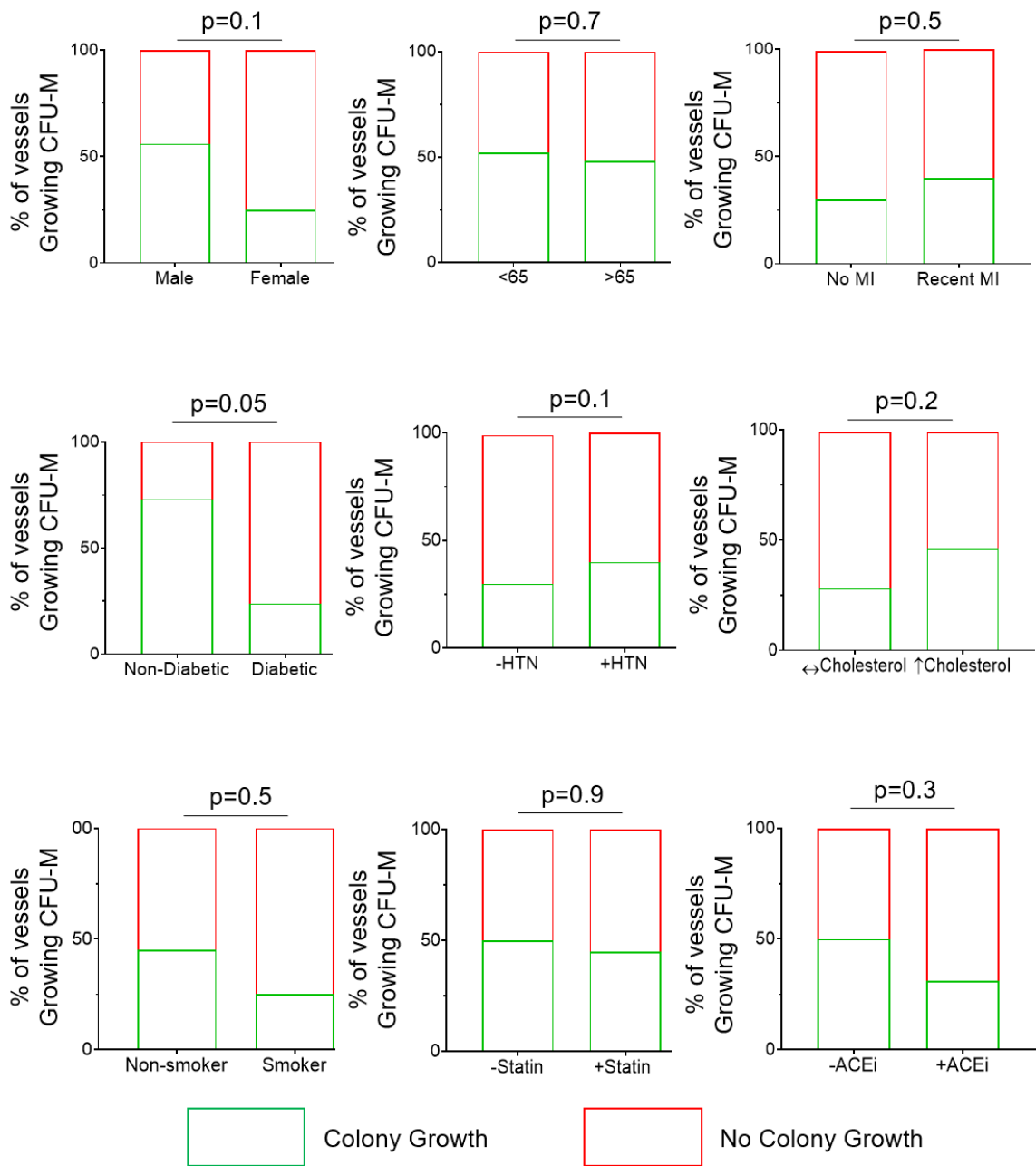
Data generated by Dr. Aaron Long (unpublished).

Figure 7-7. Discovery of CFU-M from human blood vessels.

(a) CFU-M from human SV digest. Scale bar, 100 μ m.

(b) CFU-M yield from donor-matched pairs of SV and IMA digests. N=13-16 samples. Wilcoxon test.

(c) CFU-M yield from CD45/CD31 and c-Kit/CD34 subpopulations that were FACS-isolated from SV specimens. N=2 from 2 separate experiments.



Data generated by Dr. Aaron Long (unpublished).

Figure 7-8. Impact of patient clinical factors on CFU-M recovery from SV.

Graphs showing percentage of SV specimens (n=33) that grew CFU-M after 14 d in methylcellulose, according to the presence or absence of selected clinical characteristics. Diabetes was the only clinical risk factor associated with a significant reduction in the recovery of CFU-M. All statistical comparisons were performed by Chi-square test.

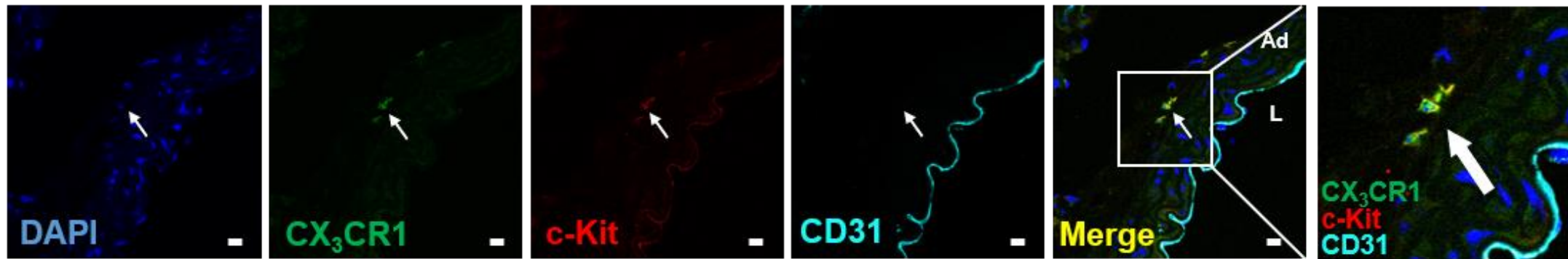
7.3.3 Impact on tissue repair

Chapters 5 and 6 indicated adaptive roles for EndoMac progenitors in mediating perfusion recovery after skeletal muscle ischaemia and skin repair post wound injury. It now remains to be seen whether this also applies to the progenitors in heart after myocardial infarction. Recent studies have shown that resident macrophages help to drive the potent regenerative ability of neonatal mouse heart, which subsequently wanes after the first week of life (167, 179, 628, 735-737). We believe that this postnatal decline in tissue recoverability may also relate to age-related depletions in tissue stores of EndoMac progenitors and accompanying attenuation of their “stemness” properties. Similar inhibitory effects of high glucose exposure may also help explain why diabetic tissues are particularly vulnerable to the effects of injury and ischaemia. Studies are now needed to examine these theories further and to ultimately develop new therapeutic strategies that can rescue or enhance these progenitors to promote effective tissue repair.

7.4 Conclusion

New blood vessel formation is central to many pathophysiological processes and is closely intertwined with inflammation. Understanding the interactions between macrophages and endothelial cells opens up new therapeutic opportunities to target different disease processes. In discovering and characterising YS-derived EndoMac progenitor cells in different tissues, this thesis provides a major conceptual advance and a new paradigm to help explain the postnatal maintenance and renewal of tissue-resident macrophages and endothelial cells under both steady state and diseased conditions.

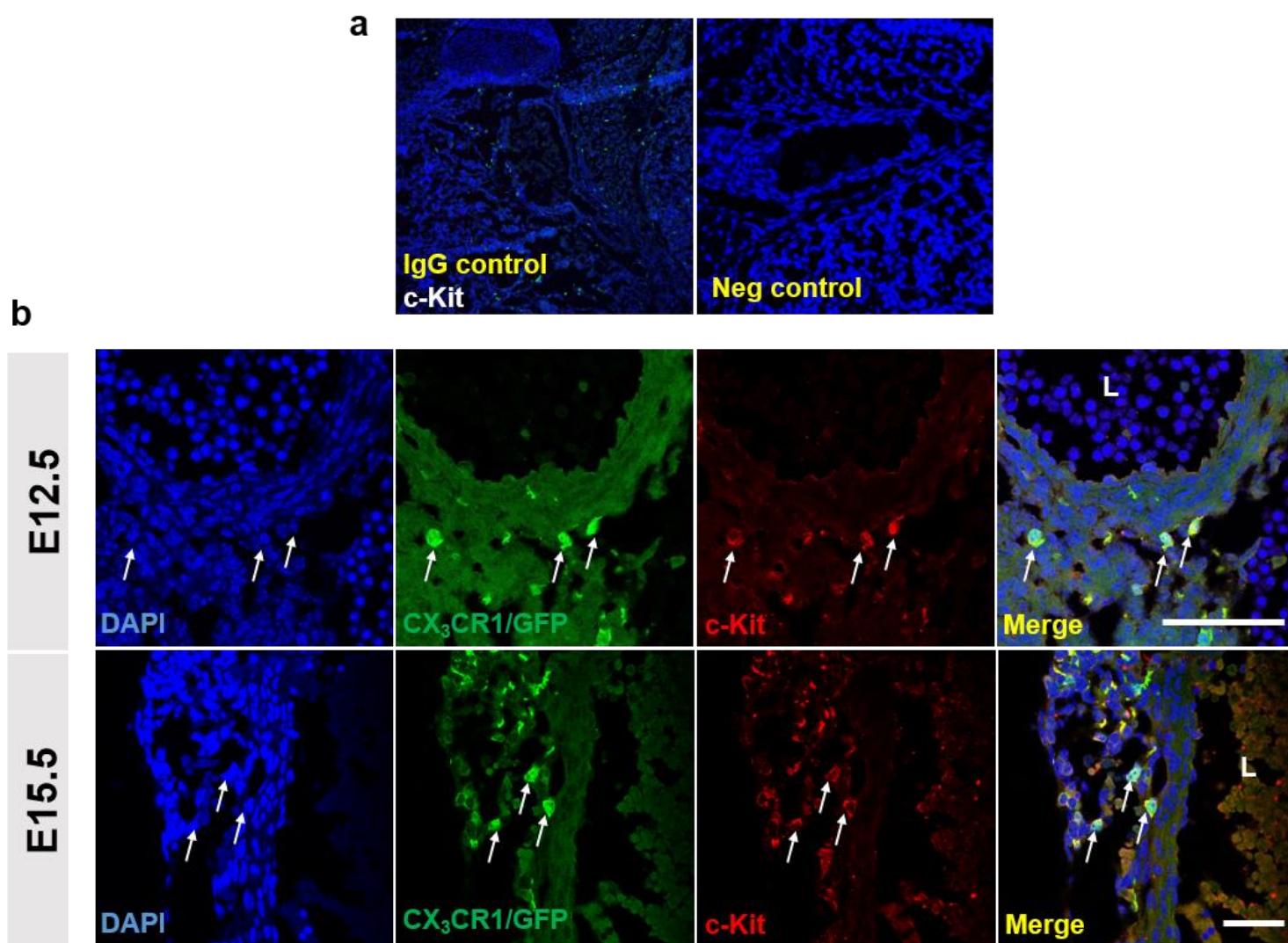
Appendices



Appendix 1. Confocal microscopy of descending aorta from 12 w *Cx3cr1*^{GFP/+} mouse shows adventitial CX₃CR1⁺c-Kit⁺ progenitors.

Image from Figure 4-1

Ad, Adventitial; L, Lumen. Scale bar, 20 μm.



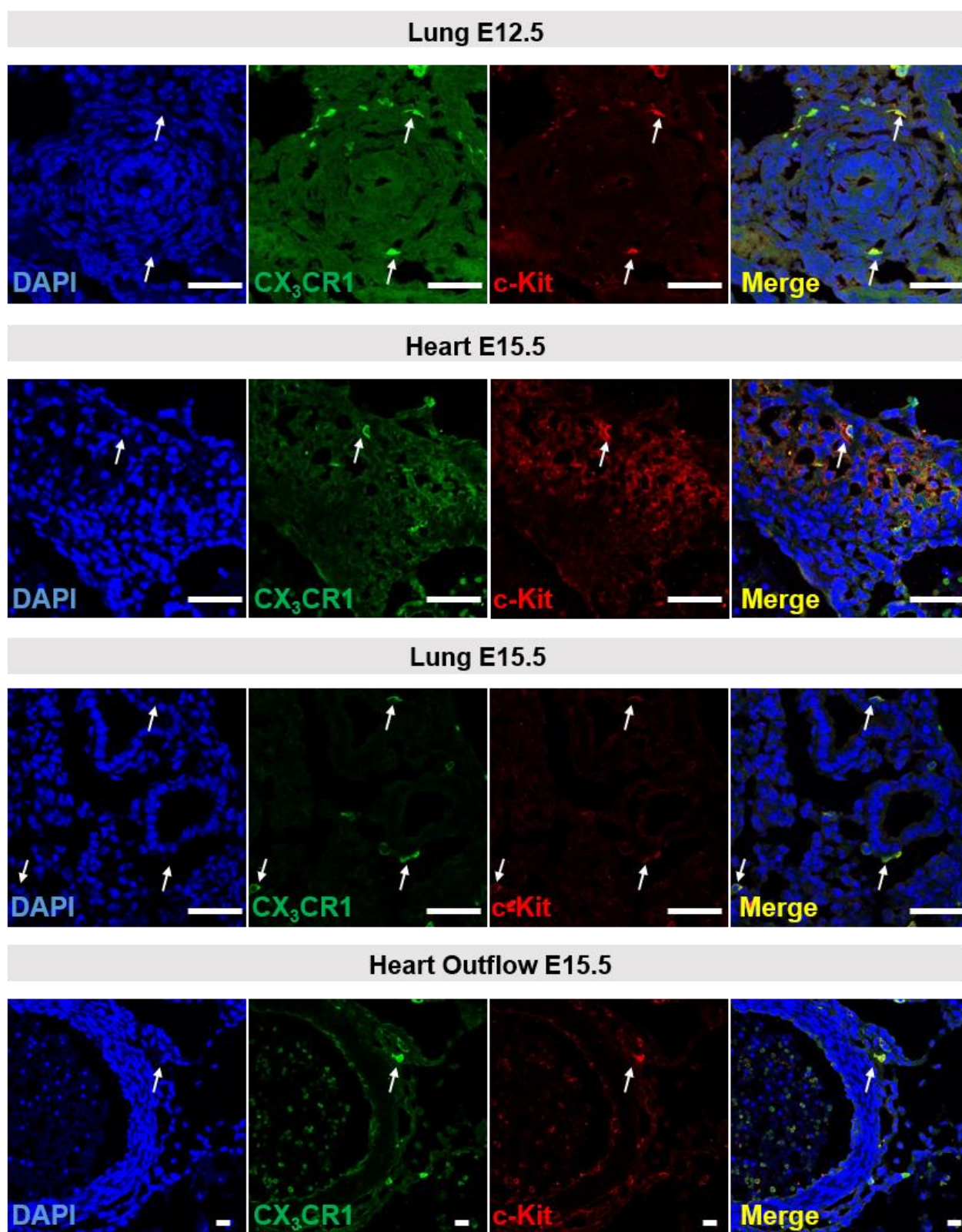
Appendix 2. Confocal microscopy of E12.5 and E15.5 AGM from *Cx3cr1*^{GFP/+} mice.

(a) Merged IgG isotype and no GFP (Neg) control staining.

(b) Confocal microscopy of E12.5 and E15.5 AGM showing adventitial CX₃CR1⁺c-Kit⁺ progenitors (arrows).

Image from Figure 4-4

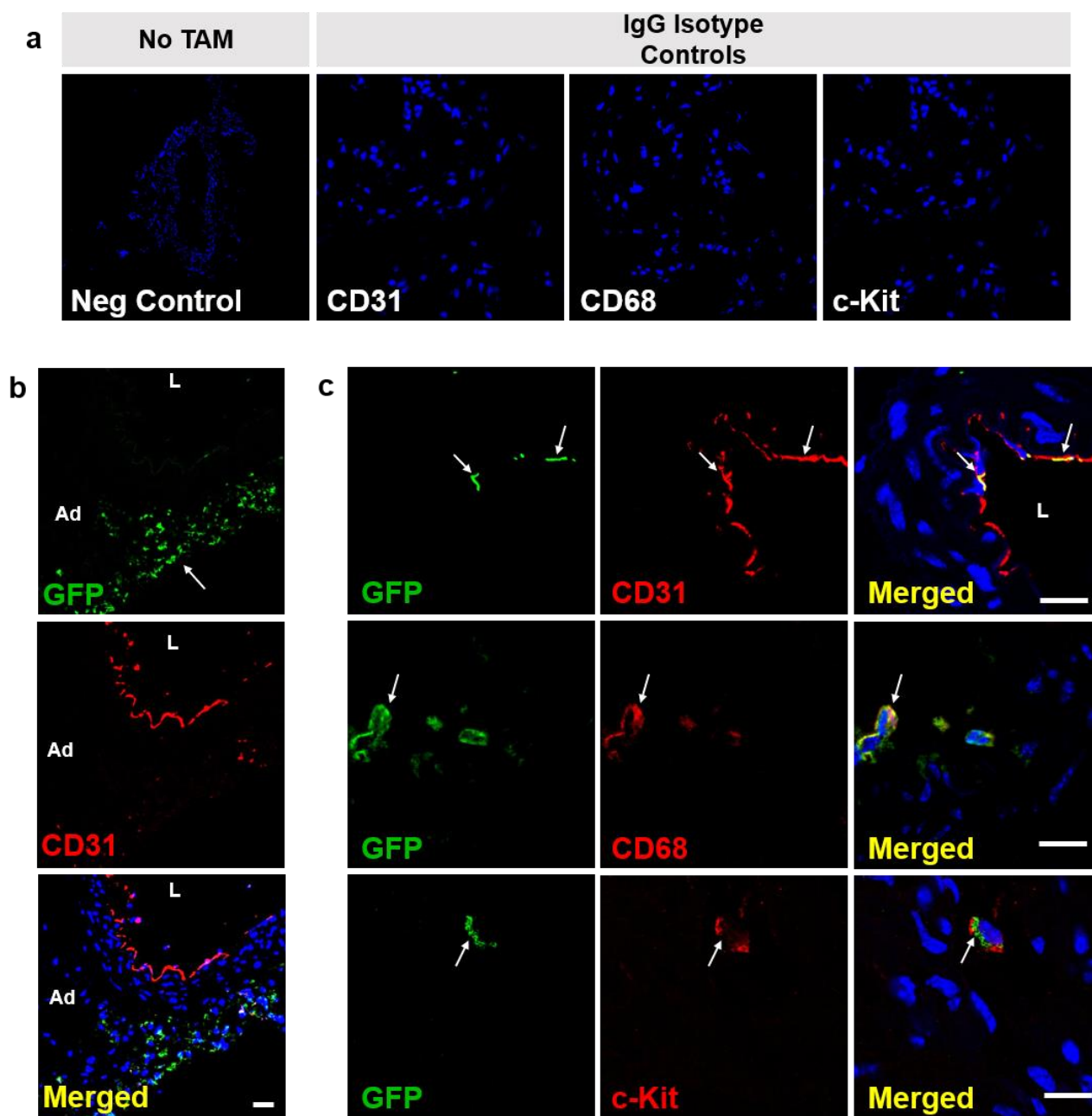
L, Lumen. Scale bar, 50 μm.



Appendix 3. Confocal microscopy of CX₃CR1⁺c-Kit⁺ progenitor cells in lung and heart at E12.5 and E15.5 from *Cx3cr1*^{GFP/+} embryos.

Image from Figure 4-6

Scale bar, 50 μm.



Appendix 4. Confocal microscopy of 12 w aorta from *Csf1r*^{MerCreMer} x *Rosa*^{GFP} mice

which were induced with TAM at E8.5.

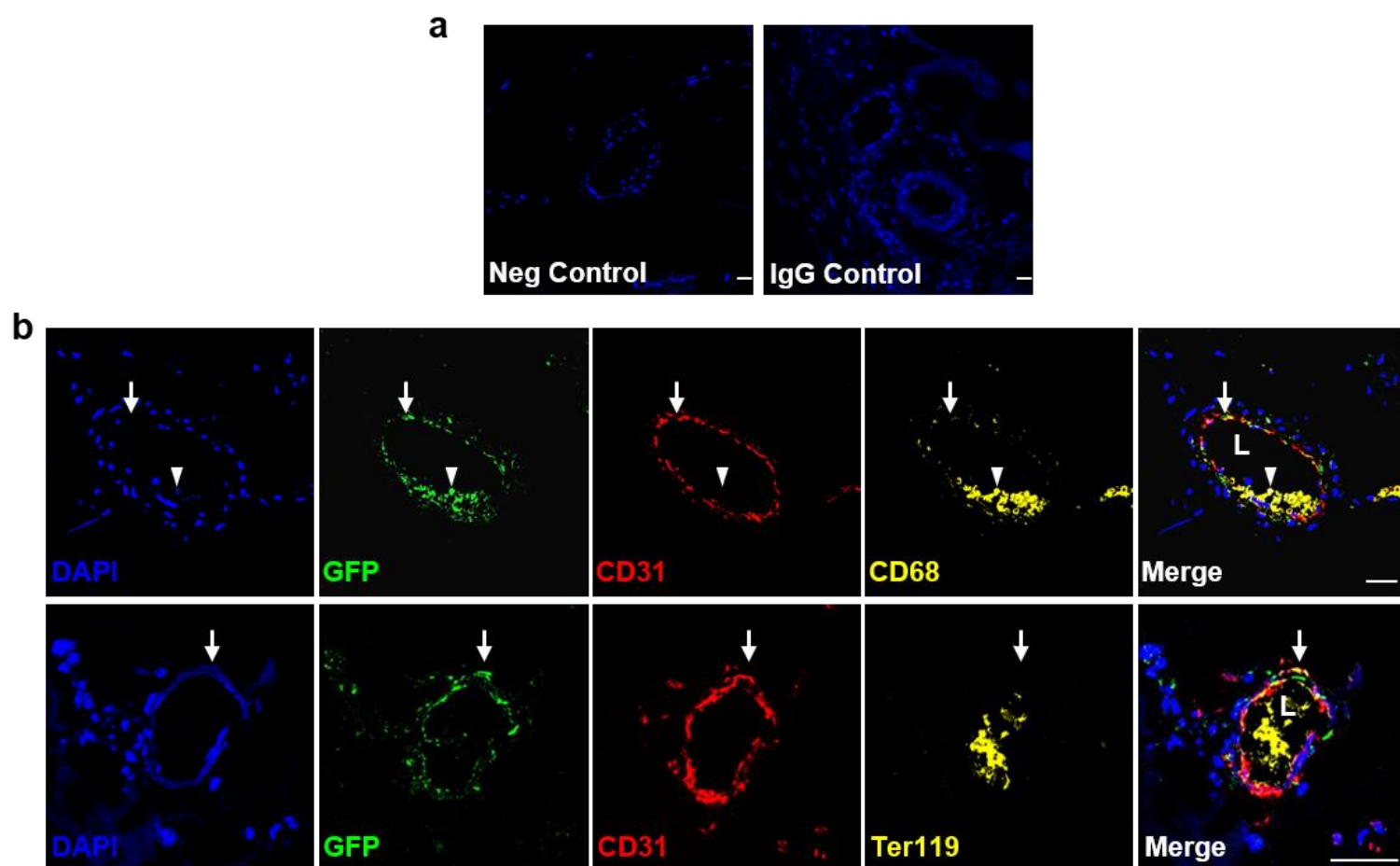
(a) No TAM control (Neg) and IgG control staining shown for each set of images.

(b) GFP⁺ cells in adventitia

(c) Magnified examples of GFP⁺ cells co-expressing CD31 in intima and CD68 and c-Kit in adventitia (arrows).

Image from 4-7

Ad, Adventitia; L, Lumen. Scale bar, 50 μ m (left) and 20 μ m (right).



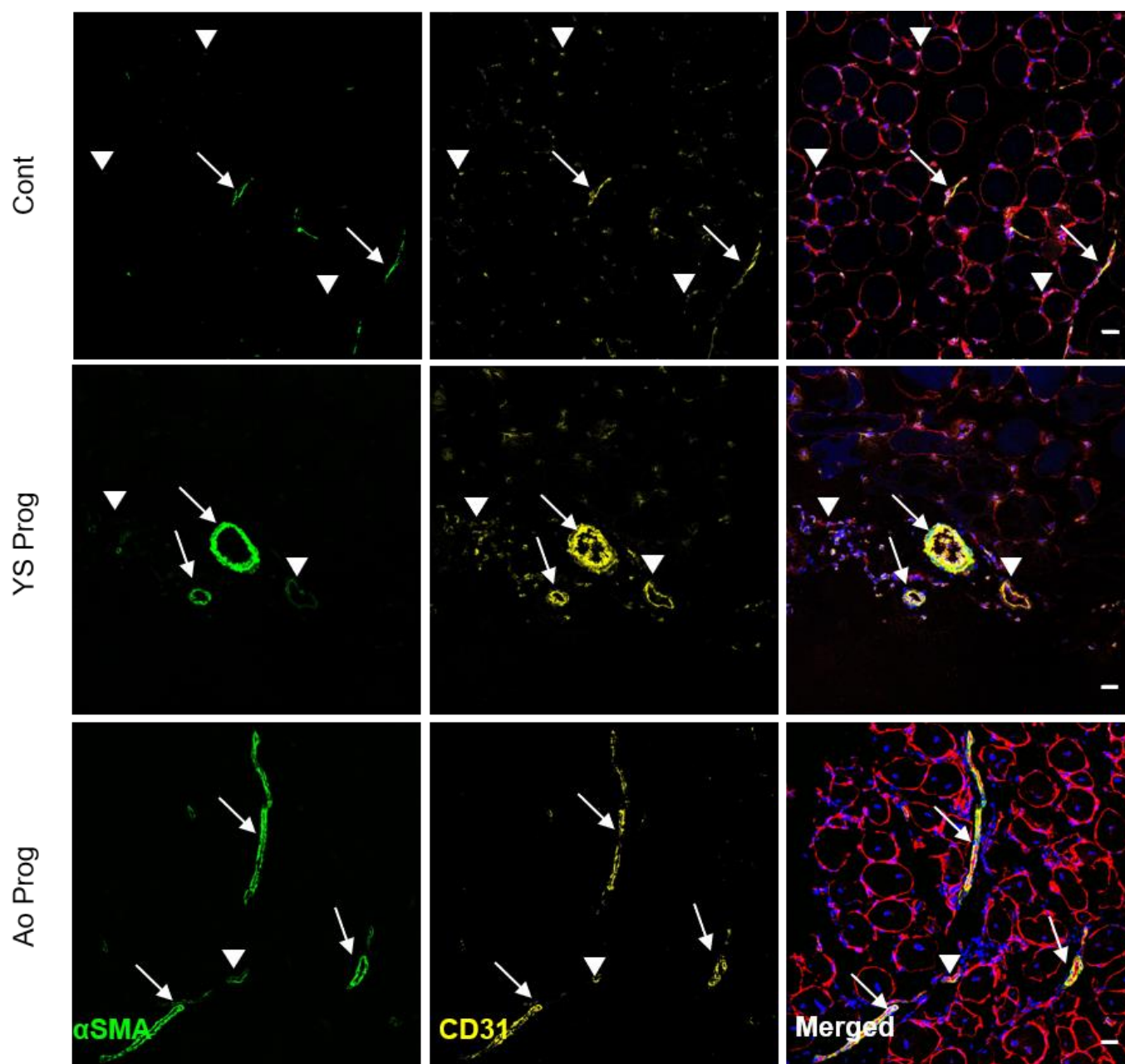
Appendix 5. Confocal microscopy of ischaemic muscle from mice receiving GFP⁺ aortic progenitors.

(a) Merged no antibody (Neg) and IgG isotype control staining.

(b) Confocal microscopy showing neovessels lined by GFP⁺CD31⁺ endothelial cells (arrows) with cluster of GFP⁺CD68⁺ Macrophages (arrowhead) (above) and perfused with host Ter119⁺ erythrocytes (below).

Image from Figure 4-10

L, Lumen. Scale bar, 20 μ m.

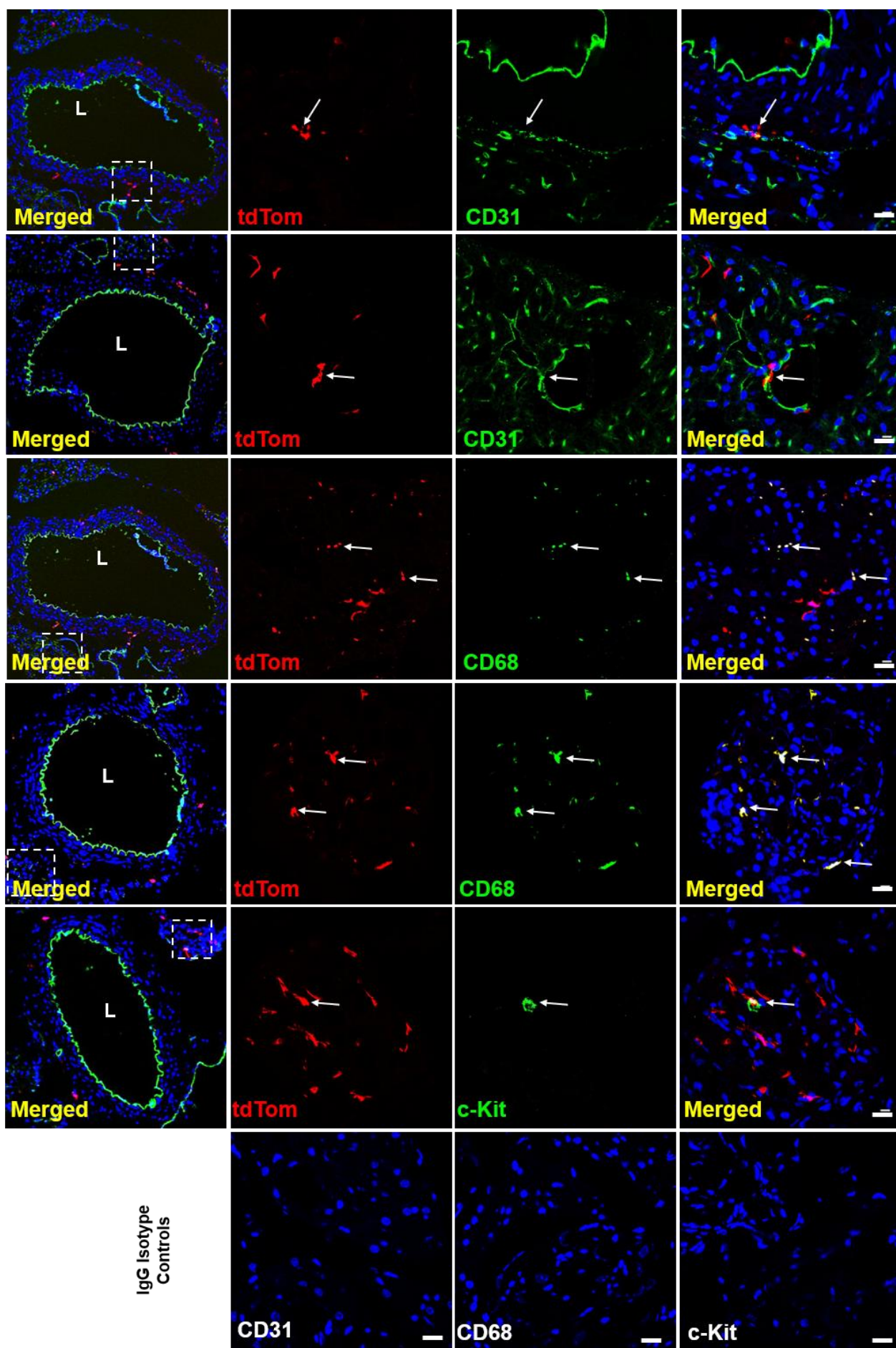


Appendix 6. Immunofluorescence microscopy of sections of hindlimb muscle at day 14 that had received injections of Matrigel™ control, E9.5 YS CFU-M progenitors or Aortic CFU-M progenitors.

Counter-staining with antibody to laminin (red) for myocytes and DAPI (blue) for nuclei.

Image from Figure 4-11

Ao, Aorta; Cont, Cell-free control; YS, Yolk Sac. Scale bar, 100 μ m.



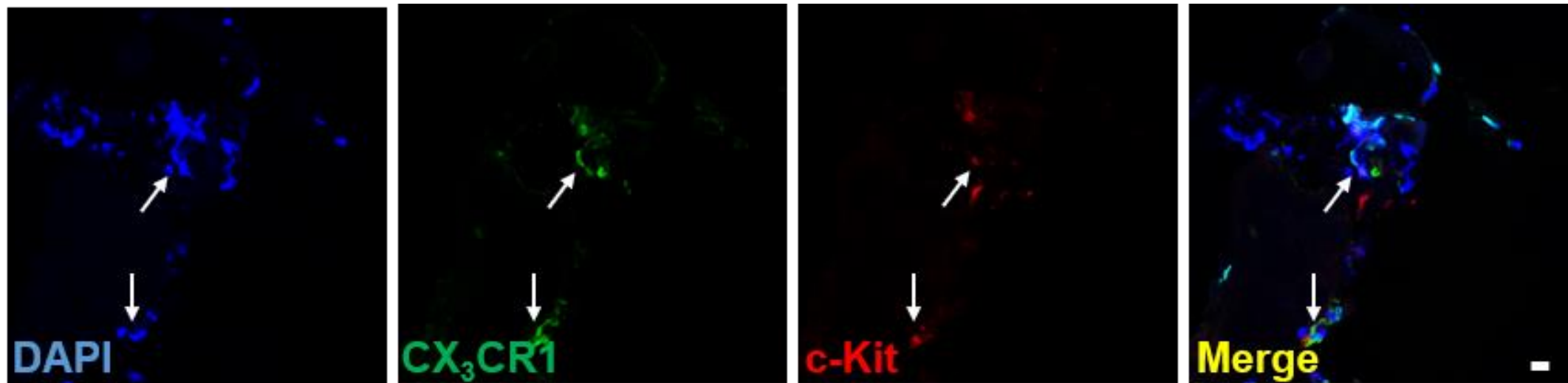
Appendix 7. Confocal microscopy images of sections of aorta from adult *Cx3cr1*^{CreER-YFP} x *Rosa*^{tdTom} mice that were induced with TAM at E9.5 and treated with AngII infusion for 48 h.

Lower power images are merged from staining for CD31 (green), tdTom (red) and DAPI (blue). Dotted boxes relate to adjacent magnified images that show tdTom⁺ cells in adventitia co-staining for CD31, CD68 or c-Kit (arrows).

IgG control staining shown for each set of images.

Image from 4-14

L, Lumen. Scale bar, 50 μ m.

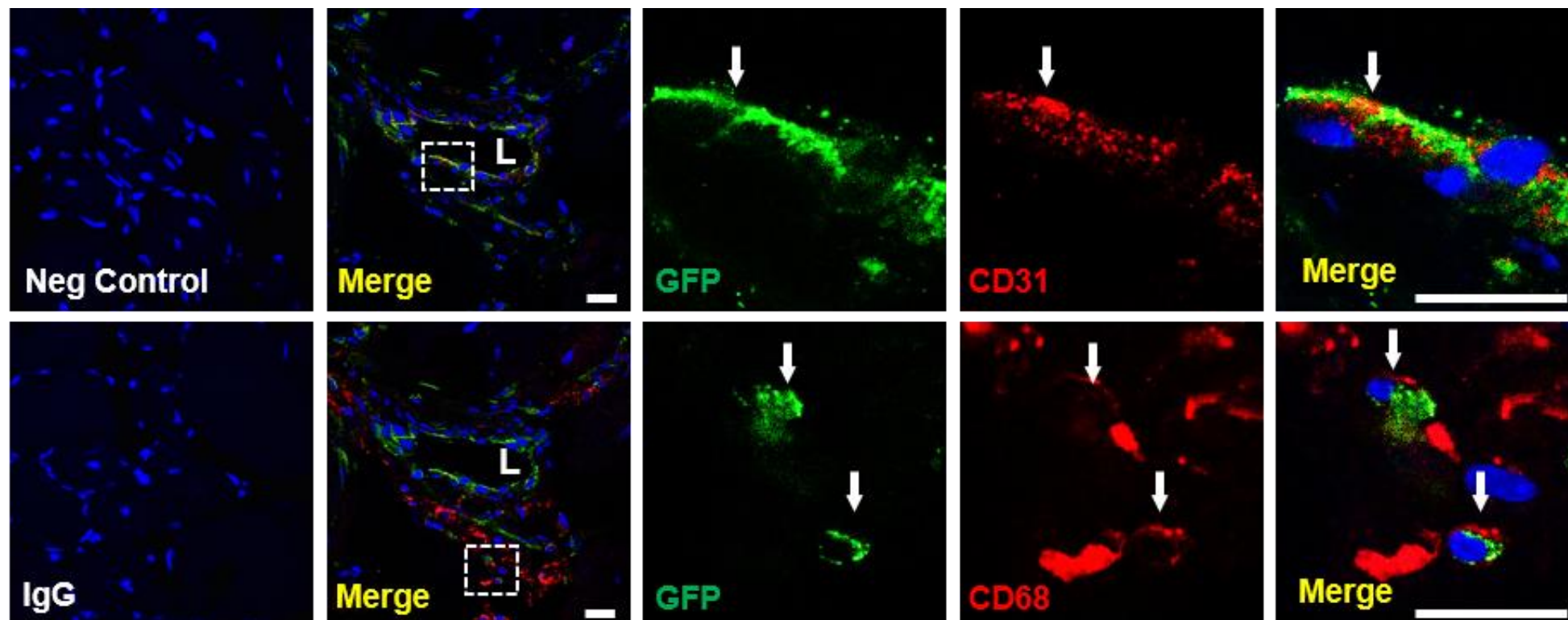


Appendix 8. Confocal microscopy of SkM from 12 w *Cx3cr1*^{GFP/+} mouse shows CX₃CR1⁺c-Kit⁺ progenitors.

Image from Figure 5-1

Scale bar, 100 μ m.

Appendix 9. Confocal microscopy of ischaemic gastrocnemius shows neovessels lined by GFP⁺CD31⁺ endothelial cells (top) with nearby

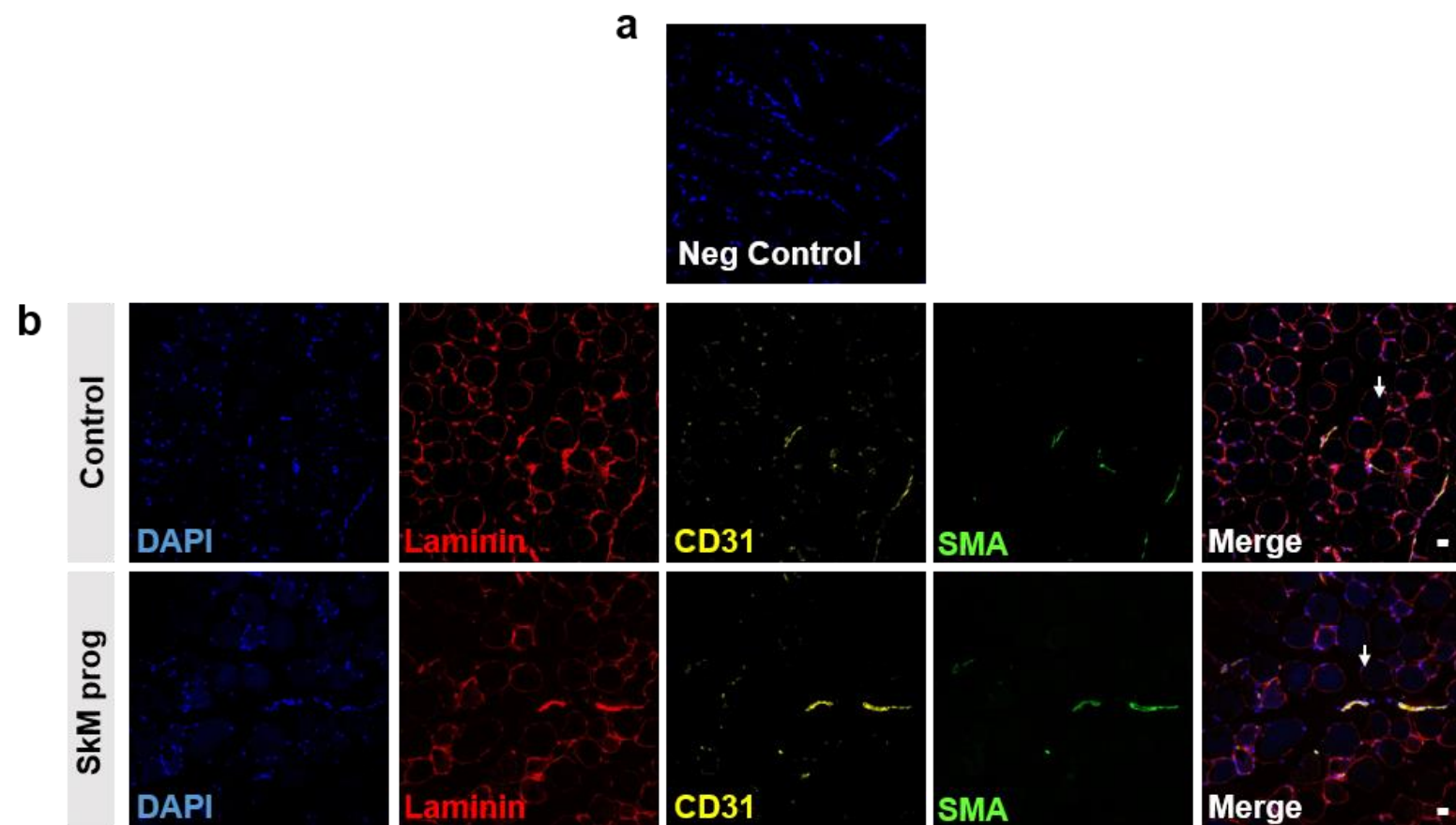


GFP⁺CD68⁺ macrophages (bottom).

Merged no antibody (Neg) and IgG isotype control staining.

Image from Figure 5-9

L, Lumen. Scale bar, 20 μ m.



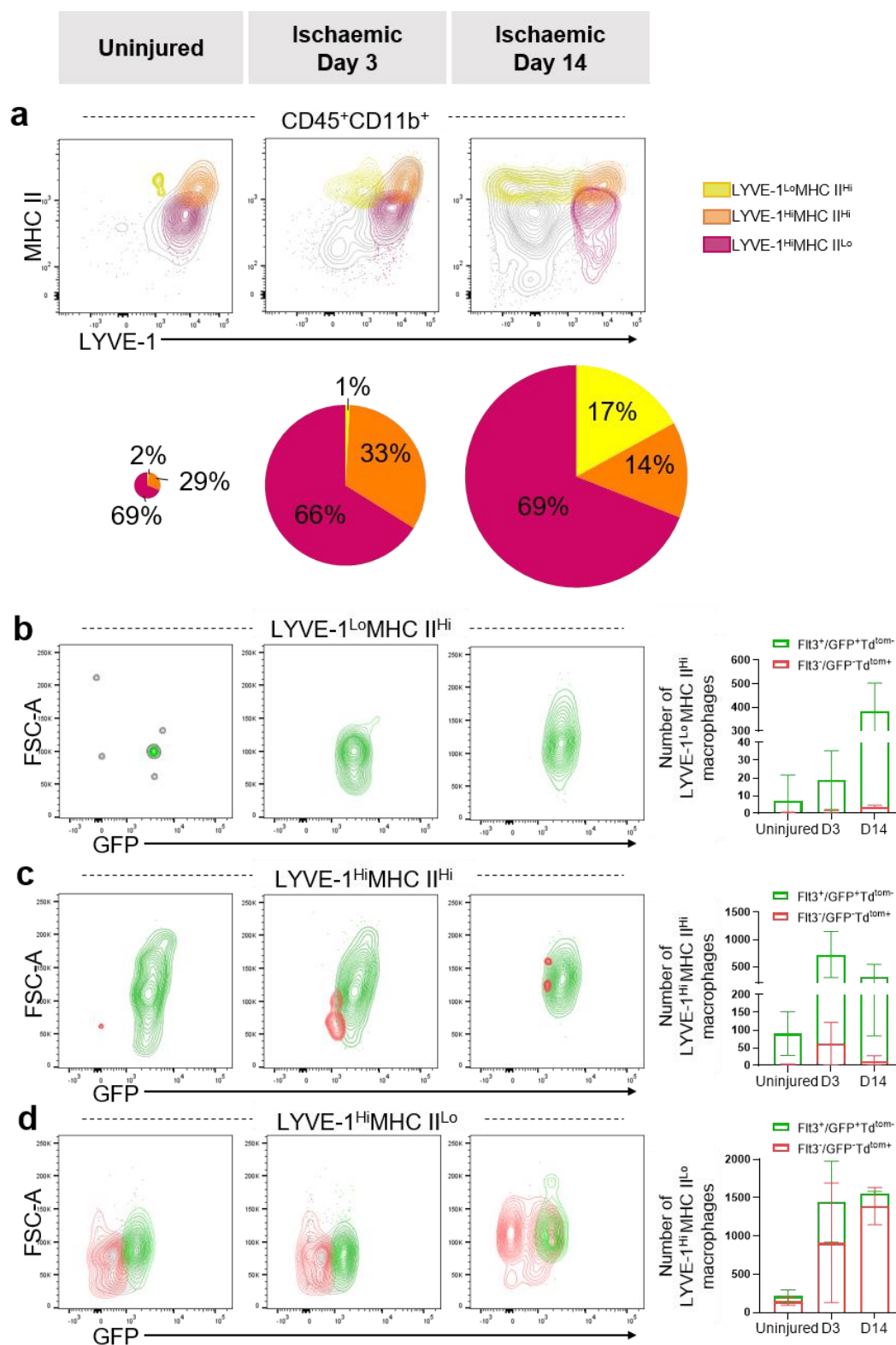
Appendix 10. Immunofluorescence microscopy of sections of gastrocnemius at day 14 that had received injections of Matrigel™ control or SkM progenitors.

(a) Merged no antibody (Neg) control staining.

(b) Counter-staining with antibody to laminin (red) for myocytes and DAPI (blue) for nuclei.

Image from Figure 5-9

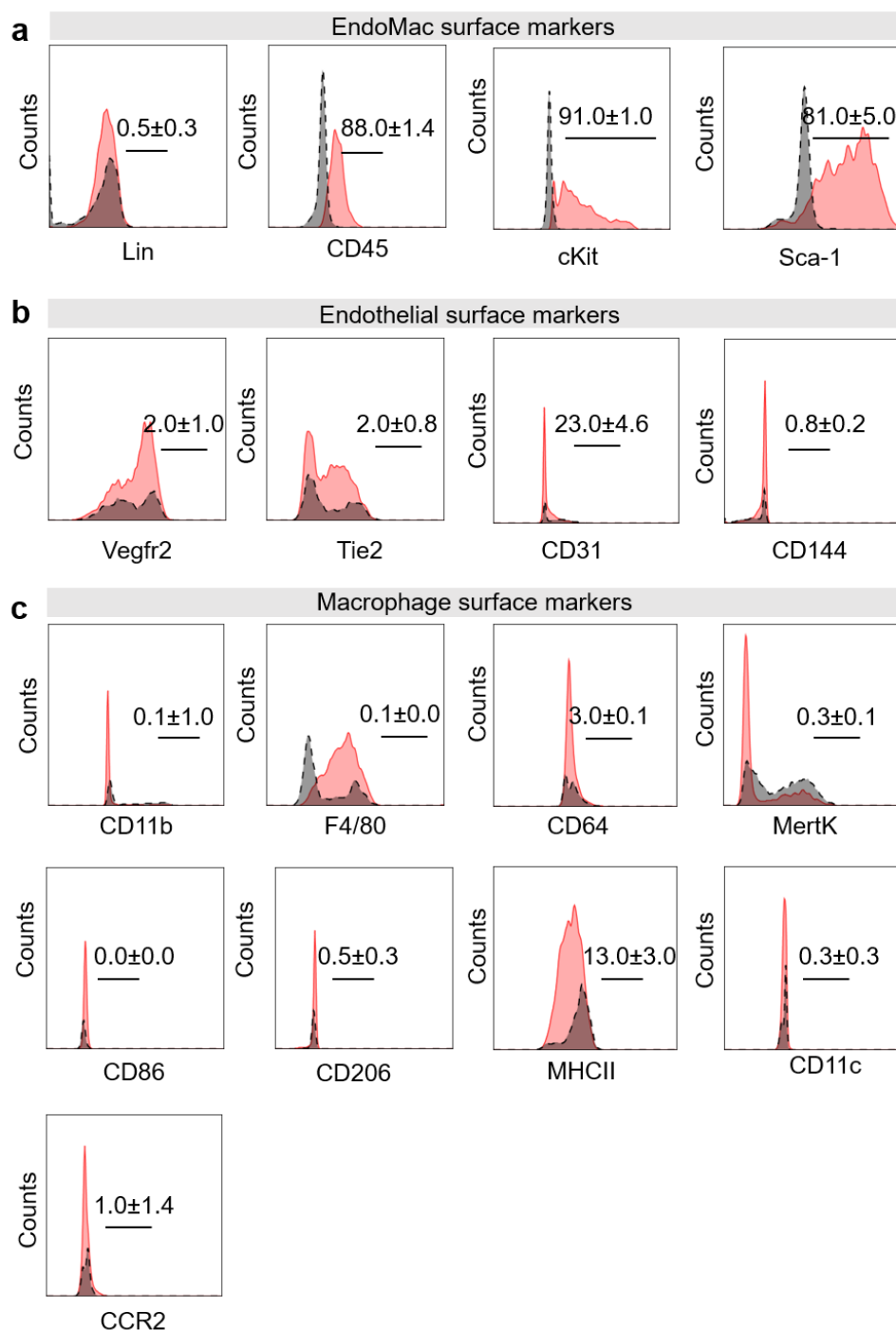
Scale bar, 100 μ m.



Appendix 11. LYVE-1^{Lo}MHCII^{Hi}, LYVE-1^{Hi}MHCII^{Hi} and LYVE-1^{Hi}MHCII^{Lo} SkM macrophage subsets arise from different origins postnatally.

(a). Flow cytometry analysis of MHCII and LYVE-1 expression in SkM of *Flt3^{Cre}* x *Rosa^{mT/mG}* mice after hindlimb ischaemia. Hindlimb muscle were harvested 3 d and 14 d after surgery. Pie charts comparing the breakdown of macrophage subsets during steady state, 3 d and 14 d after surgery from left to right. Relative sizes of pie charts are intended to absolute number from each age group. LYVE-1^{Lo}MHCII^{Hi} (yellow) LYVE-1^{Hi}MHCII^{Hi} (orange) and LYVE-1^{Hi}MHCII^{Lo} (dark red).

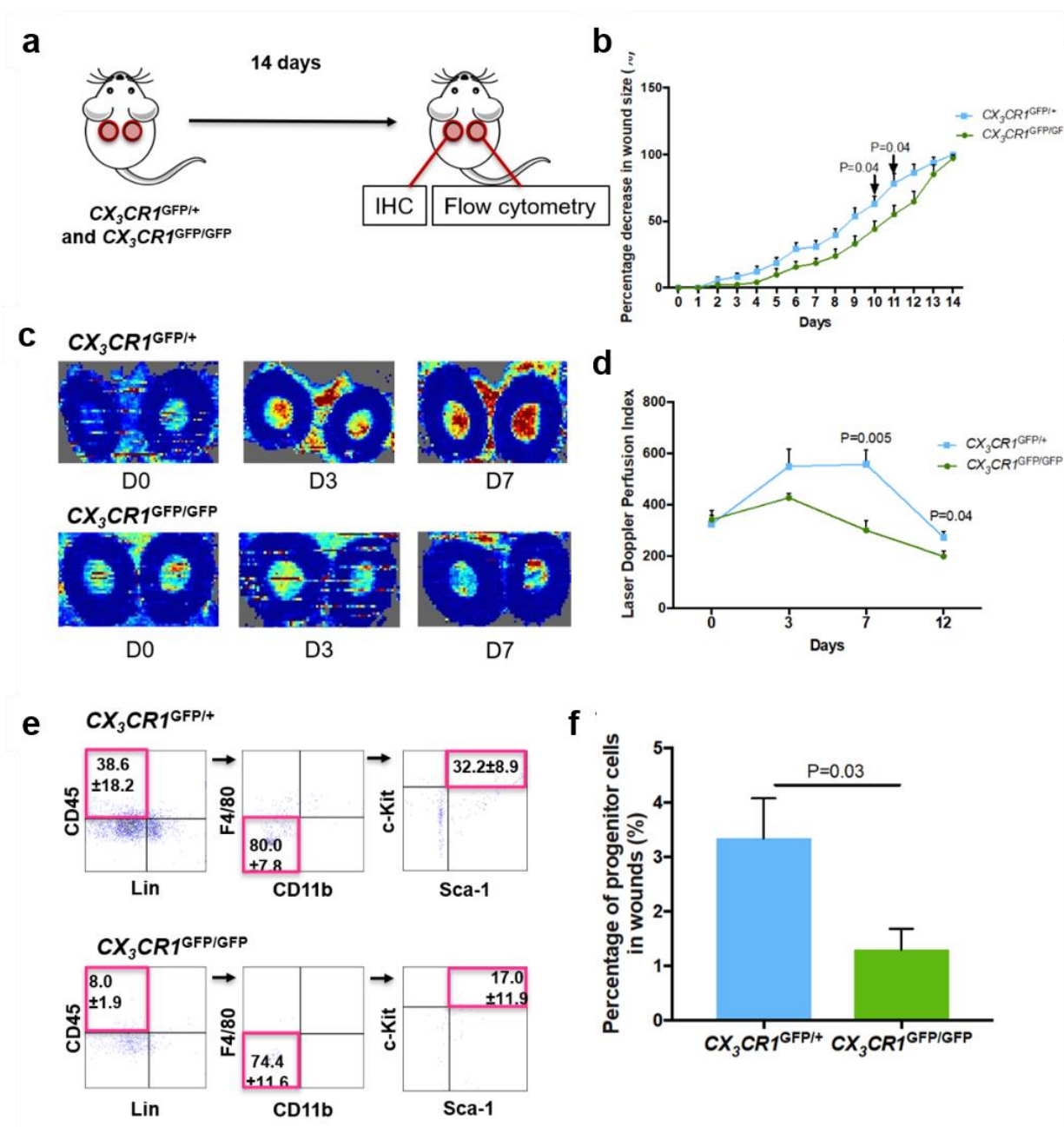
(b-d) Expression of Flt3⁻ (light red) vs Flt3⁺ (green) in **(b)** LYVE-1^{Lo}MHCII^{Hi}, **(c)** LYVE-1^{Hi}MHCII^{Hi} and **(d)** LYVE-1^{Hi}MHCII^{Lo} subpopulations ischaemic muscle 3 d vs 14 d after surgery.



Appendix 12. Phenotypic characterisation of skin CFU-M progenitors.

Data generated by Ms. Sanuri Liyanage (unpublished).

(a-c) Representative flow cytometry histograms (pink) depict the expression of **(a)** EndoMac **(b)**, endothelial and **(c)** myeloid markers on skin CFU-M progenitors. Numbers show mean ± SEM % expression for each marker relative to its FMO control (grey histogram) from n=3 mice.



Appendix 13. CX_3CR1 deficiency results in delayed wound healing.

Data generated by Ms. Sanuri Liyanage (unpublished).

(a) Study schematic.

(b) Graph showing comparison of the percentage of wound closure achieved in the $Cx3cr1^{GFP/+}$ and $Cx3cr1^{GFP/GFP}$ groups over time. N=5 mice/gp.

(c) Representative doppler perfusion images of wounds from both groups at different time-points.

(d) Summary of doppler perfusion results at different time-points. N=5 mice/gp.

(e) Representative flow cytometry dot plots used to quantify the content of LIN⁺CD45^{+/Lo}CD11b⁺F4/80⁺Sca-1⁺c-Kit⁺ progenitor cells in the day 14 healed wounds of both groups of mice. Initial gating was performed off the viable, single cell population.

(f) Summary of percentage progenitor cells. N=5 mice/gp.

For all data, mean±SEM is shown, with inter--group statistical comparisons performed by unpaired Student *t*-tests, including at each time-point shown in **(b)** and **(d)**

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