IMMUNOREGULATORY EFFECTS OF VITAMIN D₃ ON MAST CELLS DURING IMMUNOGLOBULIN E-DEPENDENT IMMUNE RESPONSES

A thesis submitted in partial fulfilment of the PhD degree

in

School of Molecular & Biomedical Science
Faculty of Sciences
The University of Adelaide

by

Chunping (Anastasia) Yu

April 2014
# Table of contents

IMMUNOREGULATORY EFFECTS OF VITAMIN D₃ ON MAST CELLS DURING IMMUNOGLOBULIN E-DEPENDENT IMMUNE RESPONSES ........................................ 1  
Table of contents ................................................................................................................. 2  
Declaration .......................................................................................................................... 8  
Abbreviations .................................................................................................................... 10  
Acknowledgments ............................................................................................................. 14  
Thesis summary ................................................................................................................. 15  
CHAPTER 1 ...................................................................................................................... 17  
LITERATURE REVIEW .................................................................................................................. 17  
1.1. Mast Cells (MCs) ................................................................................................................. 18  
1.2. Basic Biology of MCs ........................................................................................................... 18  
1.2.1. MC Ontogeny and Tissue Distribution ......................................................................... 18  
1.2.2. Homing of MCPs ......................................................................................................... 19  
1.2.3. Trafficking of Mature MCs .......................................................................................... 20  
1.2.4. MC Heterogeneity ...................................................................................................... 22  
1.2.5 MC Development and Maturation ............................................................................. 23  
1.2.6. MC Activation .......................................................................................................... 24  
1.2.7. MC-derived Mediators ............................................................................................... 25  
1.2.8. FcεRI Signalling in MCs ............................................................................................ 26  
1.3. Biological Functions of MCs in IgE-dependent Allergic Reactions ................................. 28  
1.3.1. IgE-dependent Allergic Reactions and MCs ................................................................. 28  
1.3.2. Functions of MCs during Early-phase Allergic Reactions ............................................ 29  
1.3.3. Functions of MCs during Late-phase Allergic Reactions ............................................. 30  
1.3.4. Functions of MCs during Chronic Allergic Reactions ............................................... 31  
1.3.5. Treatment Strategies for Allergic Reactions and MCs ............................................... 33  
1.4. Other Biological Functions of MCs ................................................................................. 34  
1.4.1. Roles of MCs in Homeostasis ...................................................................................... 34  
1.4.2. Roles of MCs in Parasitic Infections .......................................................................... 35  
1.4.3. Roles of MCs in Bacterial and Viral Infections ........................................................... 36  
1.4.4. Roles of MCs in Cancers ............................................................................................ 37  
1.4.5. Roles of MCs in Chronic Obstructive Pulmonary Diseases (COPD) ......................... 38
1.4.6. Negative Immunoregulatory Roles of MCs ................................................................. 39

1.5. Experimental Tools for MC Research ........................................................................ 41

1.5.1. In Vitro Tools ........................................................................................................... 41

1.5.2. In Vivo Tools ............................................................................................................ 43

1.6. VitD₃ (Cholecalciferol) ............................................................................................... 46

1.7. Biosynthesis and Metabolism of VitD₃ ...................................................................... 47

1.8. Biological Activities of VitD₃: UVB Protection and Immunoregulation ...................... 48

1.8.1. The Genomic Pathway (Figure 1.5a) ..................................................................... 48

1.8.2. The Non-genomic Pathway (Figure 1.5b) ................................................................. 51

1.9. VitD₃ and MCs ............................................................................................................. 52

1.9.1. VitD₃-mediated Immunoregulation of MCs ............................................................. 52

1.9.2. Potential Mechanisms for VitD₃-mediated Immunoregulation of MCs .................... 53

1.10. Rationales for this Study .......................................................................................... 54

1.11. Hypothesis ................................................................................................................ 55

1.12. Project Aims .............................................................................................................. 56

1.13. Expected Outcomes and Significance ...................................................................... 60

CHAPTER 2 ...................................................................................................................... 62

MATERIALS & GENERAL METHODS ............................................................................... 62

2.1. Commercial Reagents ............................................................................................... 63

2.1.1. Common Tissue Culture Reagents ......................................................................... 63

2.1.2. Vitamin D₃ Analogues ............................................................................................ 64

2.1.3. Cytokines and Recombinant Proteins .................................................................... 64

2.1.4. Hybridomas ........................................................................................................... 64

2.1.5. Antibodies for Tissue Culture ................................................................................. 64

2.1.6. Reagents for SPE-7 Purification .............................................................................. 65

2.1.7. β-Hexosaminidase Release Assay Reagents ............................................................ 65

2.1.8. ELISA/EIA Kits and Reagents ................................................................................ 65

2.1.9. Flow Cytometry Reagents ....................................................................................... 66

2.1.10. Histochemistry & Immunohistochemistry Reagents ............................................. 66

2.1.11. SDS-PAGE & Western Blotting Reagents ............................................................ 67

2.1.12. Primers (all used at 5 µg/mL): Geneworks ......................................................... 68

2.1.13. Molecular Biology Reagents .................................................................................. 68
2.1.14. In Vivo Experiment Reagents

2.2. Solutions and Buffers

2.2.1. Tissue Culture Media and Solutions

2.2.2. Buffers and Solutions for SPE-7 Purification

2.2.3. Buffers and Solutions for β-Hexosaminidase Release Assay

2.2.4. ELISA Buffers and Solutions

2.2.5. Flow Cytometry Buffers and Solutions

2.2.6. Histology/Immunohistology Solutions

2.2.7. Buffers and Solutions for SDS-PAGE & Western Blotting

2.2.8. Molecular Biology Buffers and Solutions

2.2.9. Buffers and Solutions for In Vivo Experiments

2.3. Purification of Mouse α-DNP IgE (SPE-7 clone)

2.3.1. Conjugation of DNP and BSA

2.3.2. Coupling of DNP-BSA to HiTrap NHS-activated HP column

2.3.3. De-activation of Excess Active Groups

2.3.4. Purification of SPE-7 from Hybridoma Culture Supernatant

2.4. Mice

2.5. General Methods

2.5.1. Culturing of mBMCMCs

2.5.2. Culturing of hCBMCs

2.5.3. Culturing of WEHI-3 hybridoma

2.5.4. Culturing of SPE-7 hybridoma

2.5.2. Flow Cytometry

2.5.2.1. Surface Ag Labelling

2.5.2.2. Intracellular Ag Labelling

2.5.3. In Vitro Procedures

2.5.3.1. β-hexosaminidase Release Assay

2.5.3.2. Histamine Release Assay

2.5.3.3. IgE + sAg Stimulation of mBMCMCs

2.5.3.4. IgE-mediated stimulation of hCBMCs

2.5.3.5. Cytokine Measurement by ELISA

2.5.3.6. VitD3 Treatment (without stimulation)
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5.3.7</td>
<td>Measurement of $1\alpha,25(OH)_2D_3$</td>
<td>95</td>
</tr>
<tr>
<td>2.5.4</td>
<td>Molecular Biology</td>
<td>96</td>
</tr>
<tr>
<td>2.5.4.1</td>
<td>Genotyping of WT and $VDR^{-/-}$ Mice</td>
<td>96</td>
</tr>
<tr>
<td>2.5.4.2</td>
<td>RNA Extraction</td>
<td>97</td>
</tr>
<tr>
<td>2.5.4.3</td>
<td>Complementary DNA (cDNA) Synthesis</td>
<td>98</td>
</tr>
<tr>
<td>2.5.4.4</td>
<td>qRT-PCR</td>
<td>98</td>
</tr>
<tr>
<td>2.5.5</td>
<td>SDS-PAGE and Western Blot Analysis</td>
<td>99</td>
</tr>
<tr>
<td>2.5.5.1</td>
<td>Preparation of Total Cell Lysates</td>
<td>99</td>
</tr>
<tr>
<td>2.5.5.2</td>
<td>SDS-PAGE</td>
<td>100</td>
</tr>
<tr>
<td>2.5.5.3</td>
<td>Protein Transfer and Western Blot Analysis</td>
<td>100</td>
</tr>
<tr>
<td>2.5.6</td>
<td>In Vivo and Related Procedures</td>
<td>101</td>
</tr>
<tr>
<td>2.5.6.1</td>
<td>Engraftment of mBMCMCs</td>
<td>101</td>
</tr>
<tr>
<td>2.5.6.2</td>
<td>PCA with Topical Application of $1\alpha,25(OH)_2D_3$ or $25OHD_3$</td>
<td>101</td>
</tr>
<tr>
<td>2.5.6.3</td>
<td>Ear Tissue Histology and Numeration of Tissue MCs</td>
<td>102</td>
</tr>
<tr>
<td>2.5.6.4</td>
<td>Ear Tissue Lysate Preparation and Analysis</td>
<td>102</td>
</tr>
<tr>
<td>2.5.6.5</td>
<td>Total RNA Extraction from Ear Tissues</td>
<td>103</td>
</tr>
<tr>
<td>2.5.6.6</td>
<td>Serum Preparation and Analysis</td>
<td>103</td>
</tr>
<tr>
<td>2.5.7</td>
<td>Histochemistry and Immunohistochemistry</td>
<td>103</td>
</tr>
<tr>
<td>2.5.7.1</td>
<td>Cytospin Slide Preparation</td>
<td>103</td>
</tr>
<tr>
<td>2.5.7.2</td>
<td>May-Grünwald Giemsa Staining</td>
<td>104</td>
</tr>
<tr>
<td>2.5.7.3</td>
<td>Kimura Staining of hCBMCs</td>
<td>104</td>
</tr>
<tr>
<td>2.5.7.4</td>
<td>Toluidine Blue Staining</td>
<td>104</td>
</tr>
<tr>
<td>2.5.7.5</td>
<td>Immunohistochemistry</td>
<td>104</td>
</tr>
<tr>
<td>2.5.8</td>
<td>Centrifugation</td>
<td>105</td>
</tr>
<tr>
<td>2.5.9</td>
<td>Statistical Analyses</td>
<td>105</td>
</tr>
</tbody>
</table>

CHAPTER 3

$1\alpha,25(OH)_2D_3$ POTENTIATES THE NEGATIVE IMMUNOREGULATORY PROPERTIES OF MAST CELLS DURING IgE+sAg-MEDIATED ACTIVATION IN VITRO | 107 |

3.1. Introduction | 108 |

3.2. Results | 110 |

3.2.1. WT and VDR$^{-/-}$ mBMCMCs exhibit similar phenotypical and functional characteristics | 110 |
3.2.2. $1\alpha,25(OH)_2D_3$ does not affect the efficiency of IgE preload, the expression of surface molecules, and survival of mBMCMCs

3.2.3. $1\alpha,25(OH)_2D_3$ reduces IgE + sAg-mediated mBMCMC degranulation in a VDR-dependent manner

3.2.4. $1\alpha,25(OH)_2D_3$ VDR-dependently down-regulates the de-novo synthesis and secretion of pro-inflammatory cytokines but up-regulates that of the anti-inflammatory IL-10, from IgE + sAg-activated mBMCMCs

3.2.5. Curcumin reduces IgE + sAg-mediated cytokine production by mBMCMCs in a VDR- and non-genomic VitD3 pathway-dependent manner

3.3. Discussion

CHAPTER 4

MAST CELLS CAN CONVERT 25OHD3 TO $1\alpha,25(OH)_2D_3$ AND THUS ENABLE 25OHD3 TO IMMUNOSUPPRESS IgE + sAg-ACTIVATED MAST CELLS IN VITRO

4.1. Introduction

4.2. Results

4.2.1. Mouse BMCMCs constitutively express CYP27B1 in their cytosol

4.2.2. 25OHD3 up-regulates CYP27B1 expression in mBMCMCs in a VDR-dependent manner

4.2.3. 25OHD3 dose-dependently induces endogenous synthesis of $1\alpha,25(OH)_2D_3$ by mBMCMCs

4.2.4. 25OHD3 treatment reduces IgE + sAg-mediated degranulation and pro-inflammatory cytokine production of mBMCMCs in a dose- and VDR-dependent manner

4.3. Discussion

CHAPTER 5

TOPICALLY APPLIED $1\alpha,25(OH)2D3$ AND 25OHD3 CAN REDUCE THE CUTANEOUS PATHOLOGY ASSOCIATED WITH IgE-MEDIATED PASSIVE CUTANEOUS ANAPHYLAXIS IN A MAST CELL VITAMIN D RECEPTOR-DEPENDENT MANNER IN VIVO

5.1. Introduction

5.2. Results

5.2.1. Topical $1\alpha,25(OH)2D3$ application alone does not affect ear thickness of WT mice

5.2.2. Topical $1\alpha,25(OH)_2D_3$ application at the site of inflammation can suppress PCA-associated ear swelling

5.2.3. Topical $1\alpha,25(OH)_2D_3$ application reduced the extent of MC degranulation and the expression of various MC-derived mediators associated with PCA in vivo
5.2.4. VDR expression is required for the suppressive effect of 1α,25(OH)₂D₃ on PCA-associated cutaneous pathology ................................................................. 141

5.2.5. The PCA-suppressing effect of topical 1α,25(OH)₂D₃ treatment requires specifically VDR expression by cutaneous MCs ................................................................. 142

5.2.6. Topical 25OHD₃ can suppress PCA-associated ear swelling via similar mechanisms to 1α,25(OH)₂D₃ .............................................................................................. 145

5.2.7. The suppressive effect of topical 25OHD₃ on PCA-associated cutaneous pathology also requires MC expression of VDR .............................................................................. 147

5.3. Discussion ......................................................................................................................... 148

CHAPTER 6 .................................................................................................................... 155
FROM THE MOUSE TO THE HUMAN – TRANSLATIONAL ASPECTS .............................................. 155

6.1. Introduction ...................................................................................................................... 156

6.2. Results ............................................................................................................................... 158

6.2.1. 1α,25(OH)₂D₃ reduces IgE + α-IgE-mediated TNFα but not IL-10 production by hCBMCs .................................................................................................................. 158

6.3.2. Human CBMCs constitutively express CYP27B1, which can be up-regulated by 25OHD₃ treatment ............................................................................................................. 159

6.3.3. Human CBMCs produce endogenous 1α,25(OH)₂D₃ following 25OHD₃ treatment in a dose-dependent manner .................................................................................. 159

6.3. Discussion ......................................................................................................................... 160

CHAPTER 7 .................................................................................................................... 164
CONCLUDING REMARKS & FUTURE DIRECTIONS ................................................................ 164

References ....................................................................................................................... 171
Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to me (Chunping Yu) 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. I give consent to this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying according to the provisions of the Copyright Act 1968. I also give permission for the digital version of my thesis to be made available on the web, via the digital research repository of the University of Adelaide, the Library catalogue, the Australasian Digital Theses Program and also through web search engines, unless permission has been granted by the University to restrict access for any unforeseen reasons. Finally, I acknowledge that the copyright of the published works listed below resides with the copyright holder(s) of those works.

List of publications


Abbreviations

1α,25(OH)₂D₃: 1α,25-dihydroxyvitamin D₃
25OHD₃: 25-hydroxyvitamin D₃
6C: 6-s-cis
6T: 6-s-trans
Ab: antibody
ACD: anticoagulant citrate dextrose
Ag: antigen
APS: ammonium persulphate
ASM: airway smooth muscle
A1AT: α₁-antitrypsin
BM: bone marrow
BSA: bovine serum albumin
BTK: Bruton’s tyrosine kinase
cAMP: cyclic AMP
CCL: CC-chemokine ligand
CHS: contact hypersensitivity
COPD: chronic obstructive pulmonary disease
CTMC: connective tissue mast cell
CYP24A1: 25-hydroxyvitamin D₃-24-hydroxylase
CYP27B1: 25-hydroxyvitamin D-1α-hydroxylase enzyme
DAG: Diacylglycerol
DBP: VitD₃-binding protein
DC: dendritic cell
DMEM: Dulbecco’s modified eagle medium
DMSO: dimethyl sulphoxide
DNP: dinitrophenyl
DT: diphtheria toxin
ECL: enhanced chemiluminescence
EIA: enzyme immunoassay
ELISA: enzyme-linked immunosorbent assay
EMTU: epithelial-mesenchymal trophic unit
ERK: extracellular signal regulated kinase
FCS: fetal bovine serum
FceRI: high affinity IgE receptor
FDNB: 1-fluoro-2,4,-dinitrobenzene
GAB: growth-factor-receptor-bound protein
GMP: granulocyte/macrophage progenitor
hCBMC: human cord-blood-derived MCs
HDC: histidine decarboxylase
HIV: human immunodeficiency virus
HMEM: Hank’s MEM
HRP: horse radish peroxidase
HS: horse serum
HSA: human serum albumin
HSC: haematopoietic stem cell
IFN: interferon
Ig: immunoglobulin
IL: interleukin
IMDM: Iscove’s modified Dulbecco’s medium
InsP3: inositol-1,4,5,-triphosphate
ITAM: immunoreceptor tyrosine-based activation motif
LN: lymph node
LT: leukotriene
MAPK: mitogen-activated protein kinase
mBMCMC: mouse bone marrow-derived cultured mast cell
MC: mast cell
MCP: mast cell progenitor
MC_T: tryptase positive mast cell
MC_TC: tryptase and chymase positive mast cell
MEK: MAPK kinase
miR: microRNA
MKP: mitogen-activated protein kinase phosphatase
MMC: mucosal mast cell
MMCP: mouse mast cell protease
MMP: matrix metalloproteinase
NEAA: non-essential amino acid
NF-κB: nuclear factor-κB
PBS: phosphate buffered saline
PCA: passive cutaneous anaphylaxis
PCR: polymerase change reaction
Pen/Strep: Penicillin/Streptomycin
PG: prostaglandin
PI3K: phosphatidylinositol-3-OH kinase
PKB (AKT): protein kinase B
PKC: protein kinase C
PL: phospholipase
PLA_2: phospholipase A_2
PLC_γ: phospholipase C_γ
PMA: phorbol-12 myristate-13 acetate
PtdIns(3,4,5)P₃: phosphatidylinositol-3,4,5,-trisphosphate

PTH: parathyroid hormone

qRT-PCR: quantitative real-time PCR

RIA: radioimmunoassay

RT: room temperature

RT-PCR: real-time PCR

RXR: retinoid X receptor

sAg: specific antigen

SCF: stem cell factor

SDS-PAGE: sodium dodecyl sulfate - polyacrylamide gel electrophoresis

TCR: T cell receptor

TGF: transforming growth factor

Th: T helper

TLR: toll-like receptor

TNF: tumor necrosis factor

T<sub>reg</sub>: T regulatory cell

TSLP: thymic stromal lymphopoietin

UV: ultraviolet

VDR: vitamin D receptor

VDRE: vitamin D response element

VDR<sub>mem</sub>: plasma membrane-associated vitamin D receptor

VitD: vitamin D

WT: wild-type
Acknowledgments

First and foremost, I would like to thank my supervisors, Dr. Michele Grimbaldeston, Prof. Shaun McColl, and Prof. Angel Lopez, for their constant guidance and support throughout my PhD study, especially Michele for offering me such a stimulating and challenging project and for demonstrating to me personally what scientific research is really about. A big thank also goes to Shaun, for securing me a scholarship that allows me to begin my PhD study in the first place and for helping with reviewing my thesis towards the end.

I also want to thank everyone in the lab, Dr. Boris Federic, Lisa Biggs, Zhen Liu, Houng Taing and Renee Gilbey, as well as Jyotsna, Kiwi and Samantha in our neighbour lab. It was your friendship and unconditional encouragement that kept me sane and able to continuously moving forward. A special thank you must go to Boris, who has generously offered help with quite a few large-scale animal experiments. For everyone else in the Centre for Cancer Biology, I would like to express my appreciation for making me feel like a part of a big family.

Finally, I want to dedicate this thesis to my husband, Yang Lu, and all my family overseas, as without your deep understanding and unconditional love and support, I would not be able to stay focused and complete my experiments in a three-year time frame.
Thesis summary

Mast cells (MCs) can exert anti-inflammatory effects via production of interleukin (IL)-10 in a number of Immunoglobulin (Ig)E-independent immune responses. Recently, we reported that 1α,25-dihydroxyvitamin D₃ (1α,25(OH)₂D₃), the biologically active form of vitamin D₃ (VitD₃), can induce IL-10 production from mouse bone marrow-derived cultured MCs (mBMCMCs). For the current project, we further investigated if the well-recognised pro-inflammatory properties of MCs in IgE-dependent immune settings can be reduced upon 1α,25(OH)₂D₃ administration and, if so, which mechanisms are likely to be responsible. In the presence of 1α,25(OH)₂D₃, IgE + specific antigen (sAg)-stimulated mBMCMCs exhibited reduced degranulation, as well as decreased production of the pro-inflammatory cytokines, TNFα and IL-6, in a vitamin D receptor (VDR)-dependent manner. Concomitantly, 1α,25(OH)₂D₃ significantly up-regulated the production of IL-10. In addition, we demonstrated for the first time the expression of CYP27B1, the enzyme that generates 1α,25(OH)₂D₃ from its inactive precursor 25-hydroxyvitamin D₃ (25OHD₃) in both mBMCMCs and human cord-blood-derived MCs (hCBMCs). This enables mBMCMCs to produce endogenous 1α,25(OH)₂D₃ and thus granting 25OHD₃ similar VDR-dependent immunosuppressive effects to 1α,25(OH)₂D₃ on activated MCs either directly or indirectly.

By employing a mouse IgE-mediated MC-dependent passive cutaneous anaphylaxis (PCA) model as well as four mouse groups with different cutaneous MC profiles in the ears, including wild-type (WT) C57BL/6 mice, MC-deficient C57BL/6-Kiₜₗ⁻/⁻ mice and C57BL/6-Kiₜₗ⁻/⁻ mice engrafted with either WT or VDR-deficient (VDR⁻⁻)
mBMCMCs, we found that topical application of either 1α,25(OH)₂D₃ or 25OHD₃ significantly curtailed the magnitude of PCA-associated ear swelling, potentially by reducing the extent of MC degranulation and/or the secretion of various MC-derived cytokines. Notably, these PCA-suppressive effects required the presence of dermal MCs and their expression of VDR.

Taken together, data presented in this thesis provide evidence that 1α,25(OH)₂D₃ and 25OHD₃, the latter likely via its conversion to the active metabolite by MCs, can suppress IgE + sAg-mediated MC activation in a VDR-dependent manner both in vitro and in vivo. This suggests the therapeutic potential for various VitD₃ analogues to treat MC-dependent IgE-associated allergic disorders.
CHAPTER 1

LITERATURE REVIEW
1.1. Mast Cells (MCs)

First discovered as granular cells by Dr von Recklinghausen in 1863 and named “mastzellen” (i.e. well-fed cells) by Dr Paul Ehrlich in 1878, MCs can be identified as FceRI (the high affinity immunoglobulin [Ig]E receptor) and c-kit (CD117; receptor for stem cell factor [SCF]) double positive cells with numerous metachromatic granules in their cytoplasm that stain with aniline dyes such as toluidine blue. (1, 2) Since their initial discovery over a century ago, MCs have gained increasing attention, due largely to their wide distribution throughout the body, their high plasticity in phenotypical and functional properties, and their substantial involvement in a large array of physiological and pathophysiological processes including, in particular, IgE-dependent allergic reactions. (3, 4) The recent findings that MCs are capable of playing anti-inflammatory as well as pro-inflammatory roles during a number of innate and acquired immune responses have sparked further interest in seeking strategies which can drive the functionality of MCs towards the anti-inflammatory end of the spectrum. (5-8) The ultimate aim of the current project is to investigate alternatives in IgE-mediated MC activation by anti-inflammatory exogenous stimuli with a particular emphasis on biologically active Vitamin D3 (VitD3).

1.2. Basic Biology of MCs

1.2.1. MC Ontogeny and Tissue Distribution

Human MCs originate from CD34+ pluripotent haematopoietic stem cells (HSCs) in the bone marrow (BM), which differentiate to form CD34+CD117+CD13+ MC progenitors (MCPs). (3) MCPs then enter the circulation and migrate to tissues where they take up
residence and undergo maturation in the presence of cytokines such as IL-6 and SCF. In mice, the situation is more complicated as mouse HSCs give rise to granulocyte/macrophage progenitors (GMPs) in the BM, and MCPs can develop either from GMPs or from HSCs directly. Moreover, a group of GMP-derived common basophil/MC progenitors are found in mouse spleen and have been shown to also give rise to MCPs. (4, 9) According to Chen et al. (10), a population of Lin- c-kit+ Sca-1’ Ly6c- FceRI CD27 T1/ST2+ cells exist in adult mouse BM, which gave rise only to MCs in culture and could reconstitute the MC compartment when transferred into MC-deficient mice. Similar to human MCPs, mouse MCPs then circulate through blood and do not mature until they reach the anatomical location in which they will ultimately reside. Unlike their human counterparts, however, mouse MCPs require IL-3 and/or SCF for maturation and differentiation. Typically, mature MCs are found throughout vascularised tissues, often in close proximity to blood and lymphatic vessels and/or at surfaces that are exposed to the environment, such as the skin, airway and gastrointestinal tract. (4, 11) Although currently unknown for humans, in mice at least, committed MCPs (besides mature MCs) are also abundant in the small intestine (12, 13) and at sites of chronic inflammation; which, in the latter case, is due largely to inflammation-induced up-regulation of MC-attracting chemokines. (14).

1.2.2. Homing of MCPs

The wide anatomical distribution of mature MCs implies the migration of MCPs to a large variety of tissues occurs after leaving the BM. Although the precise mechanism(s) for such tissue-specific homing process remains to be fully understood, it has been suggested that there is a sequential expression of surface molecules, such as adhesion
molecules and chemokine receptors, on MCPs. Only the progenitors expressing an appropriate set of these molecules are able to enter the corresponding tissue and/or interact with cells within that tissue. Thus the tissue-specificity in these scenarios is conferred by the specific receptor-ligand interaction between MCPs and resident cells of a particular tissue. The constitutive recruitment of MCPs to the small intestine in mice, for instance, is directed by tissue-derived CXCR2 ligands (i.e. chemokines) as both human and mouse MCPs express the corresponding chemokine receptor, CXCR2. Interaction between CXCR2 and their ligands subsequently facilitates the adhesion between α4β7 integrin (expressed on MCPs) and vascular cell adhesion molecule-1 and/or mucosal addressin cell adhesion molecule-1 (both are counter-ligands for α4β7 integrin expressed on intestinal endothelial cells), thus further promoting the homing and retainment of MCPs in the tissue. The recruitment of MCPs to asthmatic lungs, on the other hand, relies on the inducible changes in the chemotaxis-adhesion pathways upon the onset of the allergic reaction, as normal lungs are not common residence for either MCPs or mature MCs. In this case, although the nature of the chemoattractants responsible remains to be identified, the adhesive interaction between vascular cell adhesion molecule-1 (up-regulated on endothelial cells due to inflammation) and α4β7 and α4β1 integrins (both expressed on MCPs) has been demonstrated to be crucial.

1.2.3. Trafficking of Mature MCs

Mature MC populations are not generally found in peripheral blood of healthy individuals. They can, however, be readily observed under c-kit gain-of-function mutation disease settings, such as mastocytosis. In addition, the discovery of a large variety of MC chemotactic factors, including chemokines (e.g. monocytes chemoattractant protein-1 and...
CC-chemokine ligand [CCL]5/RANTES, anaphylatoxins (e.g. C3a and C5a), angiogenic factors (e.g. platelet-derived growth factor and vascular endothelial growth factor) and a number of cytokines (e.g. IL-3 and transforming growth factor [TGF]-β), urges the reconsideration of the possibility that mature MCs do exist in peripheral blood under physiological and/or pathological conditions. (17) Furthermore, interaction between SCF and its receptor c-kit has been shown to alter the functional state of integrins on the surface of tissue MCs. This, together with the evidence that chemokine receptor expression on these cells also vary, suggests that mature MCs may detach from their original residence via changes in adhesion molecule expression and migrate towards particular chemotactic factor(s) at other sites. (18)

Supporting evidence for this notion has been observed in vivo. Early work done by Wang et al. (17) showed MC migration from the skin to the draining lymph nodes (LN) and subsequently to the spleen during the sensitization phase of dinitrofluorobenzene-induced contact hypersensitivity (CHS) in mice. A more recent study by Byrne et al. (19) also demonstrated CXCR4/CXCL12-mediated migration of mature MCs from the skin to the B cell-rich areas of draining LN upon ultraviolet (UV) irradiation. During liver regeneration in rats after partial hepatectomy, MC migration into the liver was shown to be crucial for the restoration of hepatic MC population. (20) Furthermore, human MCs expressing CCR1 and CCR4 are able to migrate towards the chemokine CCL5/RANTES, both in vitro and in vivo. (21, 22) Recent findings that the pro-inflammatory cytokines tumor necrosis factor (TNF)α and IL-6 can induce chemotaxis and chemokinesis of tissue MCs, respectively, provide a plausible explanation for the accumulation of MCs at sites of inflammation. (23)
1.2.4. MC Heterogeneity

To date, two major subsets of MCs have been described in rodents: mucosal MCs (MMCs) and connective tissue MCs (CTMCs); which show differences in many aspects such as tissue distribution, granule contents, histochemical staining properties, and response to certain stimuli or inhibitors.\(^4,24\) For example, MMCs predominate in the lung, intestinal and other mucosae, and preferentially express chymases including mouse MC protease (MMCP)-1 and -2; whereas CTMCs predominate in connective tissues and serosal cavities (e.g. peritoneal cavity), and express chymases MMCP-4, and -5 (also MMCP-9 in the case of uterine MCs), tryptases MMCP-6, and -7, and carboxypeptidase A.\(^25\) Human MCs also exhibit heterogeneity and are classified, based on their serine protease contents, as tryptase positive MCs (MC\(_T\)) and tryptase and chymase double positive MCs (MC\(\_TC\)), with the latter also containing carboxypeptidase A.\(^26-28\)

Although the tissue distribution of human MC subsets is not as distinctive as their mouse counterparts, MC\(_T\) and MC\(\_TC\) do share some characteristics with MMCs and CTMCs, respectively. Because MC heterogeneity in different tissues is largely influenced by the microenvironment presented, it is likely to be more diverse and dynamic than merely two or three polarized phenotypes. The plasticity of MCs is conferred by their ability to express certain profiles of proteases according to the particular microenvironmental conditions they encounter in the tissue that they reside.\(^4\) For example, when mBMCMCs and cultured peritoneal MCs from (WB/ReJ×C57BL/6)\(F_1\) (WBB6F\(_1\)) WT mice (both expressing MMCP-1, -2, -4, -5, and -6) were transplanted into the stomach wall of MC-deficient WBB6F\(_1\)-Kit\(^{W/W}\) mice, they appeared in both the mucosa, where they had down-regulated MMCP-1, -4, -5 and -6, and muscularis propria, where they had
down-regulated MMCP-1 and -2. On the other hand, when fully differentiated peritoneal MCs from WT mice (expressing MMCP-2, -4, -5 and -6) were transplanted, they also adopted to the stomach mucosa by down-regulating MMCP-4, -5 and -6 when injected at a considerably low number to allow local proliferation.

1.2.5 MC Development and Maturation

As mentioned previously, circulating MCPs do not undergo maturation until they have reached their destined anatomical location. It seems natural, therefore, that MC development and maturation should be driven by growth factors and cytokines present in the local microenvironment. (24, 29) SCF, produced primarily by fibroblasts, is probably the most critical MC growth factor. Interaction between SCF and its receptor c-kit, provides vital signals for the development of murine MCs as both the W and Sl mice, which have mutations in the chromosomal loci coding for c-kit and SCF, respectively, have profound deficiency in tissue MCs. (30, 31) Sawai et al. (32) reported that SCF can also promote the development of human MCs, as demonstrated by its proliferative effect during MC culturing from human umbilical cord-blood.

In addition to SCF, a number of cytokines are also proven important for MC development and/or maturation in mice and/or humans. T cell-derived cytokine IL-3 alone, for instance, is required to generate mBMCMCs. These mBMCMC have been used extensively in research of MC biology, although they resemble more closely MMCs or immature MCs, with respect to size, granularity and functional properties. (33) To obtain more CTMC-like cells in vitro, other cytokine(s) such as SCF, or IL-4, or both together, are also required. (34-36) It is worth mentioning that IL-10, a well-known anti-inflammatory cytokine is
another enhancer for MC growth, as whilst it alone does not induce MC colony formation, it demonstrates synergistic action with IL-3, SCF, or IL-4 in the proliferation of mBMCMC.\(^{(36)}\) Culturing human MCs \textit{in vitro}, on the other hand, requires SCF and IL-6. In the presence of both cytokines, MCs can be generated from human cord blood-derived mononuclear cells as well as CD34\(^+\) cells. \(^{(36, 37)}\)

\textbf{1.2.6. MC Activation}

There are many mechanisms by which MCs can be activated but perhaps the most well recognised is IgE-dependent activation. Most, if not all, MCs in the body are sensitised with IgE molecules of different specificities bound via their high-affinity FceRI receptors. Cross-linking of these IgE molecules by their corresponding Ag leads to aggregation of FceRI receptors and ultimately MC activation. Upon such IgE + sAg-mediated activation, MCs generally undergo an immediate and highly regulated granule-emptying process called degranulation. Depending on the strength of the stimuli, MCs can either release all their granule contents at once through compound and/or multi-vesicular exocytosis, or gradually empty their granules via piecemeal exocytosis.\(^{(38)}\) More importantly, MCs are capable of participating in multiple cycles of activation for mediator release, although the regeneration of a fully reconstituted granular compartment can take up to 72 h. \(^{(39)}\)

In addition to IgE receptor cross-linking, MCs express a wide array of receptors and can be activated by a variety of other stimuli, including growth factors (e.g. SCF) \(^{(40)}\), cytokines (e.g. IL-1, IL-3 and IL-33) \(^{(41)}\), chemokines (e.g. macrophage inflammatory protein-1α) \(^{(42)}\), anaphylatoxins (e.g. C3a and C5a) \(^{(43)}\), neuropeptides (e.g. substance P and calcitonin gene-related peptide) \(^{(44, 45)}\), Ig free light-chains \(^{(46)}\), and pathogen-derived
Ags (i.e. signalling through toll-like receptor (TLR)s, TLR-2, -3, -4, -7 and -9) (47-50).

Unlike IgE + sAg-mediated activation, activation of MC by non-IgE-related stimuli does not always lead to degranulation. Even when it does, the degranulation is usually piecemeal in nature, leading to a differential and selective, rather than a “wholesale”, release of MC-derived mediators (Section 1.2.7). (51) For example, IL-1 and granulocyte/macrophage colony stimulating factor induce the release, only in the picomolar range, of histamine; whereas calcitonin gene-related peptide triggers piecemeal secretion of MMCP-1 from mBMCMCs. (45, 52) Furthermore, monomeric IgE, in the absence of sAg, can also stimulate mBMCMCs. In this case, however, it does not induce degranulation or leukotriene synthesis by the cells but rather a more potent production of proinflammatory cytokines, such as TNFα, IL-6, IL-4 and IL-13. (53)

1.2.7. MC-derived Mediators

MC-derived mediators (summarized in Table 1.1) fall into three main categories: pre-formed mediators, lipid-derived mediators, and cytokines, chemokines and growth factors. Most of these mediators are both pleiotropic and redundant, meaning that each mediator has more than one function, whilst more than one mediator may exhibit the same biological effect. (3, 54, 55)

Pre-formed mediators are synthesized and stored in the cytoplasmic granules. They are generally released within seconds to minutes of MC activation, especially of the IgE-dependent type (Section 1.2.6). Depending on the microenvironment for maturation of any particular MC, its detailed profile of pre-formed mediators may vary. Nevertheless, three broad classes of molecules are always present, including histamine, neutral
Table 1.1. Mast cell-derived immunological mediators and their functions (24, 54, 55, 57)*

<table>
<thead>
<tr>
<th>Pre-formed Mediators</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histamine</td>
<td>Vasodilation; Muscle contraction</td>
</tr>
<tr>
<td>Proteoglycans (e.g. heparin)</td>
<td>Protease packaging and stabilization; Angiogenesis</td>
</tr>
<tr>
<td>Neutral proteases</td>
<td>Matrix degradation; Leukocyte recruitment</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Lipid-derived Mediators</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prostaglandins (e.g. PGD₂)</td>
<td>Neutrophil recruitment; Vasodilation; Edema</td>
</tr>
<tr>
<td>Leukotrienes (e.g. LTB₄)</td>
<td>Leukocyte recruitment; Bronchoconstriction</td>
</tr>
<tr>
<td>PAF</td>
<td>Bronchoconstriction</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cytokines, Chemokines, and Growth Factors</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF**</td>
<td>Leukocyte recruitment; Angiogenesis; Fibroblast activation</td>
</tr>
<tr>
<td>IL-1</td>
<td>Leukocyte recruitment; Angiogenesis</td>
</tr>
<tr>
<td>IL-2</td>
<td>Lymphocyte activation</td>
</tr>
<tr>
<td>IL-3</td>
<td>Leukocyte growth factor</td>
</tr>
<tr>
<td>IL-4</td>
<td>Immunomodulatory (Th2)</td>
</tr>
<tr>
<td>IL-6</td>
<td>Activation of leukocyte and fibroblasts</td>
</tr>
<tr>
<td>IL-8</td>
<td>Neutrophil recruitment</td>
</tr>
<tr>
<td>IL-10</td>
<td>Immunomodulatory (Th2; anti-inflammatory)</td>
</tr>
<tr>
<td>IL-13</td>
<td>Immunomodulatory (Th2); B cell activation</td>
</tr>
<tr>
<td>IL-15**</td>
<td>T cell recruitment and activation</td>
</tr>
<tr>
<td>IL-18</td>
<td>Lymphocyte activation; Angiogenesis</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Activation of macrophages; Immunomodulatory (Th1)</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Immunomodulatory (anti-inflammatory); Angiogenesis</td>
</tr>
<tr>
<td>VEGF &amp; FGF₂</td>
<td>Angiogenesis</td>
</tr>
<tr>
<td>Chemokines (e.g. CCL2, 3, 4, 5, 11, 20 and CXCL1, 2, 8, 9, 10, 11)</td>
<td>Leukocyte recruitment</td>
</tr>
<tr>
<td>Growth factors (e.g. SCF, GM-CSF, GnRH-1, b-FGF, NGF)</td>
<td>Development, differentiation, maturation and proliferation of various types of cells including MCs</td>
</tr>
</tbody>
</table>

PAF, platelet-activating factor; IL, interleukin; IFN, interferon; TGF, transforming growth factor; VEGF, vascular endothelial growth factor; FGF, fibroblast growth factor; SCF, stem cell factor; GM-CSF, Granulocyte-macrophage colony stimulating factor; GnRH, gonadotropin-releasing hormone; NGF, nerve growth factor.

* The functions listed are immunology-related, with particular regards to during allergic reactions.

** TNF and IL-15 can also be pre-formed and stored in MC granules (see text for more details).
proteases, and proteoglycans, where the former two are generally embedded in a matrix consisted of the latter inside MC granules. (56) Certain mouse MC populations also contain pre-formed TNFα, which serves as an immediate source of this cytokine during IgE-mediated immune responses. (57) In general, the generation of lipid-derived mediators begins within minutes to hours of MC activation, through the cleavage of arachidonic acid from membrane phospholipids. (24) Examples of this category of mediators are prostaglandins (PGs) such as PGD₂ and PGE₂, leukotrienes (LTs) such as LTB₄ and LTC₄, and platelet-activating factor. Within hours of activation, MCs start the de novo synthesis of a wide range of cytokines, chemokines and growth factors (Table 1.1). (54) Again, depending on the individual development and activation circumstances, the quality as well as quantity of these mediators generated and secreted may differ from MC to MC. It is noteworthy that very few cytokines such as TNFα and IL-15 can be both pre-formed and newly synthesized at later stages of an immune response (57, 58), possibly due to their rapid effects upon release, including vasodilation and/or leukocyte recruitment. (59, 60)

1.2.8. FcεRI Signalling in MCs

FcεRI on MCs is expressed as a heterotetrameric receptor composed of an IgE-binding α-subunit, a signal-amplifying membrane-tetraspanning β-subunit and two identical disulfide-linked γ-subunits, each containing an immunoreceptor tyrosine-based activation motif (ITAM) that is important for conveying activating signals to MCs. (61, 62) Cross-linking of FcεRI-bound IgE molecules with their sAg leads to aggregation of two or more FcεRI receptors and subsequent activation of the receptor-associated protein tyrosine kinases LYN and FYN. LYN, in turn, either phosphorylates the ITAMs in FcεRI and thus recruits and activates another protein tyrosine kinase SYK, or initiates the RAS-mitogen-
activated protein kinase (MAPK) signalling cascade. \(^{(62-64)}\) The RAS-MAPK pathway, consisted of RAS, RAF, MAPK kinase (MEK) and MAPKs (e.g. extracellular signal regulated kinase [ERK]1/2, JNK and p38), activates a variety of transcription factors as well as phospholipase A\(_2\) (PLA\(_2\), which participates in arachidonic acid metabolism), thereby regulating the synthesis of protein and lipid-derived mediators, respectively. \(^{(65-67)}\) By triggering the phospholipase C\(_\gamma\) (PLC\(_\gamma\)) pathway, SYK subsequently generates diacylglycerol (DAG) and activates protein kinase C (PKC), the latter of which is a regulator for RAS-MAPK pathway as well as MC degranulation and gene transcription. In addition to DAG, PLC\(_\gamma\) activation also generates inositol-1,4,5-trisphosphate (InsP\(_3\)), leading to regulation of intracellular calcium (Ca\(^{2+}\)) responses and, again, MC degranulation and gene transcription. \(^{(68, 69)}\) FYN, on the other hand, phosphorylates the adaptor protein growth-factor-receptor-bound protein (GAB)2, which in turn activates the phosphatidylinositol-3-OH kinase (PI(3)K) pathway. The product of this pathway, phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P\(_3\)), is an important lipid mediator that regulates the activity of various enzymes, such as Bruton’s tyrosine kinase (BTK) and protein kinase B (PKB/AKT). Whilst BTK is capable of activating PLC\(_\gamma\) and its downstream events, AKT up-regulates gene transcription either directly or indirectly via activation of nuclear factor (NF)-\(\kappa\)B. \(^{(70-73)}\) Furthermore, although not shown in Figure 1.1, several adapter proteins, in particular the linker for T cell activation and non-T-cell activation linker, also play crucial roles in IgE-dependent MC activation as they (upon phosphorylation by LYN/SYK) help assembling other signalling components in the vicinity of the engaged Fc\(\varepsilon\)RI molecules. \(^{(74-76)}\)
Figure 1.1. Simplified scheme of FcεRI signalling events in MCs.

Cross-linking of FcεRI-bound IgE with sAg induces aggregation of two or more FcεRI molecules and activates the receptor-associated protein tyrosine kinases LYN, SYN and FYN. By subsequent activation of other key signalling components, such as the RAS-MAPK pathway, PLCγ, PI(3)K, and PKC, degranulation, phospholipid metabolism and/or gene transcription could be resulted, leading to the secretion of a wide array of immunological mediators. See text for more details. Figure is adapted from Galli et al. (64)
Antigen

\[ \text{IgE} \quad \gamma \beta \quad \beta \gamma \]

\[ \text{ITAM} \]

\[ \text{LYN} \quad \text{FYN} \]

\[ \text{Ras} \quad \text{Raf} \]

\[ \text{MEK} \quad \text{Ca}^{2+} \]

\[ \text{Degranulation} \]

\[ \text{FcyRI} \]

\[ \text{Plasma Membrane} \]

\[ \text{MC-derived mediators} \]

- Biogenic amines
- Proteoglycans
- Proteases
- Certain cytokines

\[ \text{NF-\kappaB} \quad \text{Genes and Transcription} \]

\[ \text{• Cytokines} \]

\[ \text{• Chemokines} \]

\[ \text{• Growth factors} \]

\[ \text{• Prostaglandins} \]

\[ \text{• Leukotrienes} \]

\[ \text{• Platelet-activating factor} \]

\[ \text{PLA}_2 \quad \text{PLC}_\gamma \quad \text{PI(3)K} \]

\[ \text{DAG} \quad \text{PKC} \]

\[ \text{InsP}_3 \]

\[ \text{Phospholipid metabolism} \]

\[ \text{• Biogenic amines} \]

\[ \text{• Proteoglycans} \]

\[ \text{• Proteases} \]

\[ \text{• Certain cytokines} \]
1.3. Biological Functions of MCs in IgE-dependent Allergic Reactions

1.3.1. IgE-dependent Allergic Reactions and MCs

According to the recent national health survey (77), over 25% of Australian population (approximately 5 million people) suffer from IgE-dependent allergic responses. Among children (under the age of 15) and young adults (aged between 15 and 24) in particular, asthma (12%) and hayfever/allergic rhinitis (19%) represent the most prevalent conditions, respectively. What is more alarming, there seems to be stronger overall growth in the number of allergy-affected older Australians in recent years and, if the trend continues, there will be a 70% increase in the number of allergy suffers (to approximately 8 million) by 2050. (78) In addition to reduced quality of life for individuals with allergy, the financial cost of allergies in 2007 alone reached AUS$7.8 billion, with lost productivity and health system expenditure being the major contributing factors, and thus inflicted a substantial burden on the Australian economy. (78) Furthermore, similar increases in the occurrence of allergy are being observed worldwide, especially in western countries, with the marked increase of allergies in children and young adults being the major concern. (79) As a consequence, extensive amounts of research have been carried out to investigate the causes and mechanisms of allergic disorders.

The term “allergy”, first described by Clemens von Pirquet in 1906, refers to an abnormal immune response directed against non-infectious environmental substances named allergens. (64, 80) Although allergy can either involve or not involve allergen-specific IgE molecules, this thesis will focus on IgE-dependent allergic responses, some examples of which are anaphylaxis, hayfever, atopic dermatitis (eczema), allergic asthma, and some
food allergies. Most, if not all, of these reactions are triggered by Ags that induce IgE production and subsequent “sensitisation” of host MCs. Common sources of allergens include pollens, house-dust-mite faecal particles, animal dander, certain foods (e.g. peanuts, fish, milk and eggs), latex and insect venoms. The induction of IgE production by these allergens reflect their ability to elicit a T helper (Th)2 response and increase the levels of IL-4 and IL-13, thus promoting immunoglobulin class switching (i.e. towards IgE) recombination in B cells. Furthermore, MCs express CD40L on their surface and can therefore stimulate B cells through CD40. This, together with the ability of already sensitised MCs to produce IL-4 and IL-13, raises the possibility that MCs can further drive IgE production, sensitisation of additional cells, and epitope spreading. Thus well before the onset of any allergic responses, MCs have set the stage for a detrimental outcome.

In general, IgE-dependent allergic reactions can be classified into three temporal phases: early-phase reactions, late-phase reactions and chronic reactions. The key features of, as well as the roles of MCs during, each of these phases will be described in details in the following sections.

1.3.2. Functions of MCs during Early-phase Allergic Reactions

Early-phase allergic reactions (Figure 1.2), also referred to as Type I immediate hypersensitivity reactions, occur within minutes of allergen exposure and are mainly caused by MC-derived pre-formed mediators (Section 1.2.7) at the affected site. For example, rapidly released histamine, PGDs and LTs are responsible for the commonly seen vasodilation, increased vascular permeability and bronchoconstriction during early-
Figure 1.2. Early-phase allergic reactions (in the airway)

The binding of a particular allergen to its cognate FcεRI-bound IgE molecules leads to receptor aggregation, MC activation, and the secretion of all three classes of MC-derived mediators. The rapidly released mediators, such as histamine and TNFα, result in bronchoconstriction, vasodilation, increase vascular permeability and increased mucus production from goblet cells. MCs also promote an influx of other inflammatory leukocytes, by up-regulating adhesion molecules on vascular endothelial cells (e.g. via TNFα) as well as secreting chemotactic mediators (e.g. PGD₂ and LTB₄) and chemokines (e.g. IL-8 and CCL2), and thus contributing to the transition from early- to late-phase reaction. See text for more details. Figure is adapted from Galli et al. (64)
Allergen

Increased IgE vascular permeability

Leukocyte recruitment (transition to late-phase response)

Vasodilation

Increased mucus production

Histamine, TNFα, tryptase

TNFα, PGDs, LTs, tryptase

Histamine, PGDs, LTs

TNFα, PGD2, LTB4, IL-8, CCL2

Histamine, TNFα, tryptase

Increased vascular permeability

Endothelial cell

Blood vessel

Neutrophils, Eosinophil, T cell

Airway smooth muscle

Epithelial cell

Goblet cell

Bronchoconstriction

Airway lumen
phase airway inflammation. (87, 88) Several mediators, such as histamine, TNFα and tryptase, can also lead to increased mucus production by goblet cells. Moreover, MCs can initiate the recruitment and/or activation of other leukocytes, and thus contributing to the onset of late-phase reactions, both by up-regulating adhesion molecules on vascular endothelial cells through TNFα secretion and by releasing various chemotactic mediators (e.g. LTB₄ and PGD₂) and chemokines (e.g. macrophage inflammatory protein 2, IL-8 and CCL2). (64, 89, 90) Although basophils, which also express FceRI, may contribute to early-phase responses by producing similar mediators, this is less likely under normal circumstances due to a paucity of basophil populations at peripheral tissues. (91) In addition to the localised events mentioned above, early-phase allergic reactions can also be systemic (i.e. anaphylaxis), where mediators from MCs (e.g. histamine) and/or basophils (e.g. platelet-activating factor) are released into circulation via IgE- and IgG-mediated mechanisms, respectively. (87, 92-94)

1.3.3. Functions of MCs during Late-phase Allergic Reactions

In contrast to early-phase reactions, late-phase reactions (Figure 1.3) typically develop within 2-6 h of allergen exposure, and largely reflect the actions of mediators from a variety of recruited leukocytes, including firstly neutrophils followed by eosinophils, basophils and T cells. (87, 95) For example, elastase released by newly recruited neutrophils can promote the activation of matrix metalloproteinases (MMPs) and the degradation of Type III collagen (96), eosinophil-derived basic proteins are shown to cause damage to epithelial linings at allergic sites (97), and IL-13 produced by T cells and basophils can contribute to continuing mucus hyper-production and/or bronchoconstriction (91, 98). In this regard, the main roles of MCs during this stage of the responses include maintaining
Figure 1.3. Late-phase allergic reactions (in the airway)

Although sharing many features in common with early-phase reactions, last-phase allergic reactions typically occur hours after allergen challenge and are thought to reflect the actions of innate (e.g. MCs, neutrophils, basophils and eosinophils) and adaptive (e.g. allergen-specific Th2 cells) immune cells that are resident or have been recruited from the circulation, as well as the release of inflammatory mediators by tissue-resident cells (e.g. epithelial cells). Therefore, MCs contribute to a late-phase reaction predominantly by maintaining the influx of immune cells, activating certain innate cell types (e.g. neutrophils, basophils and eosinophils) and regulating the functionality of others (e.g. T cells, B cells and DCs [not shown]). See text for more details. Figure is adapted from Galli et al. (64)
Allergen → Broncho-constriction

Epithelial damage → Increased mucus production

Airway lumen

Epithelial cell

Cytokines & chemokines

IL-13

Th2 cell

IL-9

GM-CSF, IL-3, IL-5

Eosinophil

GM-CSF, IL-3, IL-5

Eosinophil basic proteins

Elastase

IL-8

Neutrophils

IL-8, TNFα

LTs

Neutrophils

IL-8

Airway smooth muscle

Broncho-constriction

MMP activation & collagen degradation

Elastase

IL-8
the influx of these other cell types, activating innate immune cells (e.g. neutrophils by TNFα and eosinophils by IL-5), and affecting many aspects of the functionality of dendritic cells, T cells and B cells (by TNFα, IL-10 and TGF-β). \(^{(11, 99)}\) It is, however, important to realise that although MCs are generally regarded as proinflammatory effector cells during allergic reactions, some mediators they secrete, such as IL-10, TGFβ and MMCP-4, do display anti-inflammatory or immunosuppressive properties. \(^{(99, 100)}\) Furthermore, certain MC-derived mediators (e.g. histamine, PGDs, LTs, TNFα, IL-4, IL-13 and tryptase) can also affect the biology of various structural cells, including vascular endothelial cells, epithelial cells, fibroblasts, smooth muscle cells and goblet cells. \(^{(64, 101)}\)

### 1.3.4. Functions of MCs during Chronic Allergic Reactions

Chronic allergic reactions (Figure 1.4) can develop as a result of repetitive exposure to an allergen. Characteristic of such reactions is the accumulation of a large variety of innate and adaptive immune cells, and the phenotypical as well as functional changes in structural cells (e.g. fibroblasts, myofibroblasts and airway smooth muscle [ASM] cells) at the affected sites. \(^{(64)}\) These reactions are often detrimental to the host as they can quickly escalate significant alterations to tissue architecture if left unrestrained. For example, patients with chronic asthma often suffer from substantial thickening of the airway walls and bronchoconstriction, which together cause severe narrowing of the airway lumen. \(^{(102, 103)}\) Increased deposition of extracellular matrix proteins (e.g. fibronectins and collagens), hyperplasia of goblet cells, and the resultant additional mucus production are some of the other hallmarks of chronic asthma. Similar tissue remodelling processes are also evident in chronic cases of allergic rhinitis \(^{(104)}\) and atopic dermatitis \(^{(105)}\), leading to the formation of nasal polyps and fibrotic papules, respectively.
Figure 1.4. Chronic stage of allergic reactions (in the airway)

In chronic allergic reactions, more MCs are recruited to and develop in the tissues. Together with other tissue-resident as well as newly recruited innate (e.g. neutrophils, basophils and eosinophils) and adaptive (e.g. Th2 cells, B cells and DCs) immune cells, they interact with epithelial cells, structural cells (e.g. fibroblasts, myofibroblasts and airway smooth muscles), blood vessels and lymphatic vessels, thus contributing to airway smooth muscle hyperplasia, bronchoconstriction and severe narrowing of airway lumen, goblet-cell metaplasia and many other symptoms of chronic airway allergy. Exacerbated by exposure to pathogens and/or environmental factors, chronic allergy-induced epithelial injury and the consequent repair response can result in the establish of EMTU. This unit helps to sustain a Th2-associated microenvironment, and thereby promoting sensitization to additional allergens or allergen epitopes and regulating the airway remodelling process. See text for more details. Figure is adapted from Galli et al. (64)

FGF, fibroblast growth factor; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; VEGF, vascular endothelial growth factor.
Allergen

Epithelial injury by viral/bacterial infection and/or environmental factors

Increased mucus production

Airway lumen

Th2 cell

Eosinophil

Basophil

B cell

DC

Broncho-constriction & severe narrowing of airway lumen

MC

CCL11, CXCL8, CXCL12

SCF

Histamine, TNF-α, IL-13

TGF-β, TNF-α, VEGFA

Formation of an EMTU

Cytokines

Inflammatory mediators

FGFs, PDGFs, EGF, VEGFs

Histamine, TNF-α, IL-13

TSLP, IL-33

GM-CSF

IL-3, IL-5

IL-3, IL-4

IL-9, IL-13

GM-CSF

IL-3, IL-5

IL-4, IL-13

IL-4, IL-13

IL-4, IL-13

IL-4, IL-13

IL-4, IL-13

IL-4, IL-13

IL-4, IL-13

IgE production

Myofibroblast Fibroblast

IL-13

IL-3, IL-4

IL-9, IL-13

GM-CSF

IL-3, IL-5

Histamine, TNF-α, IL-13

Formation of an EMTU

Airway smooth muscle hyperplasia
Furthermore, at sites of chronic allergy, damaged epithelial cells and substantial alterations in their barrier functions can result in a higher risk of infections by pathogens or environmental factors.\(^{105-107}\) Such infections, in the cases of chronic asthma, can in turn exacerbate the signs and symptoms of the disease.\(^{106}\) Although the precise mechanisms underlying such exacerbation are not yet fully elucidated, an epithelial-mesenchymal trophic unit (EMTU) is thought to be a contributor, as it is established upon repetitive epithelial damage-repair cycles caused by infections and has been shown to sustain a Th2 environment and therefore promote sensitisation to additional allergens.\(^{108}\)

In addition, disruption of the epithelial barrier can also activate keratinocytes and tissue dendritic cells (DCs) to release thymic stromal lymphopoietin (TSLP) and IL-10, respectively, both of which can then enhance Th2 cell differentiation and contribute to the chronic phase of allergic responses.\(^{105}\)

In the case of chronic asthma, MC hyperplasia is often observed near ASM linings and within the EMTU. At these sites, they further interact with structural cells and thus contribute to many of the hallmarks of the disease, including severe bronchoconstriction, mucus hypersecretion, tissue fibrosis, and the development of “non-specific airway hyperreactivity” to histamine and LTs.\(^{64}\) An interesting feature associated with chronic stages of the disease is the interaction between MCs and structural cells that becomes reciprocal as the number and functionality of the former are also affected by the latter. For example, ASM cells produce several MC chemotactic factors (e.g. CCL11, CXCL8 and CXCL12) and growth factors (e.g. SCF), resulting in further recruitment and local proliferation of MCs, respectively.\(^{101, 109}\) Epithelial cells within the epithelial-mesenchymal trophic unit also produce TSLP and IL-33, both of which are correlated
with the severity of asthma, likely due to their ability to induce DC-mediated Th2 differentiation and to recruit and activate Th2 cells, respectively. \(^{(110-112)}\) The fact that MCs express receptors for both of these cytokines indicates that they can be activated by both to exacerbate the inflammatory response and/or regulate airway remodelling. \(^{(111, 112)}\) Moreover, the ability of MCs to be activated by bacterial or viral products (e.g. via TLRs) suggests a role of these cells also during pathogen-mediated exacerbation of allergic symptoms. \(^{(101, 113, 114)}\) It should be mentioned that although dispensable for the early- and late-phase responses, basophils play a crucial role in initiating the development of IgE-mediated chronic allergic inflammation of the skin by secreting cytokines and other mediators that in turn act on tissue-resident cells (e.g. fibroblasts) as well as induce recruitment of other leukocytes. \(^{(115, 116)}\)

1.3.5. Treatment Strategies for Allergic Reactions and MCs

Although the recent increase in allergy occurrence can be, to some extent, explained by the “hygiene hypothesis”, allergy remains a multifactorial disorder, with environmental, genetic, dietary and other lifestyle factors all contributing and possibly interacting as well. \(^{(117)}\) It is, therefore, imperative that we continue to widen our understanding of the mechanisms of allergic disorders and, more importantly, continue to develop new treatment strategies.

The two fundamental elements of allergy management are allergen avoidance, both before and after sensitisation, and treatments for individuals with the disorder. \(^{(64, 118)}\) Whilst the former strategy is self-explanatory, the application of the latter requires consideration of individual circumstances. For instance, \(\alpha\)-adrenoceptor agonists and non-
sedating H1-antihistamines and topical corticosteroids are well-established treatments for allergic rhinitis sufferers; whereas inhaled corticosteroids and β2-adrenoceptor agonists are currently the mainstay of asthma management. (118)

Due to the substantial involvement of MCs in all three phases of IgE-dependent allergic reactions (Section 1.3.1), new allergy treatment strategies that either directly or indirectly alter the functionality of these cells and/or their mediators have been developed. Allergen-specific immunotherapy, via increasing the level of allergen-specific IgA and IgG4 and decreasing that of allergen-specific IgE, can inhibit MC sensitisation and mediator release, as well as reducing MC numbers in affected tissues. (118) Allergen-specific immunotherapy also induces T regulatory (T\text{reg}) cells to produce IL-10, which in turn suppress the pro-allergic effects of MCs and their mediators. (119) Similarly, the use of the IgE-specific antibody (Ab) omalizumab leads to reduced levels of free IgE molecules, decreased expression of the high affinity IgE receptor (FceRI) on MCs, and ultimately reduced secretion of MC-derived mediators. (120-122) Furthermore, a number of allergy treatments that directly target MC activation have also been reported. (123-125)

1.4. Other Biological Functions of MCs

1.4.1. Roles of MCs in Homeostasis

Although MCs are usually studied in pathological scenarios, they also contribute to a number of homeostatic processes. (4) They are important players in all three phases of wound healing: inducing recruitment of other inflammatory cells to the injured site (via corresponding chemotactic factors) during the “acute inflammatory phase”, promoting re-
epithelialisation and angiogenesis (via angiogenic factors) during the “proliferative phase”, and finally increasing proliferation of epithelial cells and fibroblasts (via vascular endothelial growth factor, platelet-derived growth factor, fibroblast growth factor and nerve growth factor) and thus contributing to tissue scarring and remodelling during the “remodelling phase”. (126) MC-derived histamine, TNFα and substance P are also involved in hair follicle recycling (127), whereas the production of osteopontin is shown to participate in bone resorption and calcification (128).

1.4.2. Roles of MCs in Parasitic Infections

IgE-dependent allergic disorders can be viewed as consequences of “misdirected” Th2 responses that have originally evolved as a host-defence mechanism against parasites. In regard to MC biology, the two scenarios do share a number of similarities. For example, during parasitic infections, MCs become heavily sensitized with parasite Ag-specific IgE that are present at high levels. MC numbers and the amount of MC-derived mediators also increase dramatically at the infected sites. (129) However, direct evidence for the involvement of MCs in protective immunity against parasites comes from only a handful of studies to date. (4, 86) For instance, it is demonstrated that both MCs and intact IgE are required for optimal resistance to a secondary infestation of mice skin with larval Ixodid Haemaphysalis longicornis ticks, although basophils may also contribute to such protection. (86, 130) In addition, MMCP-1-deficient mice are unable to expel Trichinella spiralis, suggesting the anti-helminth properties of this protease, most likely by impairing the epithelial-cell barriers and thus increasing luminal fluid flow and expulsion of the parasites. (131-133) On the other hand, MMCP-1 has little effect on host defense against Nippostrongylus brasiliensis which, unlike the intestinal-epithelial-cell-syncytia-residing
T. spiralis, inhabit the intestinal lumen. This seemingly contradicting data raises the possibility that MC-derived mediators may be more efficient at expelling tissue-dwelling nematodes, potentially by increasing vascular permeability and promoting recruitment of other leukocytes. (132, 134) Furthermore, MCs, among many other cell types such as T cells, basophils and eosinophils, are also a major source of IL-4 and IL-13, both of which play key roles in host resistance to helminth infections. (129)

1.4.3. Roles of MCs in Bacterial and Viral Infections

There has been a substantial amount of evidence indicating important sentinel and effector roles of MCs during bacterial infection, which promote clearance of the bacteria, protect the host from detrimental pathology and enhance survival. Mechanistic explanations for these roles include MC activation via TLR4 and anaphylatoxins, phagocytosis of bacteria, enhancement of the recruitment and/or function of granulocytes, and the proteolytic degradation of endogenous mediators that would otherwise accumulate to toxic levels, such as endothelin-1 and neurotensin. (135, 136) There are, however, a few instances where MCs are shown to increase mortality caused by bacterial infection, via the production of TNFα or dipeptidyl peptidase-I (which in turn reduces IL-6 levels). (137, 138) Therefore, it seems that depending on the intrinsic properties of MCs as well as genetic predispositions of the host to produce larger or smaller amounts of TNFα (137) and other cytokines, the type and/or severity of the bacterial infection can be influenced by MCs to be either beneficial or harmful.

Similarly, MCs also serve as a double-edged sword during viral infections. On one hand, upon activation via TLRs (e.g. TLR3 in response to viral double-stranded RNA), rodent
and human MCs can release an array of chemokines and cytokines, including interferon (IFN)α and IFNβ, that contribute to host defense against viruses.\(^{(48, 113)}\) On the other hand, co-stimulation of rodent and human MCs via FcεRI and certain TLR can trigger MC secretion of various pro-inflammatory mediators, suggesting a possible mechanism through which MCs might contribute to virus- (and bacteria-) mediated exacerbation of allergic asthma and other IgE and MC-associated disorders (Section 1.3.1).\(^{(113)}\)

Furthermore, some viruses such as human immunodeficiency virus (HIV) in particular, can infect MC progenitors, which maintain their viral contents during maturation at multiple tissues\(^{(139, 140)}\), thus making human MCs a reservoir of persistent HIV infection\(^{(141, 142)}\). In these cases, MC exposure to TLR2, -4, or -9 ligands can trigger HIV-1 replication in latently infected cells, and IgE-FcεRI interactions as well as viral Tat protein has been shown to up-regulate HIV-1 co-receptor (e.g. CCR3) expression by MCs, rendering them more susceptible to HIV infection.\(^{(141, 143)}\)

### 1.4.4. Roles of MCs in Cancers

There is currently controversy regarding the roles of MCs in cancers, as evidence supporting both positive and negative contribution of these cells in various tumor models is present. On one hand, MCs have been reported to accumulate in adenomatous polyps and are required for polyp formation, which is the initiating step of colon cancer.\(^{(144)}\) In this study, MC-derived TNFα is suggested responsible for the expansion of MC population at the site of tumor formation. In addition, studies on skin carcinogenesis in MC-deficient WBB6F1-Kit\(^{W/Wv}\) mice indicate the capacity of MCs to facilitate angiogenesis during early stages of tumor progression.\(^{(145)}\) Experiments with another MC-deficient mouse strain (C57BL/6-Kit\(^{W-sh/W-sh}\)) also provide evidence that MCs are
required for angiogenesis and macroscopic expansion of c-Myc-induced pancreatic β-cell tumors. However, due to the importance of c-kit signalling and the involvement of non-MC leukocyte lineages during angiogenesis, the MC-specific roles in the above Kit mutant mice remain to be validated (e.g. via WT MC engraftment experiments). On the other hand, there are certain tumor models in which MCs seem to be beneficial to the host. For example, the offspring of C57BL/6-Kit<sup>W-sh/W-sh</sup> mice and mice that bear early intestinal tumorigenesis exhibit greater frequency and size of adenomas with less tumor cell apoptosis and eosinophil infiltration, thereby indicating a protective function for MCs in colorectal tumorigenesis. One possible conclusion drawn from these seemingly contradicting studies is that, similar to the plasticity displayed during bacterial and viral infections, the net contribution of MCs in various tumor models may favour either the tumor or the host depending on the genetic variables as well as microenvironmental factors within the precise tumor model employed.

1.4.5. Roles of MCs in Chronic Obstructive Pulmonary Diseases (COPD)

Cigarette smoke-induced COPD is a debilitating disorder of the lung, with systemic effects in the skeletal muscle, heart, as well as the other organs, and is the fourth leading cause of chronic morbidity and death worldwide. Previous studies showed accumulated MCs in patients with COPD, where MC-derived tryptase-β (human) levels in sputum are also correlated with the severity of the disease. Interleukin-3-dependent MCs exposed to cigarette smoke-treated culture medium also led to increased expression of MMCP-6, a tryptase known to promote hallmark features of COPD such as inflammation, chemokine secretion, and macrophage and neutrophil chemotaxis. With the aid of a recently developed short-term mouse model of cigarette-induced COPD,
Beckett et al. showed, for the first time, indirect and yet detrimental roles for MMCP-6 and human tryptase-β by causing macrophage accumulation, and macrophage-mediated inflammation, tissue remodelling and emphysema, respectively. \(^{(158)}\) In a separate study, the same group further revealed the adverse functional roles of another mouse MC-derived tryptase, Prss31/transmembrane tryptase/tryptase-γ; as Prss31-null mice showed attenuated macrophage and neutrophil accumulation and reduced histopathology in their lungs following smoke treatment. \(^{(159)}\) These observations were consistent with previous knowledge that individuals deficient in α₁-antitrypsin (A1AT), are more susceptible to COPD development, \(^{(160)}\) and that recombinant human Prss31 can be rapidly inactivated by A1AT. \(^{(161)}\)

### 1.4.6. Negative Immunoregulatory Roles of MCs

In this thesis, a number of key effector as well as positive immunoregulatory roles of MCs have been reviewed, in the context of either IgE-mediated or non-IgE-mediated immune responses. Indeed, MCs have been historically regarded as positive contributors in these settings; and it was not until recent years that certain negative immunoregulatory effects of these cells have been identified. \(^{(11, 86, 99)}\) For example, UVB-induced histamine production by MCs can help to suppress sensitisation for CHS responses to 2,4,6-trinitrochlorobenzene in mice. \(^{(5)}\) Although the exact mechanisms/mediators involved remain to be defined, MCs have been shown to optimise peripheral tolerance to skin allografts \(^{(8)}\) and also contribute to the *Anopheles* mosquito bite-induced suppression of the development of Ag-specific T cell responses in a delayed type hypersensitivity setting \(^{(6)}\). In the former regard, however, it is recently shown that IgE-dependent MC degranulation, either within the graft or systemically, can break established peripheral
tolerance or lead to T cell-mediated acute rejection of skin allograft. The authors demonstrate that MC-derived mediators, such as MMCP-1, IL-4, IL-13 and RANTES, cause a rapid migration of both T_{reg} and MCs out of the graft as well as a transient demise in the secretion of suppressive T_{reg} cytokines.

Furthermore, a considerable number of studies have highlighted the importance of MC-derived IL-10, an anti-inflammatory cytokine, in conveying certain immunosuppressive effects, including suppression of the production of pro-inflammatory cytokines and/or chemokines by both immune (e.g. T cells and monocytes) and structural (e.g. keratinocytes) cells, and enhancement of the ability of DCs to reduce T cell proliferation and cytokine production. Recent studies by Grimbaldeston et al. and Biggs et al. demonstrated that the mechanisms leading to MC production of IL-10 may also vary in different settings, as the IgG\textsubscript{1}-Fc\gamma R signalling pathway is required for MC-IL-10-mediated suppression of many features of severe CHS responses, whereas VitD\textsubscript{3}- and MC VDR-dependence is evident for IL-10 production by skin MCs and subsequently limited pathology associated with chronic low-dose UVB irradiation. In contrast to the above beneficial roles, MC-derived IL-10 can also be harmful for the host. In particular, dermal MCs have been shown to migrate to the draining LNs upon UV irradiation and contribute to immune suppression, whilst C57BL/6-Kit\textsuperscript{W-sh/W-sh} mice engrafted with IL-10\textsuperscript{−/−} BMCMCs, unlike their WT and WT mBMCMC-engrafted counterparts, failed to demonstrate UV-induced suppression of T follicular helper cell function, germinal centre formation and humoral immune response associated with regional LNs.
1.5. Experimental Tools for MC Research

1.5.1. In Vitro Tools

MC research had been hindered until recently due to the difficulties in obtaining sizable and experimentable *ex vivo* MC cultures from *in vivo* tissues, where these cells are normally present in low numbers. Although rodent peritoneal MCs are relatively abundant, can be easily accessed and enriched to near purity \(^{(165)}\), the isolation process is usually labour-intensive and the enzymatic dispersion utilised disrupts normal cell phenotype and cell-to-cell interactions. In addition, due to the facts that there are significant species differences in MC properties and that the human peritoneal cavity has few MCs, extrapolation of results from rodent peritoneal MCs to human MCs must be carefully considered and adjusted based on individual circumstances. \(^{(4)}\) For this reason, MCs isolated from human skin, mucosae (e.g. lung and intestine), heart, uterus and kidney have also been studied. \(^{(166)}\) Unfortunately, this approach is disadvantaged by the limited source of fresh tissue specimens, low cell yields and cumbersome isolation and purification procedures involved. \(^{(167, 168)}\)

To avoid the difficulties in generating tissue-isolated MC cultures, several MC lines have been developed, such as rat RBL-2H3, mouse MC-9 and human HMC-1 and LAD-2. \(^{(4, 166)}\) Despite their abundances and ease of culture, they are transformed cells in nature and therefore could display markedly altered functions. The HMC-1 line, for example, was established from peripheral blood of a MC leukemia patient. \(^{(169)}\) As a result, these cells are immature, independent of SCF for growth, and lack well-formed granules as well as functional IgE receptors. Subsequent development of the more mature human MC lines,
LAD-1 and -2, from BM aspirates of a patient with MC sarcoma/leukemia, partially overcame these problems as these cell lines do require SCF for their survival and growth, have the ultrastructural features of human MCs with numerous granules, express surface FceRI, and degranulate readily upon IgE-mediated activation. (170)

Furthermore, since the early 1980s, primary cultures of MCs from their progenitors have been developed for both mice (e.g. mBMCMCs (171, 172), fetal skin-derived MCs (173), fetal liver-derived MCs (174) and spleen-derived MCs (175)) and humans (e.g. BM-derived MCs (176), fetal liver-derived MCs (177), cord blood-derived MCs (hCBMCs) (178) and more recently peripheral blood-derived MCs (179)). Major drawbacks with this approach are the long (usually 5-12 weeks) and expensive (due to the use of cytokine cocktails) culturing process required. (166) Moreover, although occasionally considered an advantage rather than disadvantage, *in vitro* cultured primary MCs can represent heterogeneous populations depending on their exact culturing conditions. (4) For instance, whereas IL-3 alone drives the development of typical mBMCMCs, which generally appear immature and MMC-like; the combination of IL-3 and SCF can increase the ratio of MCs to basophils during early-stage (up to 11 days) culturing as well as generate a more mature phenotype of MCs. (180, 181) Mouse BMCMCs cultured in the presence of SCF and IL-4 (i.e. no IL-3), on the other hand, give rise to a more CTMC-like population regarding their histochemical properties and response to certain stimuli, including IgE + sAg and substance P. (165)

While it is important to recognise the limitations of each of the *in vitro* MC-research tools mentioned above, they are nevertheless important sources of MCs and each provides a unique platform to study various aspects of MC biology *in vitro*.
1.5.2. In Vivo Tools

To determine whether MCs play a role in the development of a particular human disease, the number of MCs and/or the level of their mediators between normal and pathologic tissues are generally compared and any significant difference would indicate an affirmative answer. Alternatively, the c-kit mutant and thus MC-deficient WBB6F1-Kit$^{W/W-v}$ and C57BL/6-Kit$^{W-sh/W-sh}$ mice have been widely used, together with their WT counterparts, in the field of MC research. Both mouse strains are profoundly deficient in MCs but also carry other significant phenotypic abnormalities. Kit$^{W}$ is a point mutation that causes exon skipping and produces truncated c-kit that is not expressed on the cell surface; Kit$^{W-v}$ is a point mutation in the tyrosine kinase domain of c-KIT; and Kit$^{W-sh}$ is an inversion mutation that affects the transcriptional regulatory element upstream of the c-kit transcription start site on mouse chromosome 5. In addition to a profound deficiency in MC and melanocytes populations, adult WBB6F1-Kit$^{W/W-v}$ and C57BL/6-Kit$^{W-sh/W-sh}$ mice exhibit other abnormalities including, for the former, macrocytic anaemia, sterility, reduced numbers of BM and blood neutrophils, and lack of interstitial cells of Cajal as well as T cell receptor (TCR)$\gamma\delta$ cells in the small intestine; and, for the latter, enlarged spleen, mild cardiomegaly, and increased numbers of BM and blood neutrophils. Unlike the WBB6F1-Kit$^{W/W-v}$ strain, however, C57BL/6-Kit$^{W-sh/W-sh}$ mice are not anaemic, have normal populations of TCR$\gamma\delta$ cells in the intestines and did not exhibit a high incidence of idiopathic dermatitis, ulcers, or squamous papillomas of the stomach.

Due to these additional phenotypic abnormalities, differences in biological responses between WBB6F1-Kit$^{W/W-v}$ and C57BL/6-Kit$^{W-sh/W-sh}$ mice and WT controls may not
necessarily be caused by their MC deficiency. By repairing the MC deficiency through selective systemic (e.g. intravenous [i.v.]) or local (e.g. intraperitoneal [i.p.] and intradermal [i.d.]) engraftment of genetically compatible and in-vitro-derived WT or mutant MCs, so-called “MC knock-in” mice that differ from their c-kit mutant parental strains solely because of the presence of MCs, or mice that differ from each other solely because of the genotype of the MCs engrafted, have been developed and become invaluable in the field of MC research.\textsuperscript{(137, 182)} It should be noted that many MC-related studies today employed both MC-deficient mouse strains so as to confirm specific MC-dependent function(s) \textit{in vivo} irrespective of their individual genetic background and additional abnormalities.

The role of specific MC-derived mediators, on the other hand, can be examined \textit{in vivo} by using animals in which the mediator-encoding gene has been knocked out. Examples of these animals are mice that lack MMCP-1\textsuperscript{(132)}, MMCP4\textsuperscript{(28)}, tryptase β-2/MMCP-6\textsuperscript{(157)}, or mouse MC carboxypeptidase A3\textsuperscript{(184)}, all of which have been used to determine whether the absence of the corresponding protease can affect other aspects of MC phenotype and/or function. However, before definitive conclusions can be drawn, one must take into consideration the extent to which a particular mediator is selectively expressed by MCs, as well as any influence the deletion of the particular mediator has on the expression of other MC-derived products.\textsuperscript{(86)}

The most recent approach to develop new \textit{in vivo} tools for MC research led to the generation of MC-specific “Cre” mice, where Cre recombinase is expressed under the control of the promoter of the gene encoding either MMCP-5 (called Mcpt5 by the authors)\textsuperscript{(185)} or baboon α-chymase\textsuperscript{(186)}. In the former study, crossing the Mcpt5-Cre
transgenic mice with the ROSA26-EYFP Cre excision reporter strain revealed Cre-mediated recombination in MCs from the peritoneal cavity and the skin, with minimum reporter gene expression outside the MC compartment. \(^{(185)}\) In the latter case, on the other hand, the Chymase-Cre-ROSA26R mice generated showed Cre-mediated recombination in MCs from the lungs and colon tissue but not from the peritoneal cavity or in-vitro-derived BMCMCs. \(^{(186)}\) More recently, Dudeck \textit{et al.} generated mouse models of inducible or constitutive MC deficiency based on the \textit{Mcpt5-Cre} mice. \(^{(187)}\) For the inducible MC deficiency model, \textit{Mcpt5-Cre} mice are bred to the \textit{iDTR} strain \(^{(188)}\), in which Cre-mediated excision of a \textit{loxP}-flanked STOP cassette would render cells sensitive to diphtheria toxin (DT), thereby resulting in only MCs in the \textit{Mcpt5-Cre}^+\textit{iDTR}^+ offspring being sensitive to DT-induced cell death. Indeed, a single \textit{i.p.} injection of DT results in an almost complete depletion (98.7\%) of peritoneal MC population, whereas local (subcutaneous) DT treatment leads to similar extent (97.5\%) of MC depletion from the ears. \(^{(187)}\) For the constitutive MC deficiency model, on the other hand, \textit{Mcpt5-Cre} mice are crossed to the \textit{R-DTA} strain \(^{(189)}\), so that only MCs in the progenies, which have Cre-mediated deletion of the STOP cassette, would express DTA and thus undergo self-destruction. \(^{(187)}\) This is confirmed by experimental data that \textit{Mcpt5-Cre}^+\textit{R-DTA}^+ mice exhibited significant reduction in peritoneal and skin MC numbers, resembling DT-treated \textit{Mcpt5-Cre}^+\textit{iDTR}^+ mice mentioned above. \(^{(187)}\) Furthermore, by crossing \textit{Mcpt5-Cre} mice with the IL-10 mutant \textit{IL-10}^{FL/FL} strain \(^{(190)}\), a new mouse line with a selective inactivation of the \textit{IL-10} gene expression in MCs can be obtained. \(^{(187)}\) Together, the data presented above prove \textit{Mcpt5-Cre} mice a powerful tool
for studying the localised and/or systemic roles of MCs as well as particular MC-derived mediators in various disease models.

1.6. VitD₃ (Cholecalciferol)

One substance that can potentially enhance the anti-inflammatory properties of MCs is VitD₃, the “sunshine” vitamin as a significant amount can be synthesised in human skin upon UVB radiation (wavelengths: 280-320 nm). The beneficial effects of VitD₃ were first discovered over a century ago, where anecdotal evidence suggested that a lower occurrence of rickets correlated with geographical locations that received an abundance of sunshine, such as the equatorial regions. However, it was not until the 1930s that such sunshine-induced protection against rickets in children, as well as osteomalacia in adults, was due to UVB-initiated VitD₃ synthesis and its biological activities. Indeed, VitD₃ plays protective and immunoregulatory roles against many detrimental outcomes associated with excess UV exposure, including cutaneous inflammation, photoaging, and gene mutations that can lead to the development of malignancies at the affected sites. The recent findings that VitD₃ can induce MC production of IL-10 both in vitro and in mouse skin following chronic low-dose UVB irradiation in vivo, have brought our attention to the possibility that the enhancement of anti-inflammatory properties of MCs by VitD₃ exposure might represent a novel approach for reducing inflammation and tissue damage in other contexts such as during IgE-dependent allergic responses.
1.7. Biosynthesis and Metabolism of VitD₃

In addition to its dietary sources, including oily fish and certain dairy products (201), the majority of VitD₃ in the body is generated in the epidermis when pre-vitamin D₃, the photochemical product derived from 7-dehydrocholesterol (pro-vitamin D₃) in response to UVB irradiation, undergoes spontaneous thermal isomerisation. (192, 202, 203) The association with VitD₃-binding protein (DBP) allows VitD₃ to be transported to the liver, where it is hydroxylated at the C25 position by cytochrome P450 proteins (e.g. CYP2DII, CYP2D25, CYP3A4 or CYP2R1) to generate 25OHD₃ (calcidiol). DBP-bound 25OHD₃ is subsequently delivered to the kidney, where further hydroxylation at the C1α position is performed by the 25-hydroxyvitamin D-1α-hydroxylase enzyme (CYP27B1), giving rise to the biologically active form 1α,25(OH)₂D₃ (calcitriol). (192, 193, 203) It should be mentioned that whilst the level and activity of liver cytochrome P450 proteins remain relatively constant, the conversion from 25OHD₃ to 1α,25(OH)₂D₃ by CYP27B1 is tightly regulated, by levels of parathyroid hormone, calcium, phosphate, calcitonin, fibroblast growth factor-23, and 1α,25(OH)₂D₃ itself. (203) Inactivation of 1α,25(OH)₂D₃ and 25OHD₃ to 1α,24R,25(OH)₃D₃ and 24R,25(OH)₂D₃, respectively, is commonly attributed to the multifunctional 25-hydroxyvitamin D₃-24-hydroxylase (CYP24A1), which can be transcriptionally induced by the action of both VitD₃ analogues in a very sensitive manner. (204, 205) In addition, a second catabolic pathway for 1α,25(OH)₂D₃ that produces the A-ring diastereomer 1α,25(OH)2D-3epi-D₃ also exists, although the chemical reactions involved are less clear. (206)

In addition to liver and kidney cells, numerous other cell populations, including keratinocytes in the epidermis (207), possess the enzymatic machinery to process VitD₃ to
active metabolites. In the immune cell compartment, macrophages, DCs, T and B cells all express CYP27B1 upon activation and are consequently capable of autonomously producing \(1\alpha,25(OH)_2D_3\) from 25OHD3. \(^{(208-210)}\)

1.8. Biological Activities of VitD3: UVB Protection and Immunoregulation

There are two types of signalling pathways through which \(1\alpha,25(OH)_2D_3\) exerts its biological effects (Figure 1.5); one is a genomic pathway, where \(1\alpha,25(OH)_2D_3\) binds to the well characterised nucleus-residing VDR and induces transcriptional regulation; the other is a non-genomic pathway, which utilises certain plasma membrane-associated receptor(s) (VDR\(_{\text{mem}}\); yet to be fully characterised) and triggers rapid downstream cellular responses. \(^{(202, 203, 211-213)}\)

1.8.1. The Genomic Pathway (Figure 1.5a)

Most known biological functions of \(1\alpha,25(OH)_2D_3\) are mediated through VDRs which, upon ligand binding, undergo conformational changes and subsequent heterodimerization with the retinoid X receptor (RXR). \(^{(214)}\) The \(1\alpha,25(OH)_2D_3\)-VDR-RXR complex binds specifically to vitamin D response elements (VDREs) in the promoter regions of target genes, many of which play a role in VitD3-mediated UVB protection and/or immunoregulation. \(^{(193, 198, 215)}\) Ligand-bound VDR also mediates coregulator switching for chromatin remodelling and histone modifications in the promoters of target genes by inducing dissociation of corepressor complexes and recruitment of coactivator complexes, thereby stimulating the transcription of a distinct set of genes. \(^{(216)}\)
(a) Genomic VitD₃ pathways are triggered by the binding of 1α,25(OH)₂D₃ or conformationally flexible analogs to VDR, which ultimately lead to regulation of gene expression via interaction with VDRE in the promoter region of corresponding genes. Through these pathways, VitD₃ has been shown to reduce accumulation of mutations, oxidative damage as well as cutaneous inflammation caused by UVB irradiation. (b) Non-genomic VitD₃ pathways utilise an unidentified plasma membrane receptor (VDR_{mem}), which can bind to 6-s-cis locked VitD₃ analogs in addition to conventional VDR ligands. By stimulating important cellular signalling components, these pathways can lead to reduced CPD formation and gene mutation, and enhanced DNA repair and cell survival. Non-genomic activities of VitD₃ can also activate PKC and the Ras-MAPK signalling cascade, which may engage in cross-talk with the genomic pathways to stabilise VDR and to modulate gene expression, respectively. See text for more details.
1α,25(OH)2D3 or conformationally flexible analogs can regulate gene expression through VDRmem. Non-genomic pathways include GPCRs and PI(3)K. Genomic pathways involve VDR, VDRE, and components of Hh & β-catenin pathways. UVB-induced mutations and oxidative damage can be repaired by glutathione peroxidase, htrA & selenoprotein P. IL-12, IFNγ, IL-2 & IL-6, IL-4, IL-5 & IL-10 (directly/indirectly) regulate inflammation. Cell survival is promoted by components of Hh & β-catenin pathways.
Among the many genes involved in VitD$_3$-mediated UVB protection is the growth-arrest and DNA-damage gene, *GADD45α*, which encodes a protein important for normal DNA repair and maintenance of global genomic stability. Therefore, by inducing *GADD45α* expression as observed in mouse squamous cell carcinoma cells, 1α,25(OH)$_2$D$_3$ can protect the genome against UVB-induced accumulation of mutations. In addition, a number of genes that possess antioxidant properties, such as *glutathione peroxidase*, *htrA* and *selenoprotein P*, are also direct transcriptional targets of 1α,25(OH)$_2$D$_3$ and thus can confer 1α,25(OH)$_2$D$_3$ the capacity to reduce cumulative oxidative damage upon UVB irradiation. Furthermore, the ability to transcriptionally finetune the expression balance between cyclin-dependent kinase inhibitors, p21 and p27, in a murine squamous cell carcinoma model allows 1α,25(OH)$_2$D$_3$ to suppress cell proliferation in vitro and promote cell differentiation and/or apoptosis in vivo. As recently reviewed, critical components of the Hedgehog and β-catenin signalling pathways possess VDREs in the promoter region of their corresponding gene. Therefore, by direct transcriptional control of these genes and subsequent down-regulation of signalling events in both pathways, 1α,25(OH)$_2$D$_3$ can suppress the development of skin tumors.

The fact that many immune cells constitutively or inducibly express VDRs enables 1α,25(OH)$_2$D$_3$ to interact with various nuclear transcription factors in these cells and to direct transcriptional regulation of various cytokine-encoding genes, thereby granting the genomic pathways a significant role in VitD$_3$-induced immunoregulation. For instance, by blocking NF-κB activation and impeding its binding to the *p40* gene, 1α,25(OH)$_2$D$_3$ can reduce the secretion of IL-12, a major cytokine that drives the pro-inflammatory course of Th1 responses, from activated macrophages and DCs.
decreasing the production of IL-23 and IL-6 from activated DCs, both of which are important for Th17 development, 1α,25(OH)₂D₃ can also dampen the pro-inflammatory Th17 state. (221, 222) Similarly, production of the typical Th1 cytokine IFNγ is inhibited in T cells upon 1α,25(OH)₂D₃ treatment, due to interaction of the 1α,25(OH)₂D₃-VDR-RXR complex with a negative VDRE-like binding sequence in the promoter region of the gene. (223) On the other hand, the production of Th2 cytokines such as IL-4, IL-5 and IL-10 is increased because of up-regulated expression of the Th2 specific transcription factors, GATA-3 and c-Maf (224), and/or direct interaction with VDRE in the promoter region of the corresponding gene such as IL-10 (225). Findings from in vivo mouse studies suggest that the number as well as immunosuppressive activity of CD4⁺CD25⁺ Tregs cells can be elevated upon 1α,25(OH)₂D₃ treatment, leading to further increase in IL-10 levels and an anti-inflammatory response. (222, 226)

Complementing its the ability to dampen certain inflammatory responses by inducing a Th1/Th17-to-Th2 shift, 1α,25(OH)₂D₃ also has anti-proliferative functions in activated T cells via down-regulation of IL-2 production. (227) In this case, 1α,25(OH)₂D₃-VDR-RXR complex binds to the NF-AT binding site within IL-2 promoter and subsequently hinders the formation of NF-AT/AP-1 protein complex. Previous studies in prostatic epithelial cells show that 1α,25(OH)₂D₃ can also reduce IL-6 production. However, instead of a direct transcriptional regulation of the IL-6 gene, 1α,25(OH)₂D₃ targets the VDRE within the promoter of mitogen-activated protein kinase phosphatase 5 (MKP5) gene, leading to increased MKP5 expression, increased inactivation of the MAPK, p38, and ultimately reduced IL-6 production. (228) Furthermore, there are a number of other immunosuppressive properties of 1α,25(OH)₂D₃, the molecular mechanisms of which
still remain to be fully elucidated but are nevertheless VDR-dependent. Examples are the
inhibition of DC maturation and costimulatory molecule expression \(^{(222)}\) and enhanced IL-
10 production by cutaneous MCs in a chronic UVB irradiation setting as previously
mentioned \(^{(163)}\).

1.8.2. The Non-genomic Pathway (Figure 1.5b)

While much attention has focussed on the genomic pathway of VitD\(_3\) action, there is
emerging evidence that \(1\alpha,25(OH)\_2D\_3\) can also generate biological responses via rapid
(seconds to minutes), non-genomic pathways. These pathways utilise currently
unidentified VDR\(_{\text{mem}}\) \(^{(202, 215)}\), although recent data indicate the conventional VDR being a
likely candidate \(^{(229, 230)}\), to trigger the activities of important cellular signalling
components such as PKC, cyclic AMP (cAMP), intracellular Ca\(^+\) and MAPKs \(^{(211)}\).
Because \(1\alpha,25(OH)\_2D\_3\) is capable of facile rotation about its 6, 7 single carbon bond, it
can generate a continuum of potential shapes extending from the 6-s-cis (6C) to the 6-s-
trans (6T) conformation. \(^{(231)}\) Unlike the genomic pathway, which requires
conformationally flexible ligands such as \(1\alpha,25(OH)\_2D\_3\) itself, the non-genomic pathway
can utilise 6C-locked analogs as efficiently as the original ligand. \(^{(211, 231)}\) This has led to
the development of pathway-specific VitD\(_3\) agonists and antagonists, which have become
valuable tools for determining the nature of \(1\alpha,25(OH)\_2D\_3\)-elicited biological effects. For
example, with the help of the rapid acting agonists, \(1\alpha,25(OH)\_2\text{Lumisterol}_3\) and
\(1\alpha,25(OH)\_2\text{-7-dehydrocholesterol}\), and the rapid response antagonist \(1\beta,25(OH)\_2D\_3\), it
can be observed that \(1\alpha,25(OH)\_2D\_3\) treatment protects human keratinocytes against UVB-
induced CPD formation via a non-genomic pathway, both \textit{in vitro} and \textit{in vivo}, thus
promoting DNA repair and cell survival. \(^{(232, 233)}\)
Although the non-genomic VitD3 pathways do not depend on transcription, they may indirectly affect transcription through cross-talk with the genomic pathways \(^{(215)}\). It is well known that the Ras-MAPK cascade is one of several that are targeted by rapid VitD3 response. In particular, 1α,25(OH)\(_2\)D\(_3\) can activate Ras-Raf/MEK/ERK signalling in a PI(3)K-dependent manner in keratinocytes, resulting in increased AP-1 transcriptional activities and ultimately the differentiation of these cells; a UVB-protective outcome. \(^{(234)}\)

Apart from PI(3)K, Ras-Raf/MEK/MAPK pathway can also be initiated via activation of PKC or cAMP or even directly upon binding of 1α,25(OH)\(_2\)D\(_3\) to VDR\(_{mem}\) and/or the G-protein coupled receptors. \(^{(215)}\) It is therefore significant that activation of ERK can increase transcriptional activity of VDR and that activation of PCK can stabilize VDR through phosphorylation, as this suggests co-operation between the non-genomic and the genomic pathways to trans-activate VDR and elicit UVB-protective as well as immunoregulatory effects of 1α,25(OH)\(_2\)D\(_3\). \(^{(215)}\)

### 1.9. VitD\(_3\) and MCs

#### 1.9.1. VitD\(_3\)-mediated Immunoreegulation of MCs

The recent discovery that MCs, like many other immune cells, express VDR \(^{(163)}\) and are therefore 1α,25(OH)\(_2\)D\(_3\) responsive creates another regulatory axis for VitD3, where it elicits its effects by regulating the functionality of MCs. Indeed, in an IgE-dependent allergic asthma model, where MCs are known to play important roles in exacerbating the associated pathology, subcutaneous co-administration of 1α,25(OH)\(_2\)D\(_3\) with allergen is capable of reducing the airway hyperresponsiveness, potentially due to an enhanced IL-10 level in the asthmatic lung tissues. \(^{(235)}\) Although T\(_{reg}\) cells are claimed to be
responsible for the secretion of IL-10 at the time, the subsequent discovery of MCs being an alternative source of this cytokine \(^\text{(7)}\) suggests that the immunosuppressive roles of 1α,25(OH)\(_2\)D\(_3\) observed could also be due to its stimulatory effects on MC production of IL-10. Direct evidence for this notion is provided by the recent study demonstrating the ability of 1α,25(OH)\(_2\)D\(_3\) to induce VDR-dependent IL-10 production by mBMCMCs \textit{in vitro} as well as by cutaneous MCs upon UVB irradiation \textit{in vivo}. \(^\text{(163)}\)

In addition to stimulating IL-10 production, 1α,25(OH)\(_2\)D\(_3\) can also repress the pro-inflammatory properties of MCs, such as reducing calcium ionophore A23187-induced degranulation (histamine release) of mouse peritoneal MCs \(^\text{(236)}\) and decreasing the release of various pro-inflammatory mediators (e.g. MMCP-1 and LTC\(_4\)) by mBMCMCs \(^\text{(237)}\). Therefore, 1α,25(OH)\(_2\)D\(_3\) can indeed drive the functionality of MCs towards the anti-inflammatory end of the spectrum, as observed so far in IgE-independent immune settings but not yet fully understood during IgE-dependent responses. For the purpose of the current project, we aim to investigate if 1α,25(OH)\(_2\)D\(_3\)-mediated anti-inflammatory properties of MCs are also evident in IgE-dependent immune settings. Furthermore, since many other immune cell types express CYP27B1, we will determine the MC expression status of this enzyme and, if expressed, whether 25OHD\(_3\) can have the same effects as 1α,25(OH)\(_2\)D\(_3\) on MC bioactivities due to the 25OHD\(_3\) to 1α,25(OH)\(_2\)D\(_3\) conversion by MC CYP27B1.

1.9.2. Potential Mechanisms for VitD\(_3\)-mediated Immunoregulation of MCs

There are several mechanisms by which VitD\(_3\) can modulate the functions of MCs, via either transcriptional or post-transcriptional regulation of VitD\(_3\)-responsive genes, especially those encoding MC-derived mediators. One mechanism that utilises
transcriptional regulation involves fine-tuning (e.g. alteration in phosphorylation/activation status), via the rapid non-genomic pathways (section 1.8.2), of the FcεRI signalling pathway components, many of which can engage in cross-talk with the genomic pathways (Section 1.8.1) to modulate gene expression. One pathway that might bear relevance to the current project is the Ras-MAPK pathway, which involves phosphorylation of MAPKs, such as ERK1/2, and enhanced production of pro-inflammatory lipid-derived mediators and cytokines upon IgE+sAg-mediated MC activation. Another pathway of interest involves activation of the transcription factor, NF-κB, via either the serine-threonine kinase Akt or PKC, which is required for gene expression of many pro-inflammatory cytokines, especially IL-6 and TNFα, in MCs during IgE-mediated inflammation.

1.10. Rationales for this Study

In light of the new concept that MCs can negatively regulate the duration and magnitude of inflammatory responses in IgE-independent settings (Section 1.4.5), three major questions remain to be addressed: (1) Can MCs also display anti-inflammatory properties during IgE-dependent allergy-type immune responses, where they are so accustomed to being regarded as pro-inflammatory effector and immunoregulatory cells (Section 1.3)? and (2) How can the anti-inflammatory properties of MCs be potentiated in these immune settings? and (3) What cellular mechanisms may be involved in such regulation of MC functionality?

Our recent findings that the biologically active VitD₃ can induce IL-10 production by MCs and thus limit the pathology associated with chronic low-dose UVB irradiation
suggest the potential use of this well recognised immunomodulatory agent to drive a positive-to-negative-immunoregulatory functional transition of MCs during IgE + sAg-mediated immune responses. The in vivo passive cutaneous anaphylaxis (PCA) model used in this study provides a MC-dependent IgE-mediated allergy platform, which exhibits typical early- and late-phase responses including tissue swelling and leukocyte infiltration, respectively. Thus, by examining the effects of \(1\alpha,25(OH)_2D_3\) on various functional read-outs (e.g. production of pro-inflammatory and/or anti-inflammatory mediators) of in vitro-derived MCs upon IgE + sAg-mediated activation, and studying the effects of topically applied \(1\alpha,25(OH)_2D_3\) on the cutaneous pathology associated with the PCA setting using MC knock-in mice as well as WT and MC deficient controls, the first two questions mentioned above could be addressed. Subsequent mechanistic investigation into the effects of \(1\alpha,25(OH)_2D_3\) treatment on the functional status of FcεRI signalling components, as well as the functional comparison between \(1\alpha,25(OH)_2D_3\) and its non-genomic pathway-specific agonist, curcumin, could then shed light on the third question. In addition to \(1\alpha,25(OH)_2D_3\), the effects of other inactive VitD₃ metabolites, in particular 25OHD₃, will also be studied to determine if MCs possess the same capability as other immune cells to autonomously generate their own \(1\alpha,25(OH)_2D_3\).

1.11. Hypothesis

The immunoregulatory agent, VitD₃, alters IgE + sAg-mediated MC activation from a pro-inflammatory state to an anti-inflammatory one, both in vitro and during IgE-dependent PCA in vivo.
1.12. Project Aims

There are three main aims of the current project, each of which will be addressed in more details in subsequent chapters.

**Aim 1: To characterise the modulation of IgE + sAg-mediated activation of mBMCMCs by 1α,25(OH)2D3 in vitro.**

This aim, having been dissected into the following three sub-aims, will address the capacity of 1α,25(OH)2D3 to influence the immunological nature of IgE (IgE anti-dinitrophenyl [DNP] Ab) + sAg (human serum albumin [HSA]-DNP)-dependent mBMCMC activation and, at the same time, explore the potential mechanisms involved.

**Aim 1.1** To determine the nature of the effects of 1α,25(OH)2D3 on IgE + sAg-stimulated MCs, mBMCMCs from WT mice will be activated in the presence of various concentrations of 1α,25(OH)2D3 and then (a) the extent of MC degranulation will be assessed by histamine enzyme immunoassay (EIA) and β-hexosaminidase assay; (b) the gene expression for a number of MC-derived pro-inflammatory and anti-inflammatory cytokines will be examined using quantitative real-time PCR (qRT-PCR); and (c) the protein secretion of the same set of cytokines will be measured by enzyme-linked immunosorbent assay (ELISA).

**Aim 1.2** To determine the VDR-dependence of any effect of 1α,25(OH)2D3 observed during the above investigations, mBMCMCs will be generated from VDR<sup>−/−</sup> mice and analysed in parallel with their WT counterparts in the above experiments. Although VDR-dependence would generally indicate the genomic pathway for 1α,25(OH)2D3, this notion was questioned by the recent findings that conventional VDR may also serve as
VDR\textsubscript{mem} during non-genomic responses. (229, 230) To ascertain the nature of the 1\alpha,25(OH)\textsubscript{2}D\textsubscript{3} signalling pathway involved in MC immunoregulation, the commercially available curcumin, an agonist for the non-genomic pathway, will be used instead of 1\alpha,25(OH)\textsubscript{2}D\textsubscript{3} in a number of experiments mentioned above. Any effect of curcumin will be compared with that of 1\alpha,25(OH)\textsubscript{2}D\textsubscript{3}.

**Aim 2: To examine the involvement of autonomous 1\alpha,25(OH)\textsubscript{2}D\textsubscript{3} synthesis in VitD\textsubscript{3}-mediated immunoregulation of mBMCMC.**

This aim is dissected into two sub-aims as follows, which will address the potential ability of MCs to synthesise 1\alpha,25(OH)\textsubscript{2}D\textsubscript{3} from the inactive precursor 25OHD\textsubscript{3} and subsequently the effect of 25OHD\textsubscript{3} on IgE + sAg-activated mBMCMCs.

**Aim 2.1** To determine if mBMCMCs are capable of converting 25OHD\textsubscript{3} to 1\alpha,25(OH)\textsubscript{2}D\textsubscript{3}. The expression of CYP27B1 in naive mBMCMCs will firstly be examined by intracellular staining followed by flow cytometry and/or immunofluorescence microscopy. If this enzyme is indeed expressed by the cells, then any potential regulatory mechanism for its expression will be investigated. This involves treating mBMCMCs with either its substrate (i.e. 25OHD\textsubscript{3}) or its product (i.e. 1\alpha,25(OH)\textsubscript{2}D\textsubscript{3}), and noting any change in its expression level using qRT-PCR and/or western blotting. In addition, if CYP27B1 expression in MCs is confirmed, the extent of endogenous 1\alpha,25(OH)\textsubscript{2}D\textsubscript{3} synthesis from 25OHD\textsubscript{3}-treated mBMCMCs (in both culture supernatants and cell lysates) will be measured using a 1,25-(OH)\textsubscript{2}D radioimmunoassay (RIA) kit. Finally, to examine if VDR is required for CYP27B1 expression, its regulation,
and/or endogenous $1\alpha,25(OH)_2D_3$ synthesis in MCs, $VDR^{+/-}$ mBMCMCs will be analysed in parallel with their WT counterparts in all above experiments.

**Aim 2.2** To investigate the effect of 25OHD$_3$ on IgE + sAg-activated mBMCMCs, as sufficiently high concentrations of 25OHD$_3$ are able to alter the functions of mouse kidney and skin cells by binding directly to VDR. $^{243}$ To achieve this purpose, WT mBMCMs will be stimulated in the presence of various concentrations of 25OHD$_3$, in the same way as described for $1\alpha,25(OH)_2D_3$ in Aim 1.1, and the extent of degranulation and cytokine production will be assessed by β-hexosaminidase assay and ELISA, respectively. If MCs are capable of converting 25OHD$_3$ to $1\alpha,25(OH)_2D_3$ (Aim 2.1), an alternative experimental setting will also be used, where MCs are treated with 25OHD$_3$ for an certain period of time prior to IgE preload so that the cells have sufficiently time to generate their own $1\alpha,25(OH)_2D_3$. Furthermore, to determine the VDR-dependence for any potential immunoregulatory effect of 25OHD$_3$ in activated MCs, $VDR^{-/-}$ mBMCMCs will be used in addition to WT cells in all above experiments.

**Aim 3: To investigate the effects of topically applied VitD$_3$ on the cutaneous pathology associated with IgE-mediated PCA in vivo**

To address this aim, a localised PCA reaction, which provides an almost entirely MC-dependent Type I immediate hypersensitivity setting $^{89}$, will be triggered in the ear of WT C57BL/6 mice, together with topical application of either $1\alpha,25(OH)_2D_3$, 25OHD$_3$ or just the vehicle. In the case of 25OHD$_3$ application, alternative experimental settings regarding the duration of 25OHD$_3$ treatment may be used depending on the outcome of *in vitro* studies described in Aim 2.2. To determine the requirement of VDR for any potential immunoregulatory effects of VitD$_3$ analogues on PCA-associated pathology,
VDR⁻/⁻ mice will be used in parallel with their WT counterparts. The rationale for this is: if VDR is required then these mice will not be subjected to VitD₃-mediated regulation as seen in WT animals. To further verify that VitD₃ requires MC-specific VDR expression to elicit its effects, MC knock-in mice (engrafted with WT or VDR⁻/⁻ mBMCMCs) as well as the MC-deficient c-kit mutant mice will also be used together with WT controls. The rationale in this case is: if the effects of VitD₃ are indeed dependent on MC VDR expression, then whilst MC-deficient mice will not exhibit a PCA response (due to the lack of MCs), WT MC knock-in mice will mirror the response of WT controls upon all treatments (due to the presence of MCs with functional VDR in the ears) and, VDR⁻/⁻ MC knock-in mice will fail to respond to any VitD₃-mediated regulation (due to the absence of VDR expression in MCs in the ears). In all PCA-related experiments, one or more of the following assessments will be performed, in order to gain information on as many aspects of the responses:

(1) Tissue swelling: Ear thickness will be measured at multiple intervals from 30 min – 6 h after the onset of the allergic response.

(2) Local and systemic production of MC-derived mediators: Ear lysate and serum samples will be prepared from treated mice and used to detect the same set of MC-derived cytokines as for in vitro assessments (i.e. TNFa, IL-6 and IL-10). Total RNA will also be extracted from ear lysates and subjected to qRT-PCR analysis for the expression of corresponding genes.

(3) Histological detection and enumeration of MCs: Paraffin sections will be prepared from treated mouse ears and stained with toluidine blue, a MC-specific
metachramatic dye. The density, granularity and tissue-distribution of MCs will be demonstrated and determined by light microscopy. This is particularly important in “MC knock-in” mice, which will be compared to their WT and MC-deficient counterparts to ensure successful engraftment of MCs.

**Aim 4: To investigate if the immunoregulatory effects of $1\alpha,25(OH)_2D_3$ and/or $25OHD_3$ on IgE + sAg-activated mBMCMCs in vitro can be translated to hCBMCs**

To determine whether the regulatory effects of $1\alpha,25(OH)_2D_3$ are also applicable to human MCs, hCBMCs will be generated and activated (in this case by human (h)IgE preload followed by $\alpha$-hIgE Ab stimulation) in the presence of either $1\alpha,25(OH)_2D_3$ or vehicle. The production of various cytokines will be subsequently measured by ELISA. To assess the ability of hCBMCs to generate endogenous $1\alpha,25(OH)_2D_3$, the gene as well as protein expression of CYP27B1 in hCBMCs will firstly be examined. If its expression is confirmed, then culture supernatants and lysates will be collected from 25OHD3- or vehicle-treated hCBMCs, and the amount of $1\alpha,25(OH)_2D_3$ present in all samples will be analysed by the 1,25-(OH)2D RIA kit.

1.13. Expected Outcomes and Significance

It is becoming increasingly clear that depending on individual immunological circumstances, MCs can either enhance or suppress an innate or adaptive immune response. This raises the necessity to re-visit some MC-related research areas that have been intensely investigated in the past but nevertheless focused primarily on the pro-inflammatory roles of MCs. Allergic conditions that are mediated by IgE and its cognate
allergen is one of these areas, which will therefore be the ultimate focus of the current project. Given already shown attributes of VitD3 with other myeloid-derived cell populations, it is anticipated that MC functions might also be modulated by this immunoregulatory agent. Multiple mechanisms are expected to be responsible for VitD3-mediated immunosuppression via MCs, a number of which will be revealed at the completion of the study. Furthermore, the ability of MCs to synthesise 1α,25(OH)2D3 from inactive precursors, and thus their ability to respond to treatment with these precursors, will also be investigated for the first time. Together, the outcomes of this project will broaden our knowledge on the complexity of MC function in IgE-dependent allergic disorders and, more importantly, it will evaluate the therapeutic potential of VitD3 for the prevention and treatment of these disorders via its immunoregulatory effects on MCs.
CHAPTER 2
MATERIALS & GENERAL METHODS
2.1. Commercial Reagents

2.1.1. Common Tissue Culture Reagents

Dulbecco’s modified eagle medium high glucose (DMEM): Gibco

Iscove’s modified Dulbecco’s medium (IMDM): Gibco

Penicillin (10,000 U/mL)/Streptomycin (10,000 µg/mL) (Pen/Strep): Gibco

2-mercaptoethanol; 1,000× (2-ME): Gibco

Fetal bovine serum (FCS): Gibco

Horse serum (HS): Gibco

Albumin solution from bovine serum; 35% (BSA): Sigma

Dulbecco’s phosphate buffered saline; 10× (PBS): Gibco

MEM non-essential amino acid solution; 100× (NEAA): Sigma

Saponin (from Quillaja Bark): Sigma

Anticoagulant citrate dextrose solution (ACD):

Histopaque-1077: Sigma

Dextran: Pharmacosmos

Human Fc receptor blocking Ab: Miltenyi Biotec

Magnetic CD34+ beads: Miltenyi Biotec

Dimethyl sulphoxide (DMSO): BDH

Albumin, dinitrophenol (DNP-HSA): Sigma

Water for irrigation: Baxter

Charcoal: Sigma

Trypan blue: BDH
2.1.2. *Vitamin D₃ Analogues*

1α,25-Dihydroxyvitamin D₃; ≥ 99% (stock solution [1 mM] and dilutions prepared in 100% ETOH; stored at -80 °C): Sigma

25-Hydroxycholecalciferol; 98% (stock solution [1 mM] and dilutions prepared in 100% ETOH; stored at -80 °C): Sigma

Curcumin; ≥ 94% curcuminoid content (stock solution [1 mM] and dilutions prepared in 100% ETOH; stored at -80 °C): Sigma

4,4’-Diisothiocyanatostilbene-2,2’-disulfonic acid disodium salt hydrate; ≥ 80% (DIDS; stock solution [100 mM] and dilutions prepared in DMSO; stored at 4 °C): Sigma

2.1.3. *Cytokines and Recombinant Proteins*

Recombinant mouse IL-3 (rmIL-3): ProSpec-Tany

Recombinant human SCF (rhSCF): Shenandoah Biotechnology

Recombinant human IL-6 (rhIL-6): Shenandoah Biotechnology

Recombinant human IL-4 (rhL-4): ProSpec-Tany

2.1.4. *Hybridomas*

WEHI-3 Hybridoma: Stanford University, USA

SPE-7 Hybridoma: Weizmann Institute of Science, Israel

2.1.5. *Antibodies for Tissue Culture*

Monoclonal α-DNP (mouse IgE; clone SPE-7): Sigma

Monoclonal α-DNP (mouse IgE; clone H1 ε26): Stanford University, USA

Human myeloma IgE: Merck

Goat α-human IgE: eBioscience
2.1.6. Reagents for SPE-7 Purification

1-Fluoro-2,4-dinitrobenzene (FDNB): Sigma
Dioxane: BDH
HiTrap NHS-activated HP column: GE Healthcare
2,4-dinitrophenol; 97% (DNP): Sigma
Propionic Acid: BDH
Coomassie brilliant blue R-250: Thermo Scientific
VIVASPIN 20 tube: Sartorius Stedim Biotech

2.1.7. β-Hexosaminidase Release Assay Reagents

Phorbol-12 myristate-13 acetate (PMA): Sigma
Calcium ionophore A23187: Sigma
P-Nitrophenyl-N-acetyl-Beta-D-glucosaminide (p-NAG): Sigma

2.1.8. ELISA/EIA Kits and Reagents

BD OptEIA™ mouse IL-3 ELISA Set (≥ 7.81 pg/mL): BD Biosciences
BD OptEIA™ mouse IL-6 ELISA Set (≥ 15.63 pg/mL): BD Biosciences
BD OptEIA™ mouse IL-10 ELISA Set (≥ 31.25 pg/mL): BD Biosciences
BD OptEIA™ mouse TNFα ELISA Set (≥ 15.63 pg/mL): BD Biosciences
Human TNFα ELISA kit: eBioscience
Human IL-10 ELISA kit: eBioscience
EIA histamine kit: Immunotech (Beckman Coulter)
Radioimmunoassay kit (1,25-(OH)2D RIA kit; ≥ 5 pM): Immunodiagnostic Systems Ltd
3,3’,5,5’-tetramethyl-benzidine liquid substrate: Sigma
Tween-20: Sigma
2.1.9. Flow Cytometry Reagents

FITC-conjugated α-mouse FcεRIα (American Hamster IgG): eBioscience
Alexa Fluor 647-conjugated α-mouse FcεRIα (American Hamster IgG): eBioscience
PE-conjugated α-mouse CD117 (c-kit; rat IgG2b): eBioscience
Purified α-mouse CD16/32 (blocks Fc binding): eBioscience
FITC-conjugated α-mouse IgE (rat IgG1): BD Biosciences
FITC-conjugated American Hamster IgG isotype control: eBioscience
PE-conjugated rat IgG2b isotype control: eBioscience
FITC-conjugated rat IgG1 isotype control: eBioscience
Polyclonal α-CYP27B1 (rabbit IgG): Santa Cruz Biotechnology
Purified rabbit IgG isotype control: Dako
PE-conjugated α-rabbit IgG: Southern Biotech
Human Tryptase (Mouse): Millipore
1B5 mouse IgG1 isotype control: School of Molecular and Biomedical Science, University of Adelaide
PE-conjugated α-mouse IgG: Southern Biotech
NucView™ 488 Caspase-3 assay kit for live cells: Biotium
Fixation & Permeabilization Kit: eBioscience

2.1.10. Histochemistry & Immunohistochemistry Reagents

Polyclonal α-CYP27B1 (rabbit IgG): Santa Cruz Biotechnology
Rabbit polyclonal IgG (isotype control): Abcam
Alexa 594-conjugated goat α-rabbit: Molecular Probes
Human Tryptase (Mouse): Millipore
1B5 mouse IgG1 isotype control: School of Molecular and Biomedical Science, University of Adelaide

Alexa 594-conjugated goat α-mouse IgG: Molecular Probes

Accustain Geimsa and May-Grünwald stains: Sigma

Toluidine blue O: Sigma

DePex mounting medium: BDH

Fixation & Permeabilization Kit: eBioscience

DAPI (4’,6-Diamidino-2’-phenylindole dihydrochloride): Roche

Fluorescence Mounting Medium: DAKO

2.1.11. SDS-PAGE & Western Blotting Reagents

Polyclonal α-CYP27B1 (rabbit IgG): Santa Cruz Biotechnology

Rabbit α-CYP24A1: Chemicon

α-β-actin: Chemicon

Horse radish peroxidase (HRP)-conjugated α-mouse: Pierce

HRP-conjugated α-rabbit: Pierce

Bio-Rad Dc protein assay: Bio-Rad

BSA standard: New England Biolabs

Benchmark™ pre-stained protein markers: Invitrogen

Nitrocellulose membrane: Advantec

Blocking reagent: Roche

Amersham ECL Plus Western Blotting Detection System: GE Health

Stripping solution: Alpha Diagnostic International
2.1.12. Primers (all used at 5 µg/mL): Geneworks

Common forward (i.e. $VDR^{+/+}/VDR^{-/-}$): 5’ - TTCTTCAGTGCCAGCTCTT - 3’
C57/BL6 - $VDR^{+/+}$ reverse: 5’ - CTCCATCCCCCATGTGTCTTT - 3’
C57/BL6 - $VDR^{-/-}$ reverse: 5’ - CTAAACGCAGCTGCTGCTCCAGAC - 3’
Mouse β-actin forward (F): 5’ - TGGAATCCTGTGGCATCCATGAAAAC - 3’
Mouse β-actin reverse (R): 5’ - TAAAACGCAGCTGCTGCTGAAATCGATCCA - 3’
Mouse IL-10 F: 5’ - GCCCTATCGGAAATGATCAA - 3’
Mouse IL-10 R: 5’ - TTCTCACCGGGAATTCAA - 3’
Mouse TNFa F: 5’ - GATTATGGCTAGGTCCAA - 3’
Mouse TNFa R: 5’ - GAGACAGAGGCAACCTGACC - 3’
Mouse IL-6 F: 5’ - CAAAGCCAGAGTCCTTCAGA - 3’
Mouse IL-6 R: 5’ - GATGGTCTTGGTCCTTAGCC - 3’
Mouse histidine decarboxylase (HDC) F: 5’ - AGGAGGAAATCCAAAGGGAGAT - 3’
Mouse HDC R: 5’ - GGTATCCAGGCTGCACATTT - 3’
Mouse CYP27B1 F: 5’ - GCTGAAATCCCTCCTGACAC - 3’
Mouse CYP27B1 R: 5’ - GCTGAAATCCCTGACAC - 3’
Human β-actin F: 5’ - AGCACAGAGCCTCGCT - 3’
Human β-actin R: 5’ - CACGATGGAGGGGAAGAC - 3’
Human CYP27B1 F: 5’ - TGTTTGAATTTGCTCAGAGG - 3’
Human CYP27B1 R: 5’ - CGCCAATAGCAACTCTCTGTCA - 3’

2.1.13. Molecular Biology Reagents

Proteinase K: Merck
GoTaq green master mix: Promega
pUC19/HpaII DNA molecular weight marker: GeneWorks

GelRed™ Nucleic Acid Gel Stain; 10,000×: Biotium

TRIZol reagent: Invitrogen

QuantiTech reverse transcription kit: Qiagen

QuantiTech Sybr green polymerase chain reaction (PCR) master mix: Qiagen

2.1.14. In Vivo Experiment Reagents

Hank’s MEM (HMEM): Sigma

Piperazine-\(N,N'\) bis(2-ethane sulfonic acid; Pipes): Sigma

1,2-propanediol (propylene glycol); 99%: Sigma

0.9% (w/v) sodium chloride: AstraZeneca

T-PER tissue protein extraction reagent: Thermo Scientific

Complete mini EDTA-free (protease inhibitor cocktail tablets): Roche

2.2. Solutions and Buffers

2.2.1. Tissue Culture Media and Solutions

**Flushing medium**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pen/Strep</td>
<td>2%</td>
<td>10 mL</td>
</tr>
<tr>
<td>2-ME</td>
<td>0.1%</td>
<td>0.5 mL</td>
</tr>
<tr>
<td>DMEM</td>
<td></td>
<td>To 500 mL</td>
</tr>
</tbody>
</table>

**Complete DMEM medium (cDMEM)**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>FCS</td>
<td>10%</td>
<td>50 mL</td>
</tr>
<tr>
<td>Pen/Strep</td>
<td>1%</td>
<td>5 mL</td>
</tr>
<tr>
<td>2-ME</td>
<td>0.1%</td>
<td>0.5 mL</td>
</tr>
<tr>
<td>DMEM</td>
<td></td>
<td>To 500 mL</td>
</tr>
</tbody>
</table>
### Complete DMEM medium with csFCS (cDMEM-csFCS)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>csFCS</td>
<td>10%</td>
<td>50 mL</td>
</tr>
<tr>
<td>Pen/Strep</td>
<td>1%</td>
<td>5 mL</td>
</tr>
<tr>
<td>2-ME</td>
<td>0.1%</td>
<td>0.5 mL</td>
</tr>
<tr>
<td>DMEM</td>
<td></td>
<td>To 500 mL</td>
</tr>
</tbody>
</table>

### Complete BMCMC medium – early-stage culturing (cBMCMC-early)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>WEHI-3-conditioned medium</td>
<td>20%</td>
<td>100 mL</td>
</tr>
<tr>
<td>FCS</td>
<td>10%</td>
<td>50 mL</td>
</tr>
<tr>
<td>Pen/Strep</td>
<td>2%</td>
<td>10 mL</td>
</tr>
<tr>
<td>2-ME</td>
<td>0.1%</td>
<td>0.5 mL</td>
</tr>
<tr>
<td>rmIL-3 (10 µg/mL)</td>
<td>Adjust to 3 ng/mL final</td>
<td>Varies depending on the concentration of mIL-3 in the WEHI-conditioned medium used</td>
</tr>
<tr>
<td>DMEM</td>
<td></td>
<td>To 500 mL</td>
</tr>
</tbody>
</table>

### Complete BMCMC medium – late-stage culturing (cBMCMC-late)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>WEHI-3-conditioned medium</td>
<td>20%</td>
<td>100 mL</td>
</tr>
<tr>
<td>FCS</td>
<td>10%</td>
<td>50 mL</td>
</tr>
<tr>
<td>Pen/Strep</td>
<td>2%</td>
<td>10 mL</td>
</tr>
<tr>
<td>2-ME</td>
<td>0.1%</td>
<td>0.5 mL</td>
</tr>
<tr>
<td>rmIL-3 (10 µg/mL)</td>
<td>Adjust to 4 ng/mL final</td>
<td>Varies depending on the concentration of mIL-3 in the WEHI-conditioned medium used</td>
</tr>
<tr>
<td>DMEM</td>
<td></td>
<td>To 500 mL</td>
</tr>
</tbody>
</table>

### Sucrose-MgCl₂-Hepes solution

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>250 mM</td>
<td>34.23 g</td>
</tr>
<tr>
<td>MgCl₂•6H₂O</td>
<td>1.5 mM</td>
<td>0.122 g</td>
</tr>
<tr>
<td>Heps</td>
<td>10 mM</td>
<td>0.954 g</td>
</tr>
<tr>
<td>H₂O</td>
<td></td>
<td>To 400 mL</td>
</tr>
</tbody>
</table>

pH adjusted to 7.4
### Dextran-coated charcoal

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activated charcoal powder</td>
<td>0.25%</td>
<td>1 g</td>
</tr>
<tr>
<td>Dextran T-70</td>
<td>0.0025%</td>
<td>0.01 g</td>
</tr>
<tr>
<td>Sucrose-MgCl&lt;sub&gt;2&lt;/sub&gt;-Hepes solution</td>
<td>400 mL</td>
<td></td>
</tr>
</tbody>
</table>

The above were mixed together and incubated at 4°C overnight with constant agitation.

### Charcoal-stripped FCS (csFCS; heat-inactivated)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dextran-coated charcoal</td>
<td></td>
<td>400 mL</td>
</tr>
<tr>
<td>FCS</td>
<td></td>
<td>400 mL</td>
</tr>
</tbody>
</table>

Spin the Dextran-coated charcoal (500 g; 10 min) solution to pellet the charcoal. Discard the supernatant, and resuspend the pellet with equal volume of FCS. Incubate the charcoal-FCS solution at 55°C for 45 min, invert the container to mix, and incubate for a further 45 min.

### Complete BMCMC medium with csFCS and 3 ng/mL rmIL-3 (cDMEM-csFCS-3)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>csFCS</td>
<td>10%</td>
<td>50 mL</td>
</tr>
<tr>
<td>Pen/Strep</td>
<td>1%</td>
<td>5 mL</td>
</tr>
<tr>
<td>2-ME</td>
<td>0.1%</td>
<td>0.5 mL</td>
</tr>
<tr>
<td>rmIL-3 (10 µg/mL)</td>
<td>3 ng/mL</td>
<td>150 µL</td>
</tr>
<tr>
<td>DMEM</td>
<td></td>
<td>To 500 mL</td>
</tr>
</tbody>
</table>

### Complete BMCMC medium with csFCS and 4 ng/mL rmIL-3 (cDMEM-csFCS-4)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>csFCS</td>
<td>10%</td>
<td>50 mL</td>
</tr>
<tr>
<td>Pen/Strep</td>
<td>1%</td>
<td>5 mL</td>
</tr>
<tr>
<td>2-ME</td>
<td>0.1%</td>
<td>0.5 mL</td>
</tr>
<tr>
<td>rmIL-3</td>
<td>4 ng/mL</td>
<td>200 µL</td>
</tr>
<tr>
<td>DMEM</td>
<td></td>
<td>To 500 mL</td>
</tr>
</tbody>
</table>

### SPE-7 culturing medium

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>HS</td>
<td>10%</td>
<td>50 mL</td>
</tr>
<tr>
<td>Pen/Strep</td>
<td>1%</td>
<td>5 mL</td>
</tr>
<tr>
<td>2-ME</td>
<td>0.1%</td>
<td>0.5 mL</td>
</tr>
</tbody>
</table>
### hCBMC medium

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>FCS</td>
<td>10%</td>
<td>50 mL</td>
</tr>
<tr>
<td>Pen/Strep</td>
<td>2%</td>
<td>10 mL</td>
</tr>
<tr>
<td>2-ME</td>
<td>0.1%</td>
<td>0.5 mL</td>
</tr>
<tr>
<td>rhSCF</td>
<td>100 ng/mL</td>
<td></td>
</tr>
<tr>
<td>rhIL-6</td>
<td>50 ng/mL</td>
<td></td>
</tr>
<tr>
<td>NEAA</td>
<td>1×</td>
<td></td>
</tr>
<tr>
<td>IMDM</td>
<td></td>
<td>To 500 mL</td>
</tr>
</tbody>
</table>

### hCBMC medium with csFCS (hCBMC-csFCS)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>csFCS</td>
<td>10%</td>
<td>50 mL</td>
</tr>
<tr>
<td>Pen/Strep</td>
<td>2%</td>
<td>10 mL</td>
</tr>
<tr>
<td>2-ME</td>
<td>0.1%</td>
<td>0.5 mL</td>
</tr>
<tr>
<td>rhSCF</td>
<td>100 ng/mL</td>
<td></td>
</tr>
<tr>
<td>rhIL-6</td>
<td>50 ng/mL</td>
<td></td>
</tr>
<tr>
<td>NEAA</td>
<td>1×</td>
<td></td>
</tr>
<tr>
<td>IMDM</td>
<td></td>
<td>To 500 mL</td>
</tr>
</tbody>
</table>

### Dextran solution

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dextran</td>
<td>5%</td>
<td>5 g</td>
</tr>
<tr>
<td>dH₂O</td>
<td></td>
<td>To 100 mL</td>
</tr>
</tbody>
</table>

### PBS

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS (10×)</td>
<td>1 ×</td>
<td>100 mL</td>
</tr>
<tr>
<td>dH₂O</td>
<td></td>
<td>To 1 L</td>
</tr>
</tbody>
</table>

### CD34⁺ purification buffer

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>0.5%</td>
<td>7.14 mL</td>
</tr>
<tr>
<td>ACD</td>
<td>0.6%</td>
<td></td>
</tr>
</tbody>
</table>
### PBS

To 500 mL

### mBMCMC starvation medium

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pen/Strep</td>
<td>1%</td>
<td>5 mL</td>
</tr>
<tr>
<td>2-ME</td>
<td>0.1%</td>
<td>0.5 mL</td>
</tr>
<tr>
<td>BSA (35%)</td>
<td>0.1%</td>
<td>1.43 mL</td>
</tr>
<tr>
<td>DMEM</td>
<td></td>
<td>To 500 mL</td>
</tr>
</tbody>
</table>

### hCBMC starvation medium

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pen/Strep</td>
<td>1%</td>
<td>5 mL</td>
</tr>
<tr>
<td>2-ME</td>
<td>0.1%</td>
<td>0.5 mL</td>
</tr>
<tr>
<td>BSA (35%)</td>
<td>0.1%</td>
<td>1.43 mL</td>
</tr>
<tr>
<td>IMDM</td>
<td></td>
<td>To 500 mL</td>
</tr>
</tbody>
</table>

### Hybridoma freezing solution

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>10%</td>
<td>2 mL</td>
</tr>
<tr>
<td>FCS/HS</td>
<td>90%</td>
<td>18 mL</td>
</tr>
</tbody>
</table>

### 2.2.2. Buffers and Solutions for SPE-7 Purification

#### Conjugation solution

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium bicarbonate</td>
<td>0.05 M</td>
<td>1.05 g</td>
</tr>
<tr>
<td>H₂O</td>
<td></td>
<td>To 250 mL</td>
</tr>
</tbody>
</table>

#### Coupling buffer

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium bicarbonate</td>
<td>0.2 M</td>
<td>16.802 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>0.5 M</td>
<td>29.22 g</td>
</tr>
<tr>
<td>H₂O</td>
<td></td>
<td>To 1 L</td>
</tr>
</tbody>
</table>

pH adjusted to 8.3
### 1 M Propionic Acid

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propionic acid (99.5%)</td>
<td>1 M</td>
<td>37 mL</td>
</tr>
<tr>
<td>H₂O</td>
<td></td>
<td>To 500 mL</td>
</tr>
</tbody>
</table>

### Buffer A

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanolamine (99%)</td>
<td>0.5 M</td>
<td>30 mL</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>0.5 M</td>
<td>29.22 g</td>
</tr>
<tr>
<td>H₂O</td>
<td></td>
<td>To 1 L</td>
</tr>
<tr>
<td></td>
<td>pH adjusted to 8.3</td>
<td></td>
</tr>
</tbody>
</table>

### Buffer B

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium acetate</td>
<td>M</td>
<td>8.203 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>0.5 M</td>
<td>29.22 g</td>
</tr>
<tr>
<td>H₂O</td>
<td></td>
<td>To 1 L</td>
</tr>
<tr>
<td></td>
<td>pH adjusted to 4.0</td>
<td></td>
</tr>
</tbody>
</table>

### Storage solution

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disodium hydrogen phosphate</td>
<td>0.05 M</td>
<td>7.098 g</td>
</tr>
<tr>
<td>Sodium azide (10%)</td>
<td>0.1%</td>
<td>10 mL</td>
</tr>
<tr>
<td>H₂O</td>
<td></td>
<td>To 1 L</td>
</tr>
<tr>
<td></td>
<td>pH adjusted to 7.0</td>
<td></td>
</tr>
</tbody>
</table>

### Equilibration solution

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium dihydrogen phosphate</td>
<td>M</td>
<td>13.8 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>0.15 M</td>
<td>8.766 g</td>
</tr>
<tr>
<td>H₂O</td>
<td></td>
<td>To 1 L</td>
</tr>
<tr>
<td></td>
<td>pH adjusted to 7.0</td>
<td></td>
</tr>
</tbody>
</table>

### 0.1 M DNP

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

74
### Methanol (MeOH)-acetic acid-H₂O (5:4:1 ratio)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>MeOH</td>
<td>50% (v/v)</td>
<td>500 mL</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>40% (v/v)</td>
<td>400 mL</td>
</tr>
<tr>
<td>H₂O</td>
<td>10% (v/v)</td>
<td>100 mL</td>
</tr>
</tbody>
</table>

### Coomassie blue solution

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coomassie brilliant blue R-250</td>
<td>0.2% (w/v)</td>
<td>1 g</td>
</tr>
<tr>
<td>MeOH-acetic acid-H₂O</td>
<td>0.2% (w/v)</td>
<td>500 mL</td>
</tr>
</tbody>
</table>

### 2.2.3. Buffers and Solutions for β-Hexosaminidase Release Assay

#### HEPES buffer

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEPES</td>
<td>1 M</td>
<td>23.83 g</td>
</tr>
<tr>
<td>H₂O</td>
<td></td>
<td>To 100 mL</td>
</tr>
<tr>
<td>pH adjusted to 7.4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### Tyrodes buffer

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEPES buffer (1 M)</td>
<td>10 mM</td>
<td>10 mL</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>129 mM</td>
<td>7.54 g</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>5 mM</td>
<td>0.37 g</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>1.4 mM</td>
<td>0.206 g</td>
</tr>
<tr>
<td>Magnesium chloride</td>
<td>1 mM</td>
<td>0.203 g</td>
</tr>
<tr>
<td>D-glucose</td>
<td>8.4 mM</td>
<td>1.008 g</td>
</tr>
<tr>
<td>BSA</td>
<td>0.1%</td>
<td>1 g</td>
</tr>
<tr>
<td>H₂O</td>
<td></td>
<td>To 1 L</td>
</tr>
<tr>
<td>Filter sterilised</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
0.5% Triton-X

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triton-X</td>
<td>0.5%</td>
<td>0.5 mL</td>
</tr>
<tr>
<td>Tyrodes buffer</td>
<td></td>
<td>To 100 mL</td>
</tr>
</tbody>
</table>

Substrate buffer

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disodium hydrogen phosphate (0.2 M)</td>
<td>155 mM</td>
<td>70 mL</td>
</tr>
<tr>
<td>Citric acid (0.4 M)</td>
<td>88 mM</td>
<td>20 mL</td>
</tr>
<tr>
<td>pH adjusted to 4.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

p-NAG solution

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-NAG</td>
<td>4 mM</td>
<td>150 mg</td>
</tr>
<tr>
<td>Substrate buffer</td>
<td></td>
<td>45 mL</td>
</tr>
<tr>
<td>H₂O</td>
<td>67.5 mL</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Heating is applied until p-NAG is completely dissolved. The final solution is filter sterilised.</td>
<td></td>
</tr>
</tbody>
</table>

0.2 M glycine

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>0.2 M</td>
<td>15.0 g</td>
</tr>
<tr>
<td>H₂O</td>
<td></td>
<td>To 1 L</td>
</tr>
<tr>
<td></td>
<td>pH adjusted to 10.7</td>
<td></td>
</tr>
</tbody>
</table>

2.2.4. ELISA Buffers and Solutions

Blocking buffer

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>FCS</td>
<td>10%</td>
<td>100 mL</td>
</tr>
<tr>
<td>PBS</td>
<td></td>
<td>To 1 L</td>
</tr>
<tr>
<td></td>
<td>Filter sterilised</td>
<td></td>
</tr>
</tbody>
</table>

PBS-Tween (ELISA wash)
### Reagent Concentration Amount

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tween-20</td>
<td>0.05%</td>
<td>0.5 mL</td>
</tr>
<tr>
<td>PBS</td>
<td></td>
<td>To 1 L</td>
</tr>
</tbody>
</table>

**Stop solution**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulphuric acid (98%)</td>
<td>2 N (i.e. 1 M)</td>
<td>100.08 mL</td>
</tr>
<tr>
<td>H₂O</td>
<td></td>
<td>To 1 L</td>
</tr>
</tbody>
</table>

### 2.2.5. Flow Cytometry Buffers and Solutions

#### FACS buffer

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>FCS</td>
<td>2%</td>
<td>20 mL</td>
</tr>
<tr>
<td>PBS</td>
<td></td>
<td>To 1 L</td>
</tr>
<tr>
<td></td>
<td>Filter sterilised</td>
<td></td>
</tr>
</tbody>
</table>

#### FACS fixation solution (FACS-fix)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formaldehyde</td>
<td>0.1%</td>
<td>1 mL</td>
</tr>
<tr>
<td>Glucose</td>
<td>2%</td>
<td>20 g</td>
</tr>
<tr>
<td>Sodium azide (10%)</td>
<td>0.02%</td>
<td>2 mL</td>
</tr>
<tr>
<td>PBS</td>
<td></td>
<td>To 1 L</td>
</tr>
</tbody>
</table>

### 2.2.6. Histology/Immunohistology Solutions

#### 10% neutral buffered formalin

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formaldehyde</td>
<td>10%</td>
<td>100 mL</td>
</tr>
<tr>
<td>PBS</td>
<td></td>
<td>To 1 L</td>
</tr>
</tbody>
</table>

#### 1% aqueous toluidine blue

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toluidine blue</td>
<td>1%</td>
<td>1 g</td>
</tr>
</tbody>
</table>
### 0.1% acidic toluidine blue

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% aqueous toluidine blue</td>
<td>0.1% final</td>
<td>20 mL To 200 mL</td>
</tr>
<tr>
<td>1N hydrochloric acid pH adjusted to 1.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### 1% acidic toluidine blue

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toluidine blue</td>
<td>1%</td>
<td>1 g</td>
</tr>
<tr>
<td>1N hydrochloric acid pH adjusted to 0.1</td>
<td></td>
<td>100 mL</td>
</tr>
</tbody>
</table>

### 0.05% toluidine blue solution

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toluidine blue</td>
<td>0.05%</td>
<td>0.05 g</td>
</tr>
<tr>
<td>1.8% Sodium chloride solution</td>
<td></td>
<td>50 mL</td>
</tr>
<tr>
<td>80% (v/v) ETOH</td>
<td></td>
<td>22 mL</td>
</tr>
<tr>
<td>H₂O</td>
<td></td>
<td>28 mL</td>
</tr>
</tbody>
</table>

### Saturated saponin solution

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saponin</td>
<td></td>
<td>Enough to reach saturation (orange appearance)</td>
</tr>
<tr>
<td>50% ethanol</td>
<td></td>
<td>100 mL</td>
</tr>
</tbody>
</table>

### Kimura staining solution

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05% toluidine blue solution</td>
<td></td>
<td>50 mL</td>
</tr>
<tr>
<td>Saturated saponin solution</td>
<td></td>
<td>2.27 mL</td>
</tr>
<tr>
<td>0.06 M Sodium dihydrogen phosphate</td>
<td></td>
<td>22.7 mL</td>
</tr>
<tr>
<td>Filter sterilised</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### 2.2.7. Buffers and Solutions for SDS-PAGE & Western Blotting

#### NP40 lysis buffer

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>10 mM</td>
<td>1.21 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>137 mM</td>
<td>8.01 g</td>
</tr>
<tr>
<td>Glycerol</td>
<td>10%</td>
<td>100 mL</td>
</tr>
<tr>
<td>NP40</td>
<td>1%</td>
<td>10 mL</td>
</tr>
<tr>
<td>H$_2$O</td>
<td></td>
<td>To 1 L</td>
</tr>
<tr>
<td><em>pH adjusted to 7.4</em></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### Complete NP40 lysis buffer

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP40 lysis buffer</td>
<td></td>
<td>10 mL</td>
</tr>
<tr>
<td>1 M β glyceral phosphate</td>
<td>10 mM</td>
<td>100 µL</td>
</tr>
<tr>
<td>0.1 M PMSF</td>
<td>1 mM</td>
<td>100 µL</td>
</tr>
<tr>
<td>0.5 M Sodium fluoride</td>
<td>5 mM</td>
<td>100 µL</td>
</tr>
<tr>
<td>1 M Sodium vanadate</td>
<td>10 mM</td>
<td>100 µL</td>
</tr>
<tr>
<td>Aprotinin</td>
<td>0.3% (v/v)</td>
<td>30 µL</td>
</tr>
<tr>
<td>1 mg/mL Leupeptin</td>
<td>1 µg/mL</td>
<td>10 µL</td>
</tr>
</tbody>
</table>

#### 5× sample loading buffer

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 M Tris-HCl (pH = 6.8)</td>
<td>62.5 mM</td>
<td>1 mL</td>
</tr>
<tr>
<td>Glycerol</td>
<td>10%</td>
<td>0.8 mL</td>
</tr>
<tr>
<td>10% SDS</td>
<td>2%</td>
<td>1.6 mL</td>
</tr>
<tr>
<td>Bromphenol blue</td>
<td>0.5%</td>
<td>0.04 g</td>
</tr>
<tr>
<td>2-ME</td>
<td>5%</td>
<td>0.4 mL</td>
</tr>
<tr>
<td>H$_2$O</td>
<td></td>
<td>4 mL</td>
</tr>
</tbody>
</table>

#### Lower gel buffer

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>1.5 M</td>
<td>181.65 g</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.4%</td>
<td>40 mL</td>
</tr>
<tr>
<td>H$_2$O</td>
<td></td>
<td>To 1 L</td>
</tr>
<tr>
<td><em>pH adjusted to 8.8</em></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### Upper gel buffer
<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>0.5 M</td>
<td>60.55 g</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.4%</td>
<td>40 mL</td>
</tr>
<tr>
<td>H₂O</td>
<td></td>
<td>To 1 L</td>
</tr>
<tr>
<td></td>
<td><strong>pH adjusted to 6.8</strong></td>
<td></td>
</tr>
</tbody>
</table>

### 30% Acrylamide

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide</td>
<td>30%</td>
<td>146 g</td>
</tr>
<tr>
<td>N’N’-bis-acrylamide</td>
<td>0.8%</td>
<td>4 g</td>
</tr>
<tr>
<td>H₂O Filter sterilised</td>
<td></td>
<td>To 500 mL</td>
</tr>
</tbody>
</table>

### 10% ammonium persulphate (APS)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>APS</td>
<td>10%</td>
<td>1 g</td>
</tr>
<tr>
<td>H₂O</td>
<td>10%</td>
<td>To 10 mL</td>
</tr>
</tbody>
</table>

### 10% SDS-PAGE gel

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lower gel buffer</td>
<td></td>
<td>5.2 mL</td>
</tr>
<tr>
<td>30% Acrylamide</td>
<td></td>
<td>6.9 mL</td>
</tr>
<tr>
<td>10% APS</td>
<td></td>
<td>80 μL</td>
</tr>
<tr>
<td>TEMED</td>
<td></td>
<td>40 μL</td>
</tr>
<tr>
<td>H₂O</td>
<td></td>
<td>8.7 mL</td>
</tr>
</tbody>
</table>

### Stacking gel

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upper gel buffer</td>
<td></td>
<td>5 mL</td>
</tr>
<tr>
<td>30% Acrylamide</td>
<td></td>
<td>2.8 mL</td>
</tr>
<tr>
<td>10% APS</td>
<td></td>
<td>200 μL</td>
</tr>
<tr>
<td>TEMED</td>
<td></td>
<td>20 μL</td>
</tr>
<tr>
<td>H₂O</td>
<td></td>
<td>12 mL</td>
</tr>
</tbody>
</table>

### 10× SDS-PAGE running buffer

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
</table>
**Transfer buffer**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>25 mM</td>
<td>12.12 g</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.2 M</td>
<td>60.0 g</td>
</tr>
<tr>
<td>MeOH</td>
<td>15%</td>
<td>600 mL</td>
</tr>
<tr>
<td>H₂O</td>
<td></td>
<td>To 4 L</td>
</tr>
</tbody>
</table>

pH adjusted to 7.4

**Maleic acid buffer**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maleic acid</td>
<td>100 mM</td>
<td>11.6 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>150 mM</td>
<td>8.766 g</td>
</tr>
<tr>
<td>H₂O</td>
<td></td>
<td>To 1 L</td>
</tr>
</tbody>
</table>

pH adjusted to 7.5

**10% blocking solution**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blocking reagent</td>
<td>10%</td>
<td>50 g</td>
</tr>
<tr>
<td>Maleic acid buffer</td>
<td></td>
<td>To 500 mL</td>
</tr>
</tbody>
</table>

Heating is applied until blocking reagent is completely dissolved. The final solution is dispensed into 200 mL Schott bottles (100 mL/bottle) and autoclaved.

**10× TNT**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>0.5 M</td>
<td>60.57 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>1.54 M</td>
<td>90.0 g</td>
</tr>
<tr>
<td>Tween-20</td>
<td>1%</td>
<td>10 mL</td>
</tr>
<tr>
<td>H₂O</td>
<td></td>
<td>To 1 L</td>
</tr>
</tbody>
</table>

pH adjusted to 7.4

**1% blocking buffer**
0.1% blocking buffer

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% blocking solution</td>
<td>0.1% final</td>
<td>500 µL</td>
</tr>
<tr>
<td>10% sodium azide</td>
<td>0.01% final</td>
<td>50 µL</td>
</tr>
<tr>
<td>1× TNT</td>
<td></td>
<td>To 50 mL</td>
</tr>
</tbody>
</table>

2.2.8. Molecular Biology Buffers and Solutions

1 M Tris-HCl

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>1 M</td>
<td>60.57 g</td>
</tr>
<tr>
<td>H₂O</td>
<td></td>
<td>To 500 mL</td>
</tr>
</tbody>
</table>

pH adjusted to 8.0 by adding concentrated HCl

Tail lysis buffer

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 M Tris-HCl</td>
<td>100 mM final</td>
<td>100 mL</td>
</tr>
<tr>
<td>0.5 M EDTA</td>
<td>5 mM final</td>
<td>10 mL</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.2% final</td>
<td>20 mL</td>
</tr>
<tr>
<td>2 M Sodium chloride</td>
<td>200 mM final</td>
<td>100 mL</td>
</tr>
<tr>
<td>H₂O</td>
<td></td>
<td>To 1 L</td>
</tr>
</tbody>
</table>

Proteinase K (10 mg/mL stock solution) is added fresh each time (1/100 dilution) to reach a final concentration of 100 µg/mL.

TE (Tris-EDTA) buffer

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 M Tris-HCl (pH 7.5)</td>
<td>10 mM final</td>
<td>10 mL</td>
</tr>
<tr>
<td>0.5 M EDTA (pH 8.0)</td>
<td>1 mM final</td>
<td>2 mL</td>
</tr>
<tr>
<td>H₂O</td>
<td></td>
<td>To 1 L</td>
</tr>
</tbody>
</table>

Diethylpyrocarbonate (DEPC)-H₂O (RANase-free)
**Reagent Concentration Amount**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEPC</td>
<td>0.01%</td>
<td>100 µL</td>
</tr>
<tr>
<td>H₂O</td>
<td></td>
<td>To 1 L</td>
</tr>
</tbody>
</table>

This solution is prepared in autoclaved glass bottles. After the addition of DEPC, solution is allowed to stand at room temperature (RT) overnight and then autoclaved.

---

**50× TAE**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>1.6 M</td>
<td>193.8 g</td>
</tr>
<tr>
<td>Sodium acetate</td>
<td>800 mM</td>
<td>65.6 g</td>
</tr>
<tr>
<td>EDTA</td>
<td>40.27 mM</td>
<td>14.9 g</td>
</tr>
<tr>
<td>H₂O</td>
<td></td>
<td>To 100 mL</td>
</tr>
</tbody>
</table>

pH adjusted to 7.4

---

**2% agarose gel**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agarose</td>
<td>2%</td>
<td>4 g</td>
</tr>
<tr>
<td>1× TAE</td>
<td></td>
<td>To 200 mL</td>
</tr>
</tbody>
</table>

Mix and heat in microwave until agarose has completely dissolved.

---

**GelRed™ nucleic acid gel stain**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>GelRed™ (10,000× stock)</td>
<td>3×</td>
<td>30 µL</td>
</tr>
<tr>
<td>0.1 M sodium chloride</td>
<td></td>
<td>To 100 mL</td>
</tr>
</tbody>
</table>

---

### 2.2.9. Buffers and Solutions for In Vivo Experiments

**HMEM-Pipes**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pipes</td>
<td>47 mg/L</td>
<td>235 mg</td>
</tr>
<tr>
<td>Sodium hydroxide</td>
<td>105 mg/L</td>
<td>52.5 mg</td>
</tr>
<tr>
<td>HMEM</td>
<td></td>
<td>To 500 mL</td>
</tr>
</tbody>
</table>

Filter sterilised

---

**Ethanol-propylene glycol-H₂O (ETOH-PG-H₂O/Vehicle)**
<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>50% (v/v)</td>
<td>5 mL</td>
</tr>
<tr>
<td>Propylene glycol</td>
<td>25% (v/v)</td>
<td>2.5 mL</td>
</tr>
<tr>
<td>H₂O</td>
<td>25% (v/v)</td>
<td>2.5 mL</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>DNP in NaCl</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent</td>
<td>Concentration</td>
<td>Amount</td>
</tr>
<tr>
<td>DNP</td>
<td>2 mg/mL</td>
<td>100 µL</td>
</tr>
<tr>
<td>(20 mg/mL aqueous stock)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.9% NaCl</td>
<td></td>
<td>900 µL</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Tper EDTA-free lysis buffer</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent</td>
<td>Concentration</td>
<td>Amount</td>
</tr>
<tr>
<td>Complete mini EDTA-free T-PER reagent</td>
<td>1 tablet</td>
<td>10 mL</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chloroform- MeOH extraction solution</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent</td>
<td>Concentration</td>
<td>Amount</td>
</tr>
<tr>
<td>Chloroform</td>
<td>33.3% (v/v)</td>
<td>3 mL</td>
</tr>
<tr>
<td>MeOH</td>
<td>66.7% (v/v)</td>
<td>6 mL</td>
</tr>
</tbody>
</table>

2.3. Purification of Mouse α-DNP IgE (SPE-7 clone)

2.3.1. Conjugation of DNP and BSA

BSA solution (1 g dissolved in 40 mL of conjugation solution) and FDNB solution (0.1 mL of FDNB stock added to 10 mL of Dioxane) were mixed together and incubated for 2 h at RT, shielded from light. The resultant mixture was subsequently dialysed against reverse osmosis (RO) water (freshened four times) for 48 h at 4 °C. A small aliquot of the dialysed product was taken for examination of the conjugation ratio of DNP and BSA using the formula on the following page:
Once the ratio was determined to be within the normal range (2 to 9), the dialysed DNP-BSA solution was lyophilized (shielded from light in 50 mL falcon tubes at 10 mL/tube) overnight using the lyophilizing machine, and stored at 4 °C in a dark and dry place.

2.3.2. Coupling of DNP-BSA to HiTrap NHS-activated HP column

The HiTrap NHS-activated HP column was loaded, at 5 mL/min using a pump, with 30 mL of 1 mM HCl followed by 30 mL of coupling buffer. A 10 mg/mL DNP-BSA solution, prepared by diluting 0.2 g of lyophilized power with coupling buffer, was subsequently loaded onto and allowed to re-circulate the column for 90 min at RT, shielded from light. The column was then washed with 10 mL of coupling buffer, stripped off excess DNP with 10 mL of 1 M propionic acid, and rinsed with additional coupling buffer until the run-through became clear.

Ratio \( n \) = \( \frac{(M.W. \times A)}{(c \times \varepsilon \times l)} \)

- M.W. = molecular weight of BSA = 66,000 Da
- A = average absorbance at 360 nm (for DNP) obtained using a NanoDrop 1000 spectrophotometer (Thermal Scientific)
- c = concentration of BSA \( \approx \frac{1 \text{ g}}{55 \text{ mL}} \) (i.e. volume of the dialysed product) or 18.2 mg/mL
- \( \varepsilon \) = extinction coefficient of DNP at 360 nm = 17,400 M\(^{-1}\)cm\(^{-1}\)
- l = length of light path used (NanoDrop) = 0.1 cm

Once the ratio was determined to be within the normal range (2 to 9), the dialysed DNP-BSA solution was lyophilized (shielded from light in 50 mL falcon tubes at 10 mL/tube) overnight using the lyophilizing machine, and stored at 4 °C in a dark and dry place.
2.3.3. De-activation of Excess Active Groups

The following solutions were pumped through, at 5 mL/min, the coupled HiTrap column in that order:

- 30 mL of buffer A
- 30 mL of buffer B
- 30 mL of buffer A

(At this stage, the bottom of the column was capped before 30-min incubation at RT, shielded from light.)

- 30 mL of buffer B
- 30 mL of buffer A
- 30 mL of buffer B
- 15 mL of storage buffer.

After sealing the top and bottom with Parafilm, the column was stored at 4 °C (shielded from light) until further use.

2.3.4. Purification of SPE-7 from Hybridoma Culture Supernatant

After washing the DNP-BSA-coupled column with 10 mL of PBS, approximately 700 mL of SPE-7 hybridoma culture supernatant harvested (Section 2.5.4) was pumped through (5 mL/min) and allowed to re-circulate the column for 5 h (i.e. enough time for all supernatant to go through twice). The column was then washed with 25 mL of 1% NP40 in PBS followed by 100 mL of PBS (both at 5 mL/min), prior to elution with 50
mL of 0.1 M DNP solution. A total of ten fractions were collected and analysed by standard SDS-PAGE and Coomassie blue staining. All SPE-7-rich fractions identified were pooled and concentrated using a VIVASPIN 20 tube. The remaining solution (~ 5 mL) was dialysed again 1× PBS until a pale yellow colour was obtained. Finally, the purified SPE-7 was quantified using the SMART system with a Superdex 200PC 3.2/30 column (Amersham Biosciences), aliquotted and stored at -80 °C.

2.4. Mice

Female C57BL/6J (WT) mice and VDR-targeted VDR-deficient B6.129S4-Vdfrtm1Mbd/J mice that had been backcrossed to C57BL/6 mice for greater than 8 generations (VDR<sup>−/−</sup>) mice<sup>(244)</sup> were obtained from The Jackson Laboratories (Bar Harbor, Maine, USA) and bred in house at the Centre for Cancer Biology (Adelaide, Australia). Genetically MC-deficient B6-Kit<sup>W-sh/+</sup> mice were used as breeding pairs to produce MC-deficient Kit<sup>W-sh/W-sh</sup> mice and their normal WT littermates. Adult Kit<sup>W-sh/W-sh</sup> mice have a profound deficiency of MCs, including less than 1.0% the wild-type number of MCs in the dermis<sup>(182, 245)</sup>. Another WT mouse strain, namely (WB/ReJ-Kit<sup>W/+</sup> × C57BL/6J-Kit<sup>W/v</sup>)F1-Kit<sup>+/+</sup> (WBB6F1-Kit<sup>+/+</sup>), was also obtained from The Jackson Laboratory, bred in house, and used in a number of experiments as indicated. Please note that WBB6F1-Kit<sup>W/W-v</sup> mice were not used in this project. All in vivo experiments were performed with age- and sex-matched mice of at least 8 weeks of age. Experiments were performed in compliance with the ethical guidelines of the National Health and Medical Research Council of Australia.
2.5. General Methods

2.5.1. Culturing of mBMCMCs

Bone marrow extracted from femur and tibia bones of mice was cultured in cBMCMC-early medium for the first three weeks (during which period cells were inspected and medium changed 2-3 times a week), and in cBMCMC-late for the remaining of the culturing (during which period cells were inspected and expanded once or twice a week). Cells obtained at the end of 5-6 weeks were examined by flow cytometry (FcεRI and c-kit double staining) as well as May-Grünwald Geimsa staining (Section 2.5.7.2). Typical culture at this stage would consist of over 95% MCs, identified as FcεRI⁺c-kit⁺ and carrying large numbers of intracellular granules. All experiments conducted used 5- to 10-week-old cells.

2.5.2. Culturing of hCBMCs

To a 50 mL falcon tube, 5 mL of ACD was added, followed by 25 mL fresh human umbilical cord blood (Molecular Immunology Laboratory, Women and Children Hospital, Adelaide), 10 mL of dextran solution and 10 mL of PBS. Once the red blood cells have coagulated (approximately 20 min later), the top layer of cells were transferred to another tube containing 12 mL of histopaque-1077 and centrifuged at 650 g for 25 min without break. The resultant interface containing CD34⁺ progenitor cells was carefully collected and washed first with PBS and subsequently with CD34⁺ purification buffer. The cells obtained were incubated with human Fc receptor blocking Ab and magnetic CD34⁺ beads at 4 °C for 30 min. Finally, the labelled CD34⁺ cells were isolated using a magnetic separation column (Miltenyi Biotec), washed in CD34⁺ purification buffer and cultured in
hCBMC medium for the following 10-12 weeks (during which period cells were inspected and medium changed once or twice a week).

2.5.3. Culturing of WEHI-3 hybridoma

A frozen aliquot (kept in liquid nitrogen for long-term storage) was thawed and allowed to recover and expand in cDMEM medium during the initial 1-2 weeks, by the end of which over 99% of cells would be viable. To harvest WEHI-3-conditioned medium, cells were seeded at 10^5 cells/mL and incubated at 37 °C/5% CO₂ for four days. Typical cell concentration at this stage would be approximately 10^6 cells/mL. Supernatant was harvested following two centrifugation steps (first step: 250 g for 10 min at 4 °C; second stop: 350 g for 20 min at 4 °C), an aliquot was taken for analysis by IL-3 ELISA, and the remaining was stored at -20 °C. To generate more frozen WEHI-3 stocks, cells at sub-confluency concentration (where cells would exhibit optimal growth rates) were collected and resuspended in cold hybridoma freezing solution containing FCS, which was added drop-wise onto the cell pellet, to a concentration of 10^7 cells/mL. The resultant cell suspension (1 mL/aliquot in cryovials) was stored at -80 °C in “Mr. Frosty” 1 °C freezing container (Nalgene) for the initial 24 hr, and subsequently transferred into liquid nitrogen.

2.5.4. Culturing of SPE-7 hybridoma

A frozen aliquot (kept in liquid nitrogen for long-term storage) was thawed and allowed to recover and expand in SPE-7 culturing medium during the initial 1-2 weeks, by the end of which over 99% of cells would be viable. To harvest SPE-7-conditioned medium for SPE-7 purification (Section 2.3.4), cells were seeded, incubated, and supernatant harvested in the same manner as for WEHI-3 hybridoma (Section 2.5.3.). The generation
of SPE-7 frozen stocks was also the same as for WEHI-3, except that freezing solution containing HS was used in this case.

2.5.2. Flow Cytometry

2.5.2.1. Surface Ag Labelling

Cells (1-2 \times 10^6 \text{ cells/sample}) were washed with cold FACS buffer and blocked with the appropriate Fc receptor blocking Ab for 15 min on ice. In the case of mBMCMCs, cells were immediately labelled with primary Abs; whereas in the case of hCBMCs, the blocking Ab was washed off first. All primary Ab (all directly conjugated) labelling steps were carried out on ice, shielded from light, for 30 min. Labelled cells were subsequently washed twice with FACS buffer, resuspended in 500 \mu l of FACS buffer each, and analysed on the same day using a Beckman Coulter Cytomics FC 500 Flow Cytometer equipped with CXP software (version 2.2). When analysis on the same day was not feasible, cells were resuspended in FACS-fix instead and stored at 4 °C in the dark until analysis.

2.5.2.2. Intracellular Ag Labelling

Cells (1-2 \times 10^6 \text{ cells/sample}) were washed with cold FACS buffer and fixed with the 1C fixation buffer (a component of the Fixation & Permeabilization Kit) for 20 min at RT. Cells were then washed with and resuspended in 1 \times \text{permeabilization buffer} (diluted with H_2O from 10 \times \text{Permeabilization Buffer}, which is the other component of the Fixation & Permeabilization Kit), and incubated with primary Ab(s) for 30 min on ice. When unconjugated primary Abs were used, cells were washed with 1 \times \text{permeabilization buffer} following the first incubation and subsequently labelled with corresponding
fluorochrome-conjugated secondary Ab(s) for a further 30 min on ice. In all cases, labelled cells were finally washed with 1 × permeabilization buffer, resuspended in FACS buffer and analysed on a Beckman Coulter Cytomics FC 500 Flow Cytometer equipped with CXP software.

2.5.2.3. Caspase-3 Labelling

Caspase-3 labelling was carried out using the NucView™ 488 Caspase-3 assay kit as instructed by the manufacturer. Briefly, cells were washed with and resuspended in PBS to a concentration of 0.5-1 × 10^6 cells/mL. 200 µL of the resultant cell suspension was transferred to a FACS tube, where 2 µL of Caspase-3 substrate stock solution was subsequently added. After 30-min incubation at RT, 300 µL of PBS was added to the sample, which was then ready for analysis using the Beckman Coulter Cytomics FC 500 Flow Cytometer equipped with CXP software (FL1 channel).

2.5.3. In Vitro Procedures

2.5.3.1. β-hexosaminidase Release Assay

Mouse BMCMCs (10^6 cells/mL) were preloaded with α-DNP IgE (2 µg/mL) in cBMCMC-early medium. After 16-h incubation at 37 °C/5% CO₂, cells were washed with and resuspended in Tyrodes buffer, plated at a concentration of 6.25 × 10^6 cells/mL in 96-well V-bottom plates (Greiner Bio-one), and stimulated for 1 h with DNP-HSA (10 ng/mL) or, as a positive control, PMA (50 ng/mL) / A23187 (10 µM). In experiments investigating the effects of VitD₃ analogues on MC degranulation, VitD₃/ETOH control was applied to cell culture either 8 h (in the case of 25OHD₃) or immediately (in the case of 1α,25(OH)₂D₃) before IgE preload and, in all cases, immediately before DNP or
PMA/A23187 treatment. At the end of 1-h stimulation, culture supernatants and cell lysates were collected following centrifugation at 250 g for 5 min, and subsequent lysis of cell pellets using 0.5% Triton-X. All samples were then incubated with p-NAG solution for 1 h at 37 °C. β-hexosaminidase activity was detected by addition of 0.2 M glycine and absorbance at 405 nm was measured on an ELISA plate reader. Percentage of degranulation was calculated by dividing the β-hexosaminidase activity in the culture supernatant by the total β-hexosaminidase activity detected (supernatant plus lysate).

2.5.3.2. Histamine Release Assay

Mouse BMCMCs were preloaded with α-DNP IgE (SPE-7 clone) and stimulated with DNP-HSA as described in Section 2.5.3.1, except that stimulation was performed in mBMCMC starvation medium at 10^6 cells/mL for 30 min. Culture supernatants and cell lysates were collected following centrifugation at 250 g for 5 min, and subsequent lysis of cell pellets using 0.5% Triton-X in H_2O. The amount of histamine in all samples (1/20 dilution analysed for supernatants and 1/100 dilution for cell lysates) was measured using the EIA histamine kit as instructed by the manufacturer. Briefly, 12.5 µL of acylation buffer was mixed with 25 µL of sample dilutions, calibrators or controls in clean eppendorf tubes. 25 µL of acylation reagent was then added to each tube, immediately followed by vortexing. To the pre-coated plate, 50 µL of acylated samples/calibrators/controls was added per well, followed by 200 µL of histamine-alkaline phosphatase conjugate. After 2-h incubation at 4 °C with constant agitation, wells were washed and further incubated, in the dark with 200 µL of substrate per well, for 30 min at RT under constant agitation. Reaction was ended by the addition of 50 µL of stop solution per well, and absorbance at 405 nm was measured on an ELISA plate.
reader. Percentage of histamine release for each sample was calculated by dividing the amount of histamine in the culture supernatant by its total amount (supernatant plus lysate).

2.5.3.3. IgE + sAg Stimulation of mBMCMCs

For ELISA and RNA extraction (quantitative real-time PCR; qRT-PCR), mBMCMCs were preloaded with \( \alpha \)-DNP IgE as described in Section 2.5.3.1, following which cells were washed with and resuspended in mBMCMC starvation medium at \( 10^6 \) cells/mL. Subsequent stimulation was carried out with 2 ng/mL of DNP-HSA for either 6 h (in the case of cytokine measurement by ELISA; Section 2.5.3.5) or 3 h (in the case of total RNA extraction and qRT-PCR; Sections 2.5.4.2 to 2.5.4.4). For some experiments, VitD\(_3\) analogues and their corresponding vehicle solutions were applied as described in later sections.

For cell lysate preparation and western blotting, mBMCMCs \((0.5 \times 10^6 \) cells/mL\) were cultured in cDMEM-csFCS-4 medium for three days, at the end of which cells were counted and replated at \( 10^6 \) cells/mL in cDMEM-csFCS-3 medium with either ETOH or \( 1\alpha,25(\text{OH})_2\text{D}_3 \) (\( 10^{-8} \) M; 0.1% v/v ETOH) added. After 16-h incubation, cells were washed with and resuspended in cDMEM-csFCS medium at \( 10^6 \) cells/mL. ETOH and \( 1\alpha,25(\text{OH})_2\text{D}_3 \) was added to the corresponding culture, followed sequentially by 4-h incubation, the addition of SPE-7 at 2 \( \mu \)g/mL, and 4-h further incubation. Cells were then washed with and resuspended in mBMCMC starvation medium at \( 10^6 \) cells/mL. ETOH and \( 1\alpha,25(\text{OH})_2\text{D}_3 \), followed immediately by 20 ng/mL of DNP-HSA, was added to the designated treatment groups. At either 2 min or 15 min post-stimulation, cells were
washed with cold PBS twice before proceeding to total cell lysate preparation (Section 2.5.5.1).

2.5.3.4. IgE-mediated stimulation of hCBMCs

Human CBMCs (10^6 cells/mL) were cultured, for two days, with 10 ng/mL of rhIL-4 and either ETOH, 25OHD₃, or 1α,25(OH)₂D₃ in hCBMC medium. Human myeloma IgE was then added at 2.5 µg/mL, followed by 16-h further incubation. Preloaded cells were washed with and resuspended in hCBMC starvation medium at 10^6 cells/mL, and stimulated with 1 µg/mL of goat α-human IgE in the presence of ETOH or the corresponding VitD₃ analogue. Culture supernatants were collected at 6 h post-stimulation for cytokine measurement by ELISA.

2.5.3.5. Cytokine Measurement by ELISA

Freshly harvested culture supernatants from stimulated mBMCMCs and hCBMCs were used in all ELISA analysis according to manufacturers’ instructions. In the case of mouse IL-6 ELISA, appropriate dilution of samples ranging from 1/2 to 1/5 was analysed; whereas in all other cases, neat supernatants were used. Briefly, 96-well ELISA plates (Costar) were incubated with capturing Ab diluted in corresponding coating buffer overnight at 4 °C. Coated wells were washed three times with PBS-Tween before 1-h blocking with appropriate ELISA blocking buffer (provided for eBioscience kits and home-made blocking solution for BD Biosciences kits) at RT. This was followed by three washes, 2-h incubation with samples and standards at RT, and three more washes. For BD Biosciences ELISA kits, all wells were then incubated with working detection solution (containing both detection Ab and the enzyme streptavidin-HRP) for 1 h at RT;
whereas for eBioscience kits, wells were incubated with detection Ab for 1 h at RT, followed by three washes and 30-min further incubation with HRP. In all cases, after a final three-wash step, 3,3’,5,5’-tetramethyl-benzidine liquid substrate was added to all wells and reaction (RT; shielded from light) was stopped using stop solution when the top standards displayed maximum colour intensity (usually blue precipitates would be observed at the bottom of the wells). The optical density readings were obtained at 450 nm using an ELISA plate reader.

2.5.3.6. VitD3 Treatment (without stimulation)

Mouse BMCMCs and hCBMCs (0.5 × 10^6 cells/mL) were cultured in cDMEM-csFCS-4 medium and hCBMC-csFCS medium, respectively, for three days, at the end of which cells were replated at 10^6 or 2 × 10^6 cells/mL in cDMEM-csFCS-3 medium. VitD3 analogues were applied to the culture at required concentrations, and cells were incubated for the required lengths of time as indicated in later chapters.

2.5.3.7. Measurement of 1α,25(OH)2D3

Mouse BMCMCs were treated, at 2 × 10^6 cells/mL, with 25OHD3 (Section 2.5.3.6.) for 6 h before culture supernatants and cell lysates (prepared with NP40 lysis buffer) were collected, snap-frozen in liquid nitrogen, and stored, shielded from light, at -80 °C until analysis. Levels of 1α,25(OH)2D3 in all samples were measured using a radioimmunoassay kit according to the manufacturer’s instructions.
2.5.4. Molecular Biology

2.5.4.1. Genotyping of WT and $VDR^{+/-}$ Mice

The tip of tails from WT and $VDR^{+/-}$ mice were digested overnight (55 °C; with constant agitation) in tail lysis buffer with freshly added proteinase K. Tissue lysates were centrifuged (13,000 g; 10 min; 4 °C) and the liquid layer was transferred to a clean tube containing 500 µL isopropanol. DNA precipitate was formed upon gentle inversion of the tube and subsequently collected by centrifugation (maximum speed [17,000 g]; 5 min; 4 °C), washed with 1 mL of 70% (v/v) ETOH each, and collected again by centrifugation (7,500 g; 5 min; 4 °C). The DNA pellets obtained were briefly dried (on 37 °C heating block) and re-dissolved (65 °C; 30 min) in 300 µL of TE buffer each. Samples were stored at 4 °C (short-term) or -20 °C (long-term) until PCR analysis.

GoTaq Green master PCR cocktail was prepared by mixing GoTaq Green master mix (12.5 µL/sample), nuclease-free H$_2$O (6.5 µL/sample), and each of the primer pair (both at 2.5 µL/sample). The primer pair used for WT genotype consisted of C57/BL6 - $VDR^{+/+}$ and the common primer, whereas that used for $VDR^{+/-}$ genotype consisted of C57/BL6 - $VDR^{+/-}$ and the common primer (Section 2.1.12). Mouse β-actin primer pair was also used for each sample as loading controls. In 0.2 mL PCR tubes (Ultraflux), 24 µL of each PCR cocktail was mixed with 1 µL of each DNP sample, which was then subjected to thermal cycles (carried out in Veriti® 96-Well Thermal Cycler; Applied Biosystems) as described on the following page:
The resultant PCR products were loaded onto a 2% agarose gel (5 µL/lane) and subjected to gel electrophoresis at 80 volts for approximately 30 min. DNA bands were visualised, following 30-min staining in GelRed™ nucleic acid gel stain, under UV light (UVitech) using the UV photo software.

2.5.4.2. RNA Extraction

Unless otherwise stated, 5 × 10⁶ cells of each treatment group were used for RNA extraction to achieve desirable amount and quality of the final product. After collection by centrifugation (250 g, 5 min, 4 °C), cells were lysed with TRIzol reagent (1 mL/sample) by pipetting (Gilson P1000 pipette with filtered tip) up and down at least 10 times and passing through a 26 G needle twice. Samples at this stage were either stored at -80 °C (preferred) or used for immediate RNA extraction. In the former case, samples were thawed and allowed to reach equilibrium at RT for 5 min before proceeding to the next step. In all cases, 200 µL of chloroform was added to each TRIzol preparation and the tube was shaken vigorously for 15 sec, followed by 3-min incubation at RT. Samples were then centrifuged at 12,000 g for 15 min (4 °C) to achieve phase separation into the lower phenol-chloroform layer and the upper aqueous layer, which contained the RNA.
and was subsequently transferred to a clean tube. RNA precipitate was obtained upon addition of 500 µL of isopropanol to each tube and centrifugation at 12,000 g for 10 min (4 °C), washed with 1 mL of 75% (v/v) ETOH each, and briefly air-dried on a 37 °C heating block. Finally, each air-dried RNA pellet was re-dissolved in 20 µL of RNase-free H₂O (65 °C, 10 min) and quantified using a NanoDrop 1000 spectrophotometer (Thermal Scientific).

2.5.4.3. Complementary DNA (cDNA) Synthesis

QuantiTech reverse transcription kit was used to generate cDNA from all RNA samples, according to the manufacturer’s instructions. Briefly, 1 µg of RNA (adjusted to 12 µL with added RNase-free H₂O) was incubated with 2 µL of the genomic (g)DNA Wipeout Buffer for 2 min at 42 °C. The resultant reaction (14 µL) was subsequently mixed with a master stock containing 1 µL of Reverse Transcriptase, 1 µL of reverse transcription Primer Mix, and 4 µL of reverse transcription Buffer (5×); and incubated for 30 min at 42 °C followed by 3 min at 95 °C. All incubation steps mentioned above were performed using the Veriti® 96-Well Thermal Cycler. The cDNA samples obtained (20 µL each) were stored at 4 °C (short-term) or -20 °C (long-term) until analysis.

2.5.4.4. qRT-PCR

This was performed in triplicate with cDNA samples generated from cell cultures (Section 2.5.4.3) used at an appropriate dilution ranging from 1/3 to 1/10. Each final reaction (10 µL) consisted of 2 µL of cDNA dilution, 5 µL of QuantiTech Sybr green master mix, 1 µL RNase-free H₂O, and 1 µL of each of the designated primer pair (5 µg/mL working diluted used). β-actin primer pair was used at all cases to generate
loading controls. Reactions containing additional RNase-free H₂O instead of cDNA dilution were also included for each primer pair used as “no template” controls. All RT-PCR was carried out in the Rotor-Gene 6000 Real-time Rotary Analyzer (Corbett) using conditions as follows:

Holding 1:  
95°C, 15 min
95°C, 20 sec

Cycling (45 cycles):  
52°C, 30 sec
72°C, 30 sec (acquisition step)

Holding 2:  
72°C, 60 sec

Melting:  
72°C, 90 sec
72°C to 99°C at 5 sec/°C

The relative quantity of the PCR product was obtained using the “Comparative Quantitation” option of the Rotor-Gene 6000 Series Software (version 1.7).

2.5.5. SDS-PAGE and Western Blot Analysis

2.5.5.1. Preparation of Total Cell Lysates

Cell cultures (2-3 × 10⁶ cells/sample) were washed twice with cold PBS, and supernatant was removed as thoroughly as possible. Each cell pellet was subsequently lysed in 100 µL of complete NP40 lysis buffer, incubated on ice for 30 min, and centrifuged (17,000 g, 15 min, 4 °C) to remove insoluble cell debris. The resultant total cell lysates were quantitated using the Bio-Rad D₂₅ protein assay according to the manufacturer’s instructions, and stored at -20 °C until analysis.
2.5.5.2. SDS-PAGE

Total cell lysate samples (Section 2.5.5.1) were thawed, mixed with 5× sample loading buffer (i.e. in a 4:1 ratio), and boiled for 5 min before being subjected to SDS-PAGE (10% running gel; 20-50 µg of protein/lane) at 25 mAmps/gel for 60-90 min. Benchmark™ pre-stained protein markers were loaded in parallel as a reference for different protein sizes.

2.5.5.3. Protein Transfer and Western Blot Analysis

Following SDS-PAGE (Section 2.5.5.2), proteins were transferred onto a nitrocellulose membrane at 250 mAmps for 90 min. The membrane was blocked with 1% blocking buffer for 1 h at RT, and subsequently blotted with designated primary Abs (diluted in 0.1% blocking buffer) overnight at 4 °C, followed by the corresponding HRP-conjugated secondary Ab(s) for 1 h at RT. Three 15-min washes with 1× TNT were carried out after each blotting step. Finally, signals were developed using enhanced chemiluminescence (ECL) with the Amersham ECL Plus Western Blotting Detection System, detected by LAS-4000 Luminescence Analyser, and analysed using the Multi Gauge software (version 3.1). In cases where membranes were re-probed with another primary Ab, they were briefly washed with 1× TNT and stripped with the stripping solution according to the manufacturer’s instructions, prior to re-probing in the same manner as described above.
2.5.6. In Vivo and Related Procedures

2.5.6.1. Engraftment of mBMCMCs

After confirmation of over 95% purity (Section 2.5.1), 4- to 6-week-old mBMCMCs (resuspended in DMEM at $4 \times 10^7$ cells/mL) were transferred by intra-dermal injection ($i.d.$, 2 injections into each ear with $1 \times 10^6$ cells in 25 µl DMEM per injection) into both ears of 4- to 6-week-old Kit$^{W-sh/W-sh}$ mice. PCA experiments were carried out 5-8 weeks after $i.d.$ transfer of mBMCMCs.

2.5.6.2. PCA with Topical Application of $1\alpha,25(OH)_2D_3$ or 25OHD$_3$

For PCA with $1\alpha,25(OH)_2D_3$ application, the right and left ears of mice were injected $i.d.$ with 20 µl of SPE-7 (5 µg/mL; diluted in HMEM-Pipes) and 20 µL of HMEM-Pipes, respectively. Immediately following injection, both ears were topically painted (20 µL/ear, 10 µL on the outer surface and 10 µL on the inner surface) with either 3 µM of $1\alpha,25(OH)_2D_3$ diluted in ETOH-PG-H$_2$O (vehicle) or ETOH-PG-H$_2$O alone. For PCA with 25OHD$_3$ application, ears of mice were topically painted with 3 µM of 25OHD$_3$ or ETOH-PG-H$_2$O alone 8 h prior to SPE-7/HMEM-Pipes injection. Sixteen hours later in both cases, mice were injected $i.v.$ (retro-orbitally) with 100 µL of DNP-HSA (2 mg/mL) diluted in sterile 0.9% saline. Ear swelling was quantified by measurements of ear thickness using a Peacock dial thickness gauge (model G-1A) prior to (baseline) and 0.5, 1, 2, 3, or 6 h after DNP-HSA administration. At various time points post-DNP-HSA, mice were sacrificed by CO$_2$ inhalation and samples of ear were taken for histology (Section 2.5.6.3), cytokine and/or VitD$_3$ measurements (Section 2.5.6.4), or RNA
extraction and qRT-PCR (Section 2.5.6.5). Serum samples were also prepared for cytokine and/or VitD₃ measurements (Section 2.5.6.6).

2.5.6.3. Ear Tissue Histology and Numeration of Tissue MCs

Samples of ear pinna (1 mm-wide strips through the centre) were fixed in 10% buffered formalin, embedded in paraffin (with care to ensure a cross-section orientation), and 4 µm sections were cut. Ear sections were dewaxed by being immersed sequentially in Xylene (twice; 5 min each), 100% ETOH (twice; 3 min each), 70% ETOH (3 min), 50% ETOH (3 min), and H₂O (until staining); and stained with 0.1% acidic toluidine blue (Section 2.5.7.4) for detection of MCs (cytoplasmic granules appear purple). Ear pinna MCs were counted in 5-8 consecutive fixed fields of 870 µm width under 200× magnification, and MC numbers were expressed per horizontal ear cartilage field length (mm), using computer-generated image analysis (NIH Image J software, version 1.43x).

2.5.6.4. Ear Tissue Lysate Preparation and Analysis

For cytokine measurement in ear tissues, the remaining halves of each ear, following the removal of sections for histology (Section 2.5.6.3), were finely cut up on an ice cold surface. Tissue samples were each sonicated in 250 µL of Tper EDTA-free lysis buffer containing protease inhibitors, and frozen immediately at −80 ºC until analysis as follows. After thawing at RT, samples were centrifuged twice at 17,000 g (10 min each, 4 ºC) and the supernatants were collected. TNFα, IL-6 and IL-10 protein concentrations in the supernatants were measured by ELISA (eBioscience), according to the manufacturer’s instructions (lower limit of detection was 15.6 pg/mL, 7.8 pg/mL, and 31.3 pg/mL, respectively). Data obtained from each group were expressed as pg cytokine/mg protein,
where total protein concentrations in the supernatants were measured by a Bio-Rad DC protein assay, according to the manufacturer’s instructions.

2.5.6.5. Total RNA Extraction from Ear Tissues

Mouse ears were finely chopped on an ice cold surface and each sonicated in 500 µL of TRIzol reagent. Lysates were centrifuged at 17,000 g for 10 min at 4 °C to collect supernatants, to each of which a further 500 µL of TRIzol reagent was then added. After the addition of 200 µL of chloroform to each sample, RNA was prepared as described in Section 2.5.4.2.

2.5.6.6. Serum Preparation and Analysis

For cytokine measurement in serum samples, newly sacrificed mice were bled from the heart, and blood samples were incubated at RT for approximately 30 min before centrifugation twice at 13,000 g (5 min each, 4 °C) to collect serum. Serum samples were stored at -80 °C until analysis as follows. After thawing at RT, TNFα, IL-6 and IL-10 protein concentrations in the sera were measured and analysed as described previously for the ear lysates (Section 2.5.6.4).

2.5.7. Histochemistry and Immunohistochemistry

2.5.7.1. Cytospin Slide Preparation

Various MC cultures (100 µL each) were spun onto Polysine™ slides (Menzel-Gläser) at 100 g for 5 min (Shandon Cytospin 3) and allowed to air-dry prior to subsequent staining.
2.5.7.2. May-Grünwald Giemsa Staining

Air-dried cytospin slides (Section 2.5.7.1) were stained with the Accustain Giemsa and May-Grünwald stains according to manufacturer’s instructions. Briefly, slides were firstly immersed in May-Grünwald stain for 5 min and de-stained in PBS for 90 sec. The slides were then counter-stained with Giemsa stain dilution (1/20 with H2O) for 20 min, rinsed with water and air-dried. Coverslips were mounted onto samples using DePex mounting medium before microscopic observation.

2.5.7.3. Kimura Staining of hCBMCs

Human CBMC culture was mixed with Kimura staining solution in a 2:1 ratio, and incubated for 5 min at RT prior to observation of MCs (with stained granules) using a haemocytometer.

2.5.7.4. Toluidine Blue Staining

For toluidine blue staining of hCBMCs, cytospin preparation (Section 2.5.7.1) of cell culture was stained with 1% acidic toluidine blue solution for 15 min, rinsed with H2O, air-dried and finally mounted in DePex mounting medium.

For toluidine blue staining of ear tissue MCs, dewaxed ear sections (Section 2.5.6.3) were stained with 0.1% acidic toluidine blue solution for exactly 1 min, thorough rinsed with H2O, air-dried and finally mounted in DePex mounting medium.

2.5.7.5. Immunohistochemistry

Freshly prepared cytospin slides were fixed with the 1C fixation buffer (a component of the Fixation & Permeabilization Kit) for 20 min at RT. Slides were then rinsed three
times with $1 \times$ permeabilization buffer (diluted with $H_2O$ from $10 \times$ Permeabilization Buffer, which is the other component of the Fixation & Permeabilization Kit) before overnight staining with the designated primary Ab and its corresponding isotype control in a humid box at $4 \, ^\circ C$. After three 3-min washes with $1 \times$ permeabilization buffer, slides were stained with the corresponding secondary Ab at RT, shielded from light, for 1 h. Following three additional washes, slides were stained with 1 µg/mL working dilution (with MeOH) of DAPI stained at RT for 2 min, rinsed with $1 \times$ permeabilization buffer, and finally mounted with Fluorescence Mounting Medium. After drying in the dark at $4 \, ^\circ C$ for at least 4 h, slides were observed and images taken using the Nikon Spectral Imaging Confocal Microscope DIGITAL ECLIPSE C1si equipped with EZ-C1 software (version 3.20).

2.5.8. Centrifugation

Tissue culture centrifugation was performed using either a Heraeus Megafuge (1.0R) or an Eppendorf Centrifuge (5810R), whereas centrifugation of all eppendorf tubes and PCR tubes was performed using a Heraeus FRESCO 17 Centrifuge.

2.5.9. Statistical Analyses

All data shown in Chapters 3, 4, and 6 were tested for statistical significance using the unpaired, two-tailed, Student’s $t$-test (GraphPad Software, version 5.04) between each pair of different treatments. For Chapter 5, analysis of variance (ANOVA) for repeated measures was used to assess differences in ear swelling among mouse groups in all PCA experiments, and other experiments that involved repeated measurements on the same animal over a period of time. All other data were tested for statistical significance using
the unpaired, two-tailed, Student’s $t$-test between each two treatment groups. In all cases, $P$ values less than 0.05 were considered statistically significant, and data are presented as either “mean + SEM”, “mean + SD” or “median” as indicated.
CHAPTER 3

$1\alpha,25(\text{OH})_2\text{D}_3$ POTENTIATES THE NEGATIVE IMMUNOREGULATORY PROPERTIES OF MAST CELLS DURING IgE+sAg-MEDIATED ACTIVATION \textit{In Vitro}
3.1. Introduction

Our recent findings provided evidence that $1\alpha_25(OH)_2D_3$ can enhance the anti-inflammatory properties of dermal MCs during chronic low-dose UVB irradiation. (163) Although much attention has been focused on the effects of VitD$_3$ insufficiency in various settings of autoimmunity (246-248), allergy (249, 250) and the development of certain cancers (251), there is comparatively very little known about the modulatory properties of VitD$_3$ on IgE-mediated MC activation. Excessive activation of MCs in response to IgE and its cognate Ag is a hallmark feature of chronic allergic disease and immediate anaphylaxis. Therefore, it is important to establish the effects of $1\alpha_25(OH)_2D_3$ on MC degranulation and de novo cytokine production during these processes. The anti-inflammatory cytokine IL-10, although rarely mentioned in the context of IgE + sAg-mediated MC activation, is also worth examining in response to $1\alpha_25(OH)_2D_3$ treatment. The human IL-10 gene contains a VDRE in its promoter region, thus making its encoded cytokine a direct target of $1\alpha_25(OH)_2D_3$-mediated production. (225, 252) Furthermore, we recently showed that $1\alpha_25(OH)_2D_3$ can induce IL-10 production by mBMCMCs in vitro as well as by cutaneous MCs following UVB irradiation in vivo. (163)

The majority of the biological functions of $1\alpha_25(OH)_2D_3$ are exerted via the genomic pathway (Section 1.8.1), which utilises VDRs. (253) Therefore, it is important to determine the VDR-dependence and thus the pathway specificity for any potential effect of $1\alpha_25(OH)_2D_3$ on IgE + sAg-activated MCs. However, due to the recent suggestion that VDRs are also capable of certain interactions in a non-genomic VitD$_3$-dependent manner (Section 1.8.2) (229, 230), additional investigation that utilises certain pathway-specific VitD$_3$ agonists and/or antagonists is required to ascertain the involvement of either one or
both pathways. The determination of VitD$_3$ pathway specificity can also facilitate the search for potential mechanisms responsible for VitD$_3$-mediated immunoregulation of MCs in various immune settings.

The particular non-genomic pathway-specific $1\alpha_25$(OH)$_2$D$_3$ agonist utilised in the current study is curcumin (diferuloylmethane). Curcumin is a naturally occurring yellow pigment isolated from the rhizomes of the plant *Curcuma Longa* Lin found in South and Southeast Asia. The medicinal value has been well documented, highlighting specifically its antioxidant, anti-tumor, and anti-inflammatory properties and its current application in pre-clinical trials for cancer and/or inflammation treatment. For example, curcumin can inhibit tumor initiation *in vivo* as well as the proliferation and survival of tumor cells *in vitro*. It can also down-regulate the production of inflammatory cytokines by human monocytes, alveolar macrophages, and T cells. Recent evidence demonstrates that curcumin is able to bind to an alternative ligand binding pocket in VDR with a low micromolar affinity, and thus function as a non-genomic agonist of $1\alpha_25$(OH)$_2$D$_3$. Therefore, it will be illuminating to examine the effects of curcumin on IgE + sAg-activated MCs and how they compare to those of $1\alpha_25$(OH)$_2$D$_3$.

For this chapter of work, we firstly examined the effects of $1\alpha_25$(OH)$_2$D$_3$ on IgE + sAg-mediated MC degranulation, as indicated by histamine and $\beta$-hexosaminidase release, as well as the *de novo* synthesis of various cytokines, including TNF$\alpha$, IL-6 and IL-10. By employing $VDR^{-/-}$ as well as WT mBMCMCs in the above assessments, the VDR-dependence for any effect of $1\alpha_25$(OH)$_2$D$_3$ would be determined. Furthermore, with the
aid of the non-genomic-pathway-restricted VitD₃ agonist, curcumin, we investigated the pathway specificity for the effects of 1α,25(OH)₂D₃.

3.2. Results

3.2.1. WT and VDR⁻/⁻ mBMCMCs exhibit similar phenotypical and functional characteristics

The C57BL/6-VDR⁻/⁻ mice used for the current project were generated as a mouse model of VitD-dependent rickets type II by targeted ablation of the second zinc finger of the VDR DNA-binding domain (244), and have since become a valuable tool for exploring new biological functions of 1α,25(OH)₂D₃ under physiological and/or pathological conditions as well as determining the pathway specificity (i.e. genomic versus non-genomic) for these functions. Nevertheless, before using C57BL/6-VDR⁻/⁻ mBMCMCs as well as their WT counterparts to determine the VDR dependence of any potential effects of various VitD₃ analogues, we considered it essential to establish if the two types of MCs exhibit similar intrinsic phenotypical and/or functional properties. As shown in Figure 3.1, VDR⁻/⁻ mice differ from their WT counterparts in various physical aspects, such as growth retardation (Figure 3.1a), the extensive presence of grey (instead of black) hairs throughout the body (Figure 3.1a), dilation of hair follicles and signs of alopecia in the facial area (Figure 3.1b), the shorter hind limbs with abnormal morphology (Figure 3.1c) and the increased fragility of the bones due to hypercalcemia. Despite the above differences between the two mouse strains, WT and VDR⁻/⁻ mBMCMCs displayed very similar phenotypical properties (Figure 3.2). For instance, May-Grünwald Giemsa staining of 5-week-old cells revealed similar granularity between WT and VDR⁻/⁻.
Figure 3.1 Phenotypical comparisons of WT and $VDR^{-/-}$ mice. 
Body (a) and head (b) images of WT and $VDR^{-/-}$ mice. (c) Images of hind limbs (top panel) and separated tibia and femur bones (bottom panel) of WT and $VDR^{-/-}$ mice.
Figure 3.2 Phenotypical comparisons of WT and VDR\(^{-/-}\) mBMCMCs.

(a and b) May-Grünwald Giemsa staining of 5-week-old WT (a) and VDR\(^{-/-}\) (b) mBMCMCs. (c and d) 5-week-old WT (c) and VDR\(^{-/-}\) (d) mBMCMCs were stained with FITC-FceRI and PE-c-kit mAbs and analysed by flow cytometry. (e) Purity of 5-week-old WT and VDR\(^{-/-}\) mBMCMCs as obtained from flow cytometric analysis (indicated by the FceRI\(^+\)-c-kit\(^+\) population). Data are representative of ten independent cultures used in this project.
populations (Figures 3.2 a and b), surface labelling (by flow cytometry) of FcεRI and c-kit molecules on these cells also showed similar levels of expression for both (Figures 3.2 c and d), and there was no disparity in the calculated purity of WT versus VDR-/ mBMCMCs at the end of 5-week culturing (Figure 3.2e), indicating similar rates of development between the two types of cells.

From various functional aspects, WT and VDR-/ mBMCMCs exhibited similar rates of sensitization with α-DNP IgE (SPE-7 clone [Figure 3.3a] and HIN-ε26 clone [Figure 3.4a]). Upon IgE + DNP-HSA-mediated stimulation, they also demonstrated very similar extents of degranulation as evidenced by both β-hexosaminidase (Figures 3.3b and 3.4b) and histamine (Figure 3.3c) release, as well as similar production levels of pro-inflammatory cytokines, TNFα and IL-6 (Figures 3.3 d and e; 3.4 c and d), and the anti-inflammatory cytokine, IL-10 (Figure 3.3f). More importantly, these similarities were observed when two different clones of highly cytokinergic α-DNP IgE molecules, SPE-7 (Figures 3.3) and H1 DNP-ε-26 (Figure 3.4) (262), were used.

3.2.2. 1α,25(OH)2D3 does not affect the efficiency of IgE preload, the expression of surface molecules, and survival of mBMCMCs

Before investigating the effects of 1α,25(OH)2D3 on the pro-inflammatory properties of MCs during IgE + sAg-mediated activation, we first determined if this immunomodulatory agent affects MC development and differentiation, which may have intrinsic impact on their response to IgE-mediated activation. Using a flow cytometry approach, it was confirmed that 1α,25(OH)2D3 had no effect (as compared to the ETOH [0.03% final]-treated controls) on the IgE preload efficiency of both WT and VDR-/
Figure 3.3 Functional comparisons of WT and VDR<sup>-/-</sup> mBMCMCs following IgE (SPE-7 clone) + sAg-mediated activation.

Five-week-old WT and VDR<sup>-/-</sup> mBMCMCs were preloaded with 2 µg/mL of α-DNP IgE (SPE-7 clone) for 16 h. (a) Pre-loaded cells were subsequently stained with FITC-mIgE, Alexa 647-FcεRI, and PE-c-kit mAbs, and analysed by flow cytometry to assess the efficiency of the IgE preload. Non-pre-loaded cells (NT) were included as negative controls. Note that only the FcεRI<sup>+</sup>c-kit<sup>+</sup> cell population only is displayed. (b) Preloaded cells were stimulated with various concentrations of DNP-HSA for 1 h, and the extent of β-hexosaminidase degranulation was measured using β-hexosaminidase release assay. Unstimulated cells (NT) and cells that were treated with PMA/A23187 were used as negative and positive controls, respectively. (c) Preloaded cells were stimulated with 10 ng/mL of DNP-HSA for 30 min, and the extent of histamine release was measured using histamine release assay. Naive cells and cells that were preloaded only were used as negative controls. (d – f) Preloaded cells were stimulated with 20 ng/mL of DNP-HSA for 6 h, and the secretion of TNF (d), IL-6 (e), and IL-10 (f) into culture supernatant was detected using ELISA. Naive cells and cells that were preloaded only were used as negative controls. Data (b-f, mean ± SEM) are representative of at least three independent experiments.
IL-6 + SEM (pg/ml; n = 11)

Surface-bound mIgE

Counts

10^0
10^1
10^2
10^3

WT - NT
WT - IgE (SPE-7)
VDR-/ - NT
VDR-/ - IgE (SPE-7)

Isotype

% β-Hexosaminidase release; + SEM (n = 3)

DNP (ng/mL)

WT
VDR−

PMA+
A23187

% Histamine Degranulation;

Naive
IgE
IgE + DNP

WT
VDR−

TNF + SEM (pg/mL; n = 10)

Naive
IgE
IgE + DNP

WT
VDR−

IL-4 + SEM (pg/mL; n = 11)

Naive
IgE
IgE + DNP

WT
VDR−

IL-10 + SEM (pg/mL; n = 8)

Naive
IgE only
IgE + DNP

WT
VDR−
Figure 3.4 Functional comparisons of WT and VDR<sup>−/−</sup> mBMCMCs following IgE (H1N-ε26 clone) + sAg-mediated activation.

Five-week-old WT and VDR<sup>−/−</sup> mBMCMCs were preloaded with 2 µg/mL of α-DNP IgE (H1N-ε26 clone) for 16 h. (a) Pre-loaded cells were subsequently stained with FITC-mIgE, Alexa 647-FcεRI, and PE-c-kit mAbs, and analysed by flow cytometry to assess the efficiency of the IgE sensitization. Non-pre-loaded cells (NT) were included as negative controls. Note that only the FcεRI<sup>+</sup>c-kit<sup>+</sup> cell population is displayed. (b) Preloaded cells were stimulated with various concentrations of DNP-HSA for 1 h, and the extent of β-hexosaminidase degranulation was measured using β-hexosaminidase release assay. Unstimulated cells (NT) and cells that were treated with PMA/A23187 were used as negative and positive controls, respectively. (d and e) Preloaded cells were stimulated with 20 ng/mL of DNP-HSA for 6 h, and the secretion of TNF (d) and IL-6 (e) into culture supernatant was detected using ELISA. Naive cells and cells that were preloaded only were used as negative controls. Data are representative of at least three independent experiments.
mBMCMCs following 16-h incubation with SPE-7 (Figures 3.5 a and b). Six hours after DNP stimulation, the expression of key surface markers, including FcεRI and c-kit, was also comparable between 1α,25(OH)2D3 and ETOH co-treatment (Figures 3.5 c-f). Although both types of co-treatment slightly down-regulated FcεRI as well as c-kit expression as compared to naïve controls, this was likely due to Ag-triggered receptor internalisation (263, 264), a reduced health status of the cells following IL-3 and FCS deprivation during stimulation (265), and/or simply the addition of ETOH (0.03% final). Cell survival is another process that can directly affect other aspects of MC functionality, and it can be enhanced by highly cytokinergic IgE molecules alone. (262, 266) It was, therefore, significant that the survival rate, as indicated by caspase-3 labelling, of both WT and VDR-/- mBMCMCs showed no disparities between the 1α,25(OH)2D3- and ETOH-treated groups (Figures 3.5 g and h). However, the slightly higher caspase-3 expression in all stimulated cell groups when compared to the naive cells is likely also due to the presence of ETOH (0.03% final), as ETOH has been shown to induce apoptosis in HMC-1 cell lines (0.25 %) and mBMCMCs (2 %). (267)

3.2.3. 1α,25(OH)2D3 reduces IgE + sAg-mediated mBMCMC degranulation in a VDR-dependent manner

Mast cell degranulation and thus the mediators released play major roles in IgE-dependent allergic reactions, especially during the early-phase responses (Section 1.3.2). (64) Therefore, it is important to investigate the immunological effects of 1α,25(OH)2D3 on IgE + sAg-mediated MC degranulation. To achieve this, WT and VDR-/- mBMCMCs were preloaded with SPE-7 in the presence of indicated concentrations of 1α,25(OH)2D3 or ETOH (0.03 %), and subsequently stimulated with DNP together with 1α,25(OH)2D3.
Figure 3.5 $1\alpha,25$(OH)$_2$D$_3$ has no effect on the efficiency of IgE preload, the expression of surface molecules, and the survival of either WT or VDR$^-/$- mBMCMCs. WT (a, c, e, g) and VDR$^-/$- (b, d, f, h) mBMCMCs were preloaded with 2 µg/mL of $\alpha$-DNP IgE Ab (SPE-7 clone), in the presence of different doses of $1\alpha,25$(OH)$_2$D$_3$ or ETOH (0.03% final) as a negative control, for 16 h. (a and b) Surface bound IgE was detected on IgE-preloaded cells using flow cytometry to assess the efficiency of IgE preload in cells treated with $1\alpha,25$(OH)$_2$D$_3$. Naive cells were used as negative controls. (c – h) IgE-sensitized mBMCMCs were subsequently stimulated with 20 ng/mL of DNP-HSA, upon corresponding $1\alpha,25$(OH)$_2$D$_3$ or ETOH treatment, for 6 h. Surface expression of FcεRI (c and d) and c-kit (e and f) on stimulated cells was examined by flow cytometry. Naive cells were used as positive controls. (g and h) Cell survival was also examined by flow cytometry following caspase-3 labelling. Naive cells and cells that were treated with 0.1% H$_2$O$_2$ alone for 24 h were included as negative and positive controls for caspase-3 activation, respectively. Data are representative of three independent experiments using different batches of cells.
or ETOH administration. The extent of histamine release, as assessed at 30 min post-stimulation, was significantly reduced in the presence of $1\alpha,25(\text{OH})_2\text{D}_3$ treatment at the two higher concentrations tested (i.e. $10^{-7}$ and $10^{-8}$ M), as compared to the ETOH-treated controls; and such effect was only observed in WT, but not $VDR^{-/-}$ MCs (Figure 3.6a). It was to be noted that the addition of ETOH alone reduced the extent of IgE-induced histamine release, as compared to the IgE+sAg-stimulated MCs in the absence of ETOH, which was consistent with the findings that the ethanolic extract of adley testa can reduce A23187-mediated degranulation in RBL-2H3 cells (268) and that ETOH concentrations greater than 0.1% can reduce IgE-mediated $\beta$-hexosaminidase release from rat serosal MCs (269). The suppressive effect of $1\alpha,25(\text{OH})_2\text{D}_3$ on MC degranulation was confirmed by the $\beta$-hexosaminidase assay, where WT (Figure 3.6b) but not $VDR^{-/-}$ (Figure 3.6c) mBMCMCs exhibited reduced $\beta$-hexosaminidase release when treated with either concentration of $1\alpha,25(\text{OH})_2\text{D}_3$ (i.e. $10^{-7}$ or $10^{-8}$ M). More importantly, such effect of $1\alpha,25(\text{OH})_2\text{D}_3$ on $\beta$-hexosaminidase release seemed to be exclusive to IgE + sAg-mediated degranulation, as the levels of spontaneous degranulation (as represented by non-stimulated, i.e. “no DNP”, cells) and PMA/A23187-mediated degranulation remained constant for both types of cells (Figures 3.6 b and c). It is also worth noting that $1\alpha,25(\text{OH})_2\text{D}_3$ was most efficient at down-regulating MC degranulation at the highest physiological concentration applied (i.e. $10^{-7}$ M).
Figure 3.6 $1\alpha,25(\text{OH})_2\text{D}_3$ partially reduces IgE + sAg-mediated degranulation of WT but not $VDR^{-/}$ mBMCMCs.

WT (a and b) and $VDR^{-/}$ (a and c) mBMCMCs were preloaded with 2 µg/mL of IgE (SPE-7 clone) for 16 h, in the presence of indicated concentrations of $1\alpha,25(\text{OH})_2\text{D}_3$. Cells that were preloaded alone and/or in the presence of ETOH (0.03% final) were included as controls. (a) Preloaded cells were subsequently stimulated, upon corresponding $1\alpha,25(\text{OH})_2\text{D}_3$ or ETOH treatment, with 10 ng/mL of DNP-HSA for 30 min, and the percentage of histamine release was assessed using histamine EIA assay. Naive cells were included as negative controls. (b and c) Preloaded cells were stimulated, upon corresponding $1\alpha,25(\text{OH})_2\text{D}_3$ or ETOH treatment, with 10 ng/mL of DNP-HSA for 1 h, and the extent of β-hexosaminidase degranulation was measured by β-hexosaminidase assay. Naive cells and cells that were treated with PMA/A23187 were used as negative and positive controls, respectively. Data are representative of at least three independent experiments. * $p < 0.05$, ** $p < 0.01$
3.2.4. 1α,25(OH)2D3 VDR-dependently down-regulates the de-novo synthesis and secretion of pro-inflammatory cytokines but up-regulates that of the anti-inflammatory IL-10, from IgE + sAg-activated mBMCMCs

One of the hallmarks of IgE + sAg-mediated MC activation is the production of pro-inflammatory cytokines such as TNFα and IL-6, both of which are contributors of IgE-mediated allergic reactions (64, 270-274), and contain VDRE in the promoter region of their encoding gene (275, 276). Although not considered a typical product of IgE + sAg-activated MCs, the anti-inflammatory cytokine IL-10 can nevertheless be synthesised and secreted upon 1α,25(OH)2D3 treatment (163). It was therefore essential, for the purpose of the project, to determine if 1α,25(OH)2D3 can affect the cytokine production profile of IgE + sAg-activated mBMCMCs. To address this aim, WT and VDR−/− mBMCMCs were sensitized with IgE anti-DNP Ab (2 µg/mL), and stimulated in the presence of 1α,25(OH)2D3 or ETOH as described previously (Section 2.5.3.3). The production of TNFα, IL-6, and IL-10 was examined at either 3 h (for gene expression) or 6 h (for protein secretion) post-stimulation. For WT mBMCMCs, the overall mRNA and protein levels of both TNFα and IL-6 were significantly reduced in response to 1α,25(OH)2D3 treatment (10⁻⁷ M) as compared to the ETOH (0.03% final) control (Figures 3.7 a, c, e, g). The levels of IL-10, on the other hand, were significantly increased by the same concentration of 1α,25(OH)2D3 (Figures 3.8 a and c). Notably, for VDR−/− mBMCMCs, 1α,25(OH)2D3 had no effect on the production of any of the cytokines examined, whether at the level of mRNA expression or that of protein secretion (Figures 3.7 b, d, f, h; 3.8 b and d). Furthermore, 1α,25(OH)2D3 alone did not induce the de novo synthesis of TNFα or IL-6 in either WT or VDR−/− mBMCMCs (Figure 3.7 a-d). It did, however, induce that of IL-
Figure 3.7. $1a,25(OH)2D3$ dose-dependently reduces IgE + sAg-mediated production of pro-inflammatory cytokines by WT but not $VDR^{-/-}$ mBMCMCs.

WT (a, c, e, g) and $VDR^{-/-}$ (b, d, f, h) mBMCMCs were preloaded with 2 µg/mL of IgE (SPE-7 clone) for 16 h, in the presence of various concentrations of 1a,25(OH)2D3 or ETOH as a negative control. (a - d) Cells were subsequently stimulated, upon the same 1a,25(OH)2D3 or ETOH treatment, with 20 ng/mL of DNP-HSA for 3 h, and the level of TNF-encoding (a and b) and IL-6-encoding (c and d) mRNA relative to that of $\beta$-actin control was assessed using qRT-PCR. Naive cells, cells that were preloaded only, and cells that were treated with the indicated concentration of 1a,25(OH)2D3 were included as controls. (e - h) Pre-loaded cells were stimulated, upon the same 1a,25(OH)2D3 or ETOH treatment, with 20 ng/mL of DNP-HSA for 6 h, and the secretion of TNF (e and f) and IL-6 (g and h) was measured by ELISA. Non-stimulated cells were used as negative controls. Data are representative of three independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001
**WT**

- **Relative TNF mRNA + SEM (n = 3)**
  - Stim + ETOH
  - Stim + 10^(-7) M 1,25 only
  - Stim + 10^(-5) M 1,25 only
  - SPE-7 only
  - 10^(-7) M 1,25 only

- **Relative IL-6 mRNA + SEM (n = 3)**
  - Stim + ETOH
  - Stim + 10^(-7) M 1,25 only
  - Stim + 10^(-5) M 1,25 only
  - SPE-7 only
  - 10^(-7) M 1,25 only

**VDR+-**

- **Relative TNF mRNA + SEM (n = 3)**
  - Stim + ETOH
  - Stim + 10^(-7) M 1,25 only
  - Stim + 10^(-5) M 1,25 only
  - SPE-7 only
  - 10^(-7) M 1,25 only

- **Relative IL-6 mRNA + SEM (n = 3)**
  - Stim + ETOH
  - Stim + 10^(-7) M 1,25 only
  - Stim + 10^(-5) M 1,25 only
  - SPE-7 only
  - 10^(-7) M 1,25 only
Figure 3.8. 1α,25(OH)₂D₃ dose-dependently enhances IgE + sAg-mediated production of the anti-inflammatory cytokine, IL-10, by WT but not VDR⁻/⁻ mBMCMCs.

WT (a and c) and VDR⁻/⁻ (b and d) mBMCMCs were preloaded with 2 μg/mL of IgE (SPE-7 clone) for 16 h, in the presence of various concentrations of 1α,25(OH)₂D₃ or ETOH as a negative control. (a and b) Cells were subsequently stimulated, upon the same 1α,25(OH)₂D₃ or ETOH treatment, with 20 ng/mL of DNP-HSA for 3 h, and the level of IL-10-encoding mRNA relative to that of β-actin control was assessed using qRT-PCR. Naive cells, cells that were preloaded only, and cells that were treated with the indicated concentration of 1α,25(OH)₂D₃ were included as controls. (c and d) Pre-loaded cells were subsequently stimulated, upon corresponding 1α,25(OH)₂D₃ or ETOH treatment, with 20 ng/mL of DNP-HSA for 6 h, and the secretion of IL-10 was measured by ELISA. Naive cells were used as controls. Data are representative 3 – 5 independent experiments. * p < 0.05
10 in WT but not $VDR^{-/-}$ cells (Figure 3.8 a and b), which is consistent with our previous findings. \(^{163}\)

It should be pointed out that the concentration of $1\alpha,25(OH)_{2}D_3$ required to down-regulate early-stage events following IgE + sAg-mediated MC activation, such as degranulation (0.5 – 1 h post-stimulation) and mRNA expression of various cytokines (i.e. $10^{-7}$ M), was higher than that required to suppress later-stage events such as cytokine secretion (i.e. $10^{-8}$ or $10^{-9}$ M). One possible explanation for these observations is that the expression of CYP24A1, with a VDRE in the promoter region of its corresponding gene, could be up-regulated by higher concentrations of $1\alpha,25(OH)_{2}D_3$ and thus contribute to inactivation of this metabolite. \(^{205}\) Although CYP24A1 protein level did not seem to change after 3-h $1\alpha,25(OH)_{2}D_3$ treatment in either WT or $VDR^{-/-}$ mBMCMCs (Figure 3.9), other time points as well as CYP24A1 gene expression need to be examined before a definitive conclusion can be drawn.

### 3.2.5. Curcumin reduces IgE + sAg-mediated cytokine production by mBMCMCs in a VDR- and non-genomic VitD3 pathway-dependent manner

It has been established so far that VDR is required for the observed effects of $1\alpha,25(OH)_{2}D_3$ to regulate MC degranulation and cytokine production upon IgE + sAg-mediated activation, which may suggest the involvement of the genomic VitD3 pathway. Recent studies, however, reported that VDR could also play an important role in the non-genomic VitD3 pathway. \(^{229, 230}\) Therefore, to determine which VitD3 pathway is (or if both pathways are) responsible for the observed regulatory properties of $1\alpha,25(OH)_{2}D_3$, curcumin, a commercially available, non-genomic pathway-restricted agonist of
Figure 3.9. 1α,25(OH)₂D₃ has no effect on CYP24A1 expression in either WT or VDR⁻/⁻ mBMCMCs.

WT and VDR⁻/⁻ mBMCMCs were cultured first in cDMEM-csFCS-4 medium (0.5 × 10⁶ cells/mL) for 4 days, and then in cDMEM-csFCS-3 medium (10⁶ cells/mL) supplemented with alternative concentrations of 1α,25(OH)₂D₃. Naive cells (NT) and cells that were treated with ETOH were included as controls. Total cell lysates were prepared at 3 h post-treatment, and subjected to SDS-PAGE followed by western blot analysis. CYP24A1 expression, as well as that of β-actin loading controls, is shown. Data is from one experiment and represents three individual batches of cells of either genotype.
1α,25(OH)₂D₃, was used instead of 1α,25(OH)₂D₃ to assess its effect on IgE + sAg-mediated cytokine production from mBMCMCs. Unlike 1α,25(OH)₂D₃, curcumin failed to alter the mRNA expression, 3h post-stimulation, of TNF, IL-6 and IL-10 in either WT or VDR⁺/- mBMCMCs (Figure 3.10), which could be explained by its non-genomic function. Surprisingly, although mRNA expression was unaltered at the 3h time point tested, curcumin treatment resulted in a significant reduction of cytokine protein production in the supernatant of WT but not VDR⁺/- mBMCMCs at 6 h post-stimulation (Figure 3.11). Recent evidence suggests that curcumin can elicit anticancer properties by post-transcriptional regulation via certain microRNAs in cancer cells (259, 277) (discussed in more detail in the next section). Similar mechanism(s) might allow curcumin to regulate cytokine release without altering their gene expression. Furthermore, the difference between curcumin and 1α,25(OH)₂D₃ in regulating IL-10 production from MCs was highlighted as curcumin treatment decreased, rather than enhanced, IL-10 secretion from IgE + sAg-stimulated WT mBMCMCs (Figure 3.11c, compared to Figure 3.8c). At the same time, it also failed to increase the IL-10-encoding mRNA in naive WT mBMCMCs (Figure 3.10c, compare to Figure 3.7a).

3.3. Discussion

Regarded as crucial pro-inflammatory effector cells during IgE-dependent allergic reactions, MCs play important roles during all three temporal phases of these reactions (Section 1.3.1). (64) In particular, histamine that is released during MC degranulation can lead to vasodilation, increased vascular permeability and bronchoconstriction during early-phase allergic reactions, as well as contribute importantly to non-allergen-specific
Figure 3.10. Curcumin has no effect on IgE + sAg-mediated mRNA expression for various cytokines by either WT or VDR−/− mBMCMCs.

WT (a, c and e) and VDR−/− (b, d and f) mBMCMCs were preloaded with 2 µg/mL of IgE (SPE-7 clone) for 16 h, in the presence of indicated concentrations of curcumin or ETOH (0.03% final) as a control. Cells were subsequently stimulated, upon corresponding curcumin or ETOH treatment, with 20 ng/mL of DNP-HSA for 3 h, and the level of TNF- (a and b), IL-6- (c and d), and IL-10-encoding (e and f) mRNA relative to that of β-actin control was measured using qRT-PCR. Naive cells (NT), cells that were preloaded only, and cells that were treated with the indicated concentration of curcumin were included as controls. Data are representative of four independent experiments.
Figure 3.11. Curcumin dose-dependently reduces IgE + sAg-mediated secretion of TNF, IL-6 and IL-10 by WT but not VDR⁻/⁻ mBMCMCs.

WT and VDR⁻/⁻ mBMCMCs were preloaded with 2 µg/mL of IgE (SPE-7 clone) for 16 h, in the presence of indicated concentrations of curcumin or ETOH (0.03% final) as a control. Cells were subsequently stimulated, upon corresponding curcumin or ETOH treatment, with 20 ng/mL of DNP-HSA for 6 h, and the secretion of TNF (a), IL-6 (b), and IL-10 (c) was measured by ELISA. Data are representative of three independent experiments. * p < 0.05, ** p < 0.01
airway hyperreactivity at the chronic stage of the disease. Pro-inflammatory cytokines secreted by MCs during late-phase reactions, such as those investigated in this project (i.e. TNFα and IL-6), can also promote recruitment and/or activation of a large variety of immune cells (e.g. neutrophils, DCs, T cells and B cells), as well as affecting the biology of various structural cells (e.g. vascular endothelial cells, fibroblasts, smooth muscle cells and goblet cells) \(^\text{(11, 64, 99, 101, 270, 273)}\). Therefore, by suppressing IgE + sAg-mediated mBMCMC degranulation and their production of pro-inflammatory cytokines (Figures 3.6 and 3.7), it is plausible that 1\(\alpha\),25(OH)\(_2\)D\(_3\) could ease the symptoms at various stages of an allergic response, either directly or indirectly via the resultant reduction in MC-mediated leukocyte recruitment and/or activation. In addition to the latter aspect, 1\(\alpha\),25(OH)\(_2\)D\(_3\) can exhibit immunosuppressive effects on several types of leukocytes, including DCs (e.g. reducing the production of Th1 cytokine IL-12) \(^\text{(220)}\) and T cells (e.g. stimulating the development of Th2 and T\(_{\text{reg}}\) cells) \(^\text{(225, 226)}\), that may be subject to such MC-mediated recruitment, thus further providing protection against exacerbation of allergic disease.

Moreover, it should be pointed out that the effect of 1\(\alpha\),25(OH)\(_2\)D\(_3\) on MC degranulation that we observed in this study contrasts with other reports in the literature. For example, whilst our data demonstrated a suppressive effect of 1\(\alpha\),25(OH)\(_2\)D\(_3\) on IgE + sAg-mediated but not PMA/A23187(a calcium ionophore)-mediated MC degranulation, one previous study showed similar effect on A23187-mediated but not IgE + sAg-mediated MC degranulation \(^\text{(236)}\); and yet another study reported that 1\(\alpha\),25(OH)\(_2\)D\(_3\) enhances both types of MC degranulation as measured by β-hexosaminidase release assay \(^\text{(280)}\). Such apparent discrepancy among these findings can be explained by the differences in
experimental procedures, especially the type of MCs used, and the nature as well as length of 1α,25(OH)₂D₃ treatment. In the study by Toyota et al. (236), the MCs used were *ex vivo* from mouse peritoneal cavity. The same study also applied 24-hour 1α,25(OH)₂D₃ treatment after IgE preload and before sAg/A23187 stimulation, instead of including 1α,25(OH)₂D₃ during the entire preload-stimulation process. In contrast, although the second study mentioned above used mBMCMCs as well as RBL cell line, it treated all cells with 10⁻⁷ M of 1α,25(OH)₂D₃ for 24 or 48 h prior to IgE preload and subsequent stimulation. (280) Since CYP24A1 expression is up-regulated in mouse ears treated with 4 × 10⁻⁹ M of MC903, a low-calcemic analogue of 1α,25(OH)₂D₃, for 24 hours (281), 10⁻⁷ M of 1α,25(OH)₂D₃ is likely to induce CYP24A1 expression and consequently be degraded and lose effectiveness over the 24 or 48 h experimental period.

In addition to its suppressive effect on MC degranulation and production of pro-inflammatory cytokines, the ability of 1α,25(OH)₂D₃ to induce IL-10 production in IgE + sAg-activated mBMCMCs (*Figure 3.8*) is also of significance, as this well-known anti-inflammatory cytokine contributes substantially to the ability of MCs to reduce inflammation associated with allergic contact hypersensitivity responses to 2,4-dinitrofluorobenzene, urushiol and oxazolone. (7, 282) IL-10 is also required to maintain Foxp3 expression as well as suppressive functionality of T_{reg} cells which, in animal models of allergy and asthma, can produce another anti-inflammatory cytokine, TGF-β, as well as additional IL-10 and thus curtail disease progression. (283-285) Furthermore, it was recently reported that IL-10 can down-regulate FceRI expression *in vitro*, using mBMCMCs and human skin-derived MCs; as well as *in vivo*, employing *ex vivo* mouse peritoneal MCs (286, 287) Therefore, when 1α,25(OH)₂D₃ is present during IgE-mediated
allergic reactions, IL-10 produced from MCs (in addition to various other cell types) could potentially function in an autocrine and/or paracrine fashion, dampening their FceRI expression and downstream activation events.

Although our result showed no difference in CYP24A1 protein levels following 3-h $1\alpha,25$(OH)$_2$D$_3$ treatment as compared to the native and ETOH-treated controls (Figure 3.9), we cannot yet exclude the possibility that high concentrations (e.g. $10^{-7}$ M) of $1\alpha,25$(OH)$_2$D$_3$ do induce CYP24A1 gene and/or protein expression in mBMCMCs at later time points. According to Li et al. (281), the low-calcemic analogue of $1\alpha,25$(OH)$_2$D$_3$, MC903, up-regulated CYP24A1 expression in keratinocytes of mouse ears following at least 24-h treatment. Therefore, longer $1\alpha,25$(OH)$_2$D$_3$-treatment durations, as well as CYP24A1 gene expression at the end of all durations, must be examined before a definitely conclusion can be drawn as to whether CYP24A1 up-regulation can be induced by high concentrations of $1\alpha,25$(OH)$_2$D$_3$ in mBMCMCs. Furthermore, we showed here, for the first time, that naive WT and VDR$^{-/-}$ mBMCMCs express CYP24A1 protein, which is itself a significant finding as it indicates the natural ability of mBMCMCs to adjust intracellular $1\alpha,25$(OH)$_2$D$_3$ concentration. Future studies, however, are required to investigate the physiological significance of this finding.

We and others have reviewed the alternative pathways through which $1\alpha,25$(OH)$_2$D$_3$ elicits its biological effects (193, 215), including the genomic pathway which utilises the classical nuclear VDR and ultimately leads to transcriptional regulation of target genes, and the non-genomic pathway which utilises a currently unidentified VDR$_{mem}$ and/or VDR. To determine which pathway is responsible for any potential effect of $1\alpha,25$(OH)$_2$D$_3$ on IgE + sAg-activated MCs, WT and VDR$^{-/-}$ mBMCMCs were compared
to each other to verify the VDR dependency in each experiment. The rationale for this approach is that if VDR dependency can be established, then the genomic VitD$_3$ pathway would most likely be involved. Indeed, our data showed that VDR was required for the suppressive effect of 1α,25(OH)$_2$D$_3$ on gene expression and secretion of TNFα and IL-6, and its enhancive effect on that of IL-10 (Figures 3.7 and 3.8). However, it was also required for the inhibitory effect of 1α,25(OH)$_2$D$_3$ on MC degranulation (Figure 3.6), which is an immediate post-stimulation response. Furthermore, experiments using curcumin confirmed that the suppressive effect of this rapid-acting 1α,25(OH)$_2$D$_3$ agonist on IgE + sAg-mediated cytokine (TNF, IL-6 and IL-10) production from mBMCMCs was VDR dependent, but did not involve change in corresponding gene expression (Figures 3.10 and 3.11). Therefore, our findings suggest a role of the classical VDR in mediating rapid non-genomic 1α,25(OH)$_2$D$_3$ responses. This notion coincides with a previous claim that the VDR$_{mem}$ found in caveolae-enriched plasma membranes in organs of various species are in fact classical VDR in nature. (288) A more recent study further characterised two overlapping ligand binding pockets in VDR which, together with differential ligand stability and site occupancy, can selectively regulate VitD$_3$-mediated genomic and/or non-genomic cellular responses. (230) From this point of view, the current study essentially provided, for the first time, functional evidence for the involvement of MC VDR during 1α,25(OH)$_2$D$_3$-mediated non-genomic responses in IgE-dependent allergy settings.

The ability of curcumin to regulate cytokine production without affecting corresponding gene expression may appear contradictory. However, as mentioned briefly before, curcumin can post-transcriptionally modify gene expression in cancer cells by regulating
various microRNA (miR) levels. MicroRNAs represent a class of small (19- to 22-nucleotide), often phylogenetically conserved, non-coding RNAs that serve as post-transcriptional repressors of gene expression by either destabilizing target transcripts or inhibiting protein translation. Such miR-dependent gene regulation was recently demonstrated to be a reversible process, thus allowing miRs to fine-tune gene expression under different physiological/pathological circumstances. Previous studies have shown that curcumin up-regulates miR-22, with SP1 transcription factor and estrogen receptor as gene targets, in human BxPC-3 pancreatic cancer cells; as well as up-regulates the tumor suppressive miR, miR-203, in bladder cancer. Therefore, it is possible that curcumin can regulate TNF/IL-6/IL-10-targeting miRs and thus affect the translation rather than transcription of corresponding genes.

Moreover, by comparing the effects of 1α,25(OH)2D3 and curcumin, it can be concluded that the suppressive effect of 1α,25(OH)2D3 on TNFα and IL-6 production from activated MCs requires both the non-genomic VitD3 pathway, which contributes to the reduction in overall cytokine secretion (as indicated by the action of curcumin, possibly via the regulation of certain miRs), and the genomic pathway, which alone is responsible for the down-regulation in gene expression of these cytokines. Cross-talk between the two pathways may also exist in this case, but requires further investigation. The ability of 1α,25(OH)2D3 to increase IL-10 production, on the other hand, seems to rely solely on the genomic VitD3 pathway as otherwise the non-genomic pathway as utilised by curcumin would have decreased IL-10 secretion. Therefore, a differential VitD3 pathway-dependency exists for 1α,25(OH)2D3 to regulate different aspects of MC functionality in allergic settings.
The use of \( VDR^{-/-} \) mice and cells derived from them presents potential problems, as \( VDR^{-/-} \) animals do differ from their WT counterparts in a number of phenotypical characteristics (Figure 3.1)\(^{(295)}\), and \( VDR^{-/-} \) mBMCMCs obtained from a different strain of \( VDR^{-/-} \) mice showed accelerated maturation as well as higher responsiveness to IgE + sAg-mediated cytokine production (e.g. IL-6 and IL-13)\(^{(237)}\). Our data comparing WT and \( VDR^{-/-} \) MCs, however, showed little if any difference in their development, morphological properties, and functional characteristics following IgE + sAg-mediated activation (Figures 3.2, 3.3 and 3.4), which highlights the differences between alternative strains of mice carrying similar genetic defect, and perhaps also the necessity to confirm our findings using other strains of mice. Nevertheless, due to the absence of intrinsic dissimilarity between activated WT and \( VDR^{-/-} \) MCs in this study, the differences observed in the effect of \( 1\alpha,25(OH)_{2}D_{3} \) and curcumin on the two types of cells would be solely due to the action of the particular VitD\(_{3}\) analogue.

In conclusion, we demonstrated in this chapter that \( 1\alpha,25(OH)_{2}D_{3} \) can VDR-dependently suppress IgE + sAg-mediated degranulation as well as the production of pro-inflammatory cytokines from mBMCMCs, but at the same time enhance the production of IL-10. Whilst the effect of \( 1\alpha,25(OH)_{2}D_{3} \) on pro-inflammatory cytokine production requires both the non-genomic and genomic pathways; its effect on IL-10 production relies solely on the latter pathway. In the addition, although future investigation is required to determine the effects of high concentrations of \( 1\alpha,25(OH)_{2}D_{3} \) on CYP24A1 expression in mBMCMCs, we did show that mBMCMCs exhibit intrinsic CYP24A1 expression in a VDR-independent manner, the physiological significance of which is yet to be examined.
CHAPTER 4

Mast Cells Can Convert 25OHD₃ To 1α,25(OH)₂D₃ And Thus Enable 25OHD₃ To Immunosuppress IgE + sAg-Activated Mast Cells In Vitro
4.1. Introduction

The precursor metabolite of $1\alpha,25$(OH)$_2$D$_3$, 25OHD$_3$, is the major circulating form of VitD$_3$ with a serum concentration of approximately 100 nM in mice (296), and ranging from 25 nM to 200 nM in humans (297). 25OHD$_3$ is generally converted in the kidney by CYP27B1 to generate the biologically active form, $1\alpha,25$(OH)$_2$D$_3$ (Section 1.7). (203, 297) In addition to renal cells, other cell types including epidermal keratinocytes (298), epithelial cells (299), and osteoblasts (300) also express CYP27B1 and, therefore, can generate their own $1\alpha,25$(OH)$_2$D$_3$ from its inactive precursor in vitro and/or in vivo. Although such extra-renal synthesis of $1\alpha,25$(OH)$_2$D$_3$ contributes little to UVB-mediated protection against conditions such as childhood rickets and osteomalacia (softening of the bones due to defective bone mineralization) in adults, it can still locally provide protective homeostatic functions to regulate excess immune responses. (193) In addition to hepatocytes, other cell types such as keratinocytes also express CYP27A1, and therefore can carry out cutaneous synthesis of 25OHD$_3$ upon UVB irradiation and its subsequent conversion to $1\alpha,25$(OH)$_2$D$_3$. (301, 302) Dermal fibroblasts, on the other hand, express CYP27A1 but not CYP27B1 and thus function as an additional source of 25OHD$_3$ for keratinocytes and, possibly, also for the serum. (303) As mentioned previously, serum provides only minimum levels of $1\alpha,25$(OH)$_2$D$_3$. (304, 305) Therefore, it is essential for keratinocytes to generate their own $1\alpha,25$(OH)$_2$D$_3$ from cutaneous and, perhaps also circulating, 25OHD$_3$, which can then elicit autocrine and/or paracrine effects to regulate the growth, differentiation, apoptosis and other biological processes of these cells. (203) In this regard, it should be pointed out that the effect of keratinocyte-derived $1\alpha,25$(OH)$_2$D$_3$ is generally limited to the skin and not resulting in systemic outcomes, as the level
1α,25(OH)₂D₃ produced is tightly regulated by CYP24A1 expression in these cells, where substantially high levels of 1α,25(OH)₂D₃ up-regulates CYP24A1 expression, which in turn inactivates excess 1α,25(OH)₂D₃. (196)

Various immune cells, such as DCs (209), macrophages (306), T cells (209) and B cells (210) also express CYP27B1, but only when they are activated during an immune response. Unlike renal cells and keratinocytes, whose CYP27B1 expression and activity is tightly regulated by parathyroid hormone (PTH) and low levels of calcium and phosphate, immune cells do not respond to these signals but rather up-regulate CYP27B1 in response to certain cytokine(s). (196, 253) In addition, the expression and activity of CYP24A1 in macrophages and DCs is either absent or blocked, removing the feedback control as seen in keratinocytes, by the 1α,25(OH)₂D₃ produced. (209, 307, 308) Together, these findings could explain why diseases associated with immune activation, such as sarcoidosis, tuberculosis, peritonitis and inflammatory arthritis, can lead to hypercalcemia and hypercalciuria as a result of elevated levels of circulating 1α,25(OH)₂D₃. (196)

Furthermore, with the exception of DCs to date, immune cells that have been examined do not express CYP27A1 and, therefore, require 25OHD₃ produced by other cell types and/or from serum to generate their own 1α,25(OH)₂D₃. (209) Nevertheless, the ability of immune cells to synthesize 1α,25(OH)₂D₃ confers this hormone a wide range of regulatory effects on innate and adaptive immunity. (193, 196)

Despite the ever-increasing number of effector as well as immunoregulatory roles of MCs, and the established negative regulatory effects of 1α,25(OH)₂D₃ on MC functionality, the expression and activity of CYP27B1 has not been examined in these cells. Employing WT and VDR⁻/⁻ mBMCMCs, we first examined the expression of this hydroxylase in
MCs and any potential VDR-dependence for such expression. We then investigated the effect of 25OHD₃ treatment on CYP27B1 expression and subsequently 1α,25(OH)₂D₃ production by both types of mBMCMCs. If endogenous 1α,25(OH)₂D₃ production can be achieved by mBMCMCs, then the immunoregulatory ability of 25OHD₃ on IgE + sAg-activated MCs will be accessed.

4.2. Results

4.2.1. Mouse BMCMCs constitutively express CYP27B1 in their cytosol

As mentioned previously, CYP27B1 is expressed by many immune cell types, including macrophages, dendritic cells, T cells and B cells, upon activation. (196) To date, however, its expression in MCs has not been reported. By labelling naive mBMCMCs intracellularly with an α-CYP27B1 Ab, we revealed, for the first time, that MCs do indeed express this hydroxylase and that such expression is constitutively expressed in the cytosol (i.e. does not require activation of the cells) (Figures 4.1 a – e). Importantly, CYP27B1 expression in MCs is not VDR-dependent, as VDR⁻/⁻ mBMCMCs (Figures 4.1 a and e) showed an identical level of expression as their WT counterparts (Figures 4.1 a and d). These findings were further confirmed by western analysis of lysates from naive mBMCMCs (Figure 4.1f).

4.2.2. 25OHD₃ up-regulates CYP27B1 expression in mBMCMCs in a VDR-dependent manner

It has been shown previously that CYP27B1 expression can be up-regulated by 25OHD₃ treatment in rat osteoblasts. (309) To investigate if 25OHD₃ has similar effect on CYP27B1 expression in MCs, WT and VDR⁻/⁻ cells were first cultured in medium containing csFCS
Figure 4.1. Intracellular expression of CYP27B1 in naive mBMCMCs

Five- to 6-week-old WT (a, b, d) and $VDR^{-/-}$ (a, c, e) mBMCMCs ($10^6$ cells each) were labelled intracellularly with α-CYP27B1 Ab, and examined by either flow cytometry (a) or immunofluorescence staining (d and e). An isotype control (a, b, c) was included in both approaches, and the DAPI nucleus stain was used in the latter. Scale bars (b – e) represent 10 μm. (f) Lysates were prepared from WT or $VDR^{-/-}$ mBMCMCs ($3 \times 10^6$ cells each), various amount of which (as indicated) was analysed by western blotting for CYP27B1 expression. Kidney extract from a normal C57BL/6 mouse was used as a positive control. Data are representative of three independent batches of cells of either genotype.
Isotype

WT

VDR-/

CYP27B1

Count

WT

VDR KO Kidney extract (µg)

f

25 50 100

25 50 100 25

β-actin
instead of normal FCS to minimise exogenous VitD$_3$ levels. At the end of 72 h incubation, the purity of the MC populations, based on expression of key surface markers (i.e. FceRI and c-kit), was maintained (Figure 4.2a) prior to treatment with 25OHD$_3$ (10$^{-7}$ M) or ETOH (0.03%, which was applied to all subsequent ETOH treatment) and subsequent examination of CYP27B1 mRNA (at 3 h post-treatment) and protein (at 8 h post-treatment) levels, respectively. Our results showed that whilst WT mBMCMCs exhibited up-regulated gene (Figure 4.2b) as well as protein (Figures 4.2 c and d) expression of CYP27B1, VDR$^{-/-}$ cells failed to show any difference in expression on both levels. Therefore, our figures suggest that MC CYP27B1 expression can be enhanced by its 25OHD$_3$ substrate in a VDR-dependent manner. Although not having reached statistical significance, a trend towards a difference between CYP27B1 protein expression in ETOH-treated WT and VDR$^{-/-}$ mBMCMCs was noted. Further investigation is thus required to explain such observation. Interestingly, in both WT and VDR$^{-/-}$ cell types, ETOH-treated group showed a moderate reduction in CYP27B1 expression as compared to the naive control, which could due merely to the presence of ETOH (mentioned in the previous chapter).

4.2.3. 25OHD$_3$ dose-dependently induces endogenous synthesis of 1$\alpha$,25(OH)$_2$D$_3$ by mBMCMCs

Because CYP27B1 is the enzyme responsible for converting 25OHD$_3$ to 1$\alpha$,25(OH)$_2$D$_3$ in a number of other cell types$^{(196,310)}$, the logical experiment performed next investigated if it plays the same role in MCs. After 72-h incubation in medium containing csFCS, WT and VDR$^{-/-}$ mBMCMCs were treated with various concentrations of 25OHD$_3$ or ETOH for 6 h, and the level of 1$\alpha$,25(OH)$_2$D$_3$ in culture supernatant as well as cell lysate was
Figure 4.2. 25OHD$_3$ enhances CYP27B1 expression in mBMCMCs on both mRNA and protein levels

Five- to 6-week-old WT and VDR$^-$ mBMCMCs were cultured, at 5 × 10$^5$ cells/mL, in cDMEM-csFCS-4 medium for 72 h, when the expression profile of key surface markers was confirmed by flow cytometry (a). All cells were subsequently replated in cDMEM-csFCS-3 medium at 10$^6$ cells/mL, and treated with either 10$^{-7}$ M of 25OHD$_3$ or ETOH (0.03%). The level of CYP27B1 gene expression was measured at 3 h post-treatment by qRT-PCR (b), whereas the CYP27B1 protein expression was examined by western blotting at 8 h post-treatment (c and d). Lysates prepared from naive cells and normal mouse kidney extract were analysed by western blotting as controls. Data are representative of three or four independent experiments. * p < 0.05
a) Pre-culture vs. Post-culture

WT

FceRI Expression vs. c-kit Expression

VDR<sup>−/−</sup>

FceRI Expression vs. c-kit Expression

b) Relative CYP27B1 mRNA + SEM (10<sup>6</sup>; n = 3)

WT vs. VDR<sup>−/−</sup>

Naive ETOH 25OH Naive ETOH 25OH

WT 1 vs. WT 2

Naive ETOH 25OH Naive ETOH 25OH

β-actin

Naive ETOH 25OH Naive ETOH 25OH

CYP27B1

β-actin

d) Relative Band Intensity + SEM (n = 4)

Naive ETOH 25OH Naive ETOH 25OH

WT vs. VDR<sup>−/−</sup>

p = 0.09
subsequently measured. As illustrated in Figure 4.3a, treatment with the higher doses of 25OHD3 (i.e. $10^{-6}$ and $10^{-7}$ M) led to higher secretion of $1\alpha,25(OH)_2D_3$ from WT mBMCMCs as compared to the ETOH-treated control group. Although $1\alpha,25(OH)_2D_3$ level in cell lysates seemed to increase only with the highest dose (Figure 4.3b), both $10^{-6}$ M and $10^{-7}$ M of 25OHD3 succeeded in elevating the total level of the active product in WT cells (Figure 4.3c). More importantly, the highest 25OHD3 concentration (i.e. $10^{-6}$ M) was also able to induce $1\alpha,25(OH)_2D_3$ production and secretion in $VDR^{-/-}$ mBMCMCs (Figure 4.3). Furthermore, in contrast to previous findings with human keratinocytes (311), most of the endogenously synthesised $1\alpha,25(OH)_2D_3$ by MCs appeared to be secreted instead of accumulating inside the cells.

4.2.4. 25OHD3 treatment reduces IgE + sAg-mediated degranulation and pro-inflammatory cytokine production of mBMCMCs in a dose- and VDR-dependent manner

In chapter 3, evidence was provided demonstrating that $1\alpha,25(OH)_2D_3$ can elicit immunosuppressive effects on IgE + sAg-activated MCs. Since 25OHD3 can also induce endogenous $1\alpha,25(OH)_2D_3$ synthesis in MCs, we examined if 25OHD3 can affect IgE-mediated mBMCMCs activation. Our initial attempt involved preloading and stimulating WT and $VDR^{-/-}$ cells in the presence of indicated concentrations of 25OHD3 or ETOH, which was done in the same way as with $1\alpha,25(OH)_2D_3$ treatment (i.e. 25OHD3/ETOH was added immediately prior to IgE preload and DNP-HSA stimulation). As revealed by Figures 4.4 a and c, only the highest concentration of 25OHD3 tested (i.e. $10^{-6}$ M) was able to decrease the production of proinflammatory cytokines from activated WT MCs. None of the concentrations, however, managed to affect TNFα or IL-6 production by
Figure 4.3. Dose-dependent $1\alpha,25(\text{OH})_2\text{D}_3$ production by 25OHD$_3$-treated mBMCMCs

Five- to 6-week-old WT and $\text{VDR}^-$ mBMCMCs were cultured, at $5 \times 10^5$ cells/mL, in cDMEM-csFCS-4 medium for 72 h, subsequently replated at $2 \times 10^6$ cells/mL in cDMEM-csFCS-3 medium, and treated with indicated concentrations of 25OHD$_3$ or ETOH. Culture supernatant (a) as well as cell lysate (b) samples were collected at 6 h post-treatment, and the $1\alpha,25(\text{OH})_2\text{D}_3$ level in all samples was measured using the 1,25-(OH)$_2$D RIA kit. The total $1\alpha,25(\text{OH})_2\text{D}_3$ level (c) was calculated by adding together its concentration in the supernatant and that in the corresponding cell lysate. Data are representative of three independent batches of cells examined in two independent experiments, where only supernatant (not cell lysate, and therefore not used to calculate the total value) from cells treated with $10^{-8}$ M of 25OHD$_3$ was analysed. * $p < 0.05$, ** $p < 0.01$
Figure 4.4. Short-term high-dose 25OHD3 treatment reduces IgE + sAg-mediated production of pro-inflammatory cytokines by WT and VDR-/− mBMCMCs

WT (a and c) and VDR-/− (b and d) mBMCMCs were preloaded with 2 µg/mL of IgE (SPE-7 clone) for 16 h, in the presence of indicated concentrations of 25OHD3 or ETOH (0.03% final). Cells were subsequently stimulated, upon corresponding 25OHD3 or ETOH treatment, with 20 ng/mL of DNP-HSA for 6 h, and the level of TNF (a and b) and IL-6 (c and d) in culture supernatant was measured by ELISA. Non-stimulated cells were used as negative controls. Data are representative of four independent experiments.
$VDR^{-/-}$ cells (Figures 4.4 b and d). These data suggest that 25OHD$_3$ might require a longer incubation period with the MCs to be converted to 1α,25(OH)$_2$D$_3$. An alternative possibility is that only at sufficiently high concentrations could 25OHD$_3$ readily regulate the functionality of activated MCs, potentially by binding directly to VDR as demonstrated in mouse kidney and skin cells.$^{(243)}$

To examine the aforementioned possibility that, given sufficient incubation time with the cells, lower concentrations of 25OHD$_3$ may still elicit immunosuppressive effects on activated MCs upon its conversion to the active form, 1α,25(OH)$_2$D$_3$, we treated WT and $VDR^{-/-}$ cells with 25OHD$_3$ or ETOH 8 h prior to IgE preload followed by usual DNP stimulation with 25OHD$_3$/ETOH co-treatment. In this case, the level of both TNFα and IL-6 secreted following MC activation was indeed reduced by 25OHD$_3$ treatment, especially at the highest concentration (i.e. $10^{-7}$ M); and again such observation was made in WT but not $VDR^{-/-}$ cells (Figures 4.5 a – d). Similarly, long-term 25OHD$_3$ treatment also appeared capable of reducing IgE + sAg-mediated, but not spontaneous or PMA/A23187-mediated, degranulation in WT but not $VDR^{-/-}$ cells (Figures 4.5 e and f).

Furthermore, the above immunosuppressive effect of long-term 25OHD$_3$ exposure was not due to alteration of the IgE preload efficacy, assessed by the expression of key surface molecules (e.g. FcεRI and c-kit), or the viability of the cells (Figure 4.6).

4.3. Discussion

To date, there is a paucity of information in the literature on MC responses to VitD$_3$ metabolites. Here, for the first time, we demonstrate that mBMCMCs express CYP27B1 (Figure 4.1), and the resultant ability of these cells to synthesise endogenous
Figure 4.5. Long-term 25OHD$_3$ treatment dose-dependently reduces IgE + sAg-mediated pro-inflammatory cytokine production and degranulation of WT but not VDR$^{-/-}$ mBMCMCs.

WT (a, c, e) and VDR$^{-/-}$ (b, d, f) mBMCMCs were treated with indicated concentrations of 25OHD$_3$ or ETOH for 8 h, at the end of which cells were preloaded with 2 µg/mL of IgE (SPE-7 clone) for a further 16 h. Subsequently stimulation was carried out, upon corresponding 25OHD$_3$ or ETOH treatment, with 20 ng/mL of DNP-HSA for 6 h, and the level of TNF (a and b) IL-6 (c and d) was determined by ELISA. To examine the effect of 25OHD$_3$ on MC degranulation, preloaded cells were stimulated with 10ng/mL DNP for 1 h, and the extent of β-hexosaminidase degranulation was measured by β-hexosaminidase assay. Naive cells and cells that were treated with PMA/A23187 were used as negative and positive controls, respectively. Data are representative of at least three independent experiments. * p < 0.05, ** p < 0.01
Figure 4.6 25OHD₃ has no effect on the efficiency of IgE preload, the expression of surface molecules, and the survival of either WT or VDR⁻/⁻ mBMCMCs

WT (a, c, e, g) and VDR⁻/⁻ (b, d, f, h) mBMCMCs were treated with indicated concentrations of 25OHD₃ or ETOH for 8 h prior to preload with 2 µg/mL of α-DNP IgE (SPE-7 clone) for a further 16 h. (a and b) Surface bound IgE was detected on preloaded cells using flow cytometry to assess the efficiency of IgE preload. Naive cells were used as negative controls. (c - h) Preloaded cells were subsequently stimulated with 20 ng/mL of DNP-HSA, upon 25OHD₃ or ETOH treatment, for 6 h. Surface expression of FcεRI (c and d) and c-kit (e and f) on stimulated cells was examined by flow cytometry. Naive cells were used as positive controls. (g and h) Cell survival was also examined by flow cytometry following caspase-3 labelling. Naive cells and cells that were treated with 0.1% H₂O₂ alone for 24 h were included as negative and positive controls for caspase-3 activation, respectively. Data are representative of three independent batches of cells.
$1\alpha_25(OH)_2D_3$ from 25OHD$_3$ (Figure 4.3). The fact that mBMCMCs, unlike other immune cells, constitutively express this hydroxylase enzyme is significant in a number of aspects. Firstly, it allows MCs, which are abundant in the skin in close proximity to blood vessels, to readily utilise 25OHD$_3$ in the serum and/or synthesised within the tissues by structural cells such as keratinocytes under normal physiological conditions. Given that excessive MC activation can exacerbate IgE-mediated allergic responses, their ability to synthesise $1\alpha_25(OH)_2D_3$ in the naive state could potentially generate an immunosuppressive local environment well before the onset of any allergic responses. It is tantalising to speculate that one possible contributing factor to the recent allergy epidemic in Western countries is the increase in VitD$_3$ insufficiency among the populations. ($^{312}$)

Furthermore, the basal level of CYP27B1 expression seemed sufficient to allow high concentrations (e.g. $10^{-6}$ M) of 25OHD$_3$ to induce $1\alpha_25(OH)_2D_3$ production, even in $VDR^{-/-}$ mBMCMCs (Figure 4.3). Since the endogenously produced $1\alpha_25(OH)_2D_3$ was mostly secreted (Figure 4.3), it could increase the local concentration of this hormone in an autocrine and/or paracrine fashion to functional levels as determined in the previous chapter, and hence provide an additional mechanism for immune cells to negatively regulate their activation. In addition to the specific anti-allergy property as implied by our results, serum and/or tissue 25OHD$_3$ allows $1\alpha_25(OH)_2D_3$ production and, in turn, IL-10 production from naive MCs, thus conferring these cells a more general anti-inflammatory role in physiological as well as pathological circumstances.

Whilst renal CYP27B1 expression is mainly regulated by mediators of calcium and bone homeostasis, such as parathyroid hormone and $1\alpha_25(OH)_2D_3$ itself, immune cells often
adjust their CYP27B1 level differently \(^{(310)}\). Macrophage- and DC-derived CYP27B1, for example, is under the control of inflammatory signals such as interferon-\(\gamma\), and the p38 MAP Kinase/NF-\(\kappa\)B-dependent maturation, respectively; and neither is subject to the negative feedback mechanism by 1\(\alpha\),25(OH)\(_2\)D\(_3\) \(^{(219,313)}\). As mentioned above, CYP27B1 expression in mBMCMCs is up-regulatable by the 25OHD\(_3\) substrate in a VDR-dependent manner (Figures 4.2). Such a regulatory mechanism could be significant, as it allows MCs to monitor the level of 25OHD\(_3\) available in their local environment and to only up-regulate CYP27B1 expression when required and the optimal range is reached. It is possible, therefore, that to synthesize maximum amounts of the active 1\(\alpha\),25(OH)\(_2\)D\(_3\) product, MCs need to elevate their CYP27B1 expression from the basal level, which in turn depends on the availability of 25OHD\(_3\) substrate. Indeed, this notion was supported by our data that 1\(\alpha\),25(OH)\(_2\)D\(_3\) was produced by WT mBMCMCs from 25OHD\(_3\) in a dose-dependent manner (Figure 4.3). It is important to mention that the VDR-dependence for such positive regulation of CYP27B1 expression by 25OHD\(_3\) may only be significant/relevant when the substrate concentration is within physiological range (10\(^{-7}\) M), as pharmacological concentrations of 25OHD\(_3\) (e.g. 10\(^{-6}\) M) were able to induce 1\(\alpha\),25(OH)\(_2\)D\(_3\) production even in \(VDR^{-}\) MCs. However, any change in CYP27B1 expression in \(VDR^{-}\) MCs when treated with 10\(^{-6}\) M of 25OHD\(_3\) needs to be examined before a definitive conclusion in this regard can be drawn.

Our data also demonstrates that the response of mBMCMCs to 25OHD\(_3\) treatment depends on the dose of this metabolite as well as the length of time that the MCs had been pre-incubated with it. In this aspect, there is evidence to suggest that 25OHD\(_3\) can itself act as a VDR ligand, but only when its concentration is sufficiently high (i.e. \(\geq 400\))
nM). This could explain the observations from our short-term 25OHD$_3$ treatment experiment, where only $10^{-6}$ M, but not lower concentrations of this analogue, was able to reduce the production of pro-inflammatory cytokines from activated WT MCs (Figure 4.4). Such a notion can be further supported by the fact that $VDR^{-/-}$ cells failed to respond accordingly to this high 25OHD$_3$ concentration. Therefore, it appears that high levels of 25OHD$_3$ can induce immediate immunosuppressive effects on IgE + sAg-activated MCs, most likely via direct interaction with VDR, in a manner similar to responses elicited by 1$_\alpha$25(OH)$_2$D$_3$. The relatively low concentrations of 25OHD$_3$ used, on the other hand, did cause similar immunosuppressive effects on activated MCs when coupled with the long-exposure setting (Figure 4.5), which allowed sufficient time for the conversion from 25OHD$_3$ to the active 1$_\alpha$25(OH)$_2$D$_3$ form. Taken together all these findings, it is possible that when the concentration of 25OHD$_3$ available is optimal (i.e. sufficiently high), it can affect the functionality of activated MCs both promptly by direct binding to VDR and subsequently due to the induction of 1$_\alpha$25(OH)$_2$D$_3$ production; whereas when the concentration is suboptimal, 25OHD$_3$ can elicit similar regulatory effects, but in this case only via endogenously synthesised 1$_\alpha$25(OH)$_2$D$_3$. In other words, the existence of these alternative pathways to explore the immunoregulatory functions of 25OHD$_3$ allows MCs to utilise 25OHD$_3$ within a large concentration range. This ability is particularly important, as serum 25OHD$_3$ levels generally show large fluctuations and depend partly upon the nutritional status and UVB exposure of the individual.

Another significant finding from this series of experiments is the VDR-dependence for the up-regulation of CYP27B1 expression by 25OHD$_3$ and for the immunosuppressive effects of 25OHD$_3$ on activated mBMCMCs, which was consistent with the previous
report that 25OHD3 takes the same position (although at lower affinity) within the ligand binding pocket of VDR as 1α,25(OH)2D3, thus resulting in the same conformational change of the receptor. (243)

In conclusion, we demonstrated in this chapter, for the first time, the constitutive expression of CYP27B1 in mBMCMCs and the VDR-dependent up-regulation of this enzyme by 25OHD3 in its physiological concentration (i.e. 10⁻⁷ M). The functionality of this enzyme was confirmed by inducing endogenous 1α,25(OH)2D3 synthesis from 25OHD3 in $VDR^{−/−}$ as well as WT mBMCMCs in a dose-dependent manner. By potentially binding to VDR directly and/or being converted to 1α,25(OH)2D3 (in which case longer treatment time would be required), 25OHD3 can elicit immunosuppressive effects on IgE + sAg-activated MCs at a wide range of concentrations. Furthermore, functional VDRs were shown to be essential for the regulation of MC CYP27B1 expression by 25OHD3, and for the immunosuppressive effects of this VitD3 analogue on activated MCs using alternative settings. In particular, although $VDR^{−/−}$ mBMCMCs are able to synthesise 1α,25(OH)2D3 from 25OHD3, they fail to respond to 25OHD3 during IgE + sAg-mediated activation due to the lack of VDR.
CHAPTER 5

TOPICALLY APPLIED 1α,25(OH)₂D₃ AND 25OHD₃ CAN REDUCE THE CUTANEOUS PATHOLOGY ASSOCIATED WITH IgE-MEDIATED PASSIVE CUTANEOUS ANAPHYLAXIS IN A MAST CELL VITAMIN D RECEPTOR-DEPENDENT MANNER IN VIVO
5.1. Introduction

Because of the encouraging in vitro findings so far, it is important to translate these findings to corresponding in vivo settings. It is widely accepted that MCs play important effector as well as immunoregulatory roles during all three phases of IgE-dependent allergic responses (Section 1.3.1). With particular relevance to the current project, which primarily examines the early- to late-phase reactions following IgE + sAg-mediated MC activation, MCs readily release pre-formed mediators such as histamine and TNFα via degranulation, which can contribute directly to immediate type I hypersensitivity reactions and/or initiate late-phase reactions by recruiting other leukocytes. (64) The PCA model used in this study provides a MC-dependent IgE-mediated immediate hypersensitivity platform, which exhibits typical early- and late-phase responses including tissue swelling and leukocyte infiltration, respectively. (89) In this model, MC-derived TNFα contributes to the observed neutrophil recruitment. Although IL-6 and IL-10 levels are not conventionally examined in relation to IgE-mediated allergy, both cytokines can be produced by MCs, and the former has been shown to contribute to the pathogenesis of certain chronic allergic conditions (273) whereas the latter has general anti-inflammatory effects. Therefore, it is important to investigate whether these cytokines derived from MCs also play a role in the particular PCA model that we employed here.

It is well-recognised that UVB-based phototherapy can be used to treat a multitude of dermatologic disorders, such as atopic dermatitis, psoriasis, and cutaneous T cell lymphoma, and its benefits are to some extent mediated by triggering VitD3 synthesis in the skin. (314) In patients with psoriasis, for instance, previous studies have indicated insufficient basal levels of VitD3 and its metabolites in the circulation as compared to
healthy controls. Therefore, the increase in serum 25OHD₃, 24,25(OH)₂D₃ and 1α,25(OH)₂D₃ levels in these patients following broadband UVB treatment may, at least partially, explain the efficacy of this therapeutic approach. In addition, topically applied 1α,25(OH)₂D₃ (and its various analogues e.g. calcipotriol and calcipotriene) has also been proven an effective treatment for inflammatory diseases of the skin such as psoriasis and vitiligo, as well as CHS responses in a number of mouse and human experiments. A recent study by Gorman et al. further demonstrated an additive effect of topical 1α,25(OH)₂D₃ application upon UVB irradiation on suppressing CHS responses to 2,4-dinitrofluorobenzene in mice as compared to irradiation-only controls. Bearing particular relevance to the current study, 1α,25(OH)₂D₃ administration may also assist in limiting allergic diseases such as asthma, as subcutaneous immunotherapies incorporating this hormone can reduce respiratory inflammation during allergic airway disease. To date, likely mechanisms responsible for such 1α,25(OH)₂D₃-mediated immunosuppression include direct effects on keratinocyte proliferation and differentiation in psoriatic lesions, regulation of the trafficking as well as functionality of effector cells (e.g. DCs and T cells) in the diseased skin and skin-draining LNs, and enhancement of the suppressive capacity of regulatory cells such as T_{reg} cells. Despite the involvement of MCs during the pathogenesis of cutaneous disorders including psoriasis and atopic dermatitis, the effects of topically applied VitD₃ analogues on these cells have not been investigated, especially in the context of anaphylaxis. Previous chapters of this study highlighted that both 1α,25(OH)₂D₃ and 25OHD₃ could elicit immunosuppressive effects on IgE + sAg-
activated MCs in vitro. We now wanted to determine if these observed in vitro effects can be translated to an in vivo IgE-mediated PCA model.

Due to the extensive influence of VitD3 on a large variety of immune as well as structural cells, any effect of topically applied VitD3 on PCA-associated cutaneous pathology may not necessarily be mediated by MCs in the skin. To test if MCs are the target of VitD3-induced suppression of PCA responses, we used WT mice, MC-deficient *Kit*<sup>W-sh/W-sh</sup> mice and *Kit*<sup>W-sh/W-sh</sup> mice that had been engrafted selectively in to the ears with WT mBMCMCs and therefore differ from the original mutants solely because of the presence of MCs (Section 1.5.2). Furthermore, it was established in vitro that VDR is required for both 1α,25(OH)2D3 and 25OHD3 (the precursor, inactive metabolite) to elicit immunosuppressive effects on activated MCs. To examine the MC VDR-dependence for the effects of topical VitD3 on PCA, *Kit*<sup>W-sh/W-sh</sup> mice engrafted with *VDR*<sup>−/−</sup> mBMCMCs were also used, which differ from the WT mBMCMC-engrafted counterparts solely due to the lack of functional VDR in the donor MCs. Using all four groups of mice, we intended to investigate firstly the effect of topically applied 1α,25(OH)2D3 and 25OHD3 on PCA-associated cutaneous pathology and its dependency on MC VDR expression and, subsequently, the mechanisms (e.g. alteration in MC number, granularity and mediator profile) involved.
5.2. Results

5.2.1. Topical $1\alpha,25(OH)2D3$ application alone does not affect ear thickness of WT mice

A recent report by Li et al. (281) demonstrated that in WT CD1 mice treated with $1\alpha,25(OH)2D3$ or its low-calcemic analogue MC903 (calcipotriol) topically to the ears (both dissolved in ETOH, and applied at 4 nM for up to 16 consecutive days) exhibited atopic dermatitis-like features such as ear swelling and skin lesions, a process driven by the induction of keratinocyte-derived TSLP. To examine whether such a pro-inflammatory effect of $1\alpha,25(OH)2D3$ also applies to WT C57BL/6 mice, mouse ears were topically treated with either four daily doses of 3 µM (a concentration that has been routinely used (226, 323)) of $1\alpha,25(OH)2D3$ dilution or fifteen doses of 0.03 µM (a concentration that was indicated to be immunosuppressive by in vitro experiments illustrated in Chapter 3) dilution. Daily ear thickness measurement showed that these concentrations of $1\alpha,25(OH)2D3$ did not alter ear thickness compared to vehicle-treated controls (Figure 5.1).

5.2.2. Topical $1\alpha,25(OH)2D3$ application at the site of inflammation can suppress PCA-associated ear swelling

To determine the optimal dose of topical $1\alpha,25(OH)2D3$ that can suppress IgE-mediated PCA-associated ear swelling, three different concentrations were tested in WT C57BL/6 mice, out of which the highest and the most frequently used concentration (3 µM) seemed the most effective (Figure 5.2a). It should be noted that the two lower concentrations also led to a reduction in overall ear swelling response as compared to vehicle treatment, even though the differences did not reach statistical significance. Nevertheless, it was
Figure 5.1. Topically applied 1α,25(OH)₂D₃ alone has no effect on ear thickness of WT mice
Ears of WT C57BL/6 mice were topically applied with either (a) 3 μM of 1α,25(OH)₂D₃ (20 μL/ear) for four consecutive days or (b) 0.03 μM of 1α,25(OH)₂D₃ for 15 consecutive days. In both cases, vehicle (ETOH-PG-H₂O at 2:1:1 ratio)-treated mice were used as controls, and the change in ear thickness from baseline was measured and recorded every 24 h. Data are representative of one experiment using six or eight (as indicated) mice per treatment.
Figure 5.2. Optimisation of the 1α,25(OH)2D3 application strategy to study its effect on PCA-associated cutaneous pathology

Wild-type C57BL/6 (a and b) and WBB6F1 (c) mice were used in this series of experiments. (a and c) Mice were injected i.d. with 100 ng (20 µL of 5 µg/mL dilution) of α-DNP IgE (SPE-7 clone) in the right ear and equal volume of HMEM-Pipes in the left ear. Immediately following injection, both ears received topical application (20 µL/ear) of either 1α,25(OH)2D3 dilution at the indicated concentration or vehicle alone. (b) Mice were injected i.d. with either 100 ng of SPE-7 or equal volume of HMEM-Pipes in the right ear only. Immediately following injection, the left ear of the mice were topically painted with either 3 µM of 1α,25(OH)2D3 or vehicle alone. In all cases, mice were injected retro-orbitally with 200 µg of DNP-HSA (100 µL of 2 mg/mL dilution) 16 h later, and at indicated time point post-injection, the amount of ear swelling was recorded. Data in (a) are representative of three independent experiments, each using three to four mice per treatment. Data in (b and c) are representative of one experiment using the indicated number of mice per treatment. * p < 0.05, ** p < 0.01
decided that 3 µM of $1\alpha_25$(OH)$_2$D$_3$ would be used in all subsequent experiments. As expected, in all treatment groups only the IgE-preloaded ears (i.e. right ears) exhibited typical PCA-mediated swelling in response to i.v. administration of the specific antigen, DNP-HSA, which subsided by 6 h post-stimulation. By contrast, HMEM-Pipes-preloaded ears (i.e. left ears) maintained their original thickness (i.e. no swelling) throughout the observation period. To examine whether $1\alpha_25$(OH)$_2$D$_3$ needs to be applied to the same ear where PCA was induced, mice received an i.d. injection of IgE α-DNP and $1\alpha_25$(OH)$_2$D$_3$/vehicle treatment on different ears. As shown in Figure 5.2b, a systemic effect of $1\alpha_25$(OH)$_2$D$_3$ was not observed as topical $1\alpha_25$(OH)$_2$D$_3$ application to the contralateral ear that was not injected with IgE mAb could not suppress PCA-associated ear swelling in the ear that did receive IgE. Therefore, the observed PCA suppression by $1\alpha_25$(OH)$_2$D$_3$ is a localised event. To determine if these findings are restricted to the C57BL/6 background, we also tested WT WBB6F1 mice. Similar to the effects observed in C57BL/6 mice, topical $1\alpha_25$(OH)$_2$D$_3$ application (3 µM) could effectively reduce the PCA-associated ear swelling (Figure 5.2c).

5.2.3. Topical $1\alpha_25$(OH)$_2$D$_3$ application reduced the extent of MC degranulation and the expression of various MC-derived mediators associated with PCA in vivo

This series of experiments aimed at investigating potential mechanisms responsible for the observed suppressive effects of topical $1\alpha_25$(OH)$_2$D$_3$ on IgE-mediated PCA-induced ear swelling. Because $1\alpha_25$(OH)$_2$D$_3$ has been reported to directly affect the number of keratinocytes (320), Langerhans cells (324) and $T_{reg}$ cells (316) in various inflammatory settings in vivo, its effect on cutaneous MC number in the ears following PCA was examined. Unlike other cell types mentioned, the number of MCs in IgE-preloaded or
medium-preloaded ears were not significantly altered following 1α,25(OH)2D3 treatment (Figure 5.3a). Notably, PCA reaction alone did not seem to affect MC numbers in the ears 6 h after the induction of the response, as there was no difference between IgE- and medium-preloaded ears in this regard. In contrast to total dermal MC number, the extent of MC degranulation (assessed by observing the integrity of MC granules under light microscopy as outlined by Singh et al. (325)) did increase dramatically due to PCA reaction, and this was observed in both 1α,25(OH)2D3-treated (mean: 9.2% versus 34.8%) and vehicle-treated (mean: 10.6% versus 52.3%) mice (Figure 5.3b). More importantly, PCA-affected ears, but not non-PCA-affected ears, demonstrated a significant reduction (mean: 34.8% versus 52.3%) in MC degranulation upon 1α,25(OH)2D3 treatment as compared to vehicle alone. This was illustrated in Figures 5.3 c – f, where intact (red arrow) as well as degranulated (green arrow) MCs were pointed out in differently-treated ears.

On the other hand, assessment on the expression of mRNA encoding various MC-derived immunological mediators revealed that mRNA expression for HDC, the enzyme that converts histidine to histamine (326), was unchanged within 30 min (Figures 5.4 a) but moderately reduced by 3 h (Figures 5.4 b) after the onset of PCA upon 1α,25(OH)2D3 treatment (grey bars). The non-PCA affected ears, on the other hand, displayed an increase in HDC expression upon 1α,25(OH)2D3 application (white bars). Furthermore, at 3 h post-DNP administration, vehicle-treated mice exhibited a one-fold increase in HDC expression in IgE-preloaded ears as compared to medium-preloaded control ears, whereas 1α,25(OH)2D3-treated mice showed no such difference between IgE and HMEM-Pipes preloaded ears. Please note that the expression of mRNA encoding pro-inflammatory
Figure 5.3. Topically applied 1α,25(OH)₂D₃ has no effect on dermal MC number but reduces the extent of MC degranulation following PCA response

Wild-type C57BL/6 mice were injected *i.d.* with 100 ng of α-DNP IgE (SPE-7 clone) in the right ear and equal volume of HMEM-Pipes in the left. Immediately following injection, both ears received topical application (20 µL/ear) of either 3 µM of 1α,25(OH)₂D₃ or vehicle. Sixteen hours later, all mice were injected *i.v.* retro-orbitally with 200 µg of DNP-HSA. (a) The number of dermal MCs in both ear pinnae at 6 h post-injection expressed as number of MCs per mm horizontal dermal length. (b) The percentage of MC degranulation in both ears of each mouse. (c – f) Histological illustration, following toluidine blue staining, of ears that had undergone different treatments. C*, cartilage. Red arrowheads indicate intact MCs, whereas green arrowheads indicate degranulated MCs. Bars: 100 µm. Data in (a) are from three independent experiments performed, each using three to four mice per treatment group. Data in (b) are from two independent experiments performed, each using three to four mice per treatment group. Data in (c – f) are representative of one of the two independent experiments summarised in (b), which showed similar results. *** p < 0.001
Figure 5.4. Topically applied $1\alpha,25$(OH)$_2$D$_3$ reduces HDC-encoding mRNA expression following PCA response

Wild-type C57BL/6 were injected i.d. with 100 ng of SPE-7 in the right ear and equal volume of HMEM-Pipes vehicle in the left, followed immediately by topical application of either 3 µM of $1\alpha,25$(OH)$_2$D$_3$ or vehicle on both ears. Sixteen hours later, all mice were injected retro-orbitally with 200 µg of DNP-HSA, and at either 30 min (a) or 3 h (b) post-injection, total RNA was extracted from both ear pinnae of each mouse and subjected to qRT-PCR analysis for the relative expression (to that of β-actin) of HDC-encoding mRNA. Data are from three independent experiments, each using three mice per treatment. qRT-PCR was performed in triplicates for each RNA sample. * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$
cytokines TNFα and IL-6, as well as the anti-inflammatory cytokine IL-10, was also measured (data not shown) but yielded variable results.

5.2.4. VDR expression is required for the suppressive effect of 1α,25(OH)₂D₃ on PCA-associated cutaneous pathology

In chapter 3, we demonstrated that the suppressive activity of 1α,25(OH)₂D₃ on IgE-mediated MC activation in vitro requires MC expression of VDRs. To investigate its relevance in the PCA model, we examined firstly the effect of topical 1α,25(OH)₂D₃ on PCA-associated ear swelling in VDR⁻/⁻ mice in parallel with WT controls. Similar to the previous result (Figure 5.2a), WT mice in this series of experiments exhibited significantly reduced ear swelling in response to 1α,25(OH)₂D₃ topical treatment during PCA (Figure 5.5a). Mice that lack functional VDRs, on the other hand, failed to respond to topical 1α,25(OH)₂D₃ treatment. However, it is worth noting that although vehicle-treated VDR⁻/⁻ mice have normal MC numbers in their ears (Figure 5.5b), they showed significantly reduced PCA-mediated ear swelling than their WT counterparts; the reason for which requires further investigation. As observed before, the total number of dermal MCs did not alter following PCA induction in either WT or VDR⁻/⁻ mice (Figure 5.5b), and although VDR⁻/⁻ mice appeared to exhibit more MCs in their medium-preloaded ears upon 1α,25(OH)₂D₃ application, this needs to be confirmed by future repeats of the experiment. Regarding the extent of MC degranulation, WT but not VDR⁻/⁻ mice showed a 1α,25(OH)₂D₃-mediated reduction (mean: 58.2% versus 51.0%) in their PCA-affected ears (Figures 5.5c). The extent of such reduction was only moderate, possibly due to fact that the analysis was done 3 h post-DNP injection, which was after the peak of the ear swelling response. Therefore, analysis at 30 min post-DNP injection was required to
Eight- to 12-week-old sex-matched WT and VDR$^{-/-}$ C57BL/6 mice were injected i.d. with 100 ng of SPE-7 clone in the right ear and equal volume of HMEM-Pipes in the left, followed immediately by topical application of either 3 µM of 1α,25(OH)$_2$D$_3$ or vehicle on both ears. Sixteen hours later, all mice received retro-orbital injection of 200 µg of DNP-HSA, and at indicated time points post-injection, the extent of ear swelling was recorded (a). At 3 h post-injection, the number (b) and percentage degranulation (c) of dermal MCs in both ears of each mouse were measured. (d) Total RNA was also extracted, at the same time point, from both ear pinnae of each mouse and subjected to qRT-PCR analysis for the relative expression (to that of β-actin) of HDC-encoding mRNA. Data in (a) are representative of three independent experiments, each with three mice per treatment. Data in (b and c) are from one of the three experiments summarised in (a). Data in (d) are from three independent experiments, each using three mice per treatment, and qRT-PCR was performed in triplicates for each RNA sample. * p < 0.05, ** p < 0.01, *** p < 0.001
confirm this observation. Although PCA reaction seemed to induce MC degranulation in both types of mice regardless of 1\(\alpha\),25(OH\(_2\))D\(_3\) or vehicle treatment, the extent of increase was lower in vehicle-treated VDR\(^{-/-}\) mice, which was consistent with the lower level of ear swelling in these mice as compared to WT controls (Figure 5.5a).

Analysis of the expression of HDC-encoding mRNAs at 3 h post-PCA induction, on the other hand, showed similar variations between the two treatment groups for WT mice (Figures 5.5d) as previously shown (Figures 5.4b), where 1\(\alpha\),25(OH\(_2\))D\(_3\) reduced HDC expression upon PCA induction. Such reduction, however, was not observed in IgE-preloaded ears in VDR\(^{-/-}\) mice (Figures 5.5d). It is worth noting that HDC expression in vehicle-treated and PCA-affected ears in VDR\(^{-/-}\) mice was lower than that in WT controls, which may be another explanation for the reduced ear swelling in these mice upon vehicle treatment and should be investigated further by including naive (i.e. non-treated) VDR\(^{-/-}\) mice in future experiments. Nevertheless, it seemed so far that the suppressive effect of 1\(\alpha\),25(OH\(_2\))D\(_3\) on PCA-associated ear swelling, the extent of MC degranulation, and gene expression for MC-derived HDC did require functional VDRs in the animals.

5.2.5. The PCA-suppressing effect of topical 1\(\alpha\),25(OH\(_2\))D\(_3\) treatment requires specifically VDR expression by cutaneous MCs

To further establish if the VDRs specifically expressed by MCs are essential for topical 1\(\alpha\),25(OH\(_2\))D\(_3\) to suppress PCA, Kit\(^{W-sh/W-sh}\) mice and WT/VDR\(^{-/-}\) mBMCMC knock-in mice were employed with the previously stated rationale (Section 1.10). As shown in Figures 5.6a and c, WT mice again exhibited 1\(\alpha\),25(OH\(_2\))D\(_3\)-mediated reduction of overall ear swelling following PCA induction. Kit\(^{W-sh/W-sh}\) mice, due to the absence of MCs in the ears, did not exhibit swelling in either ear (vehicle or 1\(\alpha\),25(OH\(_2\))D\(_3\) treatment)
Figure 5.6. Mast cell VDR-dependence for the effect of topically applied 1α,25(OH)2D3 on PCA-associated ear swelling and cutaneous pathology

Wild-type (Kit+/+) mice (a and c), MC-deficient (KitW-sh/W-sh) mice (a and d), and MC-deficient mice that had been engrafted i.d. with either WT MCs (WT mBMCMCs → KitW-sh/W-sh; b and e) or VDR−/− MCs (VDR−/− mBMCMCs → KitW-sh/W-sh; b and f) were injected i.d. with 100 ng of SPE-7 in the right ear and equal volume of HMEM-Pipes in the left, followed immediately by topical application of either 3 µM of 1α,25(OH)2D3 or vehicle on both ears. Sixteen hours later, all mice were injected retro-orbitally with 200 µg of DNP-HSA, and at indicated time point post-injection the amount of ear swelling was recorded (a and b). In one experiment, cross sections of ears were obtained 30 min after DNP administration and subsequently stained with TB (c–f). C*, cartilage. Red arrowheads indicate MCs. Bars: 200 µm (b–e); 1,000 µm (bottom right insets in c–f); 50 µm (top right insets in c and e). Data in (a and b) are from four independent experiments performed, each with three to four mice analysed per group. Images in (c–f) were obtained from one experiment, and are representative of all mice (3–4 mice per group) with the corresponding treatment. ** p < 0.01
**a**

- **WT BMCMC** → **Kit**
  - **W-sh/W-sh - Vehicle**
  - **W-sh/W-sh - 1α,25(OH)2D3**

- **VDR-/BMCMC** → **Kit**
  - **W-sh/W-sh - Vehicle**
  - **W-sh/W-sh - 1α,25(OH)2D3**

**b**

- **WT BMCMC** → **Kit**
  - **+/+ - Vehicle**
  - **+/+ - 1α,25(OH)2D3**

- **VDR-/BMCMC** → **Kit**
  - **W-sh/W-sh - Vehicle**
  - **W-sh/W-sh - 1α,25(OH)2D3**

**c**

- **Vehicle**
- **1α,25(OH)2D3** (3 μM)

**d**

- **Kit**
- **KIH**
- **Kit**
- **KIH-WW-sh**
- **WT mBMCMCs → KIH-WW-sh**
- **VDR+/mBMCMCs → KIH-WW-sh**

**e**

- **Ear swelling** + SEM (mm; n = 6)

**f**

- **Ear swelling** + SEM (mm; n = 6)
(Figures 5.6 a and d). Importantly, repair of MC-deficiency by MC engraftment in the ears of Kit\(^{W-sh/W-sh}\) mice (i.e. WT mBMCMC knock-in mice) not only reinstated their ability to respond to PCA induction but also enabled, like the WT controls, a significant reduction in ear swelling upon 1\(\alpha\),25(OH)\(_2\)D\(_3\) treatment (Figures 5.6 b and e). By contrast, loss of functional MC VDRs in the VDR\(^{-/-}\) mBMCMC knock-in group of mice, ablated the negative regulatory ability of 1\(\alpha\),25(OH)\(_2\)D\(_3\) to reduce the IgE-PCA reactions (Figures 5.6 b and f), thereby demonstrating the suppressive effect of 1\(\alpha\),25(OH)\(_2\)D\(_3\) on PCA is MC VDR-dependent.

Confirming our previous finding (Figure 5.3a), MC number in both ears of WT mice was unaltered by 1\(\alpha\),25(OH)\(_2\)D\(_3\) treatment throughout PCA reaction (Figures 5.7 a and b). Whilst the WT mBMCMC-engrafted mice showed increased numbers of MC in PCA-affected ears at certain stage of the inflammatory response, the numbers of MC in their VDR\(^{-/-}\) mBMCMC-engrafted counterparts were decreased (Figures 5.7 a and b).

However, since similar observations were made in non-PCA-affected ears of these mice, these differences most likely reflected the effectiveness of the initial engraftment in each individual mouse rather than the effect of 1\(\alpha\),25(OH)\(_2\)D\(_3\). Therefore, future repetitions of the experiment are required before a definitely conclusion can be drawn in this regard. As expected, Kit\(^{W-sh/W-sh}\) mice lacked MCs in both ears that received either 1\(\alpha\),25(OH)\(_2\)D\(_3\) or vehicle application. The extent of MC degranulation, on the other hand, was reduced in 1\(\alpha\),25(OH)\(_2\)D\(_3\)-treated, PCA-affected ears of WT (mean at 30 min: 39.0% versus 56.9%; mean at 6 h: 34.8% versus 52.3%) and WT mBMCMC knock-in (mean at 30 min: 57.6% versus 70.7%; mean at 6 h: 52.7% versus 73.1%) mice, but was unchanged for VDR\(^{-/-}\) MC knock-in (mean at 30 min: 68.5% versus 71.5%; mean at 6 h: 66.1% versus 64.2%) mice.
Figure 5.7. Topical 1α,25(OH)₂D₃ has minimal effect on dermal MC number but reduces moderately the extent of MC degranulation in MC-engrafted KitW-sh/W-sh mice following PCA response

Wild-type (Kit⁺/⁺) mice, MC-deficient (KitW-sh/W-sh) mice, and MC-deficient mice that had been engrafted i.d. with either WT MCs (WT mBMCMCs → KitW-sh/W-sh) or VDR⁻/- MCs (VDR⁻/- mBMCMCs → KitW-sh/W-sh) were injected i.d. with 100 ng of SPE-7 in the right ear and equal volume of HMEM-Pipes in the left, followed immediately by topical application of either 3 µM of 1α,25(OH)₂D₃ or vehicle on both ears. Sixteen hours later, all mice were injected retro-orbitally with 200 µg of DNP-HSA, and at either 30 min (a and c) or 6 h (b and d) post-injection, the total number of dermal MCs (a and b) as well as the percentage of MC degranulation (c and d) in both ear pinnae were recorded. Data are representative of one of three experiments (each using three to four mice per treatment group) that showed similar ear swelling responses. * p < 0.05, ** p < 0.01, *** p < 0.001
The extent of MC degranulation in non-PCA-affected ears of all mice, however, remained constant upon $1\alpha,25(OH)_2D_3$ application. It is worth noting that although both types of MC knock-in mice appeared to have considerably lower numbers of MCs in both ears than the WT controls (Figures 5.7 a and b), they both showed higher levels of MC degranulation regardless of vehicle or $1\alpha,25(OH)_2D_3$ treatment (Figures 5.7 c and d). This could be a possible explanation for the observed higher PCA responses in these MC knock-in animals (Figures 5.6 a and b).

Analysis on mRNA expression for HDC in all four types of mice indicated similar results for WT animals as previously observed, where it was reduced upon $1\alpha,25(OH)_2D_3$ treatment (Figure 5.8). No significant difference, however, was observed between vehicle- and $1\alpha,25(OH)_2D_3$-treated ears in the other groups of mice. Moreover, HDC seemed to be expressed at similar levels in $Kit^{W-sh/W-sh}$ mice and both groups of MC knock-in mice, indicating the engraftment of MCs did not alter HDC expression. This in turns suggested that the differences in its expression between WT and $Kit^{W-sh/W-sh}$ mice were unlikely to be MC-dependent, either.

Since the gene expression analyses did not yield consistent result, the protein secretion of the proinflammatory cytokines TNF and IL-6, as well as that of the anti-inflammatory IL-10, was measured. According to Figures 5.9 a, c and e, topical $1\alpha,25(OH)_2D_3$ application did not have immediate effect on the secretion of any cytokine examined upon PCA induction in all four groups of mice. Interestingly, $Kit^{W-sh/W-sh}$ mice, which did not exhibit PCA-induced ear swelling responses, showed higher “cytokine/total protein” ratios than all three PCA-affected mouse types at this time point. This could be a reflection on the increased overall protein levels in the latter three mouse groups.
**Figure 5.8.** MC VDR expression is not required for topically applied 1α,25(OH)2D3 to reduce HDC-encoding mRNA expression following PCA induction

Wild-type (Kit+/+) mice, MC-deficient (KitW-sh/W-sh) mice, and MC-deficient mice that had been engrafted i.d. with either WT MCs (WT mBMCMCs → KitW-sh/W-sh) or VDR−/− MCs (VDR−/− mBMCMCs → KitW-sh/W-sh) were injected i.d. with 100 ng of SPE-7 in the right ear and equal volume of HMEM-Pipes in the left, followed immediately by topical application of either 3 µM of 1α,25(OH)2D3 or vehicle on both ears. Sixteen hours later, all mice were injected retro-orbitally with 200 µg of DNP-HSA, and at 3 h post-injection, total RNA was extracted from both ear pinnae of each mouse and subjected to qRT-PCR analysis for the relative expression (to that of β-actin) of HDC-encoding mRNA. Data are from three independent experiments, each with three mice analysed per treatment. qRT-PCR was performed in triplicates for each RNA sample.

* p < 0.05, *** p < 0.001
Figure 5.9. MC VDR is required, at least partially, for topically applied 1α,25(OH)2D3 to suppress cytokine secretion in PCA-affected ears

Wild-type (Kit+/+) mice, MC-deficient (KitW-sh/W-sh) mice, and MC-deficient mice that had been engrafted i.d. with either WT MCs (WT mBMCMCs → KitW-sh/W-sh) or VDR-/− MCs (VDR-/− mBMCMCs → KitW-sh/W-sh) were injected i.d. with 100 ng of SPE-7 in the right ear and equal volume of HMEM-Pipes in the left, followed immediately by topical application of either 3 µM of 1α,25(OH)2D3 or vehicle on both ears. Sixteen hours later, all mice were injected retro-orbitally with 200 µg of DNP-HSA. At either 30 min (a, c, e) or 6 h (b, d, f) post-injection, the levels of TNF (a and b), IL-6 (c and d), and IL-10 (e and f) in lysates of both ears were measured. Data were obtained from one experiment using three mice per treatment. * p < 0.05, *** p < 0.001
immediately following PCA induction, due to the secretion of other types of inflammatory mediators. At 6 h post-DNP induction, on the other hand, vehicle-treated WT mice showed the highest secretion level of all cytokines, which was significantly reduced upon 1α,25(OH)₂D₃ treatment (Figures 5.9 b, d, f). Importantly, WT but not VDR<sup>−/−</sup> mBMCMC knock-in mice, demonstrated similar 1α,25(OH)₂D₃-mediated suppression of cytokine secretion as the WT controls. Therefore, although MCs are unlikely the sole contributor to PCA-associated cytokine production, these cells, through their VDR expression, do appear to play a role in 1α,25(OH)₂D₃-mediated overall cytokine reduction. However, due to the relatively moderate (even though statistically significant) reduction in the production of measured cytokines following 1α,25(OH)₂D₃ treatment of both groups of knock-in mice, other more immediately-released MC-derived mediators such as histamine and leukotrienes need to be measured in subsequent studies. In addition to ear lysates, serum sample from all animals was also analysed for cytokine levels, so as to determine whether the effect of 1α,25(OH)₂D₃ on cytokine secretion was localised or systemic. Since the level of all three cytokines examined in all serum samples was below the detection limit of the ELISA kits (data not shown), it was unlikely that topical 1α,25(OH)₂D₃ would cause any systemic change in cytokine production.

5.2.6. Topical 25OHD₃ can suppress PCA-associated ear swelling via similar mechanisms to 1α,25(OH)₂D₃

It was demonstrated in Chapter 4 that 25OHD₃, depending on its concentration and the length of treatment, could also elicit immunosuppressive effects on IgE + sAg-activated MCs. It was, therefore, important to investigate the relevance of these findings in the
PCA model *in vivo*. In order to optimise concentration of 25OHD$_3$ for further study, a
dose response experiment was performed similar to Figure 5.2a for 1α,25(OH)$_2$D$_3$
optimisation, where various concentrations of 25OHD$_3$ was applied immediately after
IgE injection. However, unlike 1α,25(OH)$_2$D$_3$, no reduction in the swelling of IgE-
preloaded ears was observed in all 25OHD$_3$-treated groups (Figure 5.10a). Given that
MCs required a longer exposure time to 25OHD$_3$ than 1α,25(OH)$_2$D$_3$ before IgE preload
to reduce mediator release *in vitro* (Figures 4.6 and 4.7), subsequent *in vivo* experiments
were designed to provide a longer exposure time (8 h) of the ears to 25OHD$_3$ prior to IgE
injection and sensitization of the dermal MCs. Allowing more time for 25OHD$_3$ to
penetrate the ears and be converted to the active metabolite 1α,25(OH)$_2$D$_3$, enabled the
highest dose (3 µM) of topical 25OHD$_3$ to significantly impair PCA-associated ear
swelling compared to vehicle treatment (Figure 5.10b). Although the differences did not
reach statistical significance, the two lower concentrations of 25OHD$_3$ also managed to
reduce the swelling of PCA-affected ears to certain extents. As expected, all HMEM-
Pipes-preloaded ears in both short and long treatment settings showed only minimum
levels of swelling (Figures 5.10 a and b). Based on these results, longer treatment with 3
µM of topical 25OHD$_3$/vehicle was used in all subsequent experiments. Furthermore, like
topical 1α,25(OH)$_2$D$_3$, topical 25OHD$_3$ also had to be applied at the site of PCA to
suppress ear swelling, as mice that received either 25OHD$_3$ or vehicle treatment on the
different ear as to IgE preload, exhibited similar levels of swelling with both ears (Figure
5.10c). Also similar to topical 1α,25(OH)$_2$D$_3$, 25OHD$_3$-mediated suppression of ear
swelling was not restricted to the C57BL/6 background, as it gave similar results in WT
WBB6F1 mice (Figure 5.10d).
Figure 5.10. Optimisation of the *in vivo* PCA model to study the effect of topically applied 25OHD$_3$

Wild-type C57BL/6 (a – c) and WBB6F1-Kit$^{+/+}$ (e) mice were used in this series of experiments. (a) Mice were injected *i.d.* with 100 ng of α-DNP IgE (SPE-7 clone) in the right ear and equal volume of HMEM-Pipes in the left. Immediately following injection, both ears received topical application (20 µL/ear) of either 25OHD$_3$ dilution at the indicated concentration or vehicle alone. (b – d) Mice were topically painted, on both ears, with either 25OHD$_3$ dilution at the indicated concentration or vehicle 8 h prior to *i.d.* injection of 100 ng of SPE-7 in the right ear and equal volume of HMEM-Pipes in the left. In all cases, mice were injected retro-orbitally, 16 h later, with 200 µg of DNP-HSA (100 µL of 2 mg/mL dilution), and the extent of ear swelling was recorded at indicated time point post-injection. Data in (a, c and d) are representative of one experiment with the indicated number of mice per treatment. Data in (b) are representative of three independent experiments, each using three to four mice per treatment. * p < 0.05, *** p < 0.001
Subsequent experiments were performed to determine whether the potential mechanisms responsible for 1α,25(OH)2D3-mediated PCA suppression could also apply to topical 25OHD3. Indeed, topical 25OHD3 treatment did not alter the number of dermal MCs in either ear of WT mice (Figure 5.11a), but instead reduced the extent of MC degranulation in PCA-affected ears (Figure 5.11b). Analysis on the gene expression of MC-derived HDC also showed almost identical results as found with topical 1α,25(OH)2D3 (Figure 5.4), where 25OHD3 application significantly reduced the HDC expression (Figures 5.12).

5.2.7. The suppressive effect of topical 25OHD3 on PCA-associated cutaneous pathology also requires MC expression of VDR

Due to the similarity between the ability of topical 1α,25(OH)2D3 and 25OHD3 to suppress PCA-induced ear swelling in WT mice and potential mechanisms responsible, we decided to determine if MC VDR expression is required for the effects of 25OHD3. Utilising MC-deficient c-kit mutant mice and WT/VDR−/− mBMCMC knock-in mice in addition to WT controls, we demonstrated that topical 25OHD3 indeed required dermal MCs and functional VDR expression in these cells to reduce swelling of PCA-affected ears, as WT, but not VDR−/−, BMCMC knock-in mice responded to 25OHD3 treatment in the same way as WT controls (Figures 5.13).

Subsequent investigation showed no change in total MC number in either ear of all groups of mice upon 25OHD3 application (Figure 5.14a). Like 1α,25(OH)2D3, 25OHD3-treated and PCA-affected ears showed reduced extent of MC degranulation in WT (mean: 37.4% versus 52.4%) and WT MC knock-in (mean: 53.9% versus 68.2%) mice but not VDR−/− MC knock-in (mean: 63.4% versus 60.8%) mice (Figure 5.14b). The higher-than-
Figure 5.11. Topically applied 25OHD₃ has little effect on dermal MC number but reduces the extent of MC degranulation following PCA response

Wild-type C57BL/6 mice were topically painted, on both ears, with either 3 μM of 25OHD₃ or vehicle 8 h prior to i.d. injection of 100 ng of α-DNP IgE (SPE-7 clone) in the right ear and equal volume of HMEM-Pipes in the left. Sixteen hours later, all mice received retro-orbital injection of 200 μg of DNP-HSA. (a) The total number of dermal MCs in both ear pinnae at 6 h post-injection were recorded. (b) At the same time point, the percentage of MC degranulation in both ears of each mouse was calculated. Data are from three independent experiments performed, each using three to four mice per treatment group. *** p < 0.001
Wild-type C57BL/6 mice received, on both ears, topical application of either 3 µM of 
25OHD₃ or vehicle 8 h prior to i.d. injection of 100 ng of SPE-7 in the right ear and 
equal volume of HMEM-Pipes in the left. Sixteen hours later, all mice were injected 
retro-orbitally with 200 µg of DNP-HSA, and at either 30 min (a) or 3 h (b) post-
injection, total RNA was extracted from both ear pinnae of each mouse and subjected 
to qRT-PCR analysis for the relative expression (to that of β-actin) of mRNA 
encoding HDC. Data are from three independent experiments, each using three mice 
per treatment. qRT-PCR was performed in triplicates for each RNA sample. * p < 0.05, ** p < 0.01, *** p < 0.001
Figure 5.13. MC VDR expression is required for topically applied 25OHD₃ to reduce PCA-associated ear swelling
(a) Wild-type (Kit⁺/⁺) mice, MC-deficient (Kit⁺/-/-) mice, and (b) MC-deficient mice that had been engrafted i.d. with either WT MCs (WT mBMCMCs → Kit⁺/-/-) or VDR⁺ MCs (VDR⁺ mBMCMCs → Kit⁺/-/-) were topically administered, on both ears, with either 3 µM of 25OHD₃ or vehicle 8 h prior to i.d. injection of 100 ng of SPE-7 in the right ear and equal volume of HMEM-Pipes in the left. Sixteen hours later, all mice were injected retro-orbitally with 200 µg of DNP-HSA, and at indicated time point post-injection the amount of ear swelling was recorded. Data are from three independent experiments performed, each with three to four mice analysed per group.
* p < 0.05, ** p < 0.01
Figure 5.14. MC VDR expression does not convey topically applied 25OHD$_3$ the ability to change dermal MC number but is required for this metabolite to reduce the extent of MC degranulation following PCA induction

Wild-type (Kit$^{+/+}$) mice, MC-deficient (Kit$^{W-sh/W-sh}$) mice, and MC-deficient mice that had been engrafted i.d. with either WT MCs (WT mBMCMCs $\rightarrow$ Kit$^{W-sh/W-sh}$) or VDR$^{-/-}$ MCs (VDR$^{-/-}$ mBMCMCs $\rightarrow$ Kit$^{W-sh/W-sh}$) were topically painted, on both ears, with either 3 µM of 25OHD$_3$ or vehicle 8 h prior to i.d. injection of 100 ng of SPE-7 in the right ear and equal volume of HMEM-Pipes in the left. Sixteen hours later, all mice were injected retro-orbitally with 200 µg of DNP-HSA. (a) The total number of dermal MCs in both ear pinnae was recorded at 6 h post-injection. (b) At the same time point, the percentage of MC degranulation in both ears of each mouse was also calculated. Data are from two independent experiments, each using two to four mice per treatment group. * p < 0.05, ** p < 0.01, *** p < 0.001
WT percentages of degranulated MCs in MC knock-in mice as observed previously (Figures 5.7 c and d) were confirmed here, providing consistency with the stronger ear swelling responses in these animals upon PCA induction (Figure 5.13). Cytokine secretion analysis at 6 h post-DNP administration showed reduction in the level of proinflammatory cytokines, TNFα and IL-6, in WT and WT MC knock-in mice with 1α,25(OH)2D3 treatment (Figures 5.15 a and b). The level of IL-10, however, remained unchanged in all groups of mice (Figure 5.15c). Interestingly, the secretion of all three cytokines increased substantially upon 25OHD3 treatment in the non-PCA-affected ears of MC deficient mice, the explanation for which required further investigation. Taken together, our in vivo data demonstrates that MC VDR expression is required for optimal 25OHD3 suppression of PCA responses. However, more experiments are needed to establish with certainty if 25OHD3 reduces PCA-induced cytokine production in vivo.

5.3. Discussion

By using MC-deficient KitW/W-v mice and KitW-W-v mice that had been selectively engrafted with mBMCMCs into ear pinnae, Wershil et al. (89) showed that the PCA model employed for the current project is an IgE- as well as MC-dependent immediate hypersensitivity model. Our data confirmed the findings of Wershil et al., where a different MC-deficient mouse strain, KitW-sh/W-sh, showed no sign of PCA-mediated ear swelling, whereas local engraftment of WT or VDR-/- mBMCMCs to these mice restored their ability to respond to PCA induction (Figures 5.6 a and b; 5.13). In fact, it was noticeable that both types of MC knock-in mice showed considerably stronger PCA response, indicated by the higher levels of ear swelling, than the WT controls. There are
Figure 5.15. MC VDR-dependence for the effect of topical 25OHD3 on cytokine secretion in PCA-affected ears

Wild-type (Kit+/+) mice, MC-deficient (Kit\textsuperscript{W-sh/W-sh}) mice, and MC-deficient mice that had been engrafted \textit{i.d.} with either WT MCs (WT mBMCMCs $\rightarrow$ Kit\textsuperscript{W-sh/W-sh}) or VDR\textsuperscript{-/-} MCs (VDR\textsuperscript{-/-} mBMCMCs $\rightarrow$ Kit\textsuperscript{W-sh/W-sh}) were topically painted, on both ears, with either 3 µM of 25OHD\textsubscript{3} or vehicle 8 h prior to \textit{i.d.} injection of 100 ng of SPE-7 in the right ear and equal volume of HMEM-Pipes in the left. Sixteen hours later, all mice were injected retro-orbitally with 200 µg of DNP-HSA, and at 6 h post-injection the levels of TNF (a), IL-6 (b), and IL-10 (c) in lysates of both ears were measured. Data were obtained from one experiment (one of three experiments that showed similar ear swelling responses) using three mice per treatment. * $p < 0.05$, ** $p < 0.01$
several possible explanations for such observation. Firstly, as mentioned before, the higher extent of MC degranulation (Figures 5.7 c and d; 5.14b) and, consequently, the release of more pre-stored MC-derived mediators such as histamine in MC knock-in mice could contribute to more severe vasodilation and higher vascular permeability with oedema. Secondly, although total number of dermal MCs in MC knock-in mice appeared lower than WT controls, this could simply be due to the increased MC degranulation in these animals which would render some (i.e. completely degranulated) MCs unable to be detected by toluidine blue staining. (327) In other words, MC knock-in mice could in fact have more dermal MCs which, in combination with higher degranulation percentage, represent an even larger supply of histamine and other mediators that could exacerbate ear swelling. Furthermore, the more profound PCA response exhibited by MC knock-in mice could also be due to certain unknown non-MC-related intrinsic defects that these animals carry.

Despite the discovered ability of topical 1α,25(OH)2D3 to improve inflammatory conditions such as psoriasis, vitiligo and CHS (302, 316-319), its in vivo effects on IgE-mediated allergic responses, especially those that are mediated via MCs, have not been directly examined to date. Using the IgE + sAg-mediated PCA model, our data demonstrates for the first time that topical 1α,25(OH)2D3 can suppress PCA-associated ear swelling, and such effect is observed in WT mice of both C57BL/6 and WBB6F1 backgrounds (Figures 5.2 a and c). Utilising MC-deficient and MC knock-in mice, the MC-dependence for such PCA-dampening effect of 1α,25(OH)2D3 was also established (Figure 5.6). Subsequent mechanistic investigation revealed several possible explanations for the observed 1α,25(OH)2D3-mediated PCA suppression. Firstly, topical
1α,25(OH)₂D₃ treatment resulted in a lower percentage of MC degranulation in PCA-affected ears (Figure 5.3), reducing in turn the release of histamine and other pre-stored pro-inflammatory cytokines and ultimately easing the ear swelling response. This notion was supported by the finding that the expression of HDC is significantly decreased in PCA-affected ears topically treated with 1α,25(OH)₂D₃ (Figures 5.4). HDC is the enzyme that generates histamine from histidine (326), therefore, a reduction in its gene expression could indicate lower-level MC degranulation and a lesser need for additional histamine to be produced during MC regranulation (i.e. re-generation of MC granules) (328). In addition to HDC, topical 1α,25(OH)₂D₃ also reduced the protein content of pro-inflammatory cytokine, TNFα and IL-6, in mouse ears following PCA induction (5.9 b and d), thus helping further improve subsequent ear swelling response by, for instance, limiting the recruitment of other inflammatory cells. (64) Indeed, TNFα is a potent chemotactic factor for neutrophils, which counted for over 90% of infiltrating leukocytes, as shown in a similar PCA model previously. (89) Unexpectedly, the protein secretion of the anti-inflammatory cytokine, IL-10, in PCA-affected ears also seemed to decrease in response to 1α,25(OH)₂D₃ treatment (Figures 5.9 f), which indicated a decrease in MC-derived mediators, perhaps due to suppressed MC activation in general. Because of the majority of these mediators being pro-inflammatory in nature, a decrease in their levels could result in a net reduction of the overall inflammatory response. Furthermore, it should be pointed out that MCs are not the only sources of the cytokines examined in this chapter. For example, TNFα and IL-6 can be produced and secreted by newly recruited and/or resident neutrophils and DCs, respectively; whereas Treg cells are well-established sources of IL-10. Keratinocytes can also produce all three types of cytokines. (329-331)
Therefore, further investigation is required to examine the effects of topical $1\alpha,25$(OH)$_2$D$_3$ on leukocyte recruitment and the associated change in cytokine profile in PCA-affected ear pinnae, as well as its effects on structural cell types such as keratinocytes and fibroblasts.

In our *in vitro* study (Chapter 3), the immunosuppressive effects of $1\alpha,25$(OH)$_2$D$_3$ on IgE + sAg-activated MCs are dependent on the expression of functional VDR, and that VDR may play a role in non-genomic as well as genomic VitD$_3$ pathways. The initial use of $VDR^{-/-}$ mice in parallel to WT controls, which was to determine the VDR-dependence for the effects of topical $1\alpha,25$(OH)$_2$D$_3$ on PCR-associated ear swelling *in vivo*, yielded promising results as $VDR^{-/-}$ but not WT animals failed to show suppressed ear swelling upon $1\alpha,25$(OH)$_2$D$_3$ application as compared to vehicle (Figure 5.5a). The extent of MC degranulation and the gene expression for MC-derived HDC in these mice following PCA induction were also unchanged by topical $1\alpha,25$(OH)$_2$D$_3$ treatment (Figures 5.5 c and d). However, the substantially lower-than-WT ear swelling response, percentage MC degranulation, and the gene expression of HDC, in $VDR^{-/-}$ mice when topically applied with vehicle alone indicated either a lower baseline PCA response in these animals or a higher susceptibility of these animals to the vehicle contents. Future studies are, therefore, required to explain these observations. Because $VDR^{-/-}$ mice have a global deficiency of VDR, to establish the specific contribution of MC VDR, we performed the critical experiments of adoptively transferring MCs that either have (WT) or are deficient in ($VDR^{-/-}$) VDR function into MC deficient animals. Importantly, by comparing the PCA reactions of WT and $VDR^{-/-}$ mBMCMC knock-in mice with and without topical $1\alpha,25$(OH)$_2$D$_3$ treatment, we also established that VDR expression in dermal MCs is
essential for the 1α,25(OH)2D3-mediated PCA suppression in vivo (Figure 5.6). Furthermore, MC VDR expression is also required for topical 1α,25(OH)2D3 to reduce the extent of MC degranulation and the production of TNF, IL-6 and IL-10 upon PCA induction in vivo (Figures 5.7 e and f; 5.9 b, d, f). Since the observed reduction in cytokine production is relatively subtle, other MC-derived mediators such as histamine and leukotrienes will need to be assessed in subsequent studies. Nevertheless, observations made in the current study strengthened the possibility that topical 1α,25(OH)2D3 suppressed PCA-associated ear swelling at least partially via down-regulating the degranulation and overall cytokine secretion of dermal MCs. Intriguingly, the ability of topical 1α,25(OH)2D3 to VDR-dependently reduce the production of IL-10 as well as TNFα and IL-6 is similar to the effects of curcumin on activated mBMCMCs in vitro (Figure 3.11). This suggests that the non-genomic effects of 1α,25(OH)2D3 played a role in PCA suppression, and that VDRs are also important for these effect in vivo. However, further investigation utilising pathway-specific 1α,25(OH)2D3 agonists and/or antagonists, is required before definite conclusions can be drawn.

One of the most novel findings of the current project is the susceptibility of activated MCs to be negatively regulated by not only 1α,25(OH)2D3 but also its inactive precursor, 25OHD3, most likely via the production of endogenous 1α,25(OH)2D3 (Chapter 4). The in vivo relevance of such finding was confirmed in the current chapter, where topical application of 25OHD3 demonstrated very similar PCA-suppressive effects, via similar potential mechanisms, to 1α,25(OH)2D3 in a MC VDR-dependent manner (Figures 5.10 – 5.15). Again, these effects were unlikely to be direct but rather indirect via the production of endogenous 1α,25(OH)2D3 by dermal MCs, which was indicated by the
requirement of topical 25OHD₃ treatment for an extended period of time prior to IgE preload (Figure 5.10). To confirm this theory, CYP27B1⁻/⁻ mice (332) and CYP27B1⁻/⁻ mBMCMC-engrafted Kitₖ₃²/Wₖ₃² mice can be used to compare with WT and WT MC knock-in responses in the current topical 1α,25(OH)₂D₃/vehicle-incorporated PCA model. Furthermore, unlike the in vitro scenario, cell types other than MCs are also likely to respond to topical 25OHD₃ in vivo. Keratinocytes, in particular, can synthesise 1α,25(OH)₂D₃ from 25OHD₃ and therefore represent an alternative and yet perhaps more abundant source of 1α,25(OH)₂D₃ to regulate dermal MCs and PCA-associated ear swelling subsequently. (203) In this regard, the significance of MC-derived 1α,25(OH)₂D₃ can be determined by comparing the responses of WT and CYP27B1⁻/⁻ MC knock-in mice towards topical 25OHD₃ treatment followed by PCA induction. Nevertheless, the ability of MCs to respond to 25OHD₃ and to potentially generate 1α,25(OH)₂D₃ autonomously in vivo as well as in vitro, highlights the potential for serum 25OHD₃ (which is the major form of circulating VitD₃) to dampen MC over-activity throughout the body during allergic and/or other immune settings.

In conclusion, in this chapter we demonstrated that topical 1α,25(OH)₂D₃ and 25OHD₃ could suppress PCA-mediated cutaneous pathology in a MC VDR-dependent manner, although in the latter case the effects may not be direct but rather via the production of the active analogue by MCs. Potential mechanisms responsible include a reduction in PCA-induced MC-degranulation and the secretion of MC-derived TNF, IL-6 and IL-10 upon topical application of VitD₃ analogues. Although the involvement of other newly recruited as well as resident cell types requires further investigation, our findings so far nonetheless provide another possible explanation for the beneficial effects of UVB
irradiation in a number of allergic and non-allergic diseases of the skin, which is via the modulation of dermal MC activation to elevate serum 25OHD$_3$ levels and cutaneous 1α,25(OH)$_2$D$_3$ production.
CHAPTER 6
FROM THE MOUSE TO THE HUMAN – TRANSLATIONAL ASPECTS
6.1. Introduction

As mentioned previously, Vitamin D deficiency has been associated with epidemiologic patterns observed in the asthma epidemic, which is more common with obesity, African American ethnicity, and westernization of countries with higher-risk populations for asthma. In Italian children, low serum levels of 25OHD$_3$ are associated with reduced asthma control, as assessed according to the Global Initiative for Asthma guidelines and the Childhood Asthma Control Test.\(^\text{(333)}\) Other reports show a beneficial association between higher maternal VitD intake during pregnancy and protection from childhood asthma and allergic diseases in the offspring.\(^\text{(334-336)}\) As Hansdottir et al.\(^\text{(337)}\) demonstrated, primary lung epithelial cells express high baseline levels of CYP27B1 and low levels of CYP24A1, thus allowing these cells to constitutively convert serum 25OHD$_3$ to the active 1\(\alpha\),25(OH)$_2$D$_3$. 1\(\alpha\),25(OH)$_2$D$_3$ can, in turn, inhibit the proliferation of normal and asthmatic airway smooth muscle cells by inhibiting their progression to $S$ phase of the cell cycle\(^\text{(338)}\). By reducing the production of RANTES\(^\text{(339)}\), MMP-9 and a disintegrin and metalloprotease 33\(^\text{(340)}\) from airway smooth muscle cells, 1\(\alpha\),25(OH)$_2$D$_3$ can also restrain the recruitment of other leukocytes to the asthmatic lungs and ease the symptoms during asthmatic airway remodelling, respectively. Furthermore, 1\(\alpha\),25(OH)$_2$D$_3$ can increase the synthesis of IL-10 by $T_{\text{reg}}$ and DCs\(^\text{(222, 226)}\), while concurrently suppressing DC activation by down-regulating expression of costimulatory molecules CD40 and CD80/86\(^\text{(222)}\). However, it remains to be investigated if VitD$_3$ can negatively regulate human MC activity during allergic settings.

It has been known for decades that human MCs are key effector cells of allergic inflammation.\(^\text{(64)}\) In particular, they contribute to the early-phase of type I
hypersensitivity reactions by releasing histamine, LTC₄ and other mediators following
cross-linking of surface-bound IgE by allergen in sensitized individuals. (341-343) Human
MCs are also found, during late-phase allergic responses, to induce the recruitment of
neutrophils by secreting IL-8 and TNF (344, 345), induce the recruitment as well as local
activation of eosinophils by secreting IL-5 (346), and regulate T cell functions by secreting
PGD₂ (347). Furthermore, research in the past 10 years or so has re-established MCs as
cells that not only regulate allergy, but also many other physiological/biological functions,
such as blood flow and coagulation, smooth muscle contraction and peristalsis of the
intestine, mucosal secretion, wound healing, modulation of innate and adaptive immunity,
and peripheral tolerance. (8, 348, 349) Due to the difficulty in obtaining adequate numbers as
well as quality of human MC for in vitro studies, a large proportion of the available data
on MC biology and function is generated using murine primary and/or cultured MCs; an
approach that is coupled with the further advantage of the availability of multiple animal
models (e.g. transgene, knockout and adoptive transfer models) that allow the study of
MC function in complex in vivo settings, as well as the ability to access over-expression
and/or deletion of MC-derived mediators in MCs generated from gene targeted mice in
vitro. In this regard, there are many similarities between the roles of MCs in human
asthma and murine asthma models, including the ability of murine MCs to trigger early-
phase responses by releasing histamine and leukotrienes, and to contribute to late-phase
responses by recruiting other leukocytes such as neutrophils, eosinophils and T cells. (350,
351) Therefore, it will be of great clinical importance to investigate if our previous findings
in the mouse can be translated into human studies.
In this chapter, therefore, human cord blood-derived MCs were generated and used to
determine the effect of 1α,25(OH)2D3 during IgE + α-IgE-mediated activation and,
subsequently, examine CYP27B1 expression and their ability to produce 1α,25(OH)2D3
dergenously upon 25OHD3 treatment.

6.2. Results

6.2.1. 1α,25(OH)2D3 reduces IgE + α-IgE-mediated TNFα but not IL-10 production by
hCBMCs

10- to 13-week-old hCBMCs, the identity and purity of which was confirmed by their
morphological characteristics (Figure 6.1a) and intracellular tryptase expression
(Figures 6.1 b – d), were used in this series of experiments. After 2-day culturing with
rhIL-4 (10 ng/mL) to up-regulate their surface FcεRI expression (352), hCBMCs were
preloaded with IgE and stimulated, in this case, with α-IgE in the presence of alternative
concentrations of 1α,25(OH)2D3 or ETOH (0.03% final). The secretion of hTNFα and
hIL-10 was measured 6 h post-stimulation by ELISA. Similar to mBMCMCs, hCBMCs
produced significantly less TNFα when stimulated in the presence of both concentrations
(i.e. 10^-7 and 10^-8 M) of 1α,25(OH)2D3 and the reduction was more profound than that
observed with WT mBMCMCs (Figure 6.2a, compared to Figure 3.7e). On the other
hand, 1α,25(OH)2D3 treatment did not alter levels of IL-10 produced in response to IgE +
α-IgE-mediated hCBMC activation (Figure 6.2b). It is significant, however, that the
lower concentration (10^-8 M) of 1α,25(OH)2D3 alone increased the secretion of hIL-10, at
least when compared to the higher concentration. This is consistent with our previous
Figure 6.1. Morphological properties of hCBMCs
The identity and purity of 10- to 13-week-old hCBMCs was examined and confirmed by toluidine blue staining (a), and intracellular tryptase staining followed by either flow cytometry (b) or immunofluorescence staining (c and d; DAPI nucleus stain was used to locate cell populations). Scale bars (a, c and d) represent 10 µm. Data are representative of cells from two independent donors.
Ten- to 13-week-old hCBMCs were cultured in the presence of rhIL-4 (10 ng/mL) for two days, prior to 16 h preload with human IgE (2.5 µg/mL) followed by 6 h α-human IgE (1 µg/mL) stimulation. 1α,25(OH)₂D₃ (10⁻⁷ or 10⁻⁸ M) or ETOH (0.3 % final) were included in the culture throughout the experiment. The secretion of hTNF (a) and hIL-10 (b) was measured using ELISA. Data are representative of three independent experiments. * p < 0.05, ** p < 0.01
findings that 1α,25(OH)₂D₃ can dose-dependently increase IL-10 production from WT mBMCMCs in vitro. (163)

6.3.2. Human CBMCs constitutively express CYP27B1, which can be up-regulated by 25OHD₃ treatment

Due to the novelty of the previous findings regarding CYP27B1 expression in mBMCMCs, it was necessary to investigate whether similar results could be obtained in MCs derived from other species. Figure 6.3a shows hCBMCs constitutively express CYP27B1 throughout their cytosol. Similar to WT mBMCMCs, hCBMCs also exhibit up-regulated CYP27B1 protein expression at 8-h after 25OHD₃ addition (Figures 6.3 b and c). Therefore, MC expression of the CYP27B1 hydroxylase and its positive regulation by 25OHD₃ is unlikely to be species-restricted.

6.3.3. Human CBMCs produce endogenous 1α,25(OH)₂D₃ following 25OHD₃ treatment in a dose-dependent manner

Since it was established that hCBMCs express CYP27B1, we decided to examine if this enzyme enables hCBMCs to generate 1α,25(OH)₂D₃ endogenously from its precursor. Similar to the experiments using mBMCMCs (Chapter 4), hCBMCs were firstly cultured in medium containing csFCS for 72 h, and then treated with 25OHD₃ (10⁻⁶ or 10⁻⁷ M) or ETOH for 6 h. The level of 1α,25(OH)₂D₃ in culture supernatant as well as cell lysate was subsequently measured using the 1,25-(OH)₂D RIA kit. As demonstrated in Figure 6.4, treatment with both doses of 25OHD₃ led to endogenous 1α,25(OH)₂D₃ production in hCBMCs from two independent donors, and the level of 1α,25(OH)₂D₃ production was considerably higher than that in mBMCMCs (Figure 4.3). The higher
Figure 6.3. CYP27B1 expression in naive and 25OHD₃-treated hCBMCs

Cytospins of naive 12-week-old hCBMCs were prepared, which were labelled intracellularly with α-CYP27B1 Ab followed by Alexa594-conjugated secondary Ab, and examined using confocal microscopy (a). Isotype Ab labelling was included as the negative control, and the DAPI nucleus stain was used to locate cell populations. Scale bars represent 10 µm. To examine the effect of 25OHD₃ on CYP27B1 expression in hCBMCs, cells were cultured, at 5 × 10⁵ cells/mL, in hCBMC-csFCS medium for 72 h, replated at 10⁶ cells/mL, and treated with either 10⁻⁷ M of 25OHD₃ or vehicle (ETOH, 0.03%) for 8 h. Cell lysates were subsequently prepared and the CYP27B1 protein expression was examined by western blotting (b and c). Data are representative of cells from two independent donors.
Figure 6.4. Dose-dependent $1\alpha,25$(OH)$_2$D$_3$ production by 25OHD$_3$-treated hCBMCs

Nine- to 12-week-old hCBMCs were cultured, at $5 \times 10^5$ cells/mL, in hCBMC-csFCS medium for 72 h, subsequently replated at $2 \times 10^6$ cells/mL in the same medium, and treated with indicated concentrations of 25OHD$_3$ or ETOH. Culture supernatant (a) as well as cell lysate (b) samples were collected at 6 h post-treatment, and the $1\alpha,25$(OH)$_2$D$_3$ level in all samples was measured using the 1,25-(OH)$_2$D RIA kit. The total $1\alpha,25$(OH)$_2$D$_3$ level (c) was calculated by adding together its concentration in the supernatant and that in the corresponding cell lysate. Data show results from cells from two independent donors examined in one experiment.
dose (i.e. $10^{-6}$ M) of 25OHD$_3$, in particular, triggered conversion to 1α,25(OH)$_2$D$_3$ to levels 50 times higher in hCBMCs than in mBMCMCs. Like their mouse counterparts (Figure 4.3), however, hCBMCs also seemed to secrete approximately 75% of endogenously generated 1α,25(OH)$_2$D$_3$ (Figure 6.4a) and accumulate the rest inside the cells (Figure 6.4b).

6.3. Discussion

Despite the similarities between human and murine MCs mentioned at the beginning of this chapter, a number of developmental and functional differences have become obvious during the past few years. For example, whilst mouse MCs can derive from common basophil/MC progenitors and share some functional properties with murine basophils$^{(4, 9)}$; human MCs appear to develop along a different lineage from human basophils, and they morphologically as well as functionally resemble monocytes and macrophages more closely than basophils$^{(353-355)}$. In addition, although many findings on FcεRI signal transduction in MCs, such as activation of the RAS-MAPK pathway, have been confirmed in both mouse and human systems, some mechanisms that operate in murine MCs do not apply to their human counterparts.$^{(166)}$ One example is PI3K being required for FcεRI-mediated TNF synthesis by murine but not human intestinal MCs.$^{(356)}$ MCs from different species also display distinct mediator profiles. In contrast to human MCs possessing several tryptases ($\alpha$-, $\beta$-, $\gamma$-, and $\delta$-tryptases) but only a single chymase, their murine counterparts synthesise a greater array of chymases (MMCP-1, -2, -4, -5 and -9).$^{(357, 358)}$ Furthermore, many cytokines, such as IL-4, IL-5 and TNF, are produced at different levels in human MC$_T$’s and murine BMCMCs. Therefore, results from studies
using murine MCs are not always transferable to the human situation, and the relevance of findings from murine disease models is not always evident.\textsuperscript{(359, 360)} A good example set by an eosinophil-based study is that whereas anti-IL5 Ab completely blocked the airway hypersensitivity in experimental mouse models of asthma\textsuperscript{(361)}, the therapeutic application of humanized anti-IL5 Ab failed to improve the bronchial hypersensitivity of asthmatics\textsuperscript{(362)}.

Like mBMCMCs, hCBMCs also represent primary MC cultures that are derived from MCPs and, therefore, are a suitable cell type choice for the aims of the current chapter. As shown in Figure 6.2a, 1α,25(OH)\textsubscript{2}D\textsubscript{3} treatment during IgE + α-IgE-mediated hCBMC activation substantially reduced hTNFα secretion from the cells, in a manner similar to that observed with IgE-activated WT mBMCMCs (Figure 3.7e). The percentage of reduction in TNFα secretion, however, was more profound in hCBMCs (≥ 60% for both 10\textsuperscript{-7} and 10\textsuperscript{-8} M of 1α,25(OH)\textsubscript{2}D\textsubscript{3}) than in mBMCMCs (25% for 10\textsuperscript{-7} M and 40% for 10\textsuperscript{-8} M of 1α,25(OH)\textsubscript{2}D\textsubscript{3}), the reason for which requires further investigation but might merely reflect the difference between MCs from different species as discussed above. The production of hIL-10 by activated hCBMCs, on the other hand, was unchanged following 1α,25(OH)\textsubscript{2}D\textsubscript{3} treatment (Figure 6.2b). Although limited literature is available on the production of IL-10 from IgE + sAg-activated human MCs, one paper did report that human lung-derived MCs are capable of producing IL-10 in response to IgE-dependent stimulation, and that such production was highest in the presence of SCF and following at least 10 hrs of stimulation.\textsuperscript{(363)} Therefore, it is possible that the activated hCBMCs in our experiments, in the absence of any growth factor such as SCF, reached their maximum IL-10 producing capacity, which could not be further enhanced by 1α,25(OH)\textsubscript{2}D\textsubscript{3}. It is
also possible that the cells were not stimulated long enough (6 h only) with 1α,25(OH)$_2$D$_3$ for this metabolite to elicit its effects. Future experiments are, therefore, required to examine these possibilities. At any rate, even if 1α,25(OH)$_2$D$_3$ is unable to increase IL-10 production from human MCs during IgE-mediated activation, its capacity to down-regulate pro-inflammatory cytokines such as TNFα would shift the micro-environmental balance from pro-inflammatory to anti-inflammatory during allergic responses with relatively more IL-10 being present. Furthermore, despite the difference in 1α,25(OH)$_2$D$_3$-mediated IL-10 regulation between activated mBMCMCs and hCBMCs, non-activated hCBMCs treated with the lower concentration of 1α,25(OH)$_2$D$_3$ did show increased IL-10 secretion (Figure 6.2b), which is consistent with our findings in mBMCMCs both previously (163) and from the current study (Figure 3.8a).

Since there are no reports in the literature regarding human MC expression of CYP27B1 or their 1α,25(OH)$_2$D$_3$-producing capacity, it was exciting to discover these findings in hCBMCs during the current body of work (Figures 6.3 and 6.4). More importantly, hCBMCs (Figure 6.4) appear to produce over 50 times more endogenous 1α,25(OH)$_2$D$_3$ than mBMCMCs (Figure 4.3). Studies using human primary renal cultures show 1α,25(OH)$_2$D$_3$ is produced at approximately $2 \times 10^{-10}$ mol/mg protein/h (364), whereas mouse kidney cell cultures produce 1α,25(OH)$_2$D$_3$ at approximately $5 \times 10^{-10}$ mol/mg protein/h (365). The difference between these mouse and human results is relatively small, and as such could merely be a consequence of the different 25OHD$_3$ concentrations used ($10^{-8}$ M versus $5 \times 10^{-6}$ M) and/or the different 1α,25(OH)$_2$D$_3$ measurements (western versus competitive receptor assay) employed. By contrast, we observed significant differences between the amount of 1α,25(OH)$_2$D$_3$ produced by mBMCMC and hCBMC
in the current study. Further research, therefore, is required to confirm and/or explain our observations. Especially considering the recent discovery that *in vitro* human MCP-derived MC cultures have the limitation of not being able to achieve complete MC-maturation (366, 367), more physiologically relevant cellular representative of human MCs (such as primary human MC cultures) need to be examined in subsequent experiments.

In conclusion, we confirmed in this chapter that the overall immunosuppressive effect of 1α,25(OH)2D3 is applicable to IgE + α-IgE-activated hCBMCs, even though it does not alter the amount of hIL-10 produced using the current settings. We also demonstrated, again for the first time, the constitutive expression of CYP27B1 in hCBMCs and the up-regulation of this enzyme following 25OHD3 treatment. Subsequent functional investigations confirmed the ability of CYP27B1 in hCBMCs to stimulate substantial 1α,25(OH)2D3 production in a dose-dependent manner. Although further investigation is required to explain certain observations as mentioned above, this chapter nevertheless emphasizes the physiological relevance of our previous findings (especially *chapters 3 and 4*) and thus offers evidence for the application of VitD3 analogues in treating certain potentially IgE-mediated allergic disorders, such as anaphylaxis.
CHAPTER 7

CONCLUDING REMARKS & FUTURE DIRECTIONS
Despite the concurrence of increased VitD$_3$ deficiency and increased incidence of IgE-mediated allergic disorders among the Australian population$^{(368)}$, the potential effects of 1α,25(OH)$_2$D$_3$ on activated MCs in allergic settings to our knowledge has not been investigated extensively to date. Findings of the current project suggest that the increased VitD$_3$ insufficiency among the Australian population, a possible result of the recent “Slip Slop Slap” sun protection campaign by the Australian Cancer Council, can lead to more intrinsically pro-inflammatory MC populations (not only those in the skin but also throughout the body due to the lower circulating 25OHD$_3$ levels) and ultimately a higher predisposition to develop IgE-mediated allergies. In particular, we observed that 1α,25(OH)$_2$D$_3$ can down-regulate the pro-inflammatory properties of IgE + sAg-activated MCs both in vitro and in vivo. Moreover, it was revealed, for the first time, the capacity of MCs to convert 25OHD$_3$ to its active metabolite, thus conferring this inactive VitD$_3$ analogue similar immunosuppressive effects on activated MCs. Although these effects of 25OHD$_3$ seemed most likely indirect, via endogenous 1α,25(OH)$_2$D$_3$ production by MCs, it is also possible that 25OHD$_3$ can elicit direct effects on activated MCs, as demonstrated by the in vitro data using the highest concentration of this metabolite (Figures 4.4 a and c). To determine the exact nature of the action of 25OHD$_3$ in MCs, mBMCMCs from $CYP27B1^{-/-}$ mice could be generated and analysed in parallel with WT controls in in vitro experiments with 25OHD$_3$ treatment. As mentioned in Chapter 5, $CYP27B1^{-/-}$ mice and $CYP27B1^{-/-}$ MC knock-in mice will be equally valuable tools to achieve the same aim in in vivo PCA settings.

In addition to CYP27B1, DCs and macrophages also express CYP27A1, the hydroxylase that converts VitD$_3$ to 25OHD$_3$, and therefore possess the complete autonomous VitD$_3$
pathway. Since MCs also express CYP27B1, even in their naive state, it will be of great interest to examine the expression of CYP24A1 (as well as CYP2DII, CYP2D25, CYP3A4 and CYP2R1) by these cells; for if the expression is confirmed, then cutaneous MCs can theoretically utilise VitD3 generated in the skin upon UVB irradiation directly to produce endogenous 1α,25(OH)2D3. In similar regards, it will also be illuminating to repeat some of our in vivo experiments using an alternative source of 1α,25(OH)2D3. Instead of topical 1α,25(OH)2D3 application, mice could be exposed to repeated low-dose (e.g. 5 exposures to 2 kJ/m2) UVB irradiation, which we have recently reported can dramatically increase endogenous production of 1α,25(OH)2D3 in the skin (370), and followed by PCA induction. If the resultant ear swelling is reduced upon UVB exposure, then although MCs make little if any contribution to the cutaneous 1α,25(OH)2D3 production as indicated by the similar increase in 1α,25(OH)2D3 levels between WT and MC deficient mice (370), the physiological relevance of our findings will nevertheless be reinforced.

As part of the future investigation to identify potential mechanisms responsible for the immunosuppressive effects of 1α,25(OH)2D3 on activated MCs, FcεRI-initiated signalling pathways (e.g. MAPK pathway) and whether and how they are affected by 1α,25(OH)2D3 could be examined. The ultimate goals in this regard are to map out as many pathways as possible that are regulated by 1α,25(OH)2D3 treatment, and to examine the potential integration of the FcεRI-mediated and VDR/mVDR-mediated signalling networks in MCs. This can be achieved by running cDNA samples from differently treated MC groups through the newly developed RT2 Profiler™ PCR arrays to identify potential signalling molecule targets, and then validating the result by western blotting.
In addition, 1α,25(OH)2D3 seemed capable of reducing MC degranulation upon IgE + sAg-mediated activation both in vitro (Figure 3.6) and in vivo (Figure 5.3), which is an immediate response triggered by an increase in Ca2+ concentration in the cytoplasm. (371, 372) According to the literature, there are two main sources of intracellular Ca2+, one being its storage in the endoplasmic reticulum and the other its additional supply among the extracellular space (371, 372), and whereas Ca2+ is essential for the fusion of MC granules and the plasma membrane and subsequent exocytosis, the initial translocation of MC granules to the plasma membrane is in fact a Ca2+-independent and microtubule-dependent process (373). Interestingly, using VitD-deficient chicks which had been fed with either 1α,25(OH)2D3 or vehicle, it was shown that dietary supplement of 1α,25(OH)2D3 could indeed increase the synthesis of microtubule proteins by chick intestinal epithelial cells, resulting in an accelerated Ca2+ absorption from the intestines of the birds. (374) Therefore, it will be important to examine the effects of 1α,25(OH)2D3 on microtubule formation in MCs upon IgE + sAg-mediated activation, and the speed as well as magnitude of Ca2+ entry into the cytoplasm from either of the sources mentioned above.

In addition to the above mechanisms, which are most likely responsible for the non-genomic effects of 1α,25(OH)2D3, mechanisms that regulate the genomic effects of this hormone also and need to be clarified. To achieve this aim, a DNA microarray can be carried out initially, using total RNA extracted from mBMCMCs that have undergone different treatments, to pinpoint particular genes whose expression is altered with 1α,25(OH)2D3 treatment followed by activation. The importance of the top candidates can then be individually validated by qRT-PCR and/or western blotting.
Another potential mechanism of 1α,25(OH)2D3-induced MC regulation involves post-transcriptional control of relevant genes by miRs. It was discovered recently that miR-221-222, with their elevated expression, regulate cell cycle checkpoints and thus proliferation of mBMCMCs in response to acute activation stimuli. (375) Subsequent data using lentivirally transduced MCs showed that this family of miRs also have pleiotropic effects on other essential aspects of MC biology, including survival, homing and migration. (376) For the future direction of the current project, it will be interesting to determine whether IgE + sAg-mediated MC activation is subjected to the post-transcriptional regulation of miRs and, if so, which ones are affected by 1α,25(OH)2D3 treatment. This can be achieved by firstly performing a miR microarray using total RNA from differently treated MCs, and then confirming the involvement of top candidates by miR qRT-PCR and western analysis on proteins encoded by their known gene targets.

There are indeed a number of mechanisms that could explain the immunosuppressive effects of 1α,25(OH)2D3 on activated MCs in vitro, but these effects alone are unlikely to be solely responsible for the observed 1α,25(OH)2D3-mediated PCA suppression in vivo. Recently, we reported that chronic low-dose UVB irradiation, which increases 1α,25(OH)2D3 levels in ear pinnae, could limit the number of various leukocytes (e.g. granulocytes, macrophages and T cells) within the tissues in a MC VDR-dependent manner. (163) Given that 1α,25(OH)2D3 can also alter the functional properties of structural cells (e.g. keratinocytes) as well as leukocytes in various inflammatory settings (226, 316, 317, 320), the effects of topical 1α,25(OH)2D3 on the recruitment and/or functionality of these cells need to be examined to explore additional explanations for the capacity of this hormone to suppress PCA-associated cutaneous pathology. Other in vivo allergy
models, such as the mouse atopic dermatitis models \(^{(377)}\) which provide a persistent rather than transient inflammation scenario, should also be utilised to determine whether topical \(1\alpha,25(\text{OH})_2\text{D}_3\) or dietary supplements of VitD\(_3\) metabolites can also influence chronic-phase events (i.e. tissue remodelling) of allergic disorders.

Data obtained from the current project clearly demonstrated VDR-dependence for the immunosuppressive effect of \(1\alpha,25(\text{OH})_2\text{D}_3\) on IgE + sAg-mediated MC activation both \textit{in vitro} (Chapter 3) and \textit{in vivo} (Chapter 5). This, however, does not mean that such effect is genomic, as the non-genomic pathway-specific analogue, curcumin, exhibited similar effects to \(1\alpha,25(\text{OH})_2\text{D}_3\) on the secretion but not gene expression of pro-inflammatory cytokines also in a VDR-dependent manner (Figures 3.11 and 3.12). As pointed out previously, these results represent the first set of functional evidence that VDR could function as mVDR and initiate non-genomic signalling pathways. In addition, it was intriguing the fact that curcumin exhibited the opposite effect to \(1\alpha,25(\text{OH})_2\text{D}_3\) on IL-10 secretion, which suggests that whereas the non-genomic action of \(1\alpha,25(\text{OH})_2\text{D}_3\) (as represented by curcumin) can decrease IL-10 secretion (along with that of other cytokines), its genomic action counteracts with this effects and results in a net increase in IL-10 production. To substantiate this theory, other VitD\(_3\) pathway-specific agonists and antagonists will be used either instead of or in conjunction with \(1\alpha,25(\text{OH})_2\text{D}_3\), respectively, in \textit{in vitro} experiments. Furthermore, our \textit{in vivo} data demonstrated that the IL-10 level in ear tissues, as well as that of TNF\(\alpha\) and IL-6, was moderately reduced upon topical \(1\alpha,25(\text{OH})_2\text{D}_3\) treatment, which could suggest the action of non-genomic VitD\(_3\) pathways and/or post-translational modification by microRNAs. Again, other pathway specific agonists and antagonists, preferentially the ones that are low-calcemic in nature.
and therefore will not cause hypercalcemia and related symptoms \textit{in vivo}, should be tested before definite conclusions are drawn in this regard.

Taken together, the current thesis presented novel findings indicating the capacity of MCs to generate endogenous 1\(\alpha\),25(OH)\(_2\)D\(_3\) and the MC VDR-dependent immunosuppressive effects of 1\(\alpha\),25(OH)\(_2\)D\(_3\) on IgE-mediated inflammatory responses both \textit{in vitro} and \textit{in vivo}. It also provided a strong basis for future studies exploring other potential mechanisms responsible for the observed effects of 1\(\alpha\),25(OH)\(_2\)D\(_3\) on activated MCs and confirming these effects using other appropriate \textit{in vitro} and \textit{in vivo} models. Overall, the outcome of this project highlighted the complexity of MC functions in IgE-dependent allergic disorders and, more importantly, the therapeutic potential of VitD\(_3\) analogues for the prevention and treatment of these disorders via its immunoregulatory effects on MCs.
References


190


Chen, H. J., C. K. Shih, H. Y. Hsu, and W. Chiang. 2010. Mast cell-dependent allergic responses are inhibited by ethanolic extract of adlay (Coix lachryma-jobi


295. 2011. B6.129S4-Vdr<sup>tm1Mbd</sup>/J. The Jackson Laboratory.


in draining lymph nodes following skin sensitization of mice. *Immunology* 86:441-447.


203