



**Interleukin-17A Induced Human Mesenchymal Stem Cells
Are Superior Modulators Of Immunological Function**

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Thesis submitted in fulfilment for the degree of

Doctor of Philosophy

In

The Department of Medicine

Faculty of Health Sciences

The University of Adelaide

March 2016

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Thesis Abstract

Interferon-gamma (IFN- γ) preactivated mesenchymal stem cells (MSC- γ) are highly immunosuppressive but immunogenic *in vivo* due to their inherent expression of major histocompatibility (MHC) molecules. This thesis presents an improved approach where human bone-marrow derived MSC were modified with IL-17A (MSC-17) to enhance T cell immunosuppression but not their immunogenicity. It was demonstrated in **CHAPTER 3** that MSC-17, unlike MSC- γ , showed no induction or upregulation of MHC class I, MHC class II and T cell co-stimulatory molecule CD40, but maintained normal MSC morphology and phenotypic marker expression. When co-cultured with phytohemagglutinin (PHA) activated human T cells, MSC-17 potently suppressed T cell proliferation, inhibited surface CD25 expression and suppressed the elaboration of Th1 cytokines IFN- γ , TNF- α and IL-2 when compared to untreated MSC (UT-MSC). T cell suppression by MSC-17 correlated with increased IL-6 but not indoleamine 2,3-dioxygenase 1, cyclooxygenase-1 and TGF- β . In **CHAPTER 4**, it was shown that MSC-17, but not MSC- γ consistently induced CD4⁺CD25^{high}CD127^{low}FoxP3⁺ regulatory T cells (iTregs) from PHA activated CD4⁺CD25⁻ T cells. MSC-induced iTregs expressed the functional Treg markers CD39, CD73, CD69, OX40, CTLA-4 and GITR. Functionally, FACS-sorted MSC-17-induced-iTregs could suppress human T cell activation (CD154 suppression assay). **CHAPTER 5** was aimed at further dissecting mechanisms by which human MSC-17 mediate their superior modulation of T cell responses. UT-MSC, MSC- γ and MSC-17 were assessed for their gene expression profile (microarray, 3 human MSC donors). Significantly regulated genes ($p < 0.05$, fold change (FC) < -2 or > 2) were identified for their biological functions (Database for Annotation, Visualisation and Integrated Discovery, DAVID). Microarray analysis revealed that 1278 differentially expressed genes (902 upregulated; 376 downregulated) were significantly regulated between MSC- γ and UT-MSC and only 67 genes (39 upregulated; 28 downregulated)

between MSC-17 and UT-MSC. Gene ontology analysis of upregulated MSC- γ genes uncovered significant enrichment of genes involved in immune response, antigen processing and presentation, humoral responses and complement activation (eg. HLA genes, complement components and CIITA). This data is consistent with the upregulation of MHC molecules and studies showing increased MSC- γ immunogenicity. MSC-17 upregulated genes were mainly associated with chemotaxis response. This may be essential for T cell recruitment for MSC-17 immunosuppression. MMP13 was highly expressed only in MSC-17 as determined by microarray (FC 15.6) and validated by real-time PCR, hence the potential involvement of MMP13 in the superior immunomodulatory function of MSC-17. The final results **CHAPTER 6** was to translate the findings with human MSC-17 to compact bone-derived mouse MSC (CB mMSC). Unexpectedly, CB mMSC-17 unlike human MSC-17 showed no enhancement of *in vitro* immunosuppression of allogeneically induced CD4⁺ and CD8⁺ T cell proliferation. Nevertheless, CB mMSC differ from bone marrow-derived mMSC and may represent a new source to isolate mouse MSC with high purity and potent immunosuppression properties that would be beneficial in a context of allotransplantation rejection. CB mMSC, without IL-17A preconditioning mediated potent *in vitro* suppression of T cells even when used at low doses. In conclusion, human MSC-17 are superior modulators of T cells and can engender Tregs to potently suppress T cell activation with minimal immunogenicity. MSC-17 represent a potential cell therapy to modulate T cell responses for clinical application.

Thesis Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree. I give consent to this copy of my thesis when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968. The author acknowledges that copyright of published works contained within this thesis (*as listed below) resides with the copyright holder(s) of those works. I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library Search and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

Kisha Nandini Sivanathan

Acknowledgements

Firstly, I would like to thank my supervisors Professor Toby Coates, Dr. Darling Rojas-Canales and Professor Stan Gronthos for their constant mentorship and support throughout my PhD. I would also like to thank my external supervisor Associate Professor Benjamin Thierry (University of South Australia) for his supervision at the early stages of my candidature.

I would especially like to sincerely thank my principal supervisor Professor Toby Coates for his excellent mentorship, support, and the opportunity to pursue a PhD in his laboratory. I would also like to express my sincere gratitude to you, Dr. Coates, for all the opportunities you have given to me - travelling nationally and internationally for conferences, meetings and networking. These travels were always very inspiring, recharging and on the personal level, changed my perspective and views about life and all the opportunities that is available ahead. I have always admired your optimism, positive thinking approach and enthusiasm.

I would also like to specially thank Dr. Darling Rojas-Canales, my co-supervisor, for her mentorship, patience, support, motivation, advice, time, laboratory assistance and great friendship! Thanks for helping me pull through those frustrating, difficult laboratory days with positive thoughts and different perspectives.

I thank Dr. Plinio Hurtado for his invaluable intellectual input and for always being my happy, excellent and amazing friend. I also thank Professor Shane Grey (Garvin Institute, Sydney) for his intellectual input and mentorship. I thank Daniella Penko for performing islet inflations, the islet transplant procedure, assistance with other animal work and for being an amazing friend. I also extend my sincere gratitude to Sharon Paton for teaching me mesenchymal stem cell isolations; Dr. Chris Hope for assistance and contributions in the regulatory T cell experiments; The Detmold facility and Ernesto Hurtado for flow sorting; Julie Johnston for her invaluable help in the laboratory and in proof-reading this thesis; Svjetlana Kireta for teaching laboratory techniques, support, advice and also for proof-reading this thesis; The Adelaide Microarray Centre for running the microarray samples; the Department of Histology (The Queen Elizabeth Hospital) and SA Pathology for tissue sectioning and histology staining; all the bone marrow donors and the Royal Adelaide Hospital; all the buffy coat donors and the Australian Red Cross Blood Service; Dr. Michelle Lorimer (Senior Statistician, Data Management and Analysis Centre, University of Adelaide) for statistics consultation; and ALL other individuals and organisations for their support throughout this research.

I would like to acknowledge the support from the University of Adelaide for awarding me the Adelaide Graduate Research Scholarship for 3.5 years and for travel grants; The Hospital Research Foundation for extending my PhD scholarship for 6 months; The Renal Department for supporting me as a student; The Robinson Research Institute, University of Adelaide for the high impact paper funding; and all travel grants awarded by the Royal Adelaide Hospital, The Transplantation Society of Australia and New Zealand, The Transplantation Society, The American Society of Transplantation and The American Society of Transplant Surgeons.

Thank you all, to the past and present members of the laboratory and the Renal Department for everything and for the great 4 years of my PhD. For all those who had been there from the start to the end of my PhD journey: Plinio, Ernesto, Darling, Daniella, Jodie, Julie, Svjetlana and Chris, I would like to specially thank you all for your many years of friendship, memories, laughter, parties, outings and being my laboratory/Australian family; while I was thousands of miles away from home. Thank you, especially to the Hurtado family who brought lots of smiles and happiness in my life! I also thank all my friends who have supported and been there for me, particularly A'fra Usman, Nurul Efifi and Dr. Umi Salleh.

Most importantly, I would like to thank my parents and family, who have continuously supported and helped me throughout my education. I am blessed to have such supportive and encouraging parents who gave me the opportunity to study at the University of Adelaide, to pursue my degree, honours and to the end of my PhD. They have always been open to set me free and find my life in a new country. A parent's love for their child is unconditional. I thank my mother for being by my side, in Australia at the final days of my PhD and for all the delicious healthy meals! I will always remember the "Tortoise and the Hare" story which my father reminded me of as a child.

Lastly, to my PhD, I am very thankful for all the opportunities and experiences I have learnt throughout this journey, especially at a young age. Reflecting on these 4 years, achievements have come way beyond what I have imagined at the start. I have gained wisdom, travelled around the world, learnt many things about myself and the people around me; all of which helped me grow as a person. For everything that failed, what I have learnt - don't be defeated, optimism, patience, dedication, determination and persistence. Essentially, your mind is the master of your success. "You can do it, if you put your mind into it." I would like to thank an individual, a significant person in my life for many years, who was essential for my growth. I was introduced to this book called "The Monk Who Sold His Ferrari" by Robin Sharma, that truly inspired me and was a guide that helped me pull through my PhD, especially

during the difficult times. As we move to the next-phase, we take with us all the lessons we learnt from the past to create a better future. Quoted from this book “The purpose of life is a life of purpose”. The end of this PhD is a beginning of my lifelong commitment and dedication as a scientist. I truly enjoyed every step of my PhD journey and will continue to love science and research in the future. I would like to end with the most inspirational speech I have heard, back in October 2014, by Steve Jobs, while I tirelessly wished my experiments would work:

“Sometimes life hits you in the head with a brick. Don’t lose faith. I’m convinced that the only thing that kept me going was that I loved what I did. You’ve got to find what you love. And that is as true for your work as it is for your lovers. Your work is going to fill a large part of your life, and the only way to be truly satisfied is to do what you believe is great work. And the only way to do great work is to love what you do. If you haven’t found it yet, keep looking. Don’t settle. As with all matters of the heart, you’ll know when you find it. And like any great relationships, it just gets better and better as the years roll on. So keep looking until you find it. Don’t settle...Your time is limited, so don’t waste it living someone else’s life. Don’t be trapped by dogma – which is living with the results of other people’s thinking. Don’t let the noise of others’ opinions drown out your own inner voice. And most important, have the courage to follow your heart and intuition. They somehow already know what you truly want to become. Everything else is secondary.”

- Steve Jobs -

Publications

Sivanathan KN, Gronthos S, Rojas-Canales D, Thierry B, Coates PT. Interferon-gamma modification of mesenchymal stem cells: implications of autologous and allogeneic mesenchymal stem cell therapy in allotransplantation. *Stem Cell Rev.* 2014;10(3):351-375. – IF **3.214** Citations: **34** (**APPENDIX Published Paper 1**)

Lett B, **Sivanathan KN**, Coates PT. Mesenchymal stem cells for kidney transplantation. *World J Clin Urol.* 2014;3(2):87-95. (**APPENDIX Published Paper 2**)

Sivanathan KN, Rojas-Canales DM, Hope CM, et al. Interleukin-17A-Induced Human Mesenchymal Stem Cells Are Superior Modulators of Immunological Function. *Stem Cells.* 2015;33(9):2850-2863. – IF **6.523** Citations: **5** (**APPENDIX Published Paper 3**)

Press release: <http://www.adelaide.edu.au/news/news79202.html>

Stem Cell Video Highlight: (Invited) <https://www.youtube.com/watch?v=gP6GONfRP80>

Featured article in the Regenerative Medicine Weekly Newsletter (26 August 2015)

Sivanathan K.N., Rojas-Canales D, Gronthos S, Grey S.T., Coates P.T. Gene microarray comparative analysis of interferon-gamma and interleukin-17A preconditioned human Mesenchymal Stem Cells (**manuscript in preparation**)

Sivanathan K.N., Rojas-Canales D, Gronthos S, Grey S.T., Coates P.T. Immunodepletion and hypoxia preconditioning of mouse compact bone cells as a novel protocol to isolate highly immunosuppressive Mesenchymal Stem Cells (**manuscript in preparation**)

Sivanathan K.N., Rojas-Canales D, Coates P.T. Mesenchymal stem cells indirectly modulate T cell immunosuppression through the generation of regulatory T cells (**manuscript in preparation**)

Presentations

- 11th April 2016 Transplantation Society of Australia and New Zealand (TSANZ), Sydney, Australia
Sivanathan K.N., et al. Microarray gene profiling of immunosuppressive interleukin-17A preactivated human bone marrow-derived Mesenchymal stem cells (MSC-17)
- 16th Nov 2015 IPITA-IXA-CTS Joint Congress, Melbourne, Australia
Sivanathan K.N., et al. Gene microarray comparative analysis of interferon-gamma and interleukin-17A preconditioned human Mesenchymal Stem Cells (**oral presentation**)
- 16th Nov 2015 IPITA-IXA-CTS Joint Congress, Melbourne, Australia
Bron Lett, **Sivanathan K.N., et al.** Imaging of Iron nano-particle labelled Mesenchymal Stem Cells in ovine heterotopic kidney transplantation (**oral presentation**)
- 13th Nov 2015 Transplantation Science Symposium, Lorne, Australia
Sivanathan K.N., et al. Microarray gene profile study of immunosuppressive interleukin-17A preactivated human Mesenchymal Stem Cells (**oral presentation – Mentor-Mentee Award**)
- 22nd June 2015 Transplantation Society of Australia and New Zealand (TSANZ), Canberra, Australia
Sivanathan K.N., et al. IL-17A modulated human Mesenchymal Stem Cells as a novel cell therapy to engender regulatory T cells (Tregs). (**President's Prize Symposium – oral presentation – Young Investigator Award**)
- 6th May 2015 Invited speaker, Athersys Inc., Cleveland, Ohio, U.S.A
Interleukin-17A induced Mesenchymal Stem Cells are superior modulators of immunological function
- 3rd May 2015 American Transplant Congress, Philadelphia, U.S.A
Sivanathan K.N., et al. Interleukin-17A induced Mesenchymal Stem Cells are superior modulators of immunological function. (**poster presentation – Poster of Distinction – Young Investigator Award**)
- 6th Nov 2014 Robinson Institute Research Symposium, University of Adelaide, Australia
- 14th Aug 2014 Invited speaker, The Karp Laboratory, Harvard Medical School
Interferon-gamma (IFN- γ) and Interleukin-17 (IL-17) modification of Mesenchymal Stem Cells
- 11th Aug 2014 Invited speaker, The Thomas Starzl Institute, Pittsburgh
Ex vivo modification of Mesenchymal Stem Cells in Allotransplantation

- 8th Aug 2014 Invited speaker, Professor Wang, Yi-Gang Lab, University of Cincinnati
Ex vivo modification of Mesenchymal Stem Cells in Allotransplantation
- July 2014 World Transplant Congress, Moscone West Convention Centre, San Francisco
Sivanathan K.N., et al. Interferon-Gamma and onterleukin-17 modified Mesenchymal Stem Cells directly or indirectly modulate T cell responses by expressing inhibitory factors, downregulating T cell activation and inducing regulatory T cells (**oral presentation – Mentor-Mentee Award**)
- June 2014 The Transplantation Society of Australia and New Zealand (TSANZ), Canberra, Australia
Sivanathan K.N., et al. Interferon-Gamma and interleukin-17A enhance Mesenchymal Stem Cells (MSC) T cell suppressive function by mediating an increase and induction of CD4⁺CD25^{high}CD127^{low}Foxp3⁺ regulatory T cells (**oral presentation – Young Investigator Award**)
- 11th Dec 2013 Centre for Stem Cell Research, The Robinson Institute, University of Adelaide Research Day, 2013, Australia
Sivanathan K.N., et al. Mesenchymal Stem Cells preconditioned with proinflammatory cytokines enhance T cell inhibition by the downregulation of CD25 on activated T cells, expression of immunosuppressive factors and induction of regulatory T cells. (**poster presentation**)
- 17th July 2013 Invited speaker at Professor Dragan's Lab, Charite Hospital (Charité - Universitätsmedizin Berlin), Germany
Ex vivo modification of Mesenchymal Stem Cells in Allotransplantation
- 7th -11th July 2013 12th Congress of the Cell Transplant Society, Milan, Italy
Sivanathan K.N., et al. Mesenchymal Stem Cells preconditioned with proinflammatory cytokines enhance T cell inhibition by the downregulation of CD25 on activated T cells, expression of immunosuppressive factors and increase in regulatory T cells. (**oral presentation – Young Investigator Award**)
- 26th-28th June 2013 The Transplantation Society of Australia and New Zealand (TSANZ), Canberra, Australia
Sivanathan K.N., et al. *Ex vivo* modified Mesenchymal Stem Cells (MSC) enhance T cell immunosuppression by the expression of inhibitory molecules, downregulation of CD25 on activated T cells and The increase in regulatory T cells. (**oral presentation – Young Investigator Award**)
- 5th June 2013 Australian Society for Medical Research (ASMR), Adelaide, Australia
Sivanathan K.N., et al. Proinflammatory cytokine preactivated Mesenchymal Stem Cells suppress T cell proliferation by inhibiting T cell activation, increase in regulatory T cells and expression of immunosuppressive factors. (**oral presentation**)

- 21 May 2013 The Medical Staff Society Research Prize, Royal Adelaide Hospital, Adelaide, Australia (Finalist)
Sivanathan K.N., et al. Interferon-Gamma preconditioned Mesenchymal Stem Cells enhance T cell immunosuppression via the expression of inhibitory molecules, downregulation of CD25 on activated CD4⁺ or CD8⁺ T cells and increase in regulatory T cells. (**oral presentation**)
- 25th-28th Nov 2012 Australian Health and Medical Research Congress (AHMRC), Adelaide, Australia
Sivanathan K.N., et al. Interferon-Gamma and interleukin-17A modification enhance the immunomodulatory function of Mesenchymal Stem Cells (**poster presentation**)
- 31 Aug 2012 Faculty of Health Sciences Postgraduate Research Conference, University of Adelaide, Australia
Sivanathan K.N., et al. Real-Time magnetic resonance imaging localisation of superparamagnetic iron oxide nanoparticle-labelled Mesenchymal Stem Cells in ovine kidney autografts. (**poster presentation**)
- 2nd-6th June 2012 American Transplant Congress (ATC) International Conference
The John B. Hynes Convention Centre, Boston, MA.
Sivanathan K.N., et al. IFN-gamma preconditioning enhances the immunosuppressive properties of Mesenchymal Stem Cells to inhibit PHA-activated T cell proliferation (**poster presentation – presented by PT Coates**)
- Dyane A., Johnston J., **Sivanathan K.N., et al.** Magnetic resonance imaging cell tracking of Mesenchymal Stem Cells injected in kidney autografts of sheep. (**poster presentation – presented by PT Coates**)

Awards and Grants

19 Nov 2015	Royal Adelaide Hospital (RAH) Research Foundation 2016 Clinical Project Grant: “Interleukin-17A induced human Mesenchymal Stem Cells in improving transplant rejection outcomes in preclinical models of human islet transplantation – A proof-of-concept study” (AUD 50 000)
October 2015	The Hospital Research Foundation Grant Funded Scholarship (AUD 25,849 / annum)
August 2015	The International Transplantation Science Mentee-Mentor Travel Awards for the 2015 Transplantation Science Symposium, 11-13 Nov, 2015 Lorne, Australia (USD 800)
August 2015	The Robinson Research Institute High Impact Paper Funding, University of Adelaide (AUD 2680.00) (Sivanathan KN <i>et. al.</i> , 2015, Stem Cells)
June 2015	Travel grant for the Transplantation Science Symposium in Lorne, Victoria by TSANZ (AUD 900)
June 2015	TSANZ Young Investigator Award (AUD 780.92)
May 2015	Young Investigator Award, American Transplant Congress, Philadelphia, U.S.A. (USD 1000)
May 2015	Special Purpose Fund (to attend the American Transplant Congress), Royal Adelaide Hospital (AUD 5000)
May 2015	Poster of Distinction, American Transplant Congress, 2015, Philadelphia, U.S.A
June 2014	TSANZ Young Investigator award – at the 2014 TSANZ ASM (AUD 701)
June 2014	The Walter Dorothy Duncan Trust Fund, University of Adelaide – Travel Grant (AUD 1500)
25 April 2014	The International Transplantation Science Mentee-Mentor Travel Awards for the 2014 World Transplant Congress, 26-31, July, San Francisco CA, USA (USD 2000)
July 2013	The Transplantation Society Young Investigator Travel Award for the 12 th Congress of the Cell Transplant Society, Milan, Italy, 2013 (USD 2250)
June 2013	TSANZ Young Investigator award – at the 2013 TSANZ ASM (AUD 551.10)
June 2013	The Walter Dorothy Duncan Trust Fund, University of Adelaide – Travel Grant (AUD 2200)
March 2012- Sept 2015	International Adelaide Graduate Research Scholarship (AGRS), The University of Adelaide (full tuition and living allowances for 3.5 years)

Scientific Community engagement

- 2014 – 2018 (current) Cell Transplant and Regenerative Medicine (CTRMS) Society, Young Investigator Committee Member
- organising the international IPITA-IXA-CTS Joint Congress (Melbourne, Nov 2015)
 - fundraising, awards and publicity (social) involvement
 - organising the upcoming CTS joint congress in Halifax, Canada
 - joint international publications – manuscript in preparation
- 18 Nov 2015 Women in Transplantation, Networking Breakfast at the International IPITA-IXA-CTS Joint Congress – invited speaker
- only Australian representative in a panel of 5 female young investigators in the field of transplantation
 - discussed the role of women in the field of transplantation, challenges currently faced by women in this field of research and the future of women in research
 - primary focus of this event was to generate discussion and identify opportunities for women in transplantation and to support the next generation of woman leader
- 3rd July 2015 The University of Adelaide Press Release
<http://www.adelaide.edu.au/news/news79202.html>
(>5 million media views as reported on 23rd July 2015)
- July 2015 Stem Cell Journal Video highlight (Sivanathan KN *et. al.* 2015)
<https://www.youtube.com/watch?v=gP6GONfRP80>
- 2014 – current Reviewer – active reviewer for:
1 Stem Cells (2015), 1 Kidney International (2014), 1 Nephrology (2014)
- 2014-2015 HDR student engagement team, School of Medicine, University of Adelaide
- help design the future School of Medicine building space
- 2013 Committee Member of Health Science Postgraduate Association, University of Adelaide
- publicity of the newly established association at the University of Adelaide Postgraduate Conference, National Wine Centre, 2013
 - organising events for the association
- 12th Aug 2012 Science Alive, National Science Week,
Representing the Robinson Institute (University of Adelaide) at the science exhibition
- translating stem cell research to the society

Abbreviations

α -MEM - alpha Minimum Essential Medium

Ab – antibody

Ag – antigen

APC – antigen presenting cells

APC – allophycocyanin

AMR – antibody mediated rejection

ATP - adenosine triphosphate

ADP - adenosine diphosphate

AMP - adenosine monophosphate

AP-1 – activator protein 1

ANOVA – analysis of variance

AUC – area under the curve

bp – base pair

BM – bone marrow

BMMNC – bone marrow mononuclear cells

BGL – blood glucose levels

CEL – probe cell intensity files

CB – compact bones

CBA – cytokine bead array

cAMP - cyclic adenosine monophosphate

CD – cluster of differentiation

cpm – cell counts per minute

CTL – cytotoxic T cells

CTLA-4 – cytotoxic T-lymphocyte associated antigen-4

cDNA – complementary DNA

CFU-F – colony forming unit fibroblast

CMR – cell-mediated rejection

ConA – concavalin A

CXCL – chemokine C-X-C motif ligand

CXCR – C-X-C chemokine receptor

CCL – chemokine C-C motif ligand

CsA – cyclosporine A

CFSE – carboxyfluorescein succinimidyl ester

Cox – cyclooxygenase

DAVID – Database for Annotation, Visualization and Integrated Discovery

DC – dendritic cells

DMSO – dimethyl sulfoxide

DTH – delayed-type hypersensitivity

DNA – Deoxyribonucleic acid

DiOC₁₈(3) – 3,3'-Dioctadecyloxacarbocyanine perchlorate

EAE – experimental autoimmune encephalomyelitis

EDTA – Ethylenediaminetetraacetic acid

ELISA – Enzyme-linked immunosorbant assay

ECM – extracellular matrix

ERK – extracellular signal-regulated kinases

FoxP3 – forkhead box P3

FACS – fluorescence-activated cell sorting

FBS – fetal bovine serum

FMO – fluorescence minus one

FSC – forward side scatter

FASL – FAS ligand

FITC – Fluorescein isothiocyanate

GITR – glucocorticoid-induced TNFR-related protein

GARP - glycoprotein A repetitions predominant, LLRC32, Garpin

GM-CSF – granulocyte-macrophage colony-stimulating factor

GvHD – graft versus host disease

GOTERM – gene ontology term

HBSS – Hank’s Balanced Salt Solution

HO-1 – heme oxygenase-1

HLA-DR – human leukocyte antigen-DR

HPRT-1 – hypoxanthine phosphoribosyltransferase-1

H&E – Hematoxylin and eosin

HSC – haematopoietic stem cells

HGF –hepatocyte growth factor

IDO – indoleamine 2,3-dioxygenase

Ig – immunoglobulin

i.v. – intravenous

i.p. – intraperitoneal

IL - interleukin

iDC – immature DC

IPGTT – intraperitoneal glucose tolerance test

IL-17A – interleukin-17A

IFN- γ – interferon-gamma

IFNGR – interferon-gamma receptor

IGF-1 –insulin growth factor-1

iTreg – inducible regulatory T cells

JAK – Janus Kinase

LPS – Lipopolysaccharide

LAG-3 – lymphocyte-activation gene 3

LBP – lipopolysaccharide binding protein

LncRNA – long non-coding RNA

LAP - latent-associated peptide

mRNA – messenger RNA

MAPK – mitogen activated protein kinases

MSC – mesenchymal stem cells

MSC-17 – Interleukin-17A pre-treated MSC

MSC- γ – IFN- γ pre-treated MSC

mMSC – mouse MSC

mDC – mature DC

MMP – matrix metalloproteinase

MTI-MMP – membrane Type 1 MMP

MMF – mycophenolate mofetil

MFI – mean fluorescence intensity

MHC – major histocompatibility complex

MLR – mixed lymphocyte reaction

MDSC – myeloid derived suppressor cells

MST – mean survival times

miRNA – microRNA

NF- κ B – nuclear factor kappa-light-chain-enhance of activated B cells

nTreg – natural regulatory T cells

NK – natural killer

PHA – phytohemagglutinin

PCA – principal component analysis

PBMC – peripheral blood mononuclear cells

PCR – polymerase chain reaction

PD-1 – programmed death-1

PBS – phosphate-buffered saline

PE – phycoerythrin

PE-Cy7 – phycoerythrin-Cy-7

PE-Cy5.5 – phycoerythrin-Cy-5.5

PD-L1 – programmed death ligand-1, B7-H1

PGE₂ – prostaglandin-E2

POD – post operative day

RNA – ribonucleic acid

RT-PCR – real-time PCR

RBC – red blood cells

RPMI – Roswell Park Memorial Institute Medium

RMA – robust multi-array analysis

SAA1 – serum amyloid A1

Sca-1 – stem cell antigen 1

SD – standard deviation

SDF-1 – stromal cell-derived factor-1

SEM – standard error of the mean

STAT – signal transducer and activator of transcription 5

STZ – Streptozotocin

STRO-1 – stromal precursor antigen-1

snoRNA – small nucleolar RNA

SSC – side scatter

TAC – transcriptome analysis console

Th – T helper

Treg – regulatory T cells

Tr1 – T regulatory 1

Tr3 – TGF- β expressing regulatory cells

TLR – Toll-like receptor

TGF- β 1 – transforming growth factor beta 1

TCR – T cell receptors

TIMP – tissue-inhibitor of metalloproteinase

TNF- α – tumor necrosis factor-alpha

TRAF - TNF receptor-associated factors

T1D - Type 1 Diabetes

UT-MSC – untreated / unmodified MSC

VEGF – vascular endothelial growth factor

[³H]-Thymidine – tritiated thymidine

$\gamma\delta$ T cells – gamma delta T cells

Statement of Authorship

Title of Paper	Interferon-gamma Modification of Mesenchymal Stem Cells: Implications of Autologous and Allogeneic Mesenchymal Stem Cell therapy in allotransplantation
Publication Status	<input checked="" type="checkbox"/> Published <input type="checkbox"/> Accepted for Publication <input type="checkbox"/> Submitted for Publication <input type="checkbox"/> Unpublished and Unsubmitted work written in manuscript style
Publication Details	Sivanathan KN, Gronthos S, Rojas-Canales D, Thlerry B, Coates PT. Interferon-gamma modification of mesenchymal stem cells: Implications of autologous and allogeneic mesenchymal stem cell therapy in allotransplantation. <i>Stem Cell Rev.</i> 2014;10(3):351-375.

Principal Author

Name of Principal Author (Candidate)	Kisha Nandini Sivanathan
Contribution to the Paper	Manuscript writing Manuscript evaluation Acted as corresponding author
Overall percentage (%)	90%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 19/03/2016

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

- i. the candidate's stated contribution to the publication is accurate (as detailed above);
- ii. permission is granted for the candidate to include the publication in the thesis; and
- iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Stan Gronthos
Contribution to the Paper	Supervised development of the work Manuscript evaluation
Signature	Date 11/3/2016

Name of Co-Author	Darling Rojas-Canales
Contribution to the Paper	Supervised development of the work Manuscript evaluation Manuscript editing
Signature	Date 21/3/16

Name of Co-Author	Benjamin Thierry		
Contribution to the Paper	Supervised development of the work Manuscript evaluation		
Signature		Date	11/03/2016

Name of Co-Author	P. Toby Coates		
Contribution to the Paper	Supervised development of the work Manuscript evaluation		
Signature		Date	21.3.16

CHAPTER 1: LITERATURE REVIEW

Sections **1.3 - 1.5 and 1.8 - 1.11.2** are adapted and modified from the published article:

Sivanathan KN, Gronthos S, Rojas-Canales D, Thierry B, Coates PT. Interferon-gamma modification of mesenchymal stem cells: implications of autologous and allogeneic mesenchymal stem cell therapy in allotransplantation. *Stem Cell Rev.* 2014;10(3):351-375.

(APPENDIX Published Paper 1)

1.1 Allotransplantation

Allograft transplantation (allotransplantation) is a term referring to the transplantation of cells, tissues or organs between genetically dissimilar individuals. Kidney transplantation has become a standard therapy to treat patients in end-stage renal failure and in Type 1 diabetic mellitus patients; allogeneic islet transplantation provides an option for the treatment of diabetes. The high incidence of allograft rejection however is a major obstacle that limits the long-term survival of allograft and the success rate of transplantation⁵. Immunosuppressive drugs that target T cells which are key effector cells mediating graft rejection, are effective at prolonging graft survival. However, the life-long administration of these non-specific drugs poses detrimental consequences such as malignancy, opportunistic infection and organ toxicity⁶⁻⁸. Thus, strategies to develop novel therapeutic regimes that promote allograft survival and reduce or avoid the life-long dependence on immunosuppressive drugs are desirable.

1.2 Type 1 Diabetes and allogeneic islet transplantation

Type 1 Diabetes (T1D), also known as juvenile diabetes is a chronic autoimmune disease characterised by the gradual destruction of the insulin-producing beta (β)-cells in the pancreatic islet of Langerhans by autoreactive $CD4^+$ and $CD8^+$ T cells. The destruction of insulin-secreting β -cells leads to impaired glucose homeostasis and hyperglycaemia⁹. Islet allograft transplantation is a treatment to improve glycaemic control and cure T1D in patients. The Edmonton protocol is a method to transplant islets derived from a cadaveric non-diabetic donor into the portal vein of a T1D recipient. Similar to solid organ transplantation, islet allotransplantation necessitates the simultaneous administration of immunosuppressive regimes to avoid graft rejection.

1.3 Allograft rejection

Allograft rejection limits long-term patient and graft survival in clinical transplantation. Allograft rejection is primarily caused by cell-mediated antigen specific immune response to the graft. Antigen presenting cells (APC) such as immature dendritic cells (imDC) endocytose and process alloantigens, whilst undergoing maturation into mature DC in response to proinflammatory signals, allowing for efficient presentation of alloantigens¹⁰. Upon maturation, tissue resident DC migrate in response to chemokines towards T cell regions in the secondary lymphoid tissues (lymph node and spleen) where they activate naive T cells^{11,12}. The activation of antigen-specific T cells by APC initiates a donor immune response towards the allograft.

During the sensitization stage, recipient T cell receptors (TCR) on $CD4^+$ or $CD8^+$ T cells recognise foreign donor alloantigens presented on major histocompatibility complex (MHC) molecules on mature DC via distinct three pathways: **(a)** direct, **(b)** indirect or **(c)** semi-

direct pathway of allorecognition (**Figure 1.3.1**). In **(a) direct** allorecognition, recipient CD4⁺ or CD8⁺ T cells recognise intact donor antigens presented by mature donor DC on MHC class II or I, respectively. This direct pathway of allorecognition is generally associated with acute allograft rejection and occurs early after allotransplantation (**Figure 1.3.1**). The **(b) indirect** pathway of allorecognition is associated with chronic rejection and usually dominates in the later form of alloresponses post-transplantation. In this pathway, recipient DC internalises exogenous donor MHC or non-MHC proteins and process these proteins into peptides. These donor peptides are presented on recipient DC MHC molecules to recipient CD4⁺ or CD8⁺ T cells. Therefore, the indirect pathway of T cell allorecognition can occur even in the absence of donor DC or donor-derived cells that lack antigen presentation ability^{13,14} (**Figure 1.3.1**). The **(c) semi-direct** pathway of T cell allorecognition involves the acquisition of intact donor MHC-peptide complexes by recipient DC through direct-cell-to-cell contact or extracellular exosomes (microvesicles) containing donor MHC-peptide complexes. Similar to the indirect pathway of allorecognition, semi-direct T cell allorecognition could occur in the absence of donor-derived cells that lack antigen-presenting function. In these pathways, recipient TCR recognises donor peptides present on self-MHC molecules, therefore bypassing the requirement of donor-derived APC^{15,16} (**Figure 1.3.1**). Thus, in these pathways, the interaction of alloantigens on MHC molecules with recipient T-cell receptor complex (TCR-CD3) activates the Ras/MAPK (mitogen-activated protein kinase) signaling pathway in T cells. This is the first signal for T cell activation.

In addition to alloantigen presentation, T cells require co-stimulatory signals (signal 2) and cytokines (signal 3) for full naive T cell activation. Mature DC express co-stimulatory molecules B7 (CD80, CD86) and CD40 that interact with CD28 and CD40-ligand (CD154) on T cells respectively^{14,17,18}. DC-T cell interaction upregulates early T cell activation marker CD69 and increases the interleukin-2 (IL-2) receptor (CD25) expression on T cells¹². The

secretion of proinflammatory cytokines IL-12 by DC as well as interferon-gamma (IFN- γ) and IL-2 by T cells also provide additional signals that drive a T-helper 1 (Th1) immune response¹². The signals for T-cell activation are illustrated in **Figure 1.3.2**. Once activated, T cells proliferate and differentiate into donor-reactive effector and memory T cells. Immature DC that lack co-stimulatory signals are inefficient at priming a T cell immune response despite antigen-MHC interaction. TCR activation in the absence of co-stimulatory signals results in a state of T cell unresponsiveness, known as T cell anergy^{13,14,19,20}. Major pathways in which donor antigens are recognised by recipient T-, B- and NK cells following allogeneic transplantation are illustrated in **Figure 1.3.1**.

During the effector phase of cell-mediated allograft rejection (CMR), activated donor-reactive effector CD4⁺ and CD8⁺ T cells infiltrate the graft, resulting in tissue destruction. Allospecific CD4⁺ T cells are critical in initiating graft rejection. These cells are involved in the secretion of proinflammatory cytokines (IL-2) in an autocrine and paracrine manner to stimulate their own proliferation and to activate CD8⁺ T cells, respectively¹⁴. CD8⁺ T cells (cytotoxic T cells, CTL) are involved in lysis and destruction of the graft via the release of cytotoxic granule components such as perforin, granzyme B and granulysin. This process also induces graft apoptosis via cell-to-cell contact mechanisms involving the interaction of FAS (on target cells) and FAS-ligand (FAS-L) (on CD8⁺ T cells)²¹. Furthermore, alloactivated T cells secrete proinflammatory cytokines IFN- γ and tumor necrosis factor alpha (TNF- α) to activate innate immune cells such as monocytes and macrophages, further amplifying local inflammatory responses that also contribute to allograft injury and rejection²¹.

Allospecific CD4⁺ T cells are also known to contribute to B cell maturation, differentiation and alloantibody production which result in antibody-mediated allograft rejection (AMR)²¹. Alloantibodies produced by B cells are involved in complement pathway activation. Complement components (C3a, C4d, C5a and C5b) further enhance intragraft

recruitment and activation of innate immune cells, causing allograft injury and rejection (late allograft loss)^{21,22}. Apart from alloantibody production, B cells can also function as APC and activate allospecific T cell responses, thereby promoting CMR. B cells acquire donor proteins through B-cell receptors, process these proteins into peptides and present these donor peptides on MHC molecules to recipient T cells (**Figure 1.3.1**)^{22,23}.

The immunological memory response is also critical in determining the fate of the allograft following transplantation. High titres of pre-existing alloantibodies have been shown to mediate hyperacute AMR through complement activation. In addition, the presence of antigen-specific memory T or B cells following primary antigen exposure can result in accelerated allograft rejection upon re-exposure to the same antigen. Upon secondary exposure to donor antigens, antigen-specific memory cells respond more rapidly than naive cells. Memory T cells for example require less activating and co-stimulatory signals to mediate proliferation, cytokine secretion and effector functions of T cells, ultimately resulting in hyperacute (accelerated) CMR^{22,24}. Therefore, donor antigen-specific effector and memory cellular- or antibody-mediated alloimmune responses result in hyperacute, acute or chronic phase allograft rejection. Modulating T cell or B cell allorecognition of donor peptides can thus prevent allograft rejection and promote long-term allograft survival.

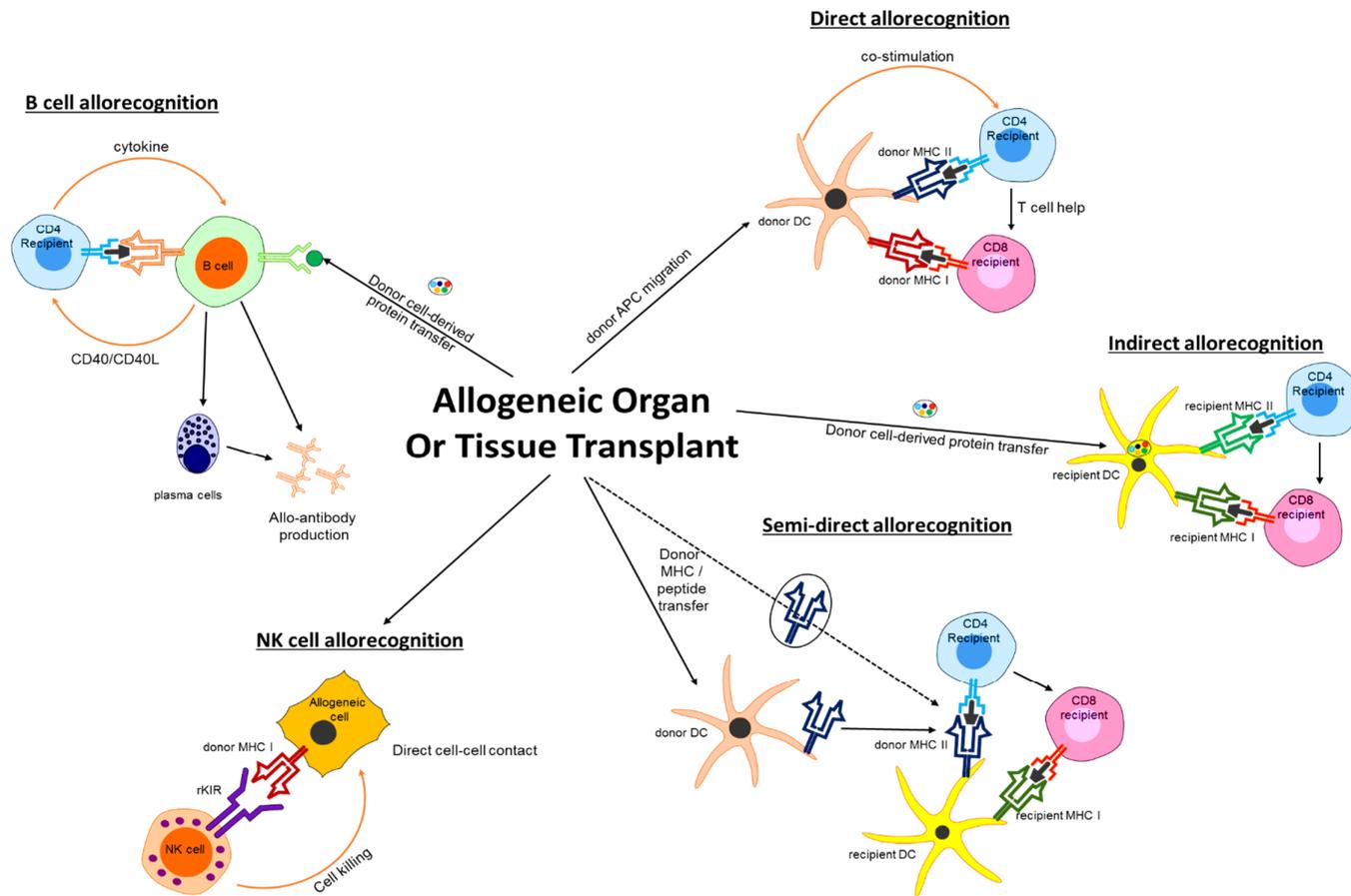


Figure 1.3.1 Pathways of allorecognition.

DC, dendritic cells; MHC, major histocompatibility complex; rKIR, recipient killer-cell immunoglobulin-like receptor. *Adapted from Griffin et al., (2013)¹.*

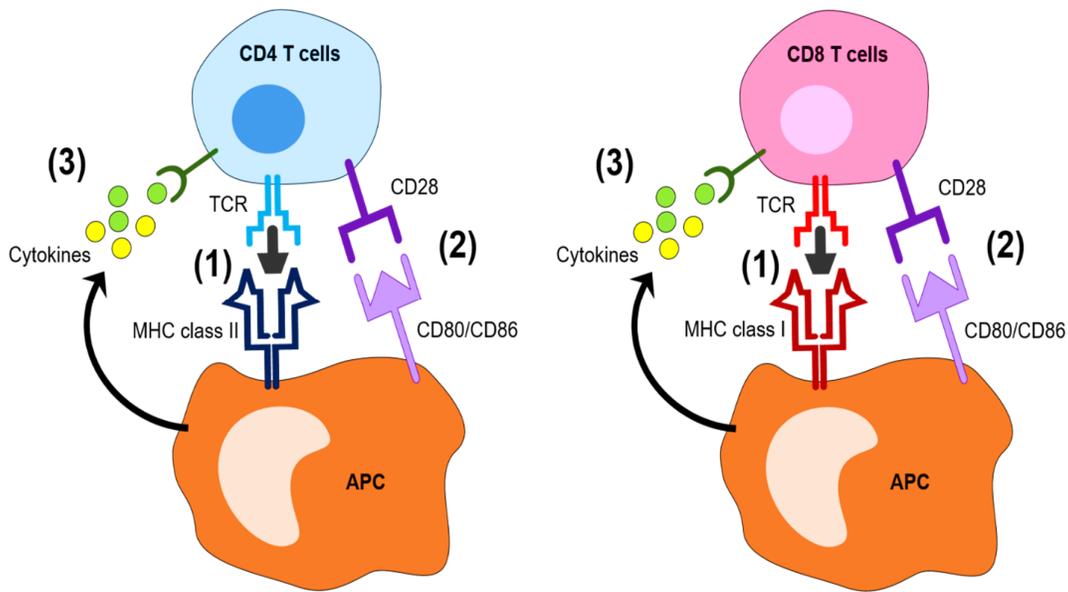


Figure 1.3.2 The three distinct signals for T cells activation.

Dendritic cells (DC) are potent antigen-presenting cells (APC) which initiate and modulate immune responses, in an antigen-specific manner. Mature DC express high levels of MHC class I or MHC class II, co-stimulatory molecules and produce cytokines that prime the activation of T cells²⁵. The first signal for T cell activation (1) is delivered via the interaction of T-cell receptor (TCR) and MHC molecules on DC. Interaction of co-stimulatory molecules CD28 and CD80/CD86 provides the second signal (2) for T cell activation. The third signal (3) involves release of cytokines mainly interleukin-12 (IL-12) and IL-2 from DC and T cells, respectively. Hence, T cell proliferation can be induced via mimicking these signals for T cell activation in presence of anti-CD3 and anti-CD28 mAb (mimic 1st signal), mitogen stimulation such as phytohemagglutinin (PHA), concavalin A (ConA) (mimic 2nd signal for activation) and also by using anti-IL-2 (mimic 3rd signal).

1.4 Immunosuppression

Systemic immunosuppressive drug regimes that target T cells are effective at prolonging graft survival but do not provide antigen-specific suppression of immune response and result in global immunosuppression. According to the Edmonton protocol, islet recipients receive induction therapy with Daclizumab (an anti-IL-2 receptor monoclonal antibody) and maintenance with Sirolimus (an mTOR inhibitor; inhibits T cell response to IL-2) and low-dose Tacrolimus (a calcineurin inhibitor; inhibits IL-2 production by T cells) to avoid islet allotransplantation rejection. The life-long administration of these non-specific drugs causes detrimental consequences such as malignancy, opportunistic infections and organ toxicity⁶⁻⁸, islet cell damage²⁶ and graft dysfunction²⁷. Furthermore, although these drug therapies are effective at preventing acute rejection, they do not ultimately prevent the occurrence of chronic allograft rejection and hence, graft loss²⁸. The applicability of allogeneic islet transplantation is therefore limited due to long-term graft dysfunction and immunosuppressive side effects, both of which consequently result in the recurrence of T1D. Thus, novel therapeutic strategies that promote allograft tolerance without ongoing use of immunosuppression are desirable. Strategies to control T cell responses can ultimately inhibit cell- and antibody-mediated allograft rejection.

1.5 Allotransplantation tolerance

Allotransplantation tolerance is defined as the long term acceptance and survival of the allograft without ongoing use of immunosuppressive drugs²⁹. This process, defined as immune tolerance was first proven experimentally by Medawar in 1953, where he demonstrated sustained alloantigen-specific unresponsiveness in the absence of chronic immunosuppression³⁰. Since then, the “Holy Grail” of transplantation is to achieve allotransplantation tolerance. Transplantation tolerance can be achieved by co-stimulatory

blockade to induce T cell anergy³¹, the deletion of donor alloantigen-reactive T cells³² and in recent years, the progress of cellular immunotherapy has paved the way for donor-specific immune regulation in transplantation³³⁻³⁵.

1.6 Cellular immunotherapy in allograft transplantation

Cell therapy is defined as the transplantation of cells developed for the purpose of restoring the function of diseased or dysfunctional cells of tissues. The application of cell-based medicinal products has emerged to be a promising way to establish immunoregulatory responses and an alternative to the current immunosuppressive drug regimens to control allotransplant rejection³⁶. There are currently several immunoregulatory cells that are being extensively studied for their therapeutic roles in preclinical and clinical trials of transplantation. These include regulatory macrophages (Mregs), myeloid derived suppressor cells (MDSC), regulatory B cells, tolerogenic DC, regulatory T cells (Tregs) and mesenchymal stem cells (MSC)^{34,36,37}. For the purpose of this thesis, Tregs and MSC cellular therapy will be discussed in detail in the following sections.

1.7 Regulatory T cells (Tregs)

Regulatory T cells (Tregs) have a central role in the maintenance of peripheral tolerance, preventing autoimmunity and controlling chronic inflammatory responses, hence maintaining normal immune homeostasis³⁸. Treg therapy has the potential to develop and function as an immunosuppressive cell therapy to promote long-term allograft function and transplantation tolerance^{36,39}. *Ex vivo* expanded Tregs were first administered as a cellular therapy to treat patients with graft-versus host disease (GvHD), a major and lethal transplant complication in patients who have undergone haematopoietic stem cell transplantation⁴⁰. Adoptive transfer of Tregs alleviated symptoms of GvHD and allowed reduction in pharmacological

immunosuppressants in these patients⁴⁰. Currently, Tregs are being investigated as an immunoregulatory cell type in clinical trials of kidney transplantation (<http://www.onestudy.org/>). The ONE study examines the potential to use both polyclonal expanded⁴¹⁻⁴⁴ and antigen-specific^{37,45,46} Tregs to prevent and treat kidney transplantation rejection in allotransplant recipients.

1.7.1 Tregs history, development and subsets

The presence of a naturally occurring thymic-derived suppressor T cell population was first described by Gershon and Kondo in 1970⁴⁷. In 1985, Sakaguchi provided direct evidence on the involvement of suppressor T cells in the induction of natural self-tolerance in mice and deficiency of these T cell subsets resulted in organ-specific autoimmune disease⁴⁸. A decade later, the pivotal discovery of Tregs was made by Sakaguchi⁴⁹. Adoptive transfer of activated CD4⁺ T cells expressing the IL-2 receptor alpha-chains (CD25) into athymic nude mice (T cell deficient mice) controlled immune response to self-antigens, suggesting the crucial role of these cells in maintaining immunological self-tolerance⁴⁹. These CD4⁺ CD25⁺ T cells not only mitigated autoimmune disease manifestation (immune response to self-antigens) but were able to suppress immune responses to non-self-antigens in mice transplanted with allografts or immunized with xenogeneic proteins^{49,50}. These naturally occurring thymic-derived Tregs (nTregs), classically characterised as CD4⁺CD25⁺ T cells constitute 5-10% of peripheral T cells and were able to suppress *in vitro* naïve T cell proliferation in an antigen-specific and non-specific manner⁵¹. The existence of a regulatory T cell population in humans was later confirmed in 2001⁵²⁻⁵⁷. Similar to mouse CD4⁺CD25⁺ Tregs as discussed in the preceding text, human Tregs confer *in vitro* antigen-specific and non-specific suppressive properties on activated effector and memory T cells⁵²⁻⁵⁷.

Forkhead box P3 (*Foxp3*) is a gene that encodes for the winged helix/forkhead family of transcription factors (Fox protein, scurfin) that is highly conserved in humans. The *Foxp3* gene product, scurfin (Foxp3 protein) is critical in the regulation of T cell activation and normal immune homeostasis⁵⁸⁻⁶⁰. Spontaneous mutations in the *Foxp3* gene were first shown to cause severe immune dysregulation in scurfy mice, an X-linked recessive mouse mutant with lethal lymphoproliferative disease that resulted in death by 4 weeks of age^{58,61,62}. The role of FoxP3 in controlling immune dysregulation was also shown in patients with IPEX (Immunodysregulation, polyendocrinopathy, enteropathy X-linked) syndrome, a disease characterised by immune dysfunction, polyendocrinopathy, enteropathy and a variety of autoimmune phenomena including allergy, thyroiditis and type 1 diabetes⁶¹⁻⁶⁵. In 2003, FoxP3 was shown to be the master regulator gene for Treg development and function⁶⁶⁻⁶⁸. Naïve CD4⁺ CD25⁻ T cells transfected with the *FoxP3* gene acquired a Treg cell phenotype and have suppressive function *in vitro* and *in vivo*, therefore confirming the role of FoxP3 in the development and function of Tregs^{66,67}.

Since the discovery of Foxp3 as the master regulator of Tregs, Tregs have been redefined as CD4⁺CD25⁺FoxP3⁺ regulatory T cells. Although CD25⁺ is a marker to characterise Tregs, activated T cells also express CD25 and hence, it was difficult to distinguish activated effector T cells and Tregs in humans⁶⁹. Baecher-Allan and colleagues reported that human peripheral CD4⁺CD25⁺ T cells comprise T cells that are CD25^{low} non-regulatory T cells and CD25^{high} regulatory T cells. CD4⁺CD25^{high} Tregs were able to potently suppress activated T cell proliferation and cytokine secretion and contained a more pure Treg population as to CD4⁺CD25^{low} non-suppressive T cells⁵³. In a human setting, FoxP3 is not only expressed in CD4⁺ Tregs but is also transiently upregulated in activated effector T cells (non-suppressive)⁷⁰ as well as in CD4⁺CD25^{low}, CD4⁺CD25⁻ and CD8⁺ T cells⁷¹⁻⁷³, thereby making it difficult for Treg immunophenotyping.

In addition, FoxP3 is an intracellular protein and cannot be used to isolate human Tregs for further expansion and functional studies. CD127, the alpha-chain of the IL-7 receptor, was later identified as a novel biomarker for human Tregs^{69,74}. These studies demonstrated that CD127 inversely correlated with FoxP3 expression and suppressor function of human CD4⁺ Tregs as well as enabling the distinction between Tregs and conventional T cells^{69,74}. Collectively, these studies described the selective enrichment of Tregs for functional studies, and has been used as a biomarker to isolate Tregs for *in vivo* patient therapy⁴⁰. CD4⁺CD25^{high}CD127^{low/-} FoxP3⁺ to date are used as standard markers for Treg immunophenotyping.

Early discovery of Tregs was centred on the existence of polyclonal thymic-derived naturally occurring Tregs (nTregs) that constitutively express FoxP3 in the periphery. During the thymic development process, FoxP3⁺ thymocytes can be detected in late double positive (CD4⁺CD8⁺) and single positive (CD4⁺CD8⁻) thymocytes⁷⁵. nTregs are developed from single positive thymocytes with higher affinity TCR interaction to thymic MHC/self-antigens while thymocytes with low affinity TCR/self-MHC-self-peptide ligands develop into CD4⁺ conventional T cells (central tolerance). nTregs that are developed intra-thymically are then delivered into the periphery to control immune responses when they encounter a specific self or foreign antigen^{38,76}. However, it was later known that Tregs can also be induced in the periphery from naïve T cells as a result of antigen-specific stimulation; known as induced Tregs (iTregs)⁷⁷. It was shown *in vitro* that CD4⁺CD25⁻ T cells upon T cell receptor (TCR) stimulation generate 2 populations: (1) CD4⁺CD25⁺ T effector cells and (2) CD4⁺CD25⁺FoxP3⁺ inducible (i)Tregs⁷⁷. These iTreg have suppressive activity, thereby suggesting that the generation of a peripheral induced Treg population is a normal consequence of immune activation and are functionally similar to nTregs⁷⁷. Peripheral iTregs can further be characterised into Tr1 (FoxP3⁻ IL-10-secreting iTregs), Tr3 (transforming growth factors-beta,

TGF- β -expressing iTregs) and iTreg (FoxP3⁺ iTregs) based on their mechanisms of immunosuppression⁷⁸. Although there are several other regulatory T cell subsets that have been identified to date, this thesis will focus on the FoxP3 expressing CD4⁺ Treg subsets: nTregs and iTregs.

In summary, Tregs have a role in maintenance of self-tolerance and immune homeostasis. Human Tregs are standardly defined as CD4⁺ CD25^{high}CD127^{low}FoxP3⁺ T cells and can be categorised into 2 subtypes: **(1)** thymus-derived naturally occurring Treg (nTreg) and **(2)** peripheral-derived inducible Treg (iTreg) that are generated from CD4⁺CD25⁻ T cells upon encountering foreign antigens. Both Treg subtypes confer suppressive activities due to their expression of regulatory cytokines and suppression of immune cell activation and proliferation⁷⁹. In the subsequent section, the basic mechanisms by which Treg mediate immunosuppression will be discussed.

1.7.2 Mechanisms of Treg immunosuppression

Attempts to distinguish human nTregs from iTregs isolated from the periphery remain a topic of debate. Nevertheless, the general methods by which Treg mediate suppression of target cells can be broadly categorised into 4 basic mechanisms: **(1)** metabolic disruption of targeted effector T cells; **(2)** Tregs derived inhibitory cytokines; **(3)** targeted cell killing (cytolysis); and **(4)** indirect T cell suppression by targeting dendritic cells maturation and function. This will be discussed in this section, irrespective of nTregs and iTregs.

(1) Metabolic disruption of targeted effector T cells:

As summarized in **Figure 1.3.2**, IL-2 is long known to be essential for effector T cell activation, proliferation and homeostasis. IL-2 binds to the IL-2 receptor (IL-2R or CD25) that consists of α -, β and γ -chain subunits⁸⁰. Mice deficient of IL-2R β developed fatal autoimmune disease due

to absence of Tregs, a condition reversible through thymic targeted expression of IL-2R β in IL-2R $\beta^{-/-}$ mice⁸¹ or by adoptive transfer of normal Tregs into IL-2R β -deficient neonatal mice⁸². These studies and others suggest the importance of the IL-2/ IL-2R signaling for Treg development, function, expansion and homeostasis⁸¹⁻⁸⁴. During Treg development, IL-2 also induces the expression of CD25 and increases FoxP3 expression in Tregs^{75,84}. Treg do not produce IL-2 but express higher levels of high-affinity CD25 (CD25^{high}) than effector cells for their survival. Tregs rely on the production of IL-2 by activated T cells. Some studies showed that in co-cultures of Tregs and effector cells, there were reduced levels of IL-2 in culture supernatants^{51,85}. This observation was a result of Treg-consumption of IL-2 instead of a decrease in IL-2 production by effector T cells⁸⁵. Thus, Tregs induce cytokine (IL-2)-deprivation-mediated apoptosis of effector T cells as one mechanism Tregs inhibit effector T cell responses⁸⁵.

Tregs also generate adenosine with immunoregulatory activity through the concordant expression of two enzymes: ecto-ATP diphosphohydrolase (**CD39**) and ecto-5'-nucleotidase **CD73** enzymes that hydrolyses extracellular nucleotides into adenosine nucleosides. Specifically, CD39 converts the ATP and ADP to 5'-AMP while CD73 catalyses 5'-AMP into adenosine nucleosides⁸⁶⁻⁸⁹. Proinflammatory ATP is known to drive the secretion of IL-17 by effector T cells. CD39 via cell-contact dependent mechanisms depletes ATP leading to the suppression of IL-17 secretion by activated T cells⁸⁹. The anti-inflammatory effect of adenosine is mediated through the adenosine A2A receptor (A2aR) expressed on activated effector T cells^{86,87}. The adenosine/A2aR receptor interaction is known to result in the suppression of T cell proliferation and cytokine production (eg. IFN- γ , IL-2, TNF- α) and was shown to be critical in preventing allograft rejection *in vivo*^{86-88,90,91}. Mechanistically, the engagement of adenosine with A2aR inhibits NF- κ B activation in responder T cells thereby reducing the expression of Th1 and Th2 cytokines as well as T cell chemotactic factors⁹¹. In addition to

effector T cell immunosuppression, CD39/CD73 have a role in the induction of peripheral iTregs. Adenosine/A2aR signaling inhibits Th1 and Th17 cell differentiation and favours the generation of FoxP3⁺ iTregs⁹⁰. iTreg generation was mediated by the inhibition of IL-6 and the expression of the Tregs promoting factor TGF- β ⁹⁰. Therefore, Treg-derived adenosine has a role in suppression of effector T cell proliferation and cytokine secretion as well as promoting the induction of iTregs.

An alternative method of Treg immunosuppression is through cyclic adenosine monophosphate (**cAMP**; another adenosine nucleoside). Tregs express high levels of cAMP and transfers cAMP to conventional T cells via cell-contact dependent gap junctions formation⁹². High levels of cAMP in effector T cells cause nuclear translocation of inducible cAMP early repressor (ICER) and cAMP response element modulator (CREM) in conventional T cells. The ICER/CREM complex then binds to the *IL-2* gene promoter, directly resulting in the transcription repression of IL-2 synthesis in responder T cells^{92,93}.

(2) Tregs derived inhibitory cytokines:

The expression of Treg-derived inhibitory cytokines including **IL-10**, **TGF- β 1** and **IL-35** is known to mediate Treg suppressor function. The role of IL-10 (CD4⁺FoxP3⁺ Tregs) and (or) TGF- β 1 in Tregs immunosuppressive function were shown in models of autoimmune diseases⁹⁴, transplantation rejection^{95,96}, allergy and asthma^{97,98} as well as in colitis⁹⁹. The adoptive transfer of IL-10 deficient (IL-10^{-/-}) Tregs or the neutralization of IL-10 and (or) TGF- β *in vivo* were shown to abrogate the inhibitory effects of Tregs in inflammatory diseases^{95-97,100}. In a humanized islet xenograft rejection model for example, adoptive transfer of *ex vivo* expanded Tregs suppressed graft rejection through IL-10 expression⁹⁶. Treg expressed IL-10 impaired T cell effector function and inhibited intragraft infiltration of conventional T cells⁹⁶. IL-10 producing Tregs were also shown to suppress T cell responses by

inhibiting DC maturation and thereby altering DC functional ability to prime T cell proliferation^{101,102}.

TGF- β has an essential role in the induction of Tregs from conventional naïve T cells through *de novo* FoxP3 expression in T cells¹⁰³⁻¹⁰⁵. TGF- β mediated induction of FoxP3 in Tregs is dependent on early CTLA-4 expression on T cells following their activation^{101,103}. TGF- β is also involved in the maintenance of Treg suppressor function and homeostasis by the sustained FoxP3 expression in both nTregs and iTregs¹⁰⁵. In addition, TGF- β is involved in Treg mediated suppression of T cell proliferation and antibody production by B cells¹⁰⁶. There are however studies suggesting that Treg suppression can occur independent of TGF- β and (or) IL-10^{77,89,107}. Although IL-10 and (or) TGF- β were required for *ex vivo* generation of suppressive Tregs, endogenous production of these anti-inflammatory cytokines was not required for Treg immunosuppression *in vitro* and *in vivo* as shown in a model of GvHD¹⁰⁷.

Apart from the expression of soluble TGF- β , membrane bound TGF- β that consist a mature form of TGF- β encased with the latent-associated peptide (**LAP**) can mediate Treg immunosuppression^{106,108}. Tregs express 2 forms of TGF- β : **(1)** latent (inactive) TGF- β that is bound to LAP and **(2)** active TGF- β (secreted TGF- β)¹⁰⁶. The latent TGF- β / LAP complex is anchored to Treg cell-surface by the leucine-rich repeat transmembrane molecule, **GARP** (glycoprotein A repetitions predominant, LLRC32, Garpin)¹⁰⁹⁻¹¹². GARP and LAP are co-expressed on activated but not resting Tregs^{109,110,112}. Therefore, latent TGF- β binding to Treg cell-surface by GARP may be a mechanism by which Tregs activate TGF- β to mediate their immunosuppression on effector T cells upon TCR stimulation¹⁰⁹. In transwell assays, Tregs were shown to require cell-cell contact to mediate their inhibitory effects on effector T cells via TGF- β ^{102,106}, suggesting the involvement of the membrane-bound TGF- β in Tregs mode of suppression. GARP not only tethers TGF- β to Treg cell surface but is also involved in retaining a Treg phenotype through the sustained expression of FoxP3 in activated Tregs^{111,112}. In a

cardiac allograft rejection model, there was evidence of high numbers of TGF- β secreting cells in the allografts consistent with the increased intragraft infiltration of LAP⁺FoxP3⁺ Tregs suggesting the potential role of LAP in *in vivo* Treg immune suppression⁹⁵.

IL-35, a heterodimer of the Epstein Bar Virus induced gene 3 (EBI3) and the IL-12 subunit p35, is another anti-inflammatory cytokine that has been implicated in Treg immunosuppressive function¹¹³⁻¹¹⁵. Murine Tregs but not effector T cells express IL-35. Tregs deficient of IL-35 (IL-35^{-/-}) failed to suppress *in vitro* effector T cell proliferation and cure inflammatory bowel disease *in vivo*¹¹⁴, suggesting the role of this cytokine in Treg inhibitory function. In addition, IL-35 was able to expand murine Tregs *in vitro* and suppress Th17 cell development *in vitro* and *in vivo*, thereby representing a novel anti-inflammatory cytokine in the modulation of inflammatory responses in collagen-induced arthritis¹¹³. It should be noted that human Tregs do not constitutively express IL-35¹¹⁶. Exogenous IL-35 however was able to induce human conventional T cells to acquire a regulatory phenotype in the absence of FoxP3 expression and mediate *in vitro* and *in vivo* suppression via IL-35 dependent mechanisms¹¹⁵.

(3) Targeted cell killing (cytolysis)

The **granzyme/perforin** pathway is commonly used by NK cells and cytotoxic CD8⁺ T cells to mediate cytolysis of viral infected and tumour cells¹¹⁷. Activated but not naïve Tregs express granzymes and perforin to mediate their cytotoxic effects on target cells via cell-contact interactions¹¹⁸⁻¹²¹. Human nTregs and iTregs preferentially express Granzyme A and Granzyme B, respectively, resulting in cytolysis of CD4⁺ T cells, CD8⁺ T cells, monocytes, immature DC and mature DC via perforin and CD18 dependent but FAS-FASL independent pathways¹¹⁸. Murine Tregs also mediate apoptosis of effector T cells by granzyme B perforin-independent¹¹⁹ or perforin-dependent pathways¹²¹. Gondek *et. al.*, 2005 additionally showed

that granzyme B dependent suppression of effector T cells by Tregs required the glucocorticoid-induced tumour necrosis factor receptor-related protein (**GITR**)¹¹⁹. GITR is a member of the TNF receptor superfamily and is another cell-surface molecule that can identify activated and functionally suppressive Tregs¹²². In this study, the upregulation of granzyme B in Tregs was regulated by GITR and the neutralization of this cell-surface molecule reversed Treg suppression and apoptosis of effector T cells¹¹⁹. In addition, granzyme B expressed by murine Tregs was shown to cause apoptosis of B cells¹²⁰. Tregs were also able to potently lyse NK cells, CD8+ T cell and DC, promoted allograft tolerance and prevented GvHD *in vivo*, thereby confirming the role of granzyme and (or) perforin as mechanisms by which Tregs mediate their cytotoxic effect^{121,123-125}.

(4) Indirect T cell suppression by targeting dendritic cells maturation and function

Tregs can indirectly exert their immunosuppressive effects on effector T cells by modulating DC. Tregs form cell-contact interactions with DC¹²⁶⁻¹²⁸ to induce DC to express immunoregulatory molecules^{129,130} as well as altering DC maturation and naïve T cell allostimulatory function^{126-128,131-135}.

Tregs can also induce DC to express immunoregulatory molecules (cell-contact or soluble factors) to indirectly mediate suppression of effector T cells¹³⁶. **CTLA-4** is a co-inhibitory molecule expressed on activated T cells that binds to B7 (CD80/CD86) co-stimulatory molecules on DC, delivering negative signals that inhibit T cell responses¹³⁷. Tregs constitutively express CTLA-4, a molecule upregulated upon Treg activation¹²⁹. CTLA-4 and B7 signaling induces the generation of IFN- γ and IDO in DC¹²⁹. The induction and secretion of IFN- γ in DC and activated Tregs, respectively, acts in a positive feedback loop to further enhance DC-derived IDO. IDO catabolises tryptophan, leading to the production of immunosuppressive kynurenine that inhibits T cells^{129,138}. In addition to the effector T cell

inhibition, IDO can promote Treg expansion, amplifying Treg immunosuppression responses^{130,139}.

Treg inhibits DC maturation evident by the downregulation of CD80 and CD86 by DC^{128,131,133,135}. The downmodulation of CD80/CD86 in DC was dependent on CTLA-4 expressed by Tregs¹³⁴. In another study, Treg were shown to form aggregates with DC and required both CTLA-4 and lymphocyte function-associated antigen 1, LFA-1 (CD11a and CD18) to inhibit DC maturation¹²⁸. Subsequently, inhibition of DC maturation by Tregs results in the decreased ability of DC to prime naïve T cell activation and proliferation^{133,135}. LAG-3 (lymphocyte-activation gene 3), a CD4 homologue expressed on Tregs was reported to bind to MHC class II expressed on DC, causing the inhibition of DC maturation (CD80) and allostimulatory function¹³⁵. The ICOS⁺FoxP3⁺ Treg population is dependent on the secretion of IL-10 to suppress DC maturation and function¹⁰².

(5) Other cell –surface Treg associated immunosuppressive molecules

CTLA-4 – a negative regulator of T cells

As discussed in the preceding text, the T cell co-inhibitory molecule CTLA-4 expressed on Tregs can act in multiple pathways to mediate Treg immunosuppression. CTLA-4 is required for TGF- β induction of FoxP3 in Tregs¹⁰¹, modulation of DC maturation¹³⁴ and induction of secretion IDO by DC¹³⁰. DC-derived IDO amplifies Treg suppression of effector cells and promote Treg expansion¹³⁹. Treg specific CTLA-4 deficiency cause systemic lymphoproliferation, developed fatal T cell mediated autoimmune disease and hyperproduction of IgE in CTLA-4 knockout mice¹⁴⁰. These CTLA-4 deficient Tregs developed and survived normally but were functionally defective as they failed to inhibit responder T cell proliferation and down-regulate CD80/CD86 on DC *in vitro* and *in vivo*¹⁴⁰. CTLA-4 is therefore a critical

molecular target to control Treg immunosuppression function mainly via the modulation of APC.

TNF-TNFR superfamily of proteins: (GITR and OX40)

GITR and OX40 are members of the tumour necrosis-factor receptor (TNFR) superfamily proteins that provide co-stimulatory signals to regulate Treg survival, function and proliferation¹⁴¹. TNFR expression in Tregs is controlled by FoxP3¹⁴². Mahmud et al., in a recent study demonstrated that the TNFR superfamily members GITR, OX40 and TNFR2 were involved in nTreg development. GITR, OX40 and TNFR2 were highly expressed in progenitor and mature Tregs while their cognate ligands, GITR-ligand, OX40-ligand and TNF, respectively, were detected on APC in the thymic medulla. Neutralization of GITR, OX40 and TNFR2 signaling with anti-GITR-ligand, anti-OX40-ligand or combination of these TNFR superfamily molecules strongly inhibited nTreg development and maturation *in vivo*¹⁴³.

GITR is constitutively expressed on activated and functionally suppressive Tregs and modulates effector cell death through the upregulation of granzyme B in Tregs^{119,122} as discussed. In initial studies, GITR was shown to have a direct role in Treg suppression. Blockade of GITR signaling with anti-GITR antibody or the specific depletion of GITR⁺Treg were shown to reverse Treg mediated suppression¹⁴⁴, cause autoimmune disease¹⁴⁵ and enhance anti-tumour immunity leading the rejection of tumours in mice¹⁴⁶. In later studies, GITR signaling was shown to favour Treg homeostasis by promoting Treg expansion and maintenance of their suppressive function¹⁴⁷⁻¹⁴⁹. High-affinity soluble Fc-GITR-ligand in presence of IL-2 and CD3 stimulation (antigen-nonspecific) provided co-stimulatory signals that enhanced Treg proliferation *in vitro* and *in vivo*. GITR-ligand specifically induced the proliferation of Tregs¹⁴⁷. In an antigen-specific context *in vivo*, the expression of GITR-ligand on DC was required in Treg homeostasis as the GITR-GITR-ligand interactions promoted Ag-

specific Treg expansion and maintenance¹⁴⁹. In other studies, GITR⁺Tregs were shown to interact with GITR-ligand on B cells to promote Treg homeostasis, proliferation and suppression of autoimmune response in mice models^{148,150}. Taken together, these studies suggest that GITR is critical for direct Treg mediated suppression of effector cells as well as in the Treg homeostasis.

Tregs constitutively express **OX40** while activation induces OX40 on conventional T cells. OX40 signaling however has distinct roles in conventional T cells and Tregs^{151,152}. OX40 deficient mice have impaired Treg survival due to increased apoptosis of Tregs *in vivo*^{143,151,153} and were incapable of inhibiting anti-CD3 induced effector T cell proliferation *in vitro*¹⁵¹. Griseri et al 2010, additionally showed that anti-OX40L blocking antibody induced activated Treg apoptosis¹⁵³. Takeda et al. demonstrated that Tregs required OX40-OX40L signaling to promote Treg expansion as the blockade of OX40-OX40L interaction using the inhibitory anti-OX40 mAb (MGP34) reversed Treg proliferation¹⁵¹. These studies suggest the critical role of OX40 signaling in Treg survival and homeostasis.

In terms of OX40 in Treg-mediated suppressor function, OX40⁺Tregs were required for Treg-mediated tumour protection¹⁵⁴ and colitis control¹⁵³. Interestingly, the role of OX40-OX40L signaling in Tregs is paradoxical. In mouse models of tumour or GvHD, triggering the OX40 pathway with the agonist anti-OX40 mAb (OX86) abrogated Treg mediated suppression evident by the enhanced anti-tumour immunity and subsequent rejection of tumours¹⁵⁴ as well as reversal of Treg protection of GvHD¹⁵⁵. Valzasina *et. al.*, also showed that OX40 triggering with the agonist anti-OX40 mAb did not revert activated Treg mediated suppression. This study suggests that OX40 triggering has differential effects on naïve and activated Tregs, whereby OX40 signaling only inhibits naïve Treg-immunosuppression¹⁵⁵. On the contrary, in a skin allograft rejection model, the blockade of OX40-OX40L pathway with the anti-OX40 mAb promoted alloreactive Treg survival by increased expression of anti-apoptotic molecules and

Treg proliferation¹⁵². Absence of OX40 signaling also enhanced Treg suppression to prevent rejection and resulted in prolonged skin allograft survival¹⁵².

CD69 – a C-type lectin receptor

CD69, a C-type lectin receptor, was initially known as an early T cell activation marker. Later studies suggest that CD69 has immunoregulatory roles¹⁵⁶ and is constitutively expressed on both iTregs and nTregs¹⁵⁷. CD69-deficient mice developed severe autoimmunity, allergy and colitis¹⁵⁷⁻¹⁵⁹, confirming the *in vivo* regulatory role of CD69. CD69⁺Tregs express higher levels of other Treg associated immunosuppressive molecules including CTLA-4, GITR and TGF- β , consistent with their enhanced suppressor function on effector cells¹⁵⁷. CD69^{-/-} Tregs were incapable of suppressing T cell proliferation and proinflammatory cytokine secretion in addition to decreased secretion of TGF- β , *in vitro* and *in vivo*^{157,159,160}. Consistent with findings from murine Tregs, Tregs derived from patients with systemic sclerosis had reduced CD69 expression by Tregs, with diminished suppressor function and reduced TGF- β production¹⁶¹. Mechanistically, CD69+ functions by phosphorylating STAT5 (signal transducer and activator of transcription 5), a signaling molecule associated with Treg development and function^{157,162}. In an earlier study, disease models that are predominantly Th17 mediated provided a link between CD69 in the balance of Th17 and Treg cells¹⁵⁸. This study also showed that CD69 activates STAT5 to inhibit Th17 cell differentiation¹⁵⁸. Contrary to STAT-5, inhibition of ERK activation in CD69⁺ Tregs was shown to promote Tregs immunosuppression¹⁵⁷.

Apart from the role of CD69 in Treg suppression, this molecule is also required for Treg differentiation. CD4⁺ T cells from CD69-deficient mice had impaired ability to differentiate into FoxP3⁺ Tregs evident by reduced Treg numbers *in vivo* and absence of *in vitro* FoxP3⁺ induction from naïve T cells¹⁵⁹. In another study, human thymic DC were shown to induce nTreg differentiation from Treg progenitor cells that were CD69^{high}CD4⁺CD8⁺

thymocytes, therefore demonstrating a link between CD69 and nTreg differentiation. Taken together, these data suggest that CD69 is essential for Treg differentiation, activation and suppressor function.

CD103 – an integrin α E

CD103, an integrin α E, is another molecule known to mediate Treg suppressive function. CD103 is expressed on activated murine effector/memory-like Tregs and is able to ameliorate inflammatory response in models of chronic GvHD¹⁶³ and antigen-induced arthritis¹⁶⁴. Zhao *et. al.*, (2008), additionally reported in their *in vivo* study that CD103⁺Tregs specially migrate to target tissues potentially due to the high levels of chemokine CCR5. Mechanistically, CD103⁺ prevented ongoing GvHD by inducing apoptosis of autoantibody secreting B cells and splenic-derived donor CD4⁺ effector T cells. These CD103⁺-expressing Tregs also suppressed pathogenic CD4⁺ T cells at GvHD target tissues¹⁶³. However, the functional regulatory role of human CD103⁺ Tregs is not well established and may differ between the human and murine system. In steady state conditions, CD103⁺ has not been detected in humans unlike in mice¹⁶⁵. Nevertheless, exogenous IL-2 and TGF- β can induce or expand CD103⁺ iTregs and nTregs, respectively, in an IL-2-dependent TGF- β pathway¹⁶⁶. Further studies are required to delineate the suppressive function of CD103 in human Tregs.

HLA-DR (MHC class II)

Tregs expressing MHC class II determinants (DR) (MHC II / HLA-DR) identifies another potent suppressive subpopulation of mature and activated Tregs, which represent 20-30% of circulating Tregs^{53,167}. HLA-DR⁺ Tregs exhibited potent *in vitro* suppression of conventional T cell proliferation by early cell-contact dependent mechanisms¹⁶⁸. This observation correlated with the increased FoxP3 gene expression in these Tregs¹⁶⁸. Moreover, monocyte-activated

nTregs upregulated MHC class II and consequently resulted in more potent suppression of effector T cells¹⁶⁷. The blockade of MHC class II in monocyte-activated nTregs abolished Tregs inhibitory function, thereby confirming the pivotal function of HLA-DR in Tregs immunosuppression¹⁶⁷.

Interestingly, recent studies have proved that Tregs can acquire MHC II molecules through a process termed as trogocytosis^{169,170}. Both activated effector T cells and Tregs can acquire peptide-MHC II complexes from APC in direct cell contact conditions¹⁷⁰. In Tregs, Ag presentation via the acquired MHC II complex results in more potent Treg suppression, while effector T cell primes naïve T cell activation (CD25) and proliferation¹⁷⁰. LAG-3 is the ligand for MHC II expressed on conventional T cells¹⁶⁹. Segal *et. al.*, proposed in their study that Tregs acquire host MHC II molecules by trogocytosis to suppress effector T cells by MHC II-LAG-3 engagement models of GvHD¹⁶⁹.

Conclusion on mechanisms of Treg mediated immunosuppression

In summary, Tregs are heterogeneous as they express various markers that define distinct Treg subpopulations. These Treg subpopulations have multifaceted modes of suppression depending on the expression of immunoregulatory molecules. It should be noted that there are other Treg subpopulations and immunosuppressive markers known to mediate Treg inhibitory function that are not discussed in this thesis. The precise mechanism of Treg immunomodulation also differs based on the source of Tregs (nTregs or iTregs), the method of Treg induction, activation and maturation, disease models as well as the vast species differences between human and mice. The role(s) of these Treg markers discussed in this section are summarized in **Table 1.7.2.1**.

Table 1.7.2.1 Role of Treg expressed molecules

Molecule	Role in Tregs
FoxP3	<ul style="list-style-type: none">• Master regulator of Tregs• Required for Treg development, maintenance and function
IL-2	<ul style="list-style-type: none">• Treg development (induces CD25, increases FoxP3), expansion, survival and homeostasis• Treg function: metabolic disruption of effector T cells through IL-2 deprivation mediated apoptosis
CD39 / CD73	<ul style="list-style-type: none">• CD39: convert ATP and ADP → AMP; suppress IL-17 secretion by activated T cells• CD73: convert AMP → adenosine• Generation of anti-inflammatory adenosine:<ul style="list-style-type: none">(a) suppress T cell proliferation(b) inhibit proinflammatory T cell cytokine secretion (eg. IFN-γ, IL-2, TNF-α)(c) decrease expression of T cell chemotactic factors(d) prevent allograft rejection(e) inhibit Th1 and Th17 cell differentiation(f) induction of FoxP3+ iTregs• CD39 / CD73: induce iTregs by promoting TGF-β secretion and inhibiting IL-6
cAMP	<ul style="list-style-type: none">• Inhibit IL-2 production by effector T cells
IL-10	<ul style="list-style-type: none">• Suppress allograft rejection• Impairs T cell effector function, inhibit intragraft infiltration of conventional T cells• Inhibit DC maturation, alters DC function to prime T cell proliferation
Soluble TGF- β	<ul style="list-style-type: none">• active/secreted TGF-β• induction of iTregs and FoxP3 expression in naïve T cells• induction of FoxP3 dependent on early CTLA-4 expression on activated T cells• maintenance of Treg suppressor function and homeostasis by sustained FoxP3• suppress T cell proliferation• inhibit B cell antibody production
Membrane bound TGF- β	<ul style="list-style-type: none">• latent TGF-β / LAP complex anchored to Tregs by GARP• LAP and GARP: co-expressed on activated not resting Tregs• T cell immunosuppression

(continued) Role of Treg expressed molecules

Molecule	Role in Tregs
GARP	<ul style="list-style-type: none">• Anchors TGB-β / LAP complex to Tregs• LAP and GARP: co-expressed on activated not resting Tregs• sustains FoxP3 expression on activated Tregs \rightarrow retaining Treg phenotype
IL-35	<ul style="list-style-type: none">• T cell immunosuppression• Treg expansion• Inhibit Th17 cell development
Granzyme / Perforin	<ul style="list-style-type: none">• expressed on activated but not naïve Tregs• Granzyme: cytolysis of CD4⁺, CD8⁺ T cells, monocytes, immature DC, mature DC via perforin• apoptosis of B cells• suppression of effector T cells required GITR
CTLA-4	<ul style="list-style-type: none">• constitutively expressed by Tregs and upregulated upon Treg activation• mediate TGF-β induction of FoxP3 in Tregs• modulates DC maturation (\downarrow CD80, CD86)• induce IDO secretion by DC (ie. IDO inhibits T cells and promote Treg expansion)
GITR	<ul style="list-style-type: none">• constitutively expressed on activated and functionally suppressive Tregs; expression controlled by FoxP3• role in nTreg development and maturation• highly expressed in progenitor and mature Tregs• Regulation of granzyme B upregulation• regulate Treg survival, function and proliferation• role in Treg function:<ol style="list-style-type: none">1. modulate effector cell death via upregulation of granzyme B in Tregs (ie. suppress T cell effector function via granzyme B)2. Treg homeostasis by promoting Treg expansion and suppressive function maintenance
OX40	<ul style="list-style-type: none">• constitutively expressed on Tregs; highly expressed in progenitor and mature Tregs; expression controlled by FoxP3• role in nTreg development and maturation• regulate Treg survival, function and proliferation and homeostasis

(continued) Role of Treg expressed molecules

Molecule	Role in Tregs
CD69	Constitutively expressed on Tregs <ul style="list-style-type: none">• role in Treg differentiation, activation and suppressor function• Function:<ol style="list-style-type: none">1. in Treg immunosuppression<ul style="list-style-type: none">- CD69⁺ Tregs – express higher levels of Treg suppressive molecules (ie. CTLA-4, GITR and TGF-β)- suppresses T cell proliferation- inhibits proinflammatory cytokine secretion- promotes Treg immunosuppression2. Treg differentiation<ul style="list-style-type: none">- link between CD69 in the balance of Th17 and Tregs- activates STAT5 to inhibit Th17 cell differentiation
CD103	Expressed on activated and memory Tregs <ul style="list-style-type: none">• Treg migration• function:<ul style="list-style-type: none">- induce apoptosis of autoantibody secreting B cells and effector T cells (*role in human Tregs not well defined)
HLA-DR (MHC class II)	Expressed on mature and activated Tregs <ul style="list-style-type: none">• suppression of T cells associated with increased FoxP3 expression• acquisition of MHC II via trogocytosis results in more potent Treg immunosuppression

Abbreviations: FoxP3, forkhead box P3; cAMP, cyclic adenosine monophosphate; TGF- β , transforming growth factors-beta; LAP, latent-associated peptide; GARP, glycoprotein A repetitions predominant, LLRC32, Garpin; CTLA-4, cytotoxic T-lymphocyte associated antigen-4; GITR, glucocorticoid-induced tumour necrosis factor receptor-related protein; HLA-DR, human leukocyte antigen-DR; ATP, adenosine triphosphate; AMP, adenosine monophosphate; ADP, adenosine diphosphate.

1.8 Mesenchymal Stem Cells

In recent years, the wide application of mesenchymal stem cells (MSC) as immune regulatory cells represents a promising approach to modulate T cell responses associated with allograft rejection and potentially induce allotransplantation tolerance. Moreover, the infusion of allogeneic MSC (Prochymal) has been approved in Canada, New Zealand and Japan as the world's first stem cell drug from Osiris Therapeutics for the treatment of paediatric patients with graft-versus-host-disease (GvHD), thus making MSC a potential “off-the-shelf” cell therapeutic product for transplantation¹⁷¹. The advantage of MSC as a cytotherapy in a transplant setting is discussed in section **1.8.1**.

1.8.1 Mesenchymal stem cells in allotransplant setting

Different populations with similar properties ascribed to bone MSC have been identified and propagated from various tissue sources including adipose tissues, dental tissue and umbilical blood. MSC-like populations from different sources are known to respond differentially in an inflammatory milieu and exert different mechanisms of immune modulation¹⁷²⁻¹⁷⁴. In this chapter, the focus is on bone marrow-derived MSC in the context of allotransplantation as they are most extensively studied and used throughout this thesis.

Bone marrow-derived MSC are multipotent non-haematopoietic stem cells that constitute 0.001-0.01% of the adult bone marrow^{175,176}. MSC hold several characteristics that would benefit their application in allotransplantation. MSC can be easily isolated from the bone marrow under minimally invasive procedures and rapidly expanded *ex vivo* prior to MSC administration *in vivo*¹⁷⁷.

A unique characteristic of MSC is their ability to specifically home to sites of inflammation. In a rat model of cardiac allograft transplantation, systemically administered MSC were reported to migrate into the allografts of animals undergoing chronic rejection¹⁷⁸.

MSC homing to site of pathology requires MSC to evade extracellular matrix (ECM) barriers and migrate along a chemokine gradient. Protein expression of MSC detected by Western blot analysis revealed MSC constitutively express matrix-metalloproteinase-2 (MMP-2), membrane-type-1 MMP (MT1-MMP) and tissue-inhibitor of metalloproteinase-2 (TIMP-2). These factors have been associated with MSC invasion capacity across ECM as knock-down of these enzymes abrogated MSC invasion in *in-vitro* cell invasion assays¹⁷⁹. Moreover, MSC have strong expression of the chemokine receptor CXCR4 that potentially drives MSC in response to stromal cell-derived factor-1 (SDF-1) gradient¹⁸⁰. SDF-1 is highly expressed at site of inflammation, thereby enabling MSC to home to the inflammatory milieu of allograft¹⁸¹ and exerting its immunomodulatory effect locally. Unlike immunosuppressive drugs which act more systemically and non-specifically on most immune-cells leading to intense immunosuppression⁶. This migratory characteristic of MSC can be exploited to locally deliver immunomodulatory or reparative molecules to prevent allograft rejection and damage, respectively.

The potential advantages of MSC in allotransplantation are in their functional ability to facilitate tissue repair, their regenerative potential and the immunomodulatory properties of these cells. MSC may elicit tissue repair through paracrine or endocrine secretion of a multitude of cytokines and growth factors such as hepatocyte growth factors (HGF), CXCL12 (stromal derived factor-1), vascular endothelial growth factor (VEGF) and insulin growth factor-1 (IGF-1)¹⁸²⁻¹⁸⁵. These MSC factors have been shown to induce angiogenesis and promote the recruitment of endogenous tissue progenitors.¹⁸⁴ Furthermore, MSC have a wide regenerative capacity with their ability to transdifferentiate into various cell types including neural cells, insulin-producing β -cells, hepatocytes, cardiomyocytes and renal cells with varying efficiencies¹⁸⁶⁻¹⁹¹. The tissue reparative and transdifferentiation function of MSC could potentially enhance their therapeutic value in allotransplantation.

The immunomodulatory activity of MSC on natural killer (NK) cells, macrophages, B cells, T cells and DC has been extensively reported in the literature^{177,192-196}. The modulation of these immune cells by MSC may prevent the augmentation of inflammatory responses involved in allograft rejection. Studies utilizing MSC in preclinical models and in clinical trials of inflammatory diseases such as GvHD^{197,198}, autoimmune disease¹⁹⁹ and allograft rejection²⁰⁰⁻²⁰³ provide evidence of the immunosuppressive function of MSC *in vivo*. The therapeutic efficacy of autologous and “off-the-shelf” (allogeneic or third-party) MSC in preclinical models of allotransplantation is summarized in **Table 1.8.1.1** and **Table 1.8.1.2**, respectively. The application of MSC as anti-rejection agents in clinical trials of solid organ allotransplantation is summarized in **Table 1.8.1.3**. In the following section, we will discuss the current literature on mechanisms by which MSC modulate T cell responses independent of MHC expression by MSC and T cells.

Table 1.8.1.1 Administration of bone marrow-derived autologous MSC in preclinical models of allotransplantation

Reference	Transplant model (species)	Drugs	Route (timing of MSC infusion)	Key findings
Nauta, 2006 ²⁰⁴	Bone marrow (mouse)	No	i.v. (POD 0 or POD 0, +3, +7, +10, +14)	• autoMSC single or 5x infusion enhance long-term engraftment of donor bone marrow by tolerance to donor and recipient antigens
Casiraghi, 2008 ²⁰⁵	Heart – semiallograft/ full mismatch allograft (mouse)	No	Intraportal (POD -7) /i.v (POD -1)	• 2x pre-transplant infusion intraportally (POD -7) and i.v. (POD -1) - prolongs survival of semi-allogeneic heart graft through expansion of Tregs - Fail to prolong fully MHC mismatched cardiac allograft survival
Solari, 2009 ²⁰⁶	Islet – omental pouch (rat)	Yes - CsA (short-term)	Co-transplant with islets	• Promote long-term islet allograft survival and induce normoglycaemic - islet + CsA → 7.13 (mean graft survival days) - Co-transplant islet + autoMSC + CsA → >51.7; ↓ IFN- γ and TNF- α levels produced by Th1 cells; enhance IL-10 secretion by CD4 ⁺ T cells
Ding, 2009 ²⁰⁷	Islet – kidney capsule (Mouse)	No	Co-transplant with islets	• prevent allograft rejection through MMP expression - <i>In vitro</i> – MMP-2, MMP-9 (↓ CD25 on activated T cells) - islet + autoMSC + MMP inhibitor → rejection
Han, 2010 ²⁰⁸	skin (mouse)	No	Adoptive transfer through i.v. (POD -7)	• <i>in vitro</i> : suppress primary and secondary alloimmune responses - inhibit proliferation and IFN- γ production of naive responder T cells and memory T cells • <i>in vivo</i> : fail to prolong allograft survival in major and minor mismatch barriers <i>in vivo</i> although demonstrate suppression in secondary alloimmune responses <i>in vitro</i>
Longoni, 2010 ²⁰⁰	Portal vein islet (rat)	No	i.v. (POD 0, +2, +4)	• prevent acute rejection and maintain glucose homeostasis

(continued) Administration of bone marrow-derived autologous MSC in preclinical models of allotransplantation

Reference	Transplant model (species)	Drugs	Route (timing of MSC infusion)	Key findings	
Kim, 2011 ²⁰⁹	Islet – portal vein (rat)	Yes – CsA	Co-transplant with islets	NA	<ul style="list-style-type: none"> • islet alone → 5 (mean survival times) • autoMSC monotherapy → (7.8) • autoMSC + CsA promote long-term graft survival (>100) - islets viable in liver - ↓ local and systemic Th1 cytokines IL-2, IFN-γ - ↑ local and systemic Th2 cytokines IL4, IL-10 - induce donor specific tolerance mediated by IL-10+ secreting CD11b⁺ cells - ↑ frequency of intragraft Tregs and Tregs in the lymph nodes
Casiraghi, 2012 ²⁰⁵	Kidney (mouse)	No	i.v. (POD +2 or POD -7 or POD -1 or POD -7, -1)	<ul style="list-style-type: none"> • autoMSC (POD+2) result in premature graft dysfunction and fail to prevent allograft rejection - allogeneic infiltration of neutrophils and complement C3 deposition indicating acute CMR • pre-transplant autoMSC administration prolongs allograft survival - mechanisms: autoMSC localization to lymphoid organs and promoted early Treg expansion 	
Seifert, 2012 ²¹⁰	kidney (rat)	Yes - MMF	i.v. (POD -4)	<ul style="list-style-type: none"> • accelerate rejection and caused kidney damage • enhance humoral immune response associated with intragraft infiltration of B cells and complement activation (complement factor CD4d deposits) 	
Xu, 2012 ²¹¹	Islet – kidney capsule (mouse)	No	i.p. (POD +2)	<ul style="list-style-type: none"> • autoMSC prolong islet allograft rejection - islet viable and secrete insulin in kidney - ↓ intragraft T cells - ↑ intragraft Tregs • autoMSC suppress effector T cell proliferation <i>in vivo</i> - inhibit T cell responses in spleen, mesenteric lymph node and in peritoneal cavity • autoMSC affect T cell differentiation <i>in vivo</i> - inhibit IFN-γ production of CD4⁺ T cells (no effect on IL-4⁺ or TGF-β⁺ CD4⁺ T cells) • autoMSC suppress alloreactivity by inducing Tregs <i>in vivo</i> - induce donor specific tolerance 	

Takahashi, 2014 ²¹²	Islet – portal vein (mouse)	No	Co-transplant with islets	<ul style="list-style-type: none"> • autoMSC alone ineffective at prolonging islet allograft survival (vs. no MSC control mice) • autoMSC + CTLA-4 Ig → 82.4 days (mean survival time) and 1 rejected graft • autoMSC + CTLA-4 Ig + anti-CD40 → indefinite graft acceptance (>100 days; 100% graft survival) - better graft function (vs. autoMSC alone and with autoMSC+CTLA-4 Ig) - presence of insulin producing cells - ↓ intragraft infiltration of CD4 and CD8 T cells (vs. autoMSC alone and with autoMSC+CTLA-4 Ig) - ↑ intragraft infiltration of Tregs - ↓ anti-donor IgG Ab response - ↓ T cell alloreactivity and ↓ IFN-γ production by recipient splenocytes and intrahepatic lymphocytes when re-exposed to donor Ag
Treacy, 2014 ²¹³	Corneal (rat)	No	i.v. (POD -7 and POD 0)	<ul style="list-style-type: none"> • autoMSC ineffective at prolonging allograft survival (100% rejection)
Ben Nasr, 2015 ²¹⁴	Islet – kidney capsule (mouse)	No	co-transplanted with islets or i.v. (POD 0)	<ul style="list-style-type: none"> • i.v. administered autoMSC ineffective at preventing allograft rejection - tracked into the spleen (not liver) • autoMSC co-transplanted with islets – prolong allograft survival and induce long-term graft survival in 30% recipients Mechanisms: - preserve islet allograft and ↑ insulin⁺ cells at site of transplant - ↓ intragraft infiltration of CD4 and CD8 T cells - promote islet revascularisation at site of transplant (ie. ↑ CD31staining) - ↓ alloimmune response (ie. ↓ proliferation to donor Ag) - abrogate Th17 immune responses - autoMSC remain within the islets graft site and also track into the spleen (not liver)

POD, post-operative day; auto, autologous (recipient); i.v., intravenous; CsA, cyclosporine A; CMR, cell-mediated rejection. *Adapted and updated from Table 1 Sivanathan KN, et. al., (2014)⁴.*

Table 1.8.1.2 Administration of bone marrow-derived allogeneic/third-party MSC in preclinical models of allotransplantation

Reference	Transplant model (species)	Drugs	Route (timing of MSC infusion)	MSC immunogenicity <i>in vivo</i>	Key findings
Bartholomew, 2002 ²⁰²	skin (baboon)	No	i.v (POD 0 and/or +3)	NA	<ul style="list-style-type: none"> • <i>in vitro</i>: -alloMSC as stimulators do not induce proliferation of allogeneic responder cells (non-immunogenic) - suppress allogeneic T cell proliferation • <i>in vivo</i>: allo/3rd party MSC prolong skin allograft survival
Inoue, 2006 ²¹⁵	heart (rat)	Yes – CsA	i.v (POD 0 and/or +3)	NA	<ul style="list-style-type: none"> • <i>in vitro</i>: alloMSC suppress responder T cell proliferation • <i>in vivo</i>: - alloMSC as a monotherapy fail to prolong allograft survival (POD 0 or POD 0 and +3) - alloMSC + low-dose CsA accelerate allograft rejection (POD 0 and +3)
Nauta, 2006 ²⁰⁴	bone marrow (mouse)	No	i.v. (POD 0)	Immunogenic – anti-donor memory T cells response (NDA)	<ul style="list-style-type: none"> • <i>in vitro</i>: - alloMSC inhibit responder T cell proliferation - alloMSC as stimulators do not induce proliferation of allogeneic responder cells (non-immunogenic) • <i>in vivo</i>: alloMSC are immunogenic and accelerate rejection by inducing memory T cell responses
Zhou, 2006 ²¹⁶	Heart (rat)	No	i.v. (POD -7, 0, +1, +2, +3)	NA	<ul style="list-style-type: none"> • <i>in vitro</i>: alloMSC suppress T cell proliferation • <i>in vivo</i>: alloMSC prolong allograft survival (mean survival 12 days) POD 12 vs. untreated mice (6 days)
Badillo, 2007 ²¹⁷	skin (mouse)	No	i.p. (POD -42 and -14)	immunogenic – anti-donor T cells and Ab response	<ul style="list-style-type: none"> • alloMSC accelerate allograft rejection • Direct assessment of alloMSC immunogenicity <i>in vivo</i> in immunocompetent mice prior to skin allotransplantation - alloMSC (but not autoMSC) induce responder CD4⁺ T cell proliferation from pre-sensitized mice - high circulating primary (IgM) and secondary (IgG) alloantibodies

(continued) Administration of bone marrow-derived allogeneic/third-party MSC in preclinical models of allotransplantation

Reference	Transplant model (species)	Drugs	Route (timing of MSC infusion)	MSC immunogenicity <i>in vivo</i>	Key findings
Itakura, 2007 ²¹⁸	islet – intraportal (rat)	Yes (DSG)	Intraportal co-administration with bone marrow (POD 0)	NA	<ul style="list-style-type: none"> • alloMSC monotherapy accelerate allograft rejection • alloMSC + BM + DSG promote long-term allograft survival (>100 days) - induce donor specific immune tolerance - 50% stable mixed chimerism - no occurrence of GvHD
Casiraghi, 2008 ²⁰⁵	heart – semiallograft (mouse)	No	Intraportal/i.v (POD -7 and/or -1 and POD +1)	NA	<ul style="list-style-type: none"> • <i>in vitro</i>: alloMSC as stimulators do not induce proliferation of allogeneic responder cells (non-immunogenic) • prolong semi allogeneic graft survival (>100days) and induce tolerance when administered 1x intraportally (POD -7) or 2x infusion (intraportally POD-7 and i.v. POD -1) - Mechanism: Expansion of donor-specific Tregs - alloMSC fail to prevent chronic rejection → mononuclear cell infiltration and mild allograft vasculopathy • 2x infusion – intraportally (POD -7) and i.v. (POD -1) of alloMSC + alloHSC fail to prevent semi allogeneic graft survival • 1x i.v. Infusion (POD +1) → accelerate allograft rejection
Popp, 2008 ²¹⁹	heart (rat)	Yes – MMF (short-term)	i.v. (POD -4)	Immunogenic (NDA)	<ul style="list-style-type: none"> • alloMSC monotherapy (POD -4) accelerate rejection - immunogenic and elicit an anti-donor immune responses (absence of alloMSC in LN, spleen, thymus, liver, lungs) • 3rd party MSC (POD -4) + MMF prolong allograft survival but not show long-term allograft survival (20 days) • alloMSC (POD -4) + MMF promote long-term allograft survival (>100 days) - mechanisms: mediated by IDO activity; interaction of MSC with DC and potentially inducing tolerogenic DC -protect allograft from acute but not chronic rejection

(continued) Administration of bone marrow-derived allogeneic/third-party MSC in preclinical models of allotransplantation

Reference	Transplant model (species)	Drugs	Route (timing of MSC infusion)	MSC immunogenicity <i>in vivo</i>	Key findings
Sbano, 2008 ²²⁰	Skin (rat)	Yes (CsA)	i.v. (POD 0 and +3)	NDA	<ul style="list-style-type: none"> • alloMSC alone accelerate allograft rejection and are immunogenic • alloMSC + CsA prolong allograft survival Mechanisms: ↓ IFN-γ, IL-2, TNF-α
Jacobson, 2008 ²²¹	Islet – kidney capsule	No	Co-transplant with islets	NA	<ul style="list-style-type: none"> • <i>in vitro</i>: 3rd party MSC suppress T cell proliferation independent of MHC • <i>in vivo</i>: <ul style="list-style-type: none"> - POD +7, +14 – no difference in graft insulin positive cells vs. no. MSC group - POD +21 – higher graft insulin positive cells vs. no MSC group
Ge, 2009 ²²²	Heart (mouse)	Rapa	i.v. (POD +1)	NDA	<ul style="list-style-type: none"> • alloMSC monotherapy prevents acute rejection (doubled allograft survival vs. untreated animals) <ul style="list-style-type: none"> - ↓ intragraft and systemic anti-donor IgG alloantibodies - ↓ intragraft infiltration of CD4⁺ and CD8⁺ T cells • alloMSC + low-dose Rapa <ul style="list-style-type: none"> - long-term survival (>100 days) and donor-specific tolerance induction - absence of intragraft and systemic anti-donor IgG alloantibodies - further ↓ intragraft infiltration of CD4⁺ and CD8⁺ T cells - ↑ frequency of tolerogenic DC and Tregs in spleen - home to lymphoid organs and allografts
Solari, 2009 ²⁰⁶	islet – omental pouch (rat)	Yes - CsA (short-term)	Co-transplant with islets	NA	<ul style="list-style-type: none"> • Promote islet allograft survival and induce normoglycaemic allo islet + CsA → graft survival up to POD +7.13 Co-transplant islet + alloMSC + CsA → prolong graft survival up to POD +11.8 but do not promote long-term allograft survival

(continued) Administration of bone marrow-derived allogeneic/third-party MSC in preclinical models of allotransplantation

Reference	Transplant model (species)	Drugs	Route (timing of MSC infusion)	MSC immunogenicity <i>in vivo</i>	Key findings
Zangi, 2009 ²²³	Bone marrow (mouse)	No	Co-administration with bone marrow (POD 0)	Immunogenic – anti-donor CD4 ⁺ and CD8 ⁺ memory T cell response	<p><i>In vitro</i>: alloMSC suppress T cell proliferation independent of MHC</p> <p><i>In vivo</i>: (T cell mediated allograft rejection model)</p> <ul style="list-style-type: none"> • allo/3rd party MSC facilitate bone marrow engraftment and prolong allograft survival (up to POD +29 vs. untreated mice) but fail to promote long-term allograft survival - alloMSC not intrinsically immunoprivileged → recognised by host CD4⁺ and CD8⁺ memory T cells (based on TCR-allogeneic MHC interaction) and are subsequently rejected <p>Direct assessment of MSC immunogenicity in immunocompetent mice:</p> <ul style="list-style-type: none"> - alloMSC (i.p) → alloMSC survival up to 40 days only (study endpoint 120 days) - alloMSC (i.v.) → reduction of alloMSC on day 20 and complete elimination by day 40 (while autoMSC - detectable up to day 120) - pre-sensitization with alloMSC → accelerate rejection of alloFibroblast (donor-antigen) re-challenge - alloMSC are immunogenic → induce CD4⁺ and CD8⁺ memory T cell against allogeneic MHC I and MHC II.
Ge, 2010 ²²⁴	kidney (mouse)	No	i.v. (POD +1)	NA	<ul style="list-style-type: none"> • alloMSC induce long-term allograft survival (>100 days) and induce donor specific tolerance <p>Mechanisms:</p> <ul style="list-style-type: none"> - ↑ IDO activity <i>in vivo</i> - ↓ circulating anti-donor Ab levels (IgG) - inhibit recipient DC maturation, function and induce generation of tolerogenic DC - ↓ T cell responsiveness to donor DC stimulation, skew T cells to Th2 cell phenotype (↓ IFN-γ, ↑ IL-4) - ↑ splenic CD4⁺CD25⁺Foxp3⁺Tregs; ↑ intragraft infiltration of Foxp3⁺ cells (generation of Tregs critical for tolerance induction) - Treg generation and allograft tolerance mediated by MSC-expressed IDO

(continued) Administration of bone marrow-derived allogeneic/third-party MSC in preclinical models of allotransplantation

Reference	Transplant model (species)	Drugs	Route (timing of MSC infusion)	MSC immunogenicity <i>in vivo</i>	Key findings
Han, 2010 ²⁰⁸	Skin (mouse)	No	Adoptive transfer through i.v. Pre-transplant MSC infusion (POD -7)	NA	<ul style="list-style-type: none"> • <i>in vitro</i>: alloMSC as stimulators do not induce proliferation of allogeneic responder (non-immunogenic); suppress primary and secondary alloimmune responses (inhibit proliferation and IFN-γ production of naive responder T cells and memory T cells) • <i>in vivo</i>: Fail to prolong allograft survival in major and minor mismatch barriers
Berman, 2010 ²²⁵	Intraportal islet (cynomolgus monkey)	Yes - Rapa	Co-transplant with islets and i.v. (POD +5 and +11)	NA	<ul style="list-style-type: none"> • alloMSC co-transplant with islets → enhance islet cell engraftment and function • alloMSC co-transplant with islets + i.v. alloMSC/3rd party MSC (POD +5 and +11) - prevent islet cell rejection and prolonged islet graft function Mechanisms: ↑ circulating Tregs
Longoni, 2010 ²⁰⁰	Islet – portal vein (rat)	Yes – Tac and Rapa	Systemic (i.v.) and locally (intraportal) (POD 0, +2, +4)	NA	<ul style="list-style-type: none"> • alloMSC + drugs: → improve glycaemic control when given at: - 3xdose (on POD 0, +2, +4) (i.v.) (best glycaemic control) or single alloMSC dose intraportally or i.v. - effective when administered locally/systemically → promote survival of transplanted islet with stable glycaemic control but ineffective at preventing chronic allograft rejection (graft survival up to POD +50) - low-grade rejection (CD3⁺ T lymphocytes infiltration on POD +10, and POD +47) - ↑ glycaemic levels – slowly return to high pre-transplant values 4 weeks after transplant • 3x alloMSC alone or 3x alloMSC + drugs: - suppression of systemic proinflammatory cytokine levels (IFN-γ and GM-CSF) - slow increase in glycaemic levels 4 weeks post-transplant - no difference between drugs alone and MSC + drugs

(continued) Administration of bone marrow-derived allogeneic/third-party MSC in preclinical models of allotransplantation

Reference	Transplant model (species)	Drugs	Route (timing of MSC infusion)	MSC immunogenicity <i>in vivo</i>	Key findings
Eggenhofer, 2011 ²²⁶	heart (mouse)	Yes	i.v. (POD -4)	NA	<ul style="list-style-type: none"> • alloMSC monotherapy → fail to prolong allograft survival • alloMSC + MMF (short-term) → prolong allograft survival graft survival up to POD +32 • <i>in vivo</i> MLR model: MMF + MSC synergistically ↓alloresponses (vs. alloMSC + CsA or alloMSC + Rapa) • <i>in vitro</i>: ineffectiveness of alloMSC + CsA could be due to disruption of IFN-γ production by activated T cells (MMF does not affect IFN-γ production)
Kim, 2011 ²⁰⁹	Islet – portal vein (rat)	Yes – CsA	Co-transplant with islets	NA	<ul style="list-style-type: none"> <i>in vitro</i>: alloMSC inhibit T cell proliferation independent of MHC <i>in vivo</i>: <ul style="list-style-type: none"> • alloMSC monotherapy accelerate allograft rejection → 3.7 (mean survival days) • alloMSC + CsA: prolong allograft survival (13.8)
Eggenhofer, 2011 ²²⁷	heart (rat)	Yes	i.v. (POD -4)	Immunogenic – anti-donor cell mediated response) (NDA)	<ul style="list-style-type: none"> • alloMSC monotherapy <ul style="list-style-type: none"> - immunogenic – evident by presence of activated CD4⁺ and CD8⁺ T cells early (POD +3) in the secondary lymphoid organs (vs. untreated group) - inhibit APC activation in secondary lymphoid organs - intragraft infiltration of T cells and evidence of accelerated allograft rejection • alloMSC + MMF <ul style="list-style-type: none"> - presence of activated T cells early after transplant (POD +3) but levels ↓ by POD +10 indicating alloMSC are immunogenic and immune response against alloMSC is controlled by MMF - delay APC activation in secondary lymphoid organs ↓ intragraft infiltration of APC and T cells by inhibiting endothelial activation that is critical for APC and T cell trafficking into the graft

(continued) Administration of bone marrow-derived allogeneic/third-party MSC in preclinical models of allotransplantation

Reference	Transplant (species)	Drugs	Route (timing of MSC infusion)	MSC immunogenicity <i>in vivo</i>	Key findings
Zhang, 2012 ²²⁸	Keratoplasty (rat)	No	i.v. (POD -3)	NA	<ul style="list-style-type: none"> • post-transplant alloMSC inhibit keratoplasty rejection and prolong allograft survival compared to pre-transplant alloMSC infusion - ↓ intra-graft infiltration of CD4⁺, CD8⁺ and CD25⁺ cells
Jia, 2012 ²²⁹	corneal (rat)	Yes	i.v. (POD -3, -2, -1 or POD 0, +1, +2, +3 or at time of rejection)	NA	<ul style="list-style-type: none"> • alloMSC monotherapy (POD -3, -2, -1) → fail to prolong allograft survival • alloMSC monotherapy (at time of rejection) → fail to prolong allograft survival • alloMSC monotherapy (POD 0, +1, +2) → prolongs allograft survival up to POD +15 - inhibits T cell responses <i>in vivo</i> - alteration of a proinflammatory to an anti-inflammatory cytokine (IL-4) secretion profile of T cells - ↑ Tregs • alloMSC (POD 0, +1, +2) + low dose CsA (1mg/kg/day) → accelerate rejection • alloMSC (POD 0, +1, +2) + CsA (2mg/kg/day) → prolong allograft survival up to POD +24
Wu, 2013 ²³⁰	Islet – kidney capsule (humanized mouse)	None	Co-transplant with islets	NA	<ul style="list-style-type: none"> • prevent rejection human islet rejection and improve islet graft function - <i>in vitro</i>: inhibit T cell proliferation and activation (↓ IFN-γ, IL-2, soluble IL-2R) by interacting with IL-10 secreting CD14⁺ monocytes; protect and improve islet graft function from proinflammatory cytokines - <i>in vivo</i>: prevent intra-graft infiltration of CD3⁺ T cells; ↑ circulating Tregs
Treacy, 2014 ²¹³	Cornea (rat)	None	i.v. (POD -7 and POD 0)	NDA	<ul style="list-style-type: none"> • allo/3rd party MSC prolong allograft survival - ↓ immune cell infiltration (ie. NK T cells, T cells and CD11b/c⁺ cells) - ↑ intra-graft infiltration of Tregs; ↑ splenic Tregs - ↓ proinflammatory cytokine expression (IL-6, IL-1β) - induce donor specific hypo-responsiveness • alloMSC are immunogenic - elicit an alloantibody response (↑ IgG1 and ↑ IgG2)

(continued) Administration of bone marrow-derived allogeneic/third-party MSC in preclinical models of allotransplantation

Reference	Transplant (species)	Drugs	Route (timing of MSC infusion)	MSC immunogenicity <i>in vivo</i>	Key findings
Wang, 2014 ²³¹	Heart (mouse)	Rapa	i.v. (POD +1)	NA	<ul style="list-style-type: none"> • alloMSC monotherapy ineffective at preventing allograft rejection • alloMSC + Rapa → prolong allograft survival dependent on PD-L1 (>100 days) Mechanisms: MSC expressed PD-L1 required for: <ul style="list-style-type: none"> - development and function of tolerogenic DC (<i>in vivo</i>) - development of Tregs and Bregs (<i>in vivo</i>) - ↓ intragraft and systemic anti-donor IgG and IgM - suppression of B cell proliferation (<i>in vitro</i>) - inhibit B cell differentiation into plasma cells and subsequent Ab production (<i>in vitro</i>)
Ben Nasr, 2015 ²¹⁴	Islet – kidney capsule (mouse)	No	Co-transplanted with islets	NA	<ul style="list-style-type: none"> • alloMSC ineffective at preventing allograft rejection

Abbreviations: i.v., intravenous; allo, allogeneic (donor); POD, post-operative day; NDA, not directly assessed; NA, Not assessed; LN, lymph node; CsA, cyclosporine A; Rapa, Rapamycin; Tac; Tacrolimus; MMF, mycophenolate mofetil; DSG, 15-deoxyspergualin. *Adapted and updated from Table 2 Sivanathan KN, et. al., (2014)*⁴.

Table 1.8.1.3 Administration of bone marrow-derived MSC in clinical trials of allotransplantation

Reference	Transplant model	Drugs	Route (timing of MSC infusion)	MSC source	Key findings
Perico, 2011 ²³²	Kidney transplantation- Pilot study: 2 patients	Yes – induction therapy: T cell-depleting Maintenance: CsA + MMF	i.v. (POD +7)	autoMSC	<p>Follow up to Day 360</p> <ul style="list-style-type: none"> - ↑ allograft function - Prevent acute rejection and maintain graft function - administration safe and feasible <p>• Mechanism:</p> <ul style="list-style-type: none"> - ↓intragraft infiltration of CD4⁺ and CD8⁺ T cells, CD14⁺ monocytes, CD20⁺ B cells, CD68⁺ macrophages - ↑ CD4⁺CD25^{high}FoxP3⁺CD127⁻ Tregs - suppression of memory CD8⁺T cell expansion and reduction in cytotoxic CD8⁺T cell function - higher ratio of Tregs to memory T cells
Tan, 2012 ²⁰¹	Kidney transplantation- Randomized controlled trial	Yes – MMF+ corticosteroids + CNI (ie. CsA or Tac)	i.v. (POD 0 and +14)	autoMSC	<ul style="list-style-type: none"> • co-administration of autoMSC + CNI (low/standard dose) - graft survival at 13- 30 months similar to control group - ↓ acute rejection (after 6 months post-transplant) - ↑ renal function (at 1 month post-transplant) (autoMSC + low dose CNI → better renal outcome vs. autoMSC + standard dose CNI) - ↓ risk of opportunistic infection and adverse side effects (at 1-year follow up)
Reinders, 2013 ²³³	Treatment of allograft rejection after kidney transplantation Pilot study: 6 patients	Yes – induction therapy: Basiliximab Maintenance: prednisone + CNI (ie. Tac or CsA)	i.v. (at the time of rejection – 2x infusion; 7 days apart)	autoMSC	<ul style="list-style-type: none"> • Clinical monitoring 24 weeks following MSC infusion - MSC infusion well tolerated with no adverse-side effects - 3/6 patients developed opportunistic viral infection and 2 patients recovered following antiviral therapy and reduction in immunosuppression - 2 patients with subclinical allograft rejection showed resolution of tubulitis and with no interstitial fibrosis or tubular atrophy <p>• Immune monitoring (12 weeks following MSC infusion)</p> <ul style="list-style-type: none"> - 5/6 patients showed donor-specific reduction of PBMC proliferation (MLR) - ↓ IFN-γ, IP-10 and MCP-1 levels in supernatant of PBMC proliferation assay following stimulation of recipient PBMC with donor-specific-PBMC in most patients <p>• MSC infusion results in systemic immunosuppression</p>

(continued) Bone marrow-derived MSC in clinical trials of allotransplantation

Reference	Transplant model	Drugs	Route (timing of MSC infusion)	MSC source	Key findings
Peng, 2013 ²³⁴	Kidney transplantation -Pilot study: 6 patients	Yes –induction therapy: Cytoxan + methylprednisolone maintenance: MMF + prednisone + low dose Tac	Renal artery (POD 0) and i.v. (POD +30)	alloMSC	<ul style="list-style-type: none"> • co-administration of alloMSC + low-dose Tac (50% of standard dose) - no short-term or long term side effects - prevent acute graft rejection - stable graft function and patient survival (at 12 months)
Perico, 2013 ²³⁵	Kidney transplantation Pilot study: 2 patients	Yes – induction therapy: low dose thymoglobulin Maintenance: CNI (CsA + MMF) + corticosteroids (methylprednisolone + prednisone)	i.v. (POD -1)	autoMSC	<ul style="list-style-type: none"> • protects patients from the development of acute cell-mediated graft dysfunction following transplant • patient 3 (1yr follow up): MSC infusion was uneventful and graft function was normal • patient 4: acute rejection 2weeks post-transplant • Patient 3 and 4 retained graft function • in MSC treated patients (vs. non-MSC treated control group) <ul style="list-style-type: none"> - ↓ in circulating memory CD8⁺ T cells - ↓ in donor-specific cytolytic CD8⁺ T cell responses - Tregs expansion similar in patients receiving induction with or without Basiliximab
Mudrabetu, 2015 ²⁰³	Kidney transplantation- Pilot study: 4 patients	Yes – induction therapy: low dose ATG Maintenance: CNI (Tac + MMF + prednisolone)	i.v. (POD -1 and POD +30)	autoMSC	<ul style="list-style-type: none"> • clinical monitoring 6 months following MSC infusion - MSC infusion is safe – no immediate or delayed adverse effects - excellent graft function in all patients - no signs of rejection or abnormalities – normal protocol biopsies at 1 and 3 months - ↑ Treg frequency - ↓ CD4⁺ T cell proliferation

Abbreviations: Auto, autologous; allo, allogeneic; CNI, calcineurin inhibitors Tac, Tacrolimus; PBMC, peripheral blood mononuclear cells; MLR,

Mixed lymphocyte reaction; IP-10, IFN- γ induced protein; MCP-1, monocyte chemotatic protein-1; ATG, anti-thymocyte globulin., MMF, mycophenolate mofetil. *Adapted and updated from Table 3 Sivanathan KN, et. al., (2014)⁴.*

1.9 MSC modulation of T cell immune responses: Independent of MHC expression

1.9.1 Direct T cell modulation

It is well established in the literature that MSC immunosuppressive phenotype can directly or indirectly modulate T cell function *in vitro* independent of MHC expression^{193,202,204,207,208,215,216,223,230,236}. MSC directly affect T cell responses by expressing an array of soluble immunosuppressive factors such as transforming growth factors-beta (TGF- β), HGF²³⁷, prostaglandin E2 (PGE₂)²³⁸, the soluble non classical molecule human leukocyte antigen-G5 (HLA-G5)¹⁹², IL-10^{239,240}, heme oxygenase-1 (HO-1)²³⁹, galectins and semaphorin-3A²⁴¹⁻²⁴⁵ as well as indoleamine 2,3-dioxygenase (IDO)^{246,247}. These MSC expressed inhibitory factors are critical to mediate the inhibition of T cell proliferation, activation or differentiation *in vitro*.

MSC also inhibit T cell responses by downregulating surface expression of early T cell activation markers CD25, CD38 and CD69^{248,249}. The soluble factors MMP-2 and MMP-9 secreted by MSC potentially cleave CD25 on T cells, thus impairing T cell activation and proliferation²⁰⁷. The administration of MMP inhibitors *in vivo* in an islet allotransplant model abrogated the suppressive effect of autologous MSC on alloreactive T cells, resulting in rejection, thereby suggesting that MMP play a crucial role in MSC immunosuppression²⁰⁷. Previous reports also show that MSC when co-cultured with T cells alter the cytokine secretion profile of activated T cells. MSC may promote an anti-inflammatory T helper 2 (Th2) cell phenotype, evident by T cell secretion of IL-3, IL-4, IL-13 and the decrease in proinflammatory Th1 cytokines IFN- γ and TNF- α ^{238,250}. Overall, these findings suggest that the expression of MMP and the alteration of a Th1 to Th2 profile of T cells are critical to MSC effect in modulating proinflammatory T cell responses that lead to allograft rejection.

MSC can also mediate T cell suppression via cell-contact dependent mechanisms involving the B7 homolog 1 (B7-H1)/ programmed death-1 (PD-1) pathway^{194,231,251,252}, the negative co-stimulatory molecule B7-H4²⁵³, the CD200/CD200 receptor (CD200R) axis²⁵⁴ toll-like receptors (TLR)²⁵⁵ and FAS-L/FAS interaction²⁵⁶. We have also reported the role of erythropoietin-producing hepatocellular tyrosine kinase receptor B subclass (EphB) and their cognate ephrinB ligands in MSC mediated T cell immunosuppression²⁴⁰. Although the CD200/200R axis was not shown to be essential in MSC-T cell mediated immunosuppression²⁵⁴, it is known that the CD200/200R interaction modulates inflammatory responses^{257,258} and induces tolerance *in vivo* in models of skin and cardiac allotransplantation²⁵⁹. Overall, the primary molecular mechanism(s) underlying the direct modulation of T cells by MSC is still unclear and may be model dependent. Some studies indicate that cell-contact dependent activation of MSC results in the expression of soluble factors that mediate T cell immunosuppression^{253,255,256}. Thus, both cell-contact and soluble factor secretion may be required for efficient MSC immunomodulation.

1.9.2 Indirect T cell modulation: MSC interaction with antigen presenting cells

MSC also indirectly modulate T cells by interacting with APC such as DC and promoting the generation of regulatory DC which are potent initiators of the immune response capable of inducing activation and proliferation of resting naive T cells²⁶⁰. MSC can prevent bone marrow-derived DC differentiation and maturation²⁶¹⁻²⁶⁵, reduce endocytosis and impair antigen-presentation ability of DC^{263,264}. MSC inhibit DC maturation and function evident by the downregulation of MHC class II expression that is required for T cell alloantigen presentation as well as the decrease in co-stimulatory molecules CD80, CD86, CD83 and CD40 on mature DC that are critical for T cell activation^{196,224,265,266}. Mature DC, in co-culture with

MSC display impaired T cell allostimulatory ability and skewed Th1 cells to an anti-inflammatory Th2 cell phenotype²⁶⁶. Some reports also indicate that MSC inhibit DC differentiation from early DC precursors. MSC inhibit the classical monocyte subset differentiation into activated monocytes, as well as inhibiting the generation of DC from CD34⁺ haematopoietic precursors and umbilical-cord blood derived haematopoietic stem cells (HSC)²⁶⁷⁻²⁶⁹. DC-like cells derived from HSC in presence of MSC were functionally impaired as they were incapable of inducing naive T cell alloresponses²⁶⁹. Moreover, DC generated in presence of MSC or MSC-conditioned media express low levels of IL-12 and IFN- γ that are required for T cell activation and were unable to induce allostimulatory T cell responses^{261,263-265,270,271}. The inhibitory effects of MSC on DC differentiation, maturation and function are mediated by MSC inhibitory factors such as IL-6^{269,272}, PGE₂^{270,273}, IL-10²⁶⁵ and growth-regulate oncogene-gamma (GRO- γ) chemokine²⁶⁴; preventing monocyte entry into G1 phase of the cell cycle¹⁹⁵ and inhibition of DC immune synapse with T cells due to altered actin distribution in DC²⁷⁴. Maturation of DC in presence of MSC were also shown to impair *in vitro* chemotactic migration ability of matured DC in response to CCL19 and CCL12 which are chemokines expressed in secondary lymphoid organs^{196,275}. *In vivo* studies demonstrate that autologous or allogeneic MSC decrease the chemotactic migratory properties of mature DC by downregulating the chemokine receptor CCR7 required for DC migration to naive T cell regions in the secondary lymphoid organs for antigen presentation and T cell activation^{196,276,277}. Collectively, MSC indirectly modulate T cell immune responses by impairing DC differentiation, maturation, function and migration to T cell activation regions in lymph nodes, which are beneficial in the context of allotransplantation and promoting allograft tolerance.

The alteration of DC phenotype also suggests that MSC may promote the generation of tolerogenic DC populations with decreased functional ability to activate alloreactive T cells

through their expression of IL-10, TGF- β , B7-H1 or Jagged-1^{231,260,271,278,279}. MSC also induce the generation of DC with immunoregulatory properties following co-culture with mature DC²⁷⁸⁻²⁸⁰. These regulatory DC were capable of suppressing *in vivo* alloantigen immune responses in a delayed-type hypersensitivity (DTH) model²⁷⁹. A subsequent study demonstrated *in vitro* that MSC skewed matured DC into tolerogenic DC that inhibit T cell responses via the expression of TGF- β . Interestingly, a study has demonstrated that MSC express the GRO- γ chemokine that inhibits DC differentiation and function. GRO- γ directly promotes monocyte-DC differentiation into MDSC with a tolerogenic phenotype. These MDSC were also capable of differentiating T cells toward the tolerogenic Th2 cell phenotype²⁶⁴.

1.9.3 Indirect modulation of T cells: MSC generate regulatory T cells

Regulatory T cells (Tregs) are potent suppressors of the immune response and are of relevance in transplantation. MSC are known to induce Tregs^{225,239,250,256,278,281,282}. The MSC-expressed soluble mediators HO-1²³⁹, PGE₂^{283,284}, TGF- β ^{283,285}, IL-10^{249,283} and Jagged-1²⁷¹ are factors involved in the induction of Tregs.

Immunoregulatory HO-1 derived from MSC was shown to induce IL-10⁺ Tr1 and TGF- β ⁺ Tr3 Treg subsets in co-cultures of alloreactive T cells²³⁹. Another study demonstrated that MSC-secreted PGE₂ and TGF- β have non-redundant roles in the induction of conventional CD4⁺CD25^{high}FoxP3⁺ Tregs, a process also mediated by cell-cell contact²⁸³. Neutralizing antibodies against TGF- β (α -TGF- β) or the antagonist to PGE₂ (indomethacin) partially reduced Treg number, while the addition of both α -TGF- β and indomethacin completely abolished MSC induction of Tregs. This study also reported that, purified MSC-induced Tregs (CD4⁺CD25⁺) were able to suppress alloantigen driven T cell proliferation in a mixed lymphocyte reaction (MLR)²⁸³. Moreover, PGE₂ has been shown to potentiate MSC to induce

IL-10⁺IFN- γ ⁺CD4⁺ Tr1-like cells²⁸⁴. Cyclooxygenase-derived PGE₂ combined with IDO expression in MSC promoted Tr1 cell development in an allogeneic MLR. These cell sorted MSC-primed Tr1 cells exhibited *in vitro* immunosuppressive activity to alloreactive T cells and were also able to inhibit *in vivo* transplant arteriosclerosis²⁸⁴. The regulatory IDO in the production and generation of Tregs was also confirmed in another study using IDO transfected MSC (IDO-MSC)²⁸⁶. These IDO-MSC derived Tregs also expressed Treg associated suppressive molecules CTLA-4, TGF- β and IL-10. *In vivo* infusion of IDO-MSC promoted rabbit kidney transplantation tolerance mediated by antigen-specific CD4⁺CD25⁺Tregs²⁸⁶.

Jagged-1 expression by MSC on the other hand not only directly expanded Tregs but was shown to generate tolerogenic DC²⁷¹. These MSC-primed tolerogenic DC induced the generation of CD4⁺CD25⁺FoxP3⁺ Tregs. MSC protective effects in mouse models of allergic airway inflammation correlated with increased Tregs and IL-10. Mouse treated with Jagged-1 knock down MSC on the contrary had significantly reduced Tregs and IL-10, confirming that Jagged-1 signaling has a role in MSC immunosuppression of *in vivo* inflammation²⁷¹. In an *in vivo* study co-administrating MSC and Rapamycin reported that B7-H1, a cell surface molecule is required for MSC generation of Tregs and subsequent tolerance induction *in vivo* in models of cardiac allotransplantation²³¹. Together, these studies suggest that MSC induction of Tregs can also be mediated by cell-contact dependent mechanisms.

Furthermore, MSC have been shown to inhibit the differentiation of naive CD4⁺ T cells into Th1 (IFN- γ expressing CD4⁺ T cells) and Th17 (IL-17 expressing CD4⁺ T cells) cell type²⁴⁹. In this study, naive CD4⁺ T cells were induced to differentiate into Th1 or Th17 cells in presence of MSC. In this co-culture system, MSC induced the generation of functional CD4⁺CD25⁺FoxP3⁺ Tregs that were capable of suppressing T cell proliferation²⁴⁹. In another study, it was shown that MSC inhibit Th1 and Th17 cells *in vivo* in mouse models of collagen-induced arthritis via glucocorticoid-induced leucine zipper (GILZ) expression²⁸². This

observation correlated with GILZ-dependent polarization of Th17 cells into CD4⁺IL-10⁺Tregs²⁸². The expression of HLA-G5 by MSC has also been shown to expand Tregs¹⁹². Taken together, these reports demonstrate that MSC can indirectly modulate T cell responses and potentially induce allograft tolerance via the induction or expansion of suppressive regulatory T cells or DC. There are several other studies that have reported that MSC infusion in preclinical models and in clinical trials of allotransplantation results in increased frequencies of Tregs (**Table 1.8.1.1**, **Table 1.8.1.2** and **Table 1.8.1.3**). These studies therefore support the notion that MSC are potent inducers of Tregs and are capable of promoting Treg function *in vivo*. MSC and Treg synergism in suppressing inflammation and promoting Treg function was further validated in studies using combined MSC and Treg therapy in models of GvHD and in colitis²⁸⁷⁻²⁸⁹. The mechanisms by which MSC directly or indirectly modulate T cell response are summarized in **Figure 1.9.1**.

Comprehensive analysis of Treg immunoregulatory molecules and the mechanisms they function can potentially identify novel markers that facilitate the interaction between MSC and Tregs. Exploring the interaction of MSC and Tregs via Treg functional associated molecules may enable the discovery of novel molecules involved in MSC-dependent Treg suppression of inflammatory responses. This can also enable specific protocol design of MSC-mediated Treg induction or expansion and consequently more potent MSC immunomodulation via indirect T cell modulation through Tregs. Understanding MSC and Treg interactions may also facilitate the use of MSC as feeder cells for Treg expansion procedures.

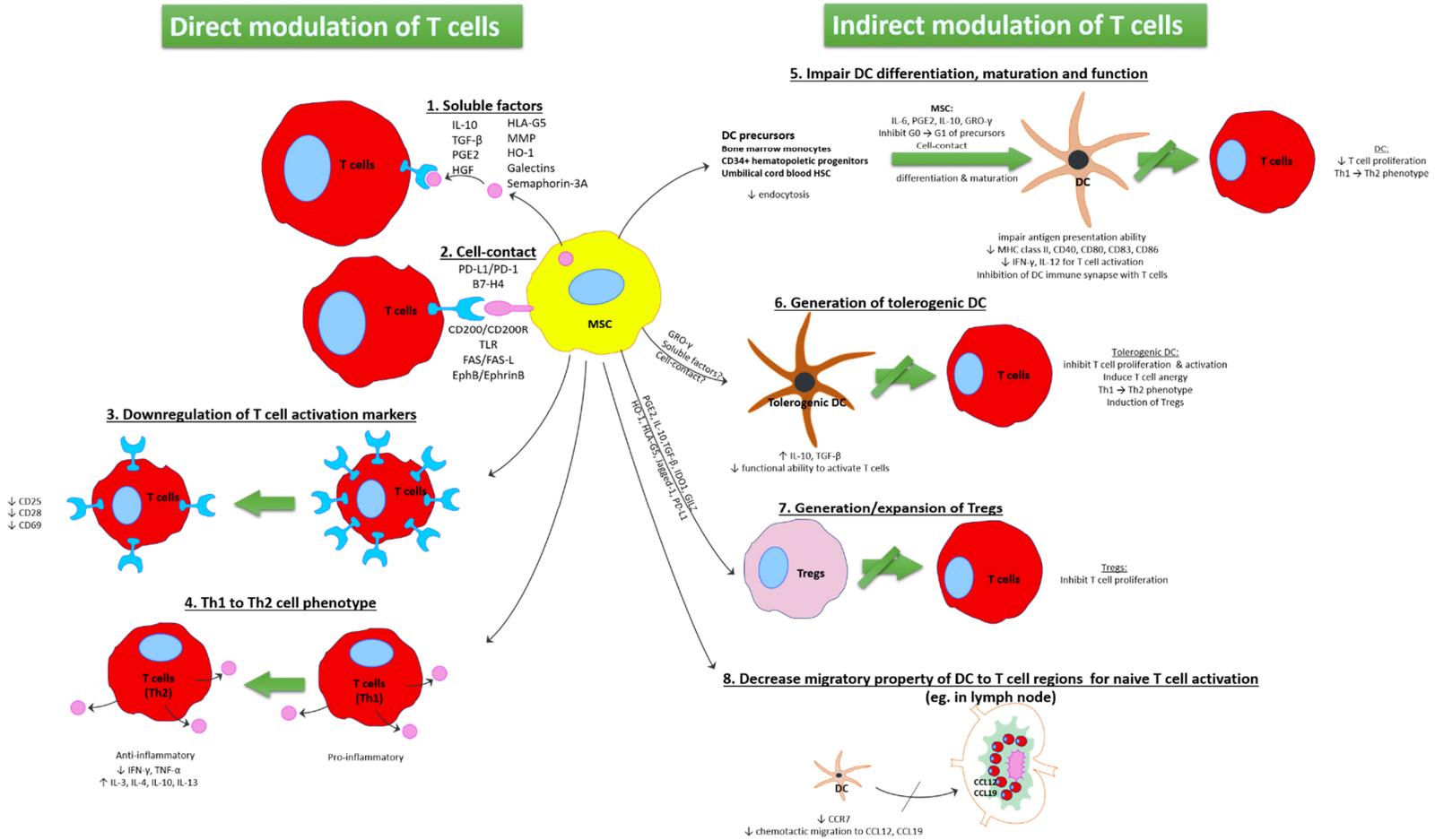


Figure 19.1 Mechanisms by which MSC directly and indirectly modulate T cell response.

Figure adapted and modified from Sivanathan KN, et al., (2014)⁴.

1.10 Immunogenicity of “off-the-shelf” MSC *in vivo* in allotransplantation

In a clinical setting of allotransplantation, the application of allogeneic (donor) or third-party MSC therapy is highly desirable compared to the derivation of autologous (patient-specific) MSC. Many clinical trials have demonstrated that the administration of autologous bone marrow MSC in various disease conditions (www.clinicaltrials.gov.au) and in solid organ transplantation (**Table 1.8.1.3**) is safe with no adverse effects. In actively ill patients however, the administration of patient-derived MSC may not be an optimal source of MSC²⁹⁰⁻²⁹⁴. In these conditions, patients would require “off-the-shelf” (allogeneic or third party) MSC therapy.

Although the administration of “off-the-shelf” MSC would be ideal in an allotransplant setting, the long-term efficacy of allogeneic (donor) or third-party derived MSC can be limited by AMR or CMR. The immunogenicity of “off-the-shelf” MSC *in vivo* is still a subject of controversy. The administration of autologous (recipient) derived MSC on the other hand could be a more efficacious option to promote long-term allograft survival and allotransplant tolerance. The therapeutic efficacy of autologous, allogeneic and third-party bone marrow-derived MSC in an allotransplant setting will be addressed in this section.

In regards to the administration of “off-the-shelf” MSC therapy, it is widely presumed in the MSC literature that these cells are non-immunogenic and are able to evade the host immune system recognition when administered in MHC-mismatched recipients²⁹⁵⁻²⁹⁷. Although several ongoing or completed clinical trials have administered “off-the-shelf” MSC in patients, there are no clear documented adverse effects related to robust anti-donor immune response following “off-the-shelf” MSC therapy²⁹⁸. Current knowledge in the MSC research field suggests that MSC-associated alloantigens are actively recognised by the recipient immune system with a range of potential outcomes^{217,299-301}. Therefore, the administration of “off-the-shelf” MSC and their long-term therapeutic efficacy *in vivo* in an allotransplant setting

is questionable due to the potential alloimmune response directed against allogeneic or third-party MSC^{1,301}. A vast majority of *in vitro* studies indicate that MSC mediated immunosuppression is independent of MHC-expression, suggesting that “off-the-shelf” MSC could be used as a non-immunogenic cellular therapy^{193,202,204,207,208,215,216,223,230,236}. However, these *in vitro* observations are not directly translatable *in vivo* into allotransplantation models. **Table 1.8.1.2** summarizes studies utilizing “off-the-shelf” MSC therapy in preclinical models of allotransplantation. The anti-donor immune response directed against allogeneic MSC in the context of allotransplantation will be discussed in this section.

1.10.1 “Off-the-shelf” MSC monotherapy

As highlighted in **Table 1.8.1.2**, there is a significant debate over the efficacy of allogeneic or third-party MSC monotherapy *in vivo* in allotransplant models. There are many studies demonstrating that allogeneic MSC monotherapy failed to prevent allograft rejection^{204,205,208,210,215,219,220,226,227,229}, whilst others support the notion of using allogeneic MSC monotherapy to prolong allograft survival in a variety of different transplant models^{202,216,222,223,227,229}.

Studies reporting on the benefits of allogeneic MSC monotherapy however only show short-term prolongation of graft survival with subsequent rejection. Casiraghi and colleagues²⁰⁵, reported that pre-transplant administration of allogeneic MSC monotherapy promotes long-term semi-allograft heart survival (> 100 days), but the presence of mononuclear cell infiltration and mild allograft vasculopathy was evident in these grafts. The longer allograft survival times in this model could relate to the fact that it was a partial MHC mismatch heart graft (semi-allograft) transplantation. Therefore, the rejection process in this model may not be as vigorous as in a full MHC mismatched allotransplant model. Together, these studies strongly

suggest that “off-the-shelf” MSC are effective at ameliorating acute but not chronic allograft rejection which occurs in the later phases post-transplantation.

Importantly, in some studies, pre- or post-transplant allogeneic MSC monotherapy accelerated allograft rejection compared to untreated animals^{204,205,215,219,220,227}. Such findings question the “immune privileged status” of “off-the shelf” MSC *in vivo* as MSC may be acting to facilitate donor specific immune responses resulting in accelerated allograft rejection. In addition, Nauta and colleagues characterised allogeneic MSC as immunogenic by their ability to induce memory T cell responses in naive immunocompetent mice, thus providing a possible explanation to the observed accelerated allograft rejection in animals of bone marrow allotransplantation²⁰⁴. In preclinical models of rat cardiac allotransplantation, Popp, 2008 and Eggenhofer, 2011, demonstrated that pre-transplant infusion of allogeneic MSC elicits an anti-donor immune response, resulting in accelerated allograft rejection. An increase in intragraft infiltration of CD4⁺ and CD8⁺ T cells as early as day 3 post transplantation suggests that allogeneic MSC induce an anti-donor cell-mediated immune response, potentially leading to allogeneic MSC rejection and hyperacute rejection of the cardiac allograft²²⁷. As discussed in section **1.3 and 1.4**, it can be postulated that the pre-transplant infusion of allogeneic MSC is recognised by the host immune response through the direct, indirect or semi-direct pathways of allorecognition to elicit cell- or antibody mediated rejection against MSC. Pre-existing alloantibodies or antigen-specific memory T or B cells following primary exposure to allogeneic MSC could have enhanced the host immune response to donor antigens, leading to increased rejection of donor heart allografts upon secondary exposure to donor alloantigens. The limitation in most of these studies is that they do not demonstrate donor specific alloimmune responses and rely on histology as readout of allograft rejection. Studies specifically investigating the alloimmune responses against allogeneic MSC in models of allotransplantation are warranted.

1.10.2 Synergistic effect of “off-the-shelf” MSC with immunosuppressive drugs

The co-administration of “off-the-shelf” MSC with immunosuppressive drugs on the other hand showed better outcome of allograft survival compared to “off-the-shelf” MSC monotherapy^{200,206,219,220,222,226,227,229,231}. Studies by Ge and colleagues²²² indicate that the infusion of allogeneic MSC monotherapy inhibits CMR and AMR. The levels of circulating and intragraft alloreactive anti-donor IgG in these animals however were higher compared to rapamycin alone or rapamycin and allogeneic MSC treated animals. The high level of alloantibodies in the MSC monotherapy treated animals may indicate that allogeneic MSC trigger an antibody-mediated response following donor antigen-infusion. The synergistic effect of rapamycin-MS therapy promoted long-term graft survival by the further inhibiting levels of immunoglobulin G (IgG) alloantibodies *in vivo*^{222,231}. MSC were additionally shown to synergise with Rapamycin to promote tolerogenic DC development and function, increase Tregs and in regulatory B cell development in models of cardiac allotransplantation²³¹. Thus, it can be postulated that the prolonged allograft survival in these studies relate to the ability of immunosuppressive drugs to control immune responses directed against allogeneic MSC, thereby, prolonging MSC survival and enabling MSC to exert their immunomodulatory effects *in vivo*. It should be noted that the duration of allograft survival post-transplantation in these models was dependent on the concentration and type of immunosuppressive drugs administered, MSC infusion timing and dose as well as the model of allotransplantation^{200,206,215,219,220,222,226,227,229}. The co-administration of allogeneic MSC with low cyclosporine A (CsA) doses in rat models of heart and corneal allotransplantation for example resulted in accelerated rejection^{215,229}, while another study reported only 57% allograft survival in rat skin allotransplantation²²⁰. These studies suggest that low doses of CsA may be insufficient to suppress anti-donor immune response against allogeneic MSC and that co-

administration of low dose CsA-MSc therapy may be ineffective to promote long-term graft survival in clinical allotransplantation.

A latter study further demonstrated that the pre-transplant infusion of allogeneic MSC and mycophenolate mofetil (MMF) accelerates allograft rejection and caused kidney damage possibly due to MSC immunogenicity *in vivo*²¹⁰. This observation correlates with high levels of systemic anti-donor IgG and intragraft depositions of B cells and complement factors compared to untreated or autologous MSC treated rats²¹⁰. It is possible that B cells activate complement pathways to further enhance the recruitment and activation of innate immune cells, therefore resulting in a more rapid kidney allograft injury and rejection in this study^{21,22}. Schu and colleagues³⁰⁰ further reported that allogeneic MSC increase anti-donor IgG antibodies in naive immunocompetent animals and these alloantibodies facilitate complement mediated lysis of allogeneic cells. This observation suggests that alloantibody mediated complement pathway activation could result in rapid kidney allograft rejection and injury following allogeneic MSC administration²¹⁰. As discussed earlier, B cells could also function as APC to further amplify allospecific CMR in this model^{22,23}.

1.10.3 Mechanisms of “off-the-shelf” MSC immunogenicity

In the allotransplant models discussed above, the immunogenicity of “off-the-shelf” MSC was not directly addressed *in vivo*. On the other hand, Zangi and colleagues²²³ have assessed the immunogenicity of allogeneic MSC by direct imaging of immune rejection and memory induction following MSC infusion in naive immunocompetent mice. The initial observation that the infusion of allogeneic MSC monotherapy failed to promote long-term allograft survival suggests that these MSC are potentially recognised by the host immune system and are subsequently rejected. They report that the survival of allogeneic MSC *in vivo* was short term as these cells were completely eliminated by day 40 while autologous MSC were detectable up

to 120 days post infusion. In addition, mice pre-sensitized with allogeneic MSC accelerated allogeneic fibroblast rejection upon donor antigen re-challenge. This study specifically showed that allogeneic MSC induce allospecific CD4⁺ and CD8⁺ memory T cells directed against allogeneic MHC I and MHC II, therefore confirming that these MSC are immunogenic *in vivo*²²³. This finding is consistent with a previous report indicating that the pre-transplant infusion of allogeneic MSC elicits an anti-donor cellular- and antibody-mediated immune response in recipients²¹⁷. CD4⁺ responder T cells from pre-sensitized animals proliferated in response of allogeneic MSC *in vitro* and high levels of IgM and IgG alloantibodies were detected in these animals. In addition, animals that were pre-sensitized with allogeneic MSC demonstrated hyperacute rejection of skin allografts upon secondary alloantigen exposure²¹⁷.

Studies directly assessing MSC immunogenicity *in vivo* suggest that MSC are susceptible to immune rejection when administered across MHC-mismatched recipients^{217,223,299-301}. As discussed in the previous section, the alloimmune response to MSC could have a negative impact on the fate of the transplanted allograft. It is likely that MSC would be administered repeatedly pre- and post-transplantation for maximal therapeutic efficacy in patients. However, the high titres of pre-existing donor specific antibodies and memory cells following MSC infusion in patients can result in increased risk of hyperacute, acute or chronic rejection of the transplanted allograft. The alloimmune response to MSC by recipient CD4⁺ and CD8⁺ T cells is directed against MHC class I and II²²³. Under normal growth conditions, MSC are known to express MHC class I but have undetectable levels of MHC class II. Although MSC lack the expression of co-stimulatory molecules to function as an APC, allogeneic MSC can potentially be recognised by the host immune system through the indirect or semi-direct pathways of allorecognition as discussed in section **1.3 and 1.4**. The derivation of MSC alloproteins or the acquisition of intact allogeneic MSC MHC-peptide complexes may further amplify CMR and AMR in recipients, leading to detrimental outcomes

for the transplanted allograft. Furthermore, it is important to consider the inflammatory milieu in an allotransplant setting. IFN- γ in particular is known to upregulate MHC class I and induce MHC class II expression of MSC^{174,236,302,303}. This could negatively impact the fate of “off-the-shelf” MSC and the subsequent effects on the allograft survival *in vivo*.

The current MSC literature therefore suggests that the infusion of autologous MSC could be more efficacious (long-term) and safer for clinical application in allotransplantation. Allogeneic MSC on the other hand could only be effective to prevent acute but not chronic rejection due to their potential immunogenicity in an allogeneic recipient which would likely represent a major challenge in an allotransplant setting. Advances to directly address the immunogenic status and fate of “off-the-shelf” MSC *in vivo* are essential especially in an inflammatory milieu to minimize the possibility of these cells being recognised and rejected in recipients, thus increasing their therapeutic benefits in patients. Enhancing the immunosuppressive function of autologous or “off-the-shelf” MSC as well as strategies to manipulate “off-the-shelf” MSC to enable them to evade host immune system recognition are the most promising approaches.

1.11 *Ex vivo* strategies to modify MSC in allotransplantation

It is becoming evident that the exposure of MSC with proinflammatory cytokines is critical to activate their immunosuppressive function^{251,304,305}. Such *ex vivo* manipulation of MSC may yield a highly immunosuppressive population of cells with enhanced regenerative properties. The development of more potent MSC would foster their application as a monotherapy or combined with low doses of standard drugs regimes in allotransplantation.

Based on the current MSC literature, several strategies can be proposed to derive novel suppressive MSC *ex vivo*. This includes the preconditioning of MSC with the IFN- γ ^{304,306} or in

combination with other proinflammatory cytokines such as TNF- α , IL-1 α or IL-1 β ³⁰⁷; the modification with immunosuppressive drugs²²² as well as culturing of MSC in 3D spheroid aggregates³⁰⁸. These *ex vivo* modification strategies have been shown to enhance MSC immunosuppression and the anti-inflammatory function of these cells. For the purpose of this thesis, the preconditioning of MSC with IFN- γ will be reviewed as it is most extensively investigated in *in vitro* and *in vivo* conditions compared to the other methods for *ex vivo* manipulation of MSC.

In this section, the potential *ex vivo* strategy to modify MSC with the proinflammatory cytokine IFN- γ , and examine more specifically the benefits of pre-activating autologous or allogeneic MSC with IFN- γ will be discussed and is summarized in **Figure 1.11.1**. The therapeutic efficacy of autologous and allogeneic MSC preactivated with IFN- γ however demonstrates conflicting effect *in vivo*, potentially due to the inherent immunogenicity of MSC. The following sections will also summarise the current literature on the *in vivo* therapeutic utility of IFN- γ preactivated autologous and allogeneic MSC (**Table 1.11.2.1**) and the potential risks associated with MSC- γ therapy **Figure 1.11.1**.

1.11.1 Interferon-gamma preactivation of MSC and immunosuppression

1.11.1.1 The emerging role of IFN- γ to preactivate MSC immunosuppression

Krampera, 2006 initially showed the role of IFN- γ in the immunosuppressive function of MSC³⁰⁵. IFN- γ is secreted at high concentrations by NK and CD8⁺ T cells, and produced at lower levels by CD4⁺ T cells. The ability of MSC to inhibit CD4⁺ T cell proliferation and to a greater extent, the suppression of CD8⁺ T and NK cells suggested that the immune cells-driven IFN- γ potentially modified the immunomodulatory function of MSC. Similarly, studies by Sheng, 2008 demonstrated that activated lymphocytes express high levels of IFN- γ and that the co-culture of MSC with IFN- γ deficient T cells abrogate the immunosuppressive effect of

MSC²⁵¹. Moreover, in an allotransplant setting, the infusion of allogeneic MSC in combination with the immunosuppressive drug MMF prolongs cardiac allograft survival^{226,227}. Eggenhofer, 2011 proposed that the enhanced immunosuppressive effect of MSC/MMF to decrease alloresponses occurs because MMF does not affect IFN- γ production by activated T cells as well as the IFN- γ serum levels *in vivo*, thus enabling IFN- γ activation of allogeneic MSC^{226,227}. The combination of allogeneic MSC and CsA on the other hand provided less effective downregulation of alloresponses as CsA completely abrogates IFN- γ production by activated T cells, thus inhibiting MSC activation of immunosuppression²²⁶. These findings suggest that the presence of IFN- γ is essential to activate the immunomodulatory function of MSC.

Recent studies supported the notion that the preactivation of MSC with IFN- γ (MSC- γ) could enhance their therapeutic efficacy in allotransplantation *in vivo*. Advances in MSC biology have now confirmed that sufficient inflammatory signals are required to activate the immunosuppressive properties of MSC. This effect was verified *in vivo* in models of GvHD and DTH^{304,307}. Allogeneic MSC when administered at the time of bone marrow transplant were ineffective due to the low levels of serum IFN- γ ³⁰⁴. It was concluded that allogeneic MSC require a sufficient threshold of IFN- γ (> 500 U/ml) to activate their immunosuppressive function *in vivo*³⁰⁴. In another study, the administration of allogeneic untreated MSC (UT-MS) on day 7 and not day 0 showed prolonged survival and reduced tissue damage in humanized mice model of acute GvHD, while allogeneic MSC- γ were effective even when infused on day 0³⁰⁹. Thus, these studies suggest that the timing of MSC administration and the proinflammatory cytokine levels *in vivo* is critical for MSC effectiveness as anti-inflammatory agents. This further supports the notion that the preactivation of MSC, prior to their administration *in vivo*, may be more effective at suppressing inflammatory T cell responses which is a potential benefit in allotransplantation.

1.11.1.2 Mechanisms of MSC- γ immunosuppression: independent of MHC expression

Several *in vitro* studies indicate that MSC- γ are more potent suppressors of T cell responses when used even at lower doses (1%) compared to UT-MS^{174,238,310}. MSC- γ profoundly inhibit mitogen, α -CD3/CD28 monoclonal antibody (mAb) and alloantigen-driven T cell proliferation despite MHC-mismatched MSC and T cells^{246,306}. A few studies have elucidated the mechanisms by which MSC- γ enhance the anti-inflammatory and immunosuppressive properties of MSC. It has been reported for instance that the blockade of IFN- γ receptor completely abrogated MSC immunosuppression, an effect partially mediated by the tryptophan catabolising enzyme IDO expressed by MSC^{305,311}. IDO is well known to suppress T cell responses to prevent rejection and induce tolerance at the fetal-maternal interface³¹². A study utilizing allogeneic IDO knockout MSC (IDO^{-/-}MSC) or co-administration of IDO inhibitor and allogeneic MSC accelerates kidney allograft rejection and inhibits donor-specific tolerance²²⁴. Allogeneic IDO^{-/-}MSC infusion resulted in the increase of circulating donor alloantibody and decrease in splenic and intragraft infiltration of Tregs compared to the wild-type allogeneic MSC treated group. Allogeneic IDO^{-/-}MSC were also incapable of inhibiting donor DC maturation and function, thus enabling donor DC to stimulate strong recipient T cell proliferative responses. These findings suggest the crucial role of IDO to mediate *in vivo* MSC immunosuppression to directly or indirectly inhibit T cell responses by the induction of Tregs and tolerogenic DC²²⁴. The accumulation of the tryptophan metabolite kynurenine is also known to mediate the anti-proliferative effects of allogeneic MSC on T cells and is shown to be elevated *in vivo* following MSC infusion^{224,313}. Therefore, the preactivation of MSC with IFN- γ can induce IDO expression in MSC and may enhance the immunosuppressive capacity of MSC- γ to prevent allotransplant rejection and potentially induce tolerance.

The effect of IFN- γ is also concentration and time dependent. Prasanna, 2010 demonstrated *in vitro* that although MSC- γ enhanced immunosuppression on mitogen-

activated T cell responses, in a MLR, MSC- γ showed similar levels of T cell inhibition as UT-MSC¹⁷⁴. The ineffectiveness of MSC- γ to enhance immunomodulation in a MLR could be explained by the lower concentration of IFN- γ (150 U/ml) used for MSC preactivation. It has previously been shown that IFN- γ induces functional IDO in a dose-dependent manner, with highest IDO activity obtained following MSC preactivation with 500 U/ml IFN- γ ³¹¹. Therefore, low concentration of IFN- γ may be insufficient to induce MSC to express the soluble inhibitory molecules required to efficiently mediate T cell suppression in the more inflammatory setting of a MLR. Another study also indicated that the level of IDO determines the extent of T cell immunosuppression³¹⁴. In a mouse model of GvHD, the co-administration of allogeneic MSC- γ (preactivated with 500 U/ml IFN- γ) at the time of bone marrow transplantation was most effective at ameliorating the disease, with 100% survival. In contrast, allogeneic UT-MSC and MSC- γ preactivated with low dose IFN- γ (5 and 50 U/ml) were ineffective at completely reversing the disease. Thus, this further confirms that a high threshold level of IFN- γ is required to initiate the immunoregulatory function of MSC³⁰⁴. There are however some contradictory effects existing in terms of allogeneic MSC immunogenicity. It is possible that allogeneic MSC- γ were ineffective at suppressing alloantigen driven T cell proliferation in a MLR *in vitro* due to enhanced immunogenicity of these cells¹⁷⁴. This will be addressed in detail in the subsequent section. As human MSC- γ showed induction in IDO, murine MSC- γ increased the inducible nitric oxide synthase (iNOS) gene expression^{174,306,315}. These inhibitory factors suggest a potential mechanism for the enhanced immune-suppression mediated by MSC- γ derived from different species. Prasanna, 2010 also showed that PGE₂ protein levels were not altered in MSC- γ compared to UT-MSC¹⁷⁴.

The role of IFN- γ has been extensively showed *in vitro* in modulating MSC function¹⁷⁴. MSC- γ , when co-cultured with mitogenically stimulated T cells diminished the CD28 co-stimulatory molecule expression on T cells, while the CD69 activation marker of T cells was

similar between UT-MSC and MSC- γ ¹⁷⁴. The reduced CD28 signaling in T cells suggests that MSC- γ potentially induce T cell anergy. In addition, an increase in the negative T cell co-stimulatory molecule cytotoxic T-lymphocyte antigen 4 (CTLA-4) was evident in these MSC- γ and T cell co-cultures¹⁷⁴. Increased expression of negative signaling molecules on T cells enhance inhibitory effect of MSC- γ on T cell proliferation^{174,316}. Moreover, MSC- γ augmented the decrease of IFN- γ , TNF- α and IL-2 and induced greater levels of IL-10 secretion in T cells^{174,316}. Consistent with these findings, another study reported that the administration of autologous or allogeneic MSC- γ *in vivo* decreased the local and systemic inflammatory responses in animal models of colitis³⁰⁶. A significant reduction in local inflammatory cytokines IFN- γ , TNF- α , IL-17A and IL-6 was observed whereas the anti-inflammatory Th2 cytokines IL-4 and IL-10 were induced in mice treated with autologous or allogeneic MSC- γ . The infusion of autologous or allogeneic UT-MSC did not prevent the disease progression³⁰⁶. The mechanisms of allogeneic MSC immunosuppression on T cells *in vivo* were studied using a humanized model of acute GvHD³⁰⁹. This study demonstrated that allogeneic MSC- γ infusion was effective at prolonging mice survival. Contradictory to previous *in vitro* findings, the benefits of MSC- γ observed in this model were not due to induction of Tregs, T cell apoptosis or induction of T cell anergy but was associated with the direct inhibition of T cell proliferation and decrease in serum TNF- α ³⁰⁹.

Allogeneic MSC- γ have previously been shown to increase CD4⁺Foxp3⁺ Tregs by up to 6 % when compared to allogeneic UT-MSC *in vitro*²⁵⁰. MSC- γ alone expressed the Treg chemokine I-309 as measured in the culture supernatants. In co-culture with naive CD4⁺ T cells, a greater induction of this chemokine was detected. The neutralisation of I-309 receptor with anti-CCR8 slightly diminished the percentage of Foxp3 positive cells in these co-cultures. Hence, this suggests a possible functional role of I-309 to partly induce the generation and recruitment of Tregs, which could indirectly mediate T cell suppression²⁵⁰. Consistent with

these findings, we also showed that MSC- γ in co-culture with mitogenically-activated CD4⁺CD25⁻ T cells induces the generation of CD4⁺CD25^{high}CD127^{low}FoxP3⁺ Tregs compared to UT-MS2. More studies nonetheless are required to verify if MSC- γ induce and expand Tregs.

The presence of IFN- γ is critical to upregulate B7-H1 expression on MSC which in turn deliver negative regulatory signals that inhibit T cell proliferation and function^{194,231,251,252,316,317}. Although these studies demonstrated that B7-H1 is a key mechanism for MSC immunosuppression, we and others have reported that the increase of B7-H1 expression following IFN- γ stimulation is not the essential mechanism for MSC to directly mediate T cell suppression^{2,318}. Nevertheless, some studies suggested that B7-H1 is critical for the induction of Treg and maintenance of islet allograft survival *in vivo*^{319,320}. Francisco and colleagues have demonstrated the role of B7-H1 in the maintenance, development and function of inducible Tregs (iTregs)³²⁰. B7-H1 promoted functional Tregs induction with enhanced suppressive ability on effector T cells compared to control generated iTregs³²⁰. Therefore, it can be hypothesized that the increase in B7-H1 expression observed on MSC following IFN- γ preactivation may potentially promote induction of functional Tregs that are highly immunosuppressive, thus enabling MSC to indirectly inhibit T cell responses. The role of MSC- γ expressing B7-H1 in the induction of Tregs has not been studied to date.

IFN- γ is also known to upregulate CD200 expression on MSC. Although the CD200/CD200R axis is not a crucial mechanism for MSC to directly inhibit T cell responses as shown *in vitro*²⁵⁴, it can be proposed that an enhanced CD200/CD200R engagement may augment the immunosuppressive properties of MSC- γ . The CD200/CD200R interaction has been shown to be critical to prevent allograft rejection by increasing intragraft infiltration of Tregs^{258,259}. In plasmacytoid DC, the interaction of CD200/CD200R increases IDO expression with enhanced functional ability of IDO to catabolise tryptophan³²¹. The implication of the

CD200/CD200R axis in MSC- γ mediated immunosuppression however is unknown and requires further investigation.

Taken together, these findings show that during alloimmune responses, MSC require activating signals from robustly proliferating T cells to induce their immunosuppressive effect. However, it is possible that the numbers of MSC, especially allogeneic MSC is drastically reduced before the required threshold of inflammatory level is reached for MSC activation and consequently the expression of inhibitory molecules to mediate immunosuppression. Therefore, we hypothesize that this lag phase for MSC activation *in vivo* can be avoided by preactivating MSC with IFN- γ *ex vivo* to enhance their immunosuppressive potential. As discussed above, the preactivation of MSC may induce or increase the expression of various immunosuppressive factors as well as enhance the inhibitory activity of these factors to directly or indirectly mediate T cell suppression. Findings on the role of IFN- γ in modulating MSC function further suggest that the derivation of MSC- γ may be more effective and feasible compared to UT-MSC to mediate immunosuppression via the secretion of tolerogenic cytokines, the induction of T cell anergy (hyporesponsiveness) and the generation of Tregs. Future research efforts should focus on elucidating other mechanisms of MSC- γ mediated enhanced immunosuppression and if these cells are capable of inducing allotransplantation tolerance. Performing microarray and proteomics analysis may enable the identification of novel MSC- γ expressed immunoregulatory molecules that may enhance the immunosuppressive effect of MSC.

1.11.1.3 MSC- γ : homing potential and tissue regeneration properties

In addition to the generation of a highly immunosuppressive MSC- γ population discussed in the previous section, MSC- γ have been shown to exhibit an enhanced homing potential to sites of inflammation and increased tissue regenerative properties^{306,322}. Culture expanded MSC

express low levels of CXCR4 and this may reduce the migratory properties of MSC to inflammatory sites, thereby potentially reducing the effectiveness of MSC *in vivo*^{180,323}. Microarray analysis revealed that IFN- γ preactivation of MSC elevates the expression of the chemokine CXCR7, MMP and the adhesion molecule lectin. The altered expression of cell trafficking molecules potentially enhanced human MSC- γ migration to inflammatory sites to mediate their anti-inflammatory effects *in vivo* in an established mouse model of colitis³⁰⁶. More recently, the administration of autologous MSC- γ in a murine wound repair model implicates that these cells were more effective for tissue regeneration compared to autologous UT-MS. This effect was thought to be mediated by the ability of autologous MSC- γ to alternatively activate host macrophages with reparative properties³²².

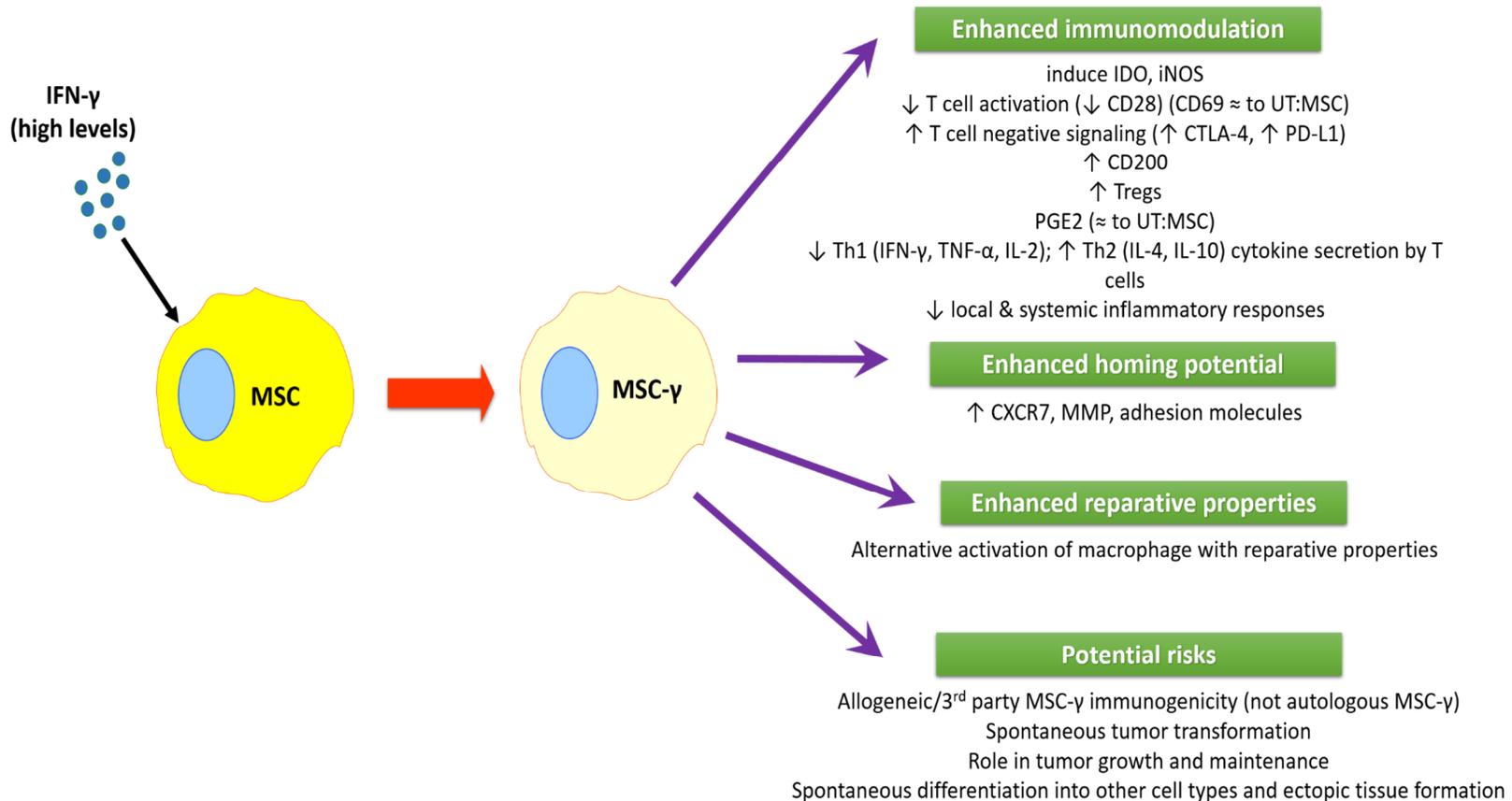


Figure 1.11.1 The potential benefits and risks of *ex vivo* IFN- γ preactivated MSC in allotransplantation.

Figure adapted and modified from Sivanathan KN, et al. (2014)⁴.

1.11.2 The immunogenic status of allogeneic MSC- γ : Implications in allotransplantation

1.11.2.1 Evidence of MSC- γ immunogenicity

Regardless of the potential therapeutic benefits of MSC- γ to enhance or induce the expression of immunosuppressive factors *in vitro* and *in vivo* as well as having an increase in homing and regenerative potential, it is important to consider whether the administration of autologous or allogeneic MSC- γ would be more efficacious in the context of allotransplantation. Based on the conflicting effects on the therapeutic benefits of allogeneic MSC application in allotransplantation (**Table 1.8.1.2** and section **1.10**), it is likely that the administration of allogeneic MSC- γ would also trigger alloimmune responses directed against these cells. In addition, *in vivo* studies reporting on the benefits of MSC- γ demonstrated short-term effects on immunosuppression and tissue repair. The long-term efficacy of MSC- γ and their potential immunogenicity, especially for allogeneic MSC- γ was not studied and is a limitation in these studies^{304,306,309,322}. There are contradictory reports indicating that administration of allogeneic MSC- γ are in fact ineffective at suppressing inflammation and are immunogenic *in vivo*^{217,299,300}, making their long-term efficacy questionable (**Table 1.11.2.1**). Interestingly, MSC- γ administration failed to treat a patient with Crohn's disease. Following a second MSC- γ infusion, this patient demonstrated elevated percentage of NK cells, suggesting that MSC- γ provoked an immune response *in vivo*³²⁴. This observation is consistent with a previous study showing that activated NKs are capable of lysing autologous or allogeneic MSC³²⁵.

Badillo, 2007 using immunocompetent mice directly demonstrated that MSC- γ enhances the primary and secondary alloantibody responses compared to UT-MSC. Higher levels of circulating anti-donor IgM and IgG alloantibodies were detected in mice administered with MSC- γ . This *in vivo* study was the first to report that MSC- γ are more immunogenic and may induced a rapid AMR of MSC- γ compared to UT-MSC²¹⁷. In a subsequent study, the

infusion of UT-MSc was effective at inhibiting inflammatory responses in mouse experimental autoimmune encephalomyelitis (EAE)²⁹⁹. Contradictory to inflammatory models of GvHD^{304,309} and colitis³⁰⁶, this study showed that MSC- γ were incapable of alleviating EAE potentially due their immunogenicity²⁹⁹. Upon secondary exposure to MSC- γ , responder cells from alloantigen presensitized recipient mice produced high levels of IFN- γ . In addition to the loss of MSC- γ tolerogenic effects on alloantigen presensitized responder cells, there was an increase of CD4⁺ T cell infiltration in the spinal cord. These findings indirectly indicate that MSC- γ are more immunogenic than UT-MSc and provoke alloimmune responses *in vivo*²⁹⁹.

Schu, 2012 reported that even though the *in vivo* survival rate of MSC- γ was similar to UT-MSc, MSC- γ were highly susceptible to cytolysis by alloantigen-specific CD8⁺ T cells *in vitro* compared to UT-MSc. This study compared the rejection rate of intraperitoneally injected UT-MSc and MSC- γ *in vivo* for short term (14 days)³⁰⁰. Zangi, 2009 reported that allogeneic UT-MSc are able to survive in immunocompetent mice for more than 20 days and are completely eliminated by day 40²²³. Similarly, another study indicated that genetically engineered allogeneic MSC overexpressing erythropoietin persist up to 54 days post-infusion in MHC-mismatched recipients³²⁶. Thus, in studies conducted by Schu, 2012, it is possible that allogeneic MSC- γ may elicit alloimmune responses in recipient and their enhanced immunosuppressive properties may have prevented acute rejection of MSC- γ , thereby enabling the short-term survival of MSC- γ in MHC-mismatched recipients. The evidence that MSC- γ are highly susceptible to T cell cytolysis could suggest that the increased immunosuppressive effect of MSC- γ may be insufficient to prevent their rejection at later phases post infusion and may induce more rapid CMR of MSC- γ ³⁰⁰. This effect could persist at later stages (>20 days) post MSC- γ infusion, which was not evaluated in this study³⁰⁰.

1.11.2.2 Mechanisms of MSC- γ immunogenicity and implications in allotransplantation

There are contradictory effects concerning IFN- γ dependent immunomodulatory effects of MSC and this could be related to the upregulation of MHC molecules as well as MSC immunogenicity^{302,327,328}. IFN- γ is known to upregulate MHC class I and induce MHC class II on MSC^{236,302,303}. The expression of MHC class II on allogeneic MSC has been previously shown to induce alloimmune responses in co-cultures with MHC-mismatched responder cells in a one-way MLR³²⁸. In another study, allogeneic MSC derived from a patient with defective IFN- γ -receptor (IFN- γ R^{-/-} MSC) inhibited T cell proliferation independent of IDO and to a similar extent to normal allogeneic MSC³²⁷. The inability of normal allogeneic MSC to enhance T cell inhibition may relate to the induction of MHC class II on MSC following IFN- γ exposure. MSC expressed MHC class II may elicit alloimmune T cell responses against normal allogeneic MSC, making them incapable of enhancing T cell inhibition. IFN- γ R^{-/-} MSC may be less immunogenic compared to normal allogeneic MSC as there was no apparent induction of MHC class II in these cells in the presence of IFN- γ . Thus, due to their possibly low immunogenicity, IFN- γ R^{-/-} MSC were capable of suppressing T cell proliferation through mechanisms independent of IFN- γ activation of MSC immunomodulatory factors.

In vivo studies by Zangi, 2009, showed that alloimmune responses against UT-MS-C were mediated by the recognition of allogeneic MHC molecules by recipient CD4⁺ and CD8⁺ memory T cells²²³. In contrast, other findings suggested that the increase in MHC class I in MSC- γ is a critical for MSC function to evade the host immune response³²⁹. The high expression of MHC class I was shown to protect MSC from activated NK cell mediated recognition and apoptosis *in vitro*, but the *in vivo* immunogenicity of MSC- γ was not evaluated in this study³²⁹. Hence, the upregulation and induction of MHC molecules in MSC- γ may increase allospecific memory T cells against MSC- γ , possibly leading to more rapid rejection of these cells. This could also provide a possible explanation for the inefficacy of MSC- γ

compared to UT-MSc in models of EAE²⁹⁹. Apart from the potential rapid rejection of MSC- γ , the induction of an immunological memory response against donor antigens could negatively impact the fate of the transplanted allograft through mechanisms discussed in section **1.3, 1.4 and 1.10.3**.

Although MSC- γ lack the expression of co-stimulatory signals (CD80, CD83, CD86) to function as APC to mediate direct T cell allorecognition and activation^{174,238,250,302}, it can be speculated that MSC- γ induce allogeneic T cell responses through the indirect or semi-direct pathway of allorecognition. Allogeneic MHC-peptide transfer from MSC- γ could be more rapid compared to UT-MSc due to high expression of MHC molecules. This enables allogeneic MHC-peptide to be recognised by recipient T cells through the semi-direct pathway. Moreover, the enhanced expression or induction of allogeneic proteins in MSC- γ (compared to UT-MSc) may activate recipient T cells by the indirect pathway of allorecognition or induce B cell activation, alloantibody production and complement mediated-lysis of donor cells in allotransplantation. It is known that the implantation of allogeneic MSC engineered to overexpress erythropoietin in MHC-mismatched recipients led to the production of anti-erythropoietin antibodies^{326,330}. Additionally Eliopoulos, 2005 showed that the overexpression of alloproteins in MSC may enable short-term survival of MSC *in vivo* but these cells were subsequently rejected³²⁶. Similarly, strategies to preactivate MSC with IFN- γ to induce or upregulate MSC-expressed immunomodulatory or reparative factors may be efficacious to prevent acute rejection in allotransplantation. The enhanced immunosuppressive capacity of MSC- γ may dampen the immune response against MSC- γ , enabling them to suppress acute allograft rejection. However, the long-term therapeutic benefit and the longevity of MSC- γ in clinical allotransplantation could be limited due to generation of antibodies against MSC- γ alloproteins. It is possible that the high levels of circulating alloantibodies could mediate a

more rapid AMR of the transplanted allograft and this is likely to occur at later stages of allograft transplantation (chronic rejection).

Furthermore, for maximal therapeutic benefits of MSC to promote long-term allograft survival, it could be envisaged that MSC- γ therapy would be administered repeatedly. The consequences of repeated allogeneic MSC- γ administration and the potential amplification of rejection against allogeneic MSC^{200,217,324,326} may also negatively impact the fate of the transplanted allograft. The mechanisms by which allogeneic MSC- γ induce alloimmune response *in vivo* and their therapeutic utility particularly in the context of allotransplantation remains to be explored.

1.11.2.3 Other safety issues concerning autologous and allogeneic MSC- γ therapy

The safety and efficacy of autologous and allogeneic MSC- γ therapy is yet to be demonstrated in preclinical models of allotransplantation. There are reports on MSC involvement in tumor maintenance³³¹⁻³³³, spontaneous malignant transformation of MSC³³⁴ and differentiation of MSC into other cell types which may lead to ectopic tissue formation³³⁵⁻³³⁷. Even though MSC- γ have enhanced capacity to specifically induce apoptosis of tumor cells *in vitro*, MSC- γ accelerated tumor growth in a malignant setting *in vivo*³³⁸. This could relate to the high expression of IDO by MSC- γ . It is known that IDO is involved in tumor-induced tolerance³³⁹. The spontaneous differentiation of allogeneic MSC- γ into other cell types could also further enhance antibody- or cell-mediated alloimmune responses in transplant recipients. Hence, future studies should investigate issues concerning autologous or allogeneic MSC- γ therapy such as in tumor transformation, spontaneous differentiation of MSC- γ into other cell types and MSC- γ ectopic tissue formation. These issues should be considered prior to the wide application of MSC- γ therapy into the clinic.

1.11.2.4 Summary of MSC- γ therapy in allotransplantation

Bone marrow-derived MSC may be beneficial for immunosuppressive and tissue reparative therapy in allotransplant patients. MSC can directly inhibit T cell proliferation, activation and function through the expression of immunomodulatory factors. The interaction of MSC with APC and their ability to promote the generation of regulatory DC and T cells can further dampen proinflammatory T cell responses. IFN- γ induce or upregulate MSC inhibitory factors to further enhance T cell inhibition by directly or indirectly interacting with T cells. Therefore, the *ex vivo* strategy to preactivate MSC with IFN- γ prior to their administration *in vivo* may be more effective than untreated MSC at ameliorating inflammatory T cell responses in allotransplantation. In addition to the generation of a highly immunosuppressive MSC- γ population, these cells have enhanced homing and tissue regenerative properties, making them a highly attractive candidate for allotransplantation cellular therapy.

The crucial choice is whether the application of autologous or allogeneic MSC would be more safe and efficacious. Allogeneic MSC induce cell- and antibody-mediated alloimmune responses which may decrease MSC longevity and effectiveness *in vivo*. The induction of donor-specific immune responses can also result in either hyperacute, acute or chronic allograft rejection depending on the route, timing (pre- or post-transplant), dose and numbers (single or multiple) of MSC infusion. Similar to models of GvHD and colitis, allogeneic MSC- γ may be effective at preventing acute inflammatory responses in allograft rejection. The potential alloimmune responses elicited by allogeneic MSC- γ suggest that these cells are more immunogenic than UT-MSC and could be detrimental when administered in allotransplant patients. The enhanced alloimmune responses following MSC- γ infusion could relate to the upregulation or induction of MHC molecules and alloproteins upon exposure of MSC to IFN- γ .

When considering the application of UT-*MSC* or *MSC-γ* monotherapy in allotransplantation, then autologous *MSC* would be most effective and safe to prevent rejection and promote long-term allograft survival. There are however concerns associated with the declined function of autologous *MSC* derived from actively ill patients. Besides that, approximately 3 months is required to generate sufficient doses of autologous *MSC* for patient infusion and this may not be ideal for newly diagnosed patients with acute disease conditions. Under these circumstances, if allogeneic *MSC* therapy is to be considered, then co-administration with immunosuppressive drugs such as MMF or rapamycin but not CsA could be most effective. Immunosuppressive drugs may control the immune responses directed against allogeneic *MSC*, prolonging *MSC* survival and enabling them to exert their beneficiary effects in allotransplantation. Understanding the mechanisms by which allogeneic UT-*MSC* and *MSC-γ* are recognised by the host immune system would enable future studies to manipulate these cells to make them less-immunogenic in MHC-mismatched recipients. This would enable the administration of “off-the-shelf” *MSC*, especially *MSC-γ* to deliver their potential immunosuppressive or regenerative factors to inhibit alloimmune rejection and promote tissue repair, respectively, following allotransplantation.

Table 1.11.2.1 Therapeutic utility of MSC- γ *in vivo*: Comparison between autologous and allogeneic MSC- γ

Reference	Experimental model (species)	Route	[IFN- γ]	Efficacy of MSC- γ vs. UT-MSC	Efficacy of UT-MSC vs. alloMSC- γ		Immunogenicity of MSC <i>in vivo</i>
					auto/alloUT-MSC	auto/alloMSC- γ	
Badillo, 2007 ²¹⁷	Immunogenicity (mouse)	i.p.	200 ng/ml (72hr)	autoUT-MSC more efficacious than autoMSC- γ alloUT-MSC and alloMSC- γ ineffective	autoUT-MSC: -low/undetectable IgG and IgM alloUT-MSC -induce primary (high IgM) and secondary (high IgG) alloantibody response	autoMSC- γ : -low/undetectable IgG or IgM alloMSC- γ : -further \uparrow primary and secondary alloantibody response (vs. alloUT-MSC)	alloUT-MSC and alloMSC- γ are immunogenic. alloMSC- γ are more immunogenic vs. alloUT-MSC – antibody-mediated response
Polchert, 2008 ³⁰⁴	GvHD (mouse)	i.v.	5 or 50 or 500 U/ml (confluency)	alloMSC- γ (500 U/ml) more efficacious while alloUT-MSC ineffective	alloUT-MSC Ineffective at preventing GvHD mortality (25% survival)	alloMSC- γ : prevent GvHD mortality (100% survival) - IFN- γ concentration dependent (ie. most effective with 500 U/ml IFN- γ preactivation)	NA
Rafei 2009 ²⁹⁹	EAE (mouse)	i.p.	2 ng/ml (80% confluency)	auto/allo UT-MSC efficacious while alloMSC- γ ineffective	alloUT-MSC - alleviate EAE - \downarrow systemic proinflammatory response (IFN- γ , IL-17) - tolerogenic effect - \downarrow CD4 ⁺ T cell infiltration in spinal cord autoUT-MSC - alleviate EAE - \downarrow systemic proinflammatory response (IFN- γ , IL-17)	alloMSC- γ - incapable of alleviating EAE - not inhibit systemic proinflammatory responses (similar IFN- γ and IL-17 levels vs. no treatment group) - loss of tolerogenic effects (are immunogenic) - higher levels of CD4 ⁺ T cell infiltration in spinal cord	alloMSC- γ is immunogenic (NDA)

(continued) Therapeutic utility of MSC- γ *in vivo*: Comparison between autologous and allogeneic MSC- γ

Reference	Experimental model (species)	Route	[IFN- γ]	Efficacy of MSC- γ vs. UT-MSC	Efficacy of UT-MSC vs. alloMSC- γ		Immunogenicity of MSC <i>in vivo</i>
					auto/alloUT-MSC	auto/alloMSC- γ	
Duijvestein, 2011 ³⁰⁶	TNBS-induced colitis – Th1 mediated colitis (mouse)	i.p.	500 U/ml (> 80% confluence)	autoMSC- γ more efficacious vs. autoUT-MSC	autoUT-MSC - prevent colitis - \uparrow survival vs. no treatment group (but similar to autoMSC- γ)	autoMSC- γ - \downarrow severity of colitis (\downarrow inflammation, infiltration of granulocytes, mononuclear cells and CD3 ⁺ T cells in colon - \uparrow survival vs. no treatment group (but similar to autoMSC- γ) - \downarrow systemic inflammation - \downarrow local inflammation in colon (\downarrow IL-6 and TNF- α) - induce a Th2 phenotype locally (\uparrow IL-10)	no apparent immunological response/rejection of MSC (NDA)
Duijvestein, 2011 ³⁰⁶	DSS-induced colitis (mouse)	i.p.	500 U/ml (>80% confluence)	alloMSC- γ efficacious while alloUT-MSC ineffective	alloUT-MSC ineffective at preventing DSS-induced colitis - not alter local inflammatory levels	alloMSC- γ prevent DSS-induced colitis - \downarrow local inflammation (\downarrow IFN and IL-17)	no apparent immunological response/rejection of MSC (NDA)
Duijvestein, 2011 ³⁰⁶	Established colitis (mouse)	i.p.	500 U/ml (6 days)	Human MSC- γ have enhanced homing potential to site of inflammation vs. human UT-MSC (24hr post injection)	Human UT-MSC -no enhanced homing potential to inflamed intestine in colitis or no colitis animals	Human MSC- γ - \uparrow homing potential to inflamed intestine in colitis animals (vs. no colitis animals)	NA
Lee, 2012 ³²²	Wound repair (mouse)	i.p.	500 U/ml (3 days)	autoMSC- γ prevent wound healing while autoUT-MSC is ineffective	autoUT-MSC - ineffective at wound healing	autoMSC- γ - \uparrow wound repair - effect dependent on host macrophage activity	NA

(continued) Therapeutic utility of MSC-γ <i>in vivo</i>: Comparison between autologous and allogeneic MSC-γ							
Reference	Experimental model (species)	Route	[IFN- γ]	Efficacy of MSC- γ vs. UT-MSC	Efficacy of UT-MSC vs. alloMSC- γ		Immunogenicity of MSC <i>in vivo</i>
					auto/alloUT-MSC	auto/alloMSC- γ	
Schu, 2012 ³⁰⁰	Immunogenicity (rat)	i.v.	100 U/ml (24hr)	alloUT-MSC and alloMSC- γ are ineffective	<p>alloUT-MSC</p> <p>- <i>in vitro</i>: slightly susceptible to cytolytic lysis by alloantigen-specific T cells (CTL)</p> <p>- <i>in vivo</i>: not induce early T cell activation and proinflammatory cytokine production</p> <p>- <i>in vivo</i>: induce IgG1 and IgG2 alloAb but low IgM</p> <p>- complement mediated lysis of MSC-induced alloAb higher vs. autoUT-MSC</p> <p>- <i>in vivo</i>: immunogenic -subjected to rejection (\downarrowsurvival times)</p>	<p>alloMSC-γ:</p> <p>- <i>in vitro</i>: highly susceptible to cytolytic lysis by alloantigen-specific T cells (CTL) (vs. alloUT-MSC)</p> <p>-alloAb response – NA</p> <p>- <i>in vivo</i>: immunogenic – subjected to rejection with similar immunogenicity vs. alloUT-MSC</p>	alloMSC- γ and alloUT-MSC are immunogenic
Tobin, 2013 ³⁰⁹	Acute GvHD (humanized mouse)	i.v.	500 U/ml (48hr)	<p>Day 0 infusion: alloMSC-γ effective while alloUT-MSC ineffective</p> <p>Day 7 infusion: alloUT-MSC similar efficacy as day 0 administered alloMSC-γ</p>	<p>alloUT-MSC (Day 0 administration)</p> <p>- ineffective at treating acute GvHD</p> <p>alloUT-MSC (Day 7 administration)</p> <p>- \downarrow acute GvHD pathology and prolong mice survival) (effective at treating acute GvHD)</p> <p>- \downarrow mononuclear cell infiltration in liver and intestine (not in lungs)</p>	<p>alloMSC-γ (D0 administration)</p> <p>- effective even when administered at Day 0 (\downarrow acute GvHD pathology and prolong mice survival)</p> <p>- \downarrow mononuclear cell infiltration in liver and intestine (not in lungs)</p> <p>- <i>in vivo</i>: \downarrow CD4⁺ T cell proliferation and \downarrow systemic TNF-α in lungs</p>	NA
Treacy, 2014 ²¹³	Cornea (rat)	i.v.	500U/ml (72hr)	autoUT-MSC and autoMSC- γ are ineffective	-	-	NA

(continued) Therapeutic utility of MSC-γ <i>in vivo</i>: Comparison between autologous and allogeneic MSC-γ							
Reference	Experimental model (species)	Route	[IFN- γ]	Efficacy of MSC- γ vs. UT-MSC	Efficacy of UT-MSC vs. alloMSC- γ		Immunogenicity of MSC <i>in vivo</i>
					auto/alloUT-MSC	auto/alloMSC- γ	
Taddio 2015 ³²⁴	Severe Crohn's disease (single case patient report)	i.v.	-	2 MSC- γ infusion (2x10 ⁶ cells/kg)	MSC- γ infusion well tolerated 10 days after 2 nd infusion: - worsening of diarrhoea - no clinical improvements		High % of NK in patient serum at 2 nd infusion; MSC- γ are immunogenic

Abbreviations: i.p, intraperitoneal; i.v., intravenous; auto, autologous; allo, allogeneic; UT-MSC, untreated MSC; MSC- γ , alloAb, alloantibodies; IFN- γ preactivated; EAE, experimental autoimmune encephalomyelitis; NDA, not directly assessed; NA, not assessed; GvHD, graft-versus host disease; TNBS, trinitrobenzene sulfonic acid; DSS, dextran sodium sulfate; NK, natural killer cells. *Adapted and updated from Table 4 Sivanathan KN, et. al., (2014)*⁴.

1.11.3 IL-17A modulation of Mesenchymal Stem Cells

1.11.3.1 IL-17A enhances MSC growth and immunosuppressive potential

It is well established that IL-17A functions as a growth factor for MSC. It has been reported that the exposure of freshly isolated MSC cultures with IL-17A increased frequency of colony-forming units fibroblast (CFU-F). IL-17A also enhanced MSC growth potential via the activation of signaling molecules Akt, Erk, MEK and p38³⁴⁰⁻³⁴². Furthermore, the presence of IL-17A did not compromise the functional differentiation and immunophenotypic marker expression as well as the fibroblastic-like morphology of MSC^{340,343}. These features suggest the IL-17A retained the mesenchymal phenotype of MSC.

Sivanathan KN, 2010 (Honours Thesis)³⁴³ for the first time demonstrated that IL-17A preconditioning of human MSC (MSC-17) were in fact more potent suppressors of mitogen induced T cell proliferation, with up to 51 % inhibition of T cells when compared to UT-MSC. MSC- γ only showed a 21.4 % inhibition under the same condition. The enhanced MSC-17 immunosuppression was also evident in a MLR and dependent on MSC dose and IL-17A concentration used for MSC preconditioning. In contrast to MSC- γ , MSC-17 showed no increase in PD-L1 expression thereby indicating other mechanisms are involved in MSC-17 mediated immunomodulation³⁴³. In this thesis we aim to further explore in detail and compare the immunomodulatory efficacy of MSC-17 to MSC- γ and UT-MSC in a context of allotransplantation.

1.12 Significance and contribution to the research

The primary goal of this study is to identify alternative *ex vivo* strategies to manipulate MSC to enhance their immunosuppressive properties while retaining them in an inert immunogenic state. In preliminary studies, we have shown that MSC pre-treated with IL-17A (MSC-17) were able to potently inhibit allogeneically and mitogenically induced T cell proliferation³⁴³. Strategies to pre-activate the immunosuppressive function of MSC with the proinflammatory cytokine IL-17A prior to their infusion *in vivo* may enhance the translation of MSC as a universal cell based therapy agent to induce allograft tolerance and prevent transplantation rejection. This proposed *ex vivo* manipulation of MSC with IL-17A may reduce or eliminate patient dependence on lifelong immunosuppression and thus prevent undesired side effects of drugs. In T1D, the administration of MSC would act to prevent islet allotransplantation rejection, facilitate islet cell regeneration, control autoimmune responses that prevent the destruction of insulin-producing β -cells and MSC transdifferentiation into insulin-producing cells. These beneficial effects of MSC can therefore prevent the recurrence of T1D in islet transplant patients. These findings would not only be beneficial in allotransplantation but also in the treatment of various inflammatory diseases such as asthma, systemic lupus erythematosus.

1.13 Thesis aims and hypotheses

The specific aims and hypotheses of this thesis are:

Aim 1: To assess the effect of 5 days IL-17A or IFN- γ treatment on human MSC immunophenotype and T cell immunosuppressive function.

Hypothesis: IL-17A modified MSC (MSC-17) show superior T cell immunosuppressive properties compared to UT-MSc and exhibit minimal immunogenicity due to their lack of expression of MHC class II molecules, in contrast to MSC- γ .

Aim 2: To evaluate the ability of MSC-17 to promote the generation of human Tregs to mediate indirect suppression of T cells, compared to MSC- γ and UT-MSc.

Hypothesis: MSC-17 mediate their potent immunosuppressive effects on effector T cells via the generation of high numbers of functionally suppressive Tregs.

Aim 3: To compare the gene expression profiles of human MSC- γ and MSC-17 by microarray and bioinformatics analyses.

Hypothesis: IFN- γ induces the expression of genes associated with increased immunogenicity, while IL-17A induces the expression of an array of chemokines and matrix metalloproteinases that increase T cell chemotaxis to MSC, mediating inhibition of T cells and induce the generation of Tregs.

Aim 4: To evaluate the efficacy of mouse compact bone derived MSC-17 to suppress *in vitro* T cell proliferation and prolong *in vivo* allograft survival in mouse models of islet allotransplantation.

Hypothesis: Compact bone MSC-17 are more potent inhibitors of T cell proliferation *in vitro*, and prolong allograft survival in mouse models of islet transplantation, compared to UT-MSC controls.

This work creates a platform from which a novel MSC-17 therapy can be developed in the future and potentially be applied to large animal models with transplantation relevance.

CHAPTER 2: MATERIALS AND METHODS

This chapter describes protocols used for *in vitro* laboratory techniques with human and animal cells as well as animal based procedures used in this thesis. Additional methodology and chapter specific protocols can be found in the relevant result chapter sections of this thesis.

2.1 Buffers and solutions

Primary MSC media: α -MEM (Gibco, Life Technologies, 12571-063), 20% FBS, 50U/ml Penicillin and 50U/ml Streptomycin, 2mM L-glutamine, 100 μ M L-ascorbate-2-phosphate, 1mM Sodium pyruvate

MSC media: α -MEM (Gibco, Life Technologies, 12571-063), 10% FBS, 50U/ml Penicillin and 50U/ml Streptomycin, 2mM L-glutamine, 100 μ M L-ascorbate-2-phosphate, 1mM Sodium pyruvate

CFU-F media: α -MEM (Gibco, Life Technologies, 12571-063), 20% FBS, 50 U/ml Penicillin and 50U/ml Streptomycin, 2mM L-glutamine, 100 μ M L-ascorbate-2-phosphate, 1mM Sodium pyruvate

Osteogenesis media: α -MEM-high glucose, 5% FBS (osteogenesis qualified FBS), 50U/ml Penicillin and 50U/ml Streptomycin, 2mM L-glutamine, 100 μ M L-ascorbate-2-phosphate, 1mM Sodium pyruvate, 10⁻⁷M Dexamethasone, 10mM Hepes, 1.8mM KH₂PO₄

Adipogenesis media: α -MEM-high glucose, 5% FBS (adipogenesis qualified FBS), 50U/ml Penicillin and 50U/ml Streptomycin, 2mM L-glutamine, 100 μ M L-ascorbate-2-phosphate, 1mM Sodium pyruvate, 0.5 μ M Isobutylmethylxanthine, 0.5 μ M Hydrocortisone, 60 μ M Indomethacin

Chondrogenesis media: α -MEM-high glucose, 50U/ml Penicillin and 50U/ml Streptomycin 2mM L-glutamine, 100 μ M L-ascorbate-2-phosphate, 1mM Sodium pyruvate, 10⁻⁷M Dexamethasone, 1x ITS + Premix (100x) (Sigma), 10ng/ml TGF- β , 1% BSA

Complete RPMI 1640 media: RPMI 1640 (Gibco, Life Technologies, 12571-063), 10% FBS, 50U/ml Penicillin and 50U/ml Streptomycin, 2mM L-glutamine, 100 μ M L-ascorbate-2-phosphate, 1mM Sodium pyruvate

DC generation media: RPMI 1640, 10% FBS, β -mercaptoethanol (2 μ L/500ml), 1000U/ml GM-CSF and 500U/ml IL-4

Islet culture media: RPMI 1640 10% FBS, 5ml GlutaMAX™-I (100X), hepes (0.01M/ml) Penicillin (0.012mg/ml), Gentamicin (0.016mg/ml)

HFF: HBSS (Sigma; H9394), 5% FBS

Cell freezing media: 10% DMSO, 90% FBS

Collagenase I / DNase I solution (2ml solution / mouse): α -MEM, 3mg/ml Collagenase II (stock 62.5 mg/ml) (Gibco), 50U/ml DNase I (stock at 20 000U/ml)

Pancreas digestion solution: 3ml cold serum-free M199 medium containing 0.67mg collagenase (Liberase TL, grade; Roche) per pancreas

0.5M EDTA Buffer: 93.06g EDTA, 500ml Milli Q Water, Adjusted to pH 8.0

iTreg sorting buffer: 2mM EDTA, 3% FBS, HBSS

Blocking buffer: HBSS, 1% BSA, 5% normal human serum (Australian Red Cross, Adelaide)

FACS Fix: 5ml 10% Formalin, 10gm glucose, 1ml 10% sodium azide

Alizarin red: 0.5g Alizarin Red, 50ml 2% Ethanol, pH 4.1 to 4.3 (adjusted with HCl or NaOH)

Oil O Red stock solution: 0.5g Oil O Red Powder, 100ml Isopropanol (*stable for 1 year)

Oil O Red Working solution: 6ml Oil O Red stock, 4ml distilled water (Allow to stand for 10mins and filter through No.46 Whatman filter paper - *stable for 2hr)

Toluidine blue stain (CFU-F Stain): 0.51g Toluidine Blue, 30ml 40% Formaldehyde , 480ml PBS

STZ buffers:

1.0M Citric acid buffer: 10.5g citric acid dissolved in 50ml sterile Baxter water (*can be prepared and stored)

0.1M tri-sodium citrate buffer pH4.5: 1.47g sodium citrate dissolved in 40ml sterile Baxter water. pH adjusted to 4.5 using 1.0M citric acid. Bring to a total volume of 50ml with sterile Baxter water and filter sterilize solution using 0.2 μ M filter (*prepared on the day of STZ induction)

STZ for induction of diabetes: STZ was solubilise with 2.5ml Tris-sodium citrate buffer, mixed for 1min and filter sterilized using a 0.2 μ M filter (*STZ was injected intraperitoneally in all mice within 15mins of drug preparation)

2.2 Isolation and culture of human Mesenchymal stem cells

2.2.1 Human plastic adherent selected MSC

Bone marrow aspirates were obtained from the posterior iliac crest of normal adults volunteers (subjects with informed consent; age 20-35 yr) according to guidelines approved by the Human Ethics Committee of the Royal Adelaide Hospital, Australia (Protocol 940911a). Human bone marrow mononuclear cells (BMMNC) at the Ficoll-Paque PLUS (Biosciences) plasma interface were isolated by density gradient centrifugation at 400xg, 30mins (no brake). BMMNC were washed twice with HFF (HBSS supplemented with 5% FBS) at 400xg, 7mins and seeded at 30 000 cells/cm² in tissue culture flasks to establish the primary plastic adherent derived MSC cell lines³⁴⁴. The MSC cultures were established in primary MSC media containing α -MEM, 20% (v/v) heat inactivated fetal bovine serum (FBS) (Gibco, Invitrogen), 2mM L-glutamine (Multicel, USA), 1mM sodium pyruvate (Sigma Aldrich), 100 μ M L-ascorbate and 1% penicillin streptomycin³⁴⁵. The primary MSC cell lines were established at 37°C in a humidified tissue culture incubator with 5% CO₂ for 1-2 weeks with first media change performed at day 7. At 80-90% confluence, adherent MSC were detached using 0.25% trypsin/EDTA (Sigma) for 4min, 37°C. MSC were re-seeded for expansion at a density of 5000 - 10 000 cells/cm² in tissue culture flasks and maintained in MSC media containing 10% heat inactivated FBS. Media change was performed every 3 days. MSC at passage 3 to 6 were used in experiments.

2.2.2 Human STRO-1 purified MSC

Immunoselection of stromal precursor antigen-1 (STRO-1) positive BMMNC by magnetic-activated cells sorting (MACS) yields a highly purified population of MSC³⁴⁶. These STRO-1⁺ MSC also have a greater efficiency to form colonies, multilineage differentiation potential and enhanced reparative capacity compared to the traditional non-selective plastic adherent

selected MSC which yield a more heterogeneous cell population^{184,347}. Thus, the MACS-based STRO-1⁺ selected MSC were also used in this study as previously described³⁴⁶. BMMNC were isolated from bone marrow aspirates as described in section 2.2.1 followed by STRO⁺ selection of human MSC by magnetic-activated cell sorting (MACS)³⁴⁶. BMMNC were incubated with the IgM mouse anti-human STRO-1 antibody for 45min followed by a 30min incubation with the biotinylated antibody, goat anti-mouse IgM Biotin for 30min. Subsequently, the cells were magnetically labelled with streptavidin microbeads for 15mins. The cell suspension was then loaded onto a MACS column that was placed in a magnetic field of a Mini-MACS separator. The magnetically labelled STRO-1⁺ BMMNC were retained in the column while the STRO-1⁻ BMMNC were eluted. STRO-1⁺ BMMNC were then eluted as the positive fraction using a plunger when the column was removed from the magnetic field. STRO-1⁺ BMMNC were seeded at 10 000 cells/cm² and established in the primary MSC media similar to generation of plastic adherent selected MSC cell lines described in section 2.2.1.

2.3 Isolation and culture of mouse Mesenchymal stem cells

2.3.1 Mouse MSC

Dissection of hind limbs: Mouse MSC were isolated from bone marrow or compact bones (tibia and femur) of C57BL/6 mice (4-6 mice per MSC isolation, 6-7 weeks old). Animals were humanely killed by carbon dioxide or isoflurane overdose followed by cervical dislocation. The animal skeleton was thoroughly rinsed in 70% ethanol. In a laminar flow hood, using sterile instruments, the mouse was skinned and both hind limbs (tibia and femur) were dissected. The hind limbs were rinsed thoroughly with cold α -MEM (Penicillin / streptomycin) to remove residual fur off the limbs. The limbs were stored in cold α -MEM (Penicillin / streptomycin) while waiting for further dissection. Once dissection of hind limbs were

completed in all mice, further dissection of the hind limbs were performed using sterile forceps and a scalpel blade. Each hind limb was bisected by cutting through the knee joint. Muscle and connective tissues from both femur and tibia were removed by scrapping the diaphysis of the bone clean, then puling the tissue towards the end of the bone. The ends of the tibia and femur were cut at the end of the marrow cavity and bones were stored in cold α -MEM (Penicillin / streptomycin) on ice until further processing.

Harvesting bone marrow (BM) cells from the tibia and femur: BM cells were harvested using a 21-gauge needle, inserted into the distal end of the femur or tibia. The bone was completely submerged in a 50ml falcon tube with 10ml cold HFF. 5 – 10ml HFF was drawn through the marrow cavity and then rapidly flushed out into the tube. This processed was repeated until the bone became completely white indicating that all marrow cells have been harvested. The resulting cell suspension was filtered through a 70 μ m nylon cell strainer to remove bone spicules or muscle and cell clumps. The viability and yields were determined by trypan blue exclusion assay. Approximately 70×10^6 bone marrow cells can be obtained from each mouse donor.

MSC isolation from compact bones (CB): Once the BM cells have been flushed from the CB, the CB was crushed into small pieces (1-2mm) using a sterile blade. Complete CB fragmentation allows subsequent enzymatic digestion to release maximum number of cells. CB cells were then incubated with collagenase II (3mg/ml) (optimized concentration and duration of collagenase digestion based on enzyme batch) and DNase I (50 U/ml) for 1hr at 37°C on a shaking platform at 240rpm. The resultant cell suspension was strained using a 70 μ m nylon cell strainer and bone chips were discarded^{348,349}. Mouse CB cells were further purified using the EasySep™ Mouse Mesenchymal Progenitor Enrichment Kit (StemCell Technologies; catalogue #19771) according to the manufacturer's instructions. CB cells were depleted of cells of haematopoietic origin using antibodies against CD45 and TER119. The enriched CB cells

were seeded at 10 000 cells/cm² in tissue culture flasks to establish the primary CB mouse MSC cell lines in primary MSC media. The primary CB mouse MSC cell lines were established at 37°C in a hypoxic environment (5% O₂, 10% CO₂, 85% N₂) in a hypoxia chamber, with media change performed every 2 days. At 70-80% confluence, adherent MSC were detached using 0.25% trypsin/EDTA (Sigma) for 4min, 37°C. MSC were re-seeded for expansion at a density of 10 000 cells/cm² in tissue culture flasks and maintained in MSC media containing 10% heat inactivated FBS. Media change was performed every 2 days. MSC at passage 3 to 6 were used in experiments.

2.4 Characterisation of human and mouse MSC

2.4.1 CFU-F assay

MSC precursors form fibroblast colony-forming unit (CFU-F) when cultured *in vitro*^{344,350}. Human BMMNC were plated in a 6-well plate with 3ml primary MSC media in triplicate wells at 1x10⁵ cells/well and 3x10⁵ cells/well. Cells were incubated at 37°C in a humidified tissue culture incubator with 5% CO₂ for 10-14 days depending on the growth of cells. For mouse CFU-F assay, bone marrow or compact bone cells were cultured in 6-well plates at 9x10⁵ cells/well or 1x10⁵ cells/well under normoxic or hypoxic conditions. At the end of these assays (ie. before colonies start to grow into each other), the plates were rinsed 3 times with PBS and the nuclei (ie. nucleic acid) of cells were stained with 1ml Toluidine blue stain. Plates were incubated on a rocker for a minimum of 2hr. The stain was rinsed gently with deionised water until water ran clear from wells and the plates were allowed to dry on bench. Colonies were counted under an inverted light microscope. A colony is defined as a cluster of ≥ 50 cells. Cobblestoned shaped colonies were excluded for CFU-F counts.

2.4.2 Surface staining and flow cytometry analysis

Immunophenotype of culture expanded MSC was characterised by flow cytometry. Following trypsinization, MSC were washed once with HFF and the single cell suspension was resuspended in blocking buffer containing HBSS supplemented with 1% bovine serum albumin (BSA; ICN Biomedicals, Aurora, OH), 5% FBS (Thermo Electron) and 5% normal human serum (Australian Red Cross, Adelaide, Australia) for 20min, 4°C. MSC were then stained with unconjugated or directly-conjugated Ab or isotype controls for 45min, 4°C. Cells were washed twice with HFF and secondary antibodies were added to appropriate tubes for 30min, 4°C. Cells were washed twice with HFF and were either acquired immediately on flow cytometer or fixed with FACS lysing solution and acquired later³¹⁵. Samples were acquired on the BD FACSCanto II (BD Biosciences) flow cytometer and analysed on FCS express V4 software.

2.4.3 Functional differentiation

MSC were induced to differentiation into adipocytes, osteocytes or chondrocytes as previously described¹⁷⁶. For osteogenesis and adipogenesis, MSC were seeded at 2×10^5 cells/T-25 flask in MSC media. **Osteogenesis:** 24hr post-seeding, MSC media was replaced with osteogenesis media containing α -MEM, 5% (v/v) heat inactivated FBS (Gibco, Invitrogen; optimized for osteogenesis), 2mM L-glutamine (Multicel, USA), 1mM sodium pyruvate (Sigma Aldrich), 100 μ M L-ascorbate, 1% penicillin streptomycin, 10^{-7} M dexamethasone (Sigma, St. Louis, MO), 10mM Hepes (Sigma St. Louis, MO) and 1.8mM KH_2PO_4 (Ajax, Taren Point, Australia). **Adipogenesis:** At 100% confluence, MSC were induced for adipogenesis in α -MEM, 5% (v/v) heat inactivated FBS (Gibco, Invitrogen; optimized for adipogenesis), 2mM L-glutamine (Multicel, USA), 1mM sodium pyruvate (Sigma Aldrich), 100 μ M L-ascorbate, 1% penicillin

streptomycin, 0.5 μ M isobutylmethylxanthine (Sigma, St. Louis, MO), 0.5 μ M hydrocortisone (Sigma, St. Louis, MO) and 60 μ M indomethacin (Sigma St. Louis, MO).

Fixing and staining: Media change was performed twice a week for 5 weeks. Cells were fixed for 1hr in 4% buffered formalin (neutral buffered formalin 3.9-6% formaldehyde, Ajax, Taren Point, Australia). Osteocytes containing calcium deposits were revealed by Alizarin Red (pH 4.2) (Sigma, St. Louis, MO) while lipid formation by adipocytes was stained with Oil Red O (Sigma St. Louis, MO). Micrographic photographs were obtained using an inverted light microscope (Nikon Eclipse TE2000-U, Nikon Eclipse Ti or Olympus IX51). **Chondrogenesis:** MSC (5×10^5 cells) in 10ml polypropylene tubes (Interpath,) were pelleted at 400xg, 10min. The MSC cell aggregates were cultured in 0.5ml serum-deprived chondrogenesis media containing α -MEM-high glucose (Gibco Life Technologies, Grand Island, NY), 2mM L-glutamine, 1mM sodium pyruvate, 1% penicillin streptomycin, 100 μ M L-ascorbate and 10^{-7} M dexamethasone, 1xITS+ Premix (BD Biosciences, San Jose, CA) 10 ng/ml TGF- β 1 (Sigma St. Louis, MO), and 1% BSA (Sigma St. Louis, MO). Media was changed twice a week for 5 weeks. The pellet aggregates were fixed in 4% buffered formalin overnight at 4°C. Formalin fixative was replaced with 70% ethanol. Samples were sent to the Department of Histopathology at The Queen Elizabeth Hospital for paraffin embedding and histology staining with Alcian blue (pH 2.5) to reveal proteoglycan synthesis by chondrocytes.

2.5 Cytokine treatment of MSC

Human MSC were seeded in tissue culture flasks at a density of 4000 cells/cm² and were allowed to adhere overnight. Fresh MSC media containing either no cytokines or recombinant human cytokines, 500U/ml IFN- γ (eBioscience) or 50ng/ml IL-17A (Peprotech) were added to the MSC cultures to derive UT-MSC, MSC- γ or MSC-17, respectively. At day 1, 2 or 5, cytokines were washed out with HBSS (Sigma) and modified MSC were used in suppressor assays, regulatory T cell assays or for gene expression analysis. For CB mouse MSC cytokine treatment, 500 000 cells were seeded into a T-75 tissue culture flask. Cells were allowed to adhere overnight and replaced with fresh MSC media containing either no cytokines or 50ng/ml recombinant mouse IL-17A (eBioscience).

2.6 Cryopreservation and log-phase growth recovery of MSC

Culture expanded human and mouse MSC (1-2x10⁶ cells/cryovial) were cryopreserved in freezing media (10% DMSO and 90% FBS). Cryovials were stored in -80°C and transferred to liquid nitrogen by day 3. Cryopreserved MSC were thawed at room temperature and transferred into a 12 ml tube (FALCON). α -MEM was added drop-wise to MSC (10 ml α -MEM / cryovial). Cells were washed twice with HFF at 400xg, 7min, 4°C and seeded in tissue culture flasks at 10 000 cells/cm² for expansion in MSC media. After 24hrs, non-adherent cells were removed by rinsing flask with HFF and adherent cells were maintained in MSC media until confluency (as described in section **2.2** and **2.3**).

2.7 T cells

2.7.1 Human T cells

PBMC were isolated by density gradient centrifugation on Ficoll-Paque PLUS (Bioscience) from buffy coats obtained from the Australian Red Cross. T cells were isolated from fresh or frozen PBMC. Nylon wool columns were used to isolate CD3⁺ T cells (>90% purity) as published previously³⁵¹. CD4⁺ T cells were isolated by negative selection using the EasySep™ CD4 isolation kit for magnetic separation (StemCell Technologies) according to the manufacturer's instructions. CD25⁺ cells were then depleted from the enriched CD4⁺ T cells using the EasySep™ CD25 positive selection kit for magnetic separation (StemCell Technologies). This separation resulted in greater than 98% purity of CD4⁺CD25⁻ T cells.

2.7.2 Mouse T cells

Isolation of T cells from splenocytes: C57BL/6 mice were humanely killed by isoflurane overdose followed by cervical dislocation and the spleen was dissected and placed in cold PBS. The spleen was rinsed in PBS in a petri dish and a 1 ml syringe plunger was used to mesh the spleen through a 70µM cell strainer until the capsule is left. Splenocytes were washed once in cold PBS (400xg, 7min, 4°C). RBC were lysed with 3ml RBC lysis buffer (37°C, 3min), washed with PBS and resuspended in column buffer for CD3⁺ T cells isolation using the Mouse T cell Enrichment Column (R&D Systems; MTCC-5/10) according to the manufacturer's instructions. This separation resulted in greater than 90% purity of CD3⁺ T cells.

CFSE-labelling of T cells: Purified T cells were labelled with carboxyfluorescein succinimidyl ester (CFSE; Invitrogen) for mouse T cell proliferation assays. T cells were resuspended at 10x10⁶ cells/ml in pre-warmed 10% FBS/PBS and stained with 0.7µl CFSE per 1ml cell suspension. Cells were incubated at 37°C for 30min, with mixing and inverting tubes at the 15min time interval and returning to incubation. The cells were then pelleted (400xg,

7min, room temperature), resuspended in 20-30ml pre-warmed 10% FBS/PBS and incubated for another 30min at 37°C. At the end of the incubation, cells were pelleted and resuspended at 2×10^6 T cells/ml in complete RPMI 1640 medium for a one-way mixed lymphocyte reaction (MLR).

2.8 Dendritic cells

2.8.1 Mouse DC propagation from bone marrow precursors

Mouse DC were generated from bone marrow cells of BALB/c mice. The protocol for DC differentiation and maturation is summarized in **Figure 2.8.1**.

Isolation of BM cells: The hind limbs (tibia and femur) were dissected and bone marrow cells from the tibia and femur were harvested as described in section **2.3.1** with the following modification: bones were washed with cold PBS and BM cells were harvested in 10% FBS/RPMI. BM cells were centrifuged at 500xg, 5min, 4°C and the pellet was resuspended in 2-3ml RBC lysis buffer for 3min, 37°C. Following RBC lysis, 20-30ml 10% FBS/RPMI was added to the BM cells and cells were centrifuged and the pellet was resuspended in 500µL 10% FBS/RPMI for the depletion of non-DC precursors by complement mediated lysis. BM cells were incubated with 5µL antibodies (**Table 2.8.1.1**) to erythroid precursors (anti-TER119), T cells (anti-CD3), B cells (anti-B220), NK cells (anti-NK-1.1), granulocytes (anti-Ly6C / anti-Ly6G) for 30min on ice, washed with 10% FBS/RPMI, centrifuged and pellet was resuspended in 4ml Low Tox^R rabbit complement (Cedarlane) for 45min at 37°C. After complement mediated lysis, 25ml 10% FBS/RPMI was added to cells, cells were centrifuged and the pellet resuspended in DC generation media containing RPMI, 10% FBS, β-mercaptoethanol (2µL/500ml), 1000U/ml recombinant mouse granulocyte macrophage colony-stimulating factor (GM-CSF; R&D Systems) and 500U/ml recombinant mouse IL-4 (R&D Systems). DC

were generated in tissue culture flasks (10-14x10⁶ BM cells/T-75 in 22ml media or 20-28x10⁶ BM cells / T-175 in 44ml media) at 37°C in a humidified tissue culture incubator with 5% CO₂.

Table 2.8.1.1 Anti-mouse antibodies for complement mediated lysis of non-DC precursors

Antibody	Clone	Company
TER119	TER-119	BD Pharmigen
CD3	17A2	BD Pharmigen
CD45R/B220	RA3-6B2	BD Pharmigen
NK-1.1	PK136	BD Pharmigen
Ly-6G and Ly-6C	RB6-8C5	BD Pharmigen

Maintenance of BM-DC in culture: At days 2 and 4, media change was performed by collecting media from the flasks into a 50ml Falcon tube, centrifuging (500xg, 5min, room temperature) and resuspending the cells in new DC generation media. On day 5, 200ng/ml lipopolysaccharide (LPS; Sigma) was added to the BM-DC culture for 24hr to induce DC maturation.

Purification of CD11c⁺ mature DC (mDC): Following 24hr maturation of DC with LPS (Day 6), the non-adherent fraction that comprises mDC was collected and CD11c⁺ mDC were purified using the EasySep™ mouse CD11c positive selection kit II (StemCell Technologies; catalogue #18780A) according to the manufacturer's instructions. Purified mDC were characterised for mDC markers by flow cytometry and cryopreserved (section 2.9) for use in MLR.

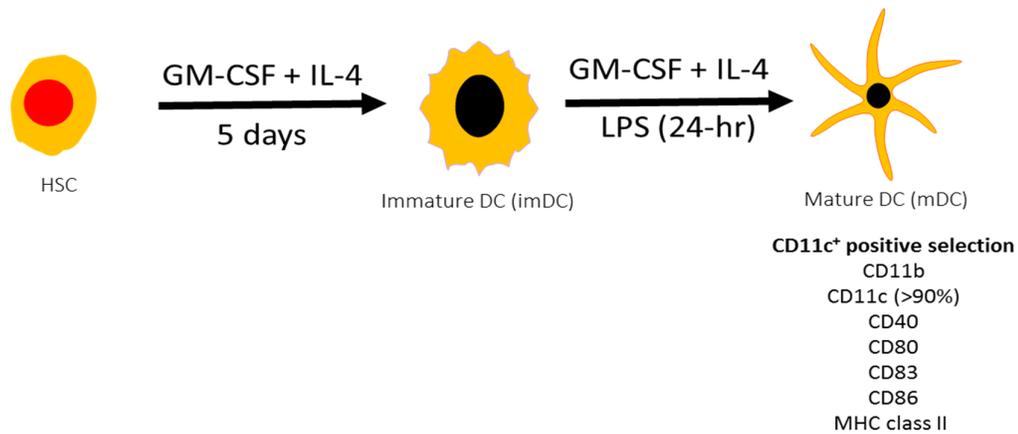


Figure 2.8.1 Generation of mouse mature DC from haematopoietic stem cells (HSC).

Bone marrow cells were harvested from BALB/c mice and cultured in DC generation media containing GM-CSF and IL-4 for 5 days to differentiate DC precursors from HSC into immature DC (imDC). LPS was added to the culture to induce imDC maturation to form mature DC. CD11c⁺ mDC from the non-adherent fraction of the cultures were purified by positive selection. Purified mDC were characterised for mDC markers by flow cytometry based on the following cell surface markers: >90% CD11b⁺, CD11c⁺, CD40⁺, CD80⁺, CD83⁺, CD86⁺ and MHC class II⁺. The negative fraction containing a heterogeneous population of cells was also evaluated for the expression of these mDC markers.

2.9 Cryopreservation and thawing of human PBMC and mouse DC

Human PBMC ($2-7 \times 10^7$ cells) and mouse DC ($1-4 \times 10^6$ cells) were frozen in cryovials in freezing media (10% DMSO and 90% FBS). Cryovials were stored at -80°C and transferred to liquid nitrogen by day 3. Cryopreserved cells were thawed at room temperature and transferred into a 12ml tube (FALCON). α -MEM was added drop-wise to cells (10ml α -MEM / cryovial). Cells were washed twice with cold PBS at 400xg, 7min, 4°C . Human PBMC were seeded into non-adherent tissue culture flasks overnight to allow cell recovery in complete RPMI 1640 media prior to use in human T cell assays while freshly thawed mouse DC were used immediately in MLR.

2.10 Human Proliferation assays

2.10.1 Human MSC proliferation assay

MSC were seeded into a 96-well flat-bottom plate at of 5×10^3 cells in 0.2ml complete RPMI 1640 medium containing 10% heat inactivated FBS, 2mM L-glutamine (Multicel, USA), 1mM sodium pyruvate (Sigma Aldrich), 100 μM L-ascorbate and 1% penicillin streptomycin. IFN- γ or IL-17A were added to wells at varying concentrations.

2.10.2 Human T cell proliferation assay

2.10.2.1 Mitogen (PHA) dependent T cell proliferation assay

CD3⁺ T cells (1×10^5 cells) were seeded into 96-well flat bottom plates with or without PHA (10 $\mu\text{g/ml}$) (Sigma) in a total volume of 0.2ml of complete RPMI 1640 medium. For suppressor assays, 30 Grays irradiated MSC at 1×10^2 (0.1%), 1×10^3 (1%) or 1×10^4 cells (10%) were seeded

overnight in 96-well flat bottom plates in complete RPMI 1640 medium. CD3⁺ T cells and PHA were added where appropriate.

2.10.2.2 Mixed lymphocyte reaction (MLR)

30 Grays irradiated MSC at 1×10^2 (0.1%), 1×10^3 (1%) or 1×10^4 cells (10%) MSC were seeded overnight into 96-well round bottom plates in a total volume of 0.2ml complete RPMI 1640 medium. MSC were co-cultured with responder PBMC in a one- or two-way MLR.

One-way MLR: MSC were co-cultured with 1×10^5 responder PBMC and 2×10^5 stimulator PBMC (30 Grays irradiated) from two different buffy coat donors.

Two-way MLR: MSC were co-cultured with 1×10^5 responder PBMC and 1×10^5 cells stimulator PBMC from two different buffy coat donors.

2.10.3 Measurement of human MSC or T cell proliferation: (³H]-Thymidine assay)

On day 4, cells were pulsed with $1 \mu\text{Ci}$ [³H]-Thymidine (Sigma Aldrich) for 18 h and harvested (Filtermate Harvester; Packard) onto glass fibre filters (PerkinElmer). Scintillation fluid (MicroscintTM20; PerkinElmer) was added to the glass fibre filters and the incorporation of [³H]-Thymidine into cellular DNA was counted using a scintillation counter (TopCount NXT; PerkinElmer). Levels of proliferation were expressed by the mean counts per minute (cpm) of three replicates.

2.11 Mouse proliferation assays

2.11.1 Mixed lymphocyte reaction (MLR)

30 Grays irradiated MSC at 1×10^2 (0.1%), 1×10^3 (1%) or 1×10^4 cells (10%) were seeded overnight into 96-well round bottom plates in a total volume of 0.2ml complete RPMI 1640 medium. CFSE-labelled responder $CD3^+$ T cells (1×10^5 cells) and 30 Grays irradiated stimulator mDC (1×10^4 cells) were added where appropriate. Cells were cultured for 72hr or 5 days and T cells were harvested for analysis of CFSE-labelled T cell proliferation. In some experiments, harvested CFSE-labelled T cells were also stained with anti-CD4 and anti-CD8 antibodies (30min, 4°C) to detect proliferation levels of $CD4^+$ and $CD8^+$ T cells, respectively.

2.12 Analysis of human T cell activation and Treg assays

Irradiated MSC (3.91×10^4 cells) were seeded overnight into 24-well flat bottom plates. $CD3^+$ or $CD4^+CD25^-$ T cells (3.91×10^6 cells) were added to appropriate wells without PHA (10 μ g/ml) in a total volume of 1.25ml complete RPMI 1640 medium. Cells were cultured for 5 days and T cells were harvested for either regulatory T cell staining or analysis of T cell activation marker expression (CD25 and CD69; **Table 2.11.1.1**). Cell culture supernatants were collected for cytokine bead array assay (CBA) or ELISA.

Table 2.11.1.1 Human T cell activation markers antibody panel

Antibody	Clone	Fluorochrome	Company
<i>T cell activation markers:</i>			
CD25	BC96	APC	eBioscience
CD69	L78	PE-Cy7	BD Biosciences

2.12.1 Transwell assays

MSC (3.91×10^4 cells) were seeded in 24-well tissue culture grade plates overnight. $0.4 \mu\text{M}$ transwell inserts (Corning) were inserted into the wells and $\text{CD4}^+ \text{CD25}^-$ T cells and PHA were added to the inserts at the same concentration as described in section 2.12.

2.12.2 Flow cytometry

T cells were stained for T cell activation or Treg markers as previously described³⁵². Briefly T cells were stained with V500 viability dye ($1 \mu\text{L} / 1 \times 10^6$ cells; Life Technologies) for 30mins, 4°C followed by staining with anti-human CD3, CD4, CD25, CD69 and CD127 for 30min, 4°C . For intracellular detection of FoxP3, cells were fixed and permeabilized using the FoxP3 staining buffer set (eBioscience) for 45mins, 4°C and subsequently blocked with normal rat serum (eBioscience) for 10min, RT. Cells were then stained with anti-human FoxP3 for 30min, 4°C . The absolute number of positively stained cells was calculated using CaliBRITE APC enumeration beads (BD Biosciences) based on the formula³⁵³:

$$\text{Absolute Treg/uL} = \frac{(\text{Number of events in region containing cells}) \times (\text{Number of bead count})}{(\text{number of events in absolute count bead region} \times \text{tested volume})}$$

Tregs were further characterised for functional markers using monoclonal anti-human antibodies to Treg as listed in **CHAPTER 4** Material and Methods section.

2.12.3 Cytokine Bead Array (CBA)

$\text{IFN-}\gamma$, $\text{TNF-}\alpha$, IL-2, IL-4, IL-6 and IL-10 in cell culture supernatants were detected using the human Th1/Th2 cytokine bead array kit II (detection range, 20 – 5000pg/ml; BD Bioscience) according to manufacturer's instruction.

2.12.4 Enzyme-linked immunosorbent assay (ELISA)

TGF- β 1 (156.3 – 10 000pg/ml; eBioscience) and PGE₂ competitive ELISA (detection range, 39 – 2500pg/ml; R&D systems) were performed according to manufacturer's instruction.

2.13 Functional human regulatory T cell assays

2.13.1 Human inducible (i)Treg sorting

iTregs were isolated from co-cultures of MSC and CD4⁺CD25⁻ T cells by labelling with anti-human CD4, CD25 and CD127 antibodies and sorting for CD4⁺ CD25^{high} (fluorescence intensity >10³) CD127^{low} population using Beckman Coulter MoFlo Astrios High Speed Cell Sorter and Summit Software version 6.2. Sorted iTregs were then stained with anti-human FoxP3 to determine purity of sorted cells. Fluorescence minus one (FMO) was used as controls to set negative gates.

2.13.2 DiOC₁₈(3) labelling of effector T cells

CD4⁺CD25⁻ T effector cells were labelled with 2mg/ml DiOC₁₈(3) (Molecular Probes, Life Technologies) in 3% FBS/PBS (45mins, 37°C) in 5% CO₂ incubator. Cells were washed with 3% FBS/PBS and adjusted to the required concentration in complete RPMI 1640 medium. The DiOC₁₈(3)-labelled CD4⁺ CD25⁻ T effector cells were then used in a iTreg CD154 suppression assay. This membrane labelling technique of effector T cells enables the measurement of iTreg suppressive function³⁵⁴.

2.13.3 Human CD154 suppression assay

Sorted iTregs were rested at 1×10^6 cells/ml in RPMI 1640 supplemented with 10% FBS and 100U/ml recombinant human IL-2 (Peprotech) for 48 h prior to use in CD154 suppression assay as previously described³⁵⁴. For CD154 suppression assay, sorted iTregs were cultured with a constant number (2.5×10^4 cells/well) of DiOC₁₈(3)-labelled CD4⁺CD25⁻ T effector cells (donor T cells) at a ratios of 1:1, 2:1, 4:1, 8:1, 16:1 Teffector:Tregs in a 96-well U-bottom plate. Effector T cells were stimulated with anti-CD3/CD28 T cell expander beads (Invitrogen) at a 4:1 bead to cell ratio to induce CD154 expression. Unstimulated T effectors were used as negative control. Anti-human CD154-APC was added to wells to determine CD154 expression on T effectors. Cultures were incubated in dark for 7-8 h at 37°C. Cells were analysed for surface expression of CD154 on the BD FACSCanto II flow cytometer.

2.14 RNA isolation and Real-time PCR analysis

Untreated, IFN- γ or IL-17A modified MSC were lysed in 350 μ L lysis buffer and cell lysates were homogenized using a QIA shredder spin columns (Qiagen). Total RNA was isolated and purified using the RNeasy mini kit (Qiagen) with the following modification: DNA was digested using the DNase I from the RNase-free DNase set (Qiagen). The quantity of total RNA was measured using NanoDrop 1000 (Thermo Scientific). For cDNA synthesis, 1000ng total RNA was transcribed with cDNA transcription reagent the Omniscript[®] Reverse Transcription kit (Qiagen), according to the manufacturer's instructions. Gene expression (10-100ng cDNA per reaction) was measured in real-time PCR (RT-PCR) with the Taqman[®] gene expression master mix and using the following gene specific Taqman[®] primers IL-6 (Hs00985639_ml), IL-10 (Hs00961622_ml), prostaglandin-endoperoxidase synthase 1 (PTGS1) (Hs00377726_ml), indoleamine 2,3-dioxygenase 1 (IDO1) (Hs00984148_ml) or

TGF- β 1 (Hs00998133_ml), IL-17A (Hs00174383_ml), IL-17F (Hs00369400_ml) IL-17RA (Hs01064648_ml) and IL-17RC (Hs00994305_ml), purchased from Applied Biosystems. Samples were run in triplicates and data were presented and normalized to the house keeping gene hypoxanthine phosphoribosyltransferase-1 (HPRT1) (Hs99999909_ml). Mean normalized expression was calculated using the Qgene Module software as previously described³⁵⁵.

2.15 *In vivo*: Islet allotransplantation model

2.15.1 Experimental mice

All experimental procedures were approved by the Animal Ethics Committee of the University of Adelaide and conform to the guidelines established by the Australian Code of Practice for the Care and Use of Animals for Scientific purposes. C57BL/6 (H2b haplotype) and BALB/c (H2d haplotype) male mice, aged 7-8 weeks were used as islet allotransplant recipients and islet donors, respectively. All animals used in this project were maintained under specific pathogen-free conditions and had free access to standard mice chow and water under a 12hr light/dark cycle at the University of Adelaide Animal House Facility (Frome Road, Adelaide, Australia). Mice were purchased from the University of Adelaide. All animal procedures were performed according to the ethics guidelines approved by the University of Adelaide ethics committee (**ethics approval: M-2013-033**).

2.15.2 Pancreatic islet isolation

Pancreatic islets were isolated from pancreata of donor male BALB/c (H2d) mice 7-8 weeks old at a ratio of 3 pancreata per transplant recipient based on previously established protocols^{356,357}. The transplantation of 300 handpicked islets per recipient was also evaluated

in this thesis. Briefly, BALB/c mice were humanely killed by isoflurane overdose followed by cervical dislocation. The pancreas was perfused with 3ml cold serum-free M199 medium (Sigma) containing 0.67mg collagenase (Liberase TL, grade; Roche) per pancreas into the pancreatic duct *in situ*. The pancreas was dissected and digested at 37°C at an optimized duration (14min 40s). Islets from the digested pancreas were isolated by Ficoll (GE Healthcare) gradient separation (2830rpm, low acceleration and no brake) for 22min, 22°C. Purified islets were washed extensively at 1250rpm, 3min and islets were cultured in RPMI 1640 supplemented with 10% FBS, 5ml GlutaMAX™-I (100X) (Gibco by Life Technologies), hepes (0.01M/ml; Media Production Unit, MPU, Royal Adelaide Hospital), Penicillin (0.012mg/ml; MPU, Royal Adelaide Hospital), Gentamicin (0.016mg/ml; MPU, Royal Adelaide Hospital) at 37°C, 5% CO₂ in ultra-low attachment culture dishes (Corning 3262) for 1 day prior to islet transplantation.

2.15.3 Islet loading for transplantation

On the day of islet transplantation, the islets were transferred into an Eppendorf tube, allowed to settle for 10min on ice and islet culture media was removed. The wash step was performed in a total of 3 times to remove any residual FBS in the islets. The islets were then loaded into a pipette tip attached to a polyethylene tubing (PE 50) using a syringe and a plunger. Once the islets were in the pipette tip, the tubing was then clamped tightly and placed upright to allow the islets to settle in the pipette tip. The clamp was removed and the islets in the pipette tube were slowly injected using the syringe plunger, until the islet clump passed the tubing junction and was in the polyethylene tubing. A bent was made to the polyethylene tubing. The islets were then centrifuged at 1300rpm, 3min, 4°C to allow the islets to settle close to the polyethylene tubing bend.

2.15.4 Mouse MSC loading for islet transplantation

Cultured compact bone mouse MSC (passage 3) were detached by trypsin-EDTA as described in section 2.3.1. MSC were washed in a total of 3 times to remove any residual FBS in MSC. MSC (1×10^6 cells) were added to the islets, and allowed to settle with the islets in an Eppendorf tube. MSC and islets were co-loaded into the polyethylene tubing similar to section 2.15.3.

2.15.5 Streptozotocin (STZ) induction of diabetes

C57BL/6 mice (male, 7-8 weeks old) under anaesthetics were injected intraperitoneally with 200mg/kg Streptozotocin (STZ; Sigma Inc.) in 10mM citrate buffer pH4.5 (BDH Chemicals, Kilsyth, VIC, Australia) for the induction of diabetes³⁵⁸. Diabetes was monitored by measurement of non-fasting daily blood glucose levels (BGL) from the tail vein using a glucometer (Optium, Abbott Diabetes Care, Victoria, Australia). Mice with BGL > 16.6mMol/L for 3 consecutive days were considered as onset of diabetes and were selected as islet transplant recipients³⁵⁶.

2.15.6 Allogeneic islet transplantation rejection model

C57BL/6 diabetic mice were transplanted with donor BALB/c islets at a ratio of three pancreata³⁵⁶ or 300 handpicked islets per transplant. The islet transplantation under the kidney capsule protocol was based on the well-established protocols at the Centre for Clinical and Experimental Transplantation (CCET) lab. Briefly, the C57BL/6 mice were anaesthetised using isoflurane, shaved and the kidneys were accessed by performing a left-flank incision. A small nick was made in the kidney capsule with a 21G needle at the inferior renal pole. Polyethylene tubing containing islets without or without MSC was then connected to a Hamilton Syringe and placed under the kidney capsule through the nick. The islets were loaded through the nick towards the superior pole of the kidney and rubbed with a forceps to reform

and seal the islet pellet. The wound was closed with sutures and the skin was stapled. The mice were given 200 μ L saline and appropriate volume of Temgesic (0.05mg/kg) subcutaneous immediately after transplant. Non-fasting BGLs were analysed on post-operative day 0 (POD) until rejection. Cure of diabetes was defined as three consecutive BGL of < 11.1mMol/L with no subsequent reversion of hyperglycaemia (3 consecutive BGL > 11.1mMol /L). Graft rejection was defined as three consecutive BGL > 16.6mMol/L after a period of normoglycaemic.

2.15.7 Intraperitoneal Glucose Tolerance Test (IPGTT)

The intraperitoneal glucose tolerance test (IPGTT) was used to test the islet graft function of transplanted mice. Mice were fasted for 4hr and injected intraperitoneally with 2g/kg glucose [d-(+) glucose solution (10%)l Sigma Inc.]. Tail vein blood samples were taken at time 0, 15, 30, 60 and 120min following glucose administration. Whole blood was analysed for BGL using a glucometer (Optium, Abbott Diabetes Care, Victoria, Australia) as an indicator of islet graft function.

2.15.8 Histology and immunohistochemistry

Kidney tissue were embedded in formalin. Tissue sections were sent to SA Pathology for tissue processing and hematoxylin and eosin (H&E) staining.

2.16 Statistics

Statistical significance ($p < 0.05$) was assessed by one-way ANOVA (representative experiment) or two-way ANOVA (pooled data) with post-Sidak multiple comparison test (in consultation with a bio-statistician) using the GraphPad Prism software.

Statement of Authorship

Title of Paper	Interleukin-17A induced human Mesenchymal Stem Cells are superior modulators of immunological function
Publication Status	<input checked="" type="checkbox"/> Published <input type="checkbox"/> Accepted for Publication <input type="checkbox"/> Submitted for Publication <input type="checkbox"/> Unpublished and Unsubmitted work written in manuscript style
Publication Details	Sivanathan KN, Rojas-Canales DM, Hope CM, Krishnan R, Carroll RP, Gronthos S, et al. Interleukin-17A-Induced Human Mesenchymal Stem Cells Are Superior Modulators of Immunological Function. Stem cells. 2015;33(9):2850-63.

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Contribution to the Paper	Conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript.	
Overall percentage (%)	85%	
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.	
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- the candidate's stated contribution to the publication is accurate (as detailed above);
- permission is granted for the candidate to include the publication in the thesis; and
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CHAPTER 3:

CHARACTERISATION OF MODIFIED MSC

This chapter is adapted and modified from the published article:

Sivanathan KN, Rojas-Canales DM, Hope CM, et al. Interleukin-17A-Induced Human Mesenchymal Stem Cells Are Superior Modulators of Immunological Function. *Stem Cells*. 2015;33(9):2850-2863.

(APPENDIX Published Paper 3)

3.1 INTRODUCTION

Bone marrow-derived mesenchymal stem cells (MSC) are multipotent non-haematopoietic stem cells that constitute 0.001 - 0.01% of the adult bone marrow^{176,359}. MSC can be easily isolated from the bone marrow under minimally invasive procedures and rapidly expanded *ex vivo* to the desired dose prior to MSC infusion *in vivo*. MSC have tissue reparative properties as they secrete a multitude of growth factors, cytokines and chemokines such as stromal derived factor-1, insulin growth-factor I, hepatocyte growth factor (HGF) and vascular endothelial growth factor^{182-184,323}. The reparative properties of MSC are enhanced by their capacity to home specifically to sites of inflammation, a property attributed to the expression of matrix metalloproteinases (MMPs) facilitating their ability to invade the extracellular matrix¹⁷⁹⁻¹⁸¹.

Apart from their homing and tissue reparative properties, the ability of MSC to suppress immune responses make them novel candidates for the application of cell-based therapies to prevent and treat inflammatory conditions including allotransplant rejection. A substantial body of literature has shown that MSC modulate immune responses through interaction with

various immune cells such as T cells, B cells, dendritic cells (DC) and natural killer cells^{177,192-196}. Preclinical models and clinical trials of inflammatory disease such as graft versus host disease (GvHD)^{197,198}, autoimmune disease¹⁹⁹ and allograft rejection²⁰⁰⁻²⁰² provide evidence on the *in vivo* immunosuppressive function of MSC. Mechanistically, the inhibition of T cell responses is mediated by the direct or indirect interaction of MSC with T cells⁴. MSC directly modulate T cell activation, proliferation and function through the expression of soluble immunosuppressive factors such as indoleamine-2,3-dioxygenase (IDO), prostaglandin-E2 (PGE₂), MMP, HGF, heme oxygenase-1 (HO-1), human leukocyte antigen-G5, transforming growth factor-beta (TGF- β), IL-6, IL-10, galectins and semaphorin-3A^{192,207,237-239,244-246,270,272}.

Cell-contact dependent mechanisms involving the CD200/CD200R, toll-like receptor, FASL/FAS, EphB/ephrin B, programmed death ligand-1 (PD-L1) and the negative co-stimulatory molecule B7-H4 have also been implicated in MSC-mediated T cell modulation^{194,240,253-256}. MSC also directly downregulate early T cell activation markers CD25, CD38 and CD69 as well as promoting an anti-inflammatory Th2 cell phenotype following co-culture with T cells^{207,238,248,250}.

Exposure of MSC with proinflammatory cytokines such as interferon-gamma (IFN- γ), tumor necrosis factor-alpha (TNF- α), IL-1 β or IL-1 α are known to enhance the immunosuppressive properties of MSC^{174,251,304,307,318}. The modification of MSC with IFN- γ (MSC- γ) in particular yields an immunosuppressive population of cells with increased reparative and migratory properties^{304,306,322}. MSC- γ however demonstrate conflicting effect *in vivo* due to their high expression of major histocompatibility (MHC) molecules that may increase MSC- γ immunogenicity^{217,299}. Other safety issues concerning MSC- γ therapy include malignant transformation in MSC and MSC- γ role in tumor growth and maintenance in a malignant setting^{338,360}.

In this chapter, we sought to identify alternative strategies to manipulate MSC to enhance their immunosuppressive properties whilst retaining them in an inert immunogenic state. IL-17A is potent mediator of proinflammatory responses and is produced predominantly by T helper 17, a subset of CD4⁺ T helper cells³⁶¹. IL-17A is known to function as a MSC growth factor^{340,342} and can act synergistically with IFN- γ and TNF- α in enhancing MSC immunosuppression *in vivo*³⁶². This thesis, for the first time, demonstrated that IL-17A preconditioned human MSC (MSC-17) function as effective as MSC- γ at suppressing T cell activation and proliferation whilst conforming to the untreated MSC (UT-MSC) phenotype. These findings highlight the therapeutic application of MSC-17 as superior cells for modulation of T cell mediated disease states.

3.2 MATERIALS AND METHODS

Materials and methods of this chapter have been described in **CHAPTER 2: MATERIALS AND METHODS**. In this section, additional chapter specific materials for human MSC immunophenotyping are shown.

3.2.1 Human MSC antibody panel

Table 3.2.1.1 Human MSC antibody panel

Antibody	Clone	Fluorochrome	Company
CD44	H9H11	Unconjugated	Lab derived
CD73	AD2	Unconjugated	BD Pharmigen
CD90	5E10	Unconjugated	BD Pharmigen
CD105	266	Unconjugated	BD Pharmigen
CD106	QE.4G9	Unconjugated	Lab derived
CD146	CC9	Unconjugated	Lab derived
CD166	3A6	Unconjugated	BD Pharmigen
STRO-1	STRO-1	Unconjugated	In-house
STRO-4	HYB H	Unconjugated	In-house
CD11a	HB202	Unconjugated	In-house
CD14	RMO52	FITC	Beckman Coulter
CD34	581	FITC	Beckman Coulter
CD40	MAB89	PE	Beckman Coulter
CD45	J33	FITC	Beckman Coulter
CD80	L307.4	FITC	BD Pharmigen
CD83	HB15e	FITC	BD Pharmigen
CD86	2331 (FUN-1)	FITC	BD Pharmigen
MHC class I	W6.32	Unconjugated	In-house
MHC class II	3.54	Unconjugated	In-house
<i>Isotype controls:</i>			
IgM	1A6.12	Unconjugated	In-house
IgG1	1B5	Unconjugated	In-house
IgG2	1D4.4	Unconjugated	In-house
IgG1	MOPC-21	FITC	BD Pharmigen
IgG2b	27-35	FITC	BD Pharmigen
IgG1	MOPC-21	PE	BD Pharmigen
<i>Secondary Ab:</i>			
Goat anti-Mouse IgG	-	FITC	Abcam

3.3 RESULTS

3.3.1 MSC inhibit T cell proliferation in a dose-dependent manner

The baseline capability of MSC to inhibit T cell proliferation *in vitro* was evaluated. Both plastic adherent selected and STRO-1 purified UT-MSC inhibited PHA- or allogeneically-stimulated T cell proliferation in a dose-dependent manner (**Figure 3.3.1** and **Figure 3.3.2**). Although maximal inhibition of T cell proliferation was obtained at 10% MSC dose, the suboptimal 1% MSC dose was selected for subsequent experiments with MSC and T cell co-cultures to better define changes in suppressive capacity of modified MSC.

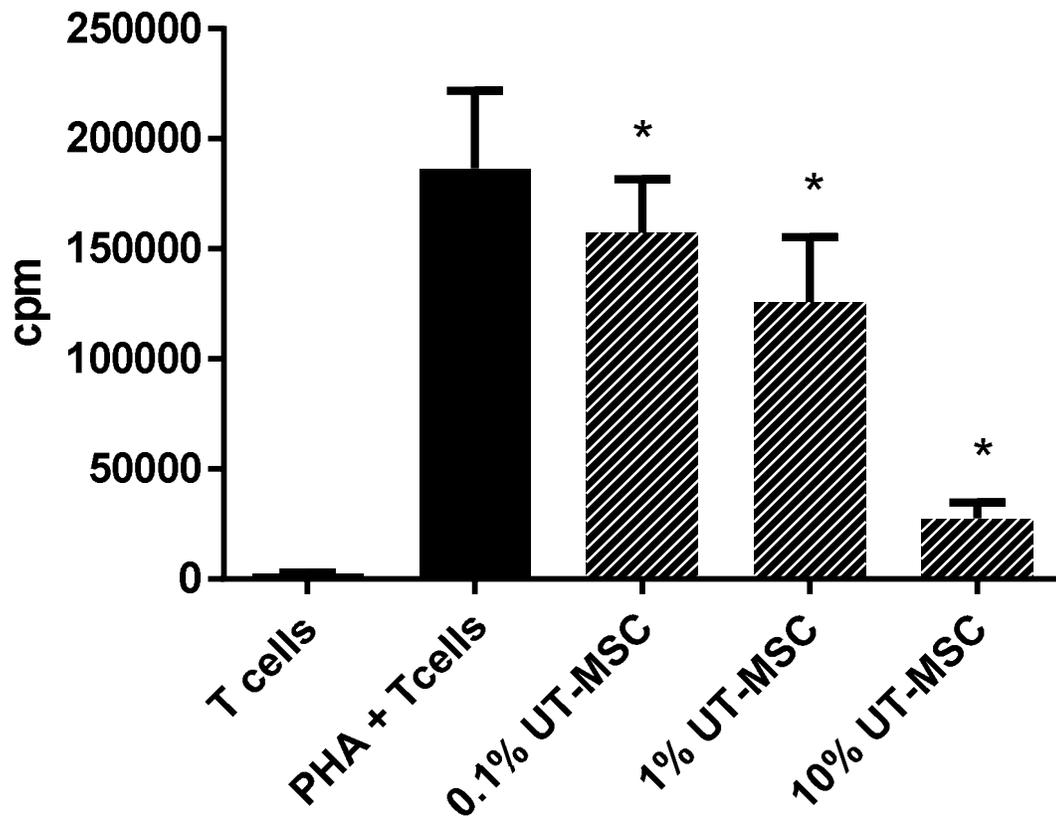


Figure 3.3.1 MSC inhibit T cell proliferation in a dose-dependent manner.

UT-MSC were co-cultured with PHA activated CD3⁺ T cells at 0.1% UT-MSC, 1% UT-MSC and 10% UT-MSC. T cell proliferation was measured in a [³H]-Thymidine assay. Data are pooled from 6 independent experiments. *p < 0.05 vs. PHA + T cells was determined by two-way ANOVA post-Sidak multiple comparison test. Error bars depict means of triplicates ± SEM. Adapted from Figure 2A Sivanathan KN, et. al., (2015)².

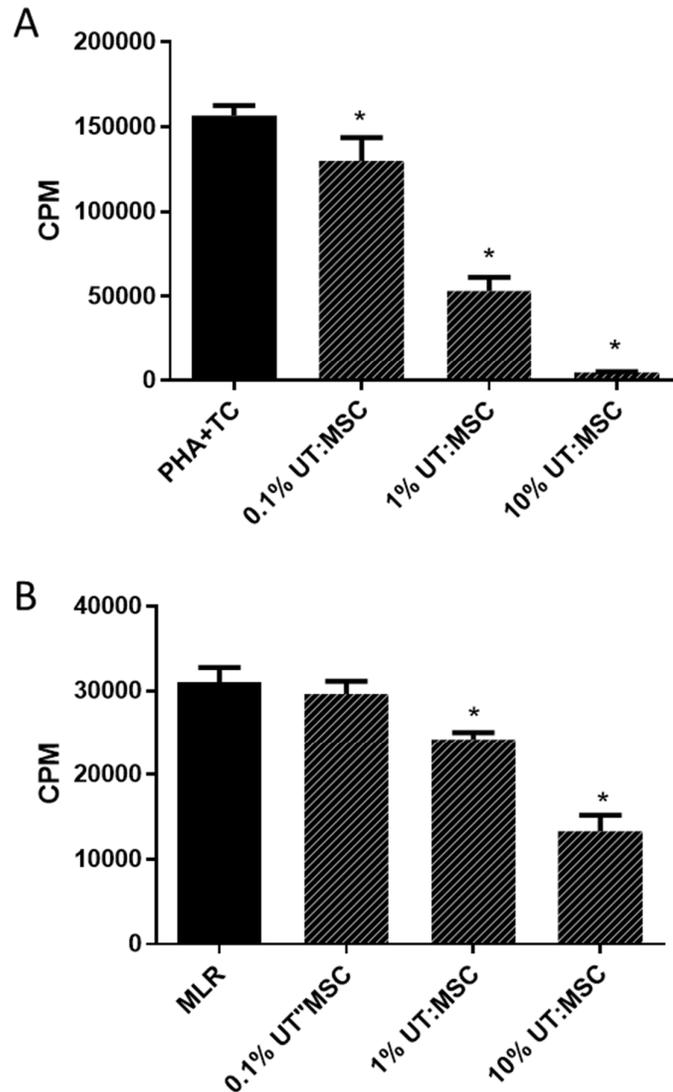


Figure 3.3.2 STRO-1 MSC inhibit T cell proliferation in a dose-dependent manner.

UT-MSC were co-cultured with either (A) PHA-activated CD3⁺ T cells or (B) responder PBMC and stimulator PBMC (30 Grays irradiated) from two different buffy coat donors in a one-way mixed lymphocyte reaction (MLR) at 0.1% UT-MSC, 1% UT-MSC and 10% UT-MSC. T cell proliferation was measured in a [³H]-Thymidine assay. Data is (A) one experiment and (B) one representative of 2 independent experiments. *p < 0.05 vs. PHA + T cells or MLR determined by one-way ANOVA post-Sidak multiple comparison test. Error bars depict means of triplicates ± SD. (*unpublished*).

3.3.2 Preliminary findings: IL-17A enhanced MSC suppress human T cells

In preliminary studies, we for the first time demonstrated that 5 day IL-17A pre-treated MSC (MSC-17) showed highest suppression of PHA-induced T cell proliferation compared to IL-1 β (MSC-IL1 β), TNF- α (MSC-TNF) and IFN- γ (MSC- γ) treated MSC (**Figure 3.3.3**). The enhanced MSC-17 immunosuppression was also evident in MLR and dependent on MSC dose and IL-17A concentration used for MSC preconditioning (**Sivanathan KN, Honours Thesis 2010**). Therefore, MSC-17 was further evaluated for the immunomodulatory ability and was compared to UT-MSC in this thesis. MSC- γ was also evaluated in this thesis as it is the most extensively studied proinflammatory cytokine to enhance MSC immunosuppression⁴.

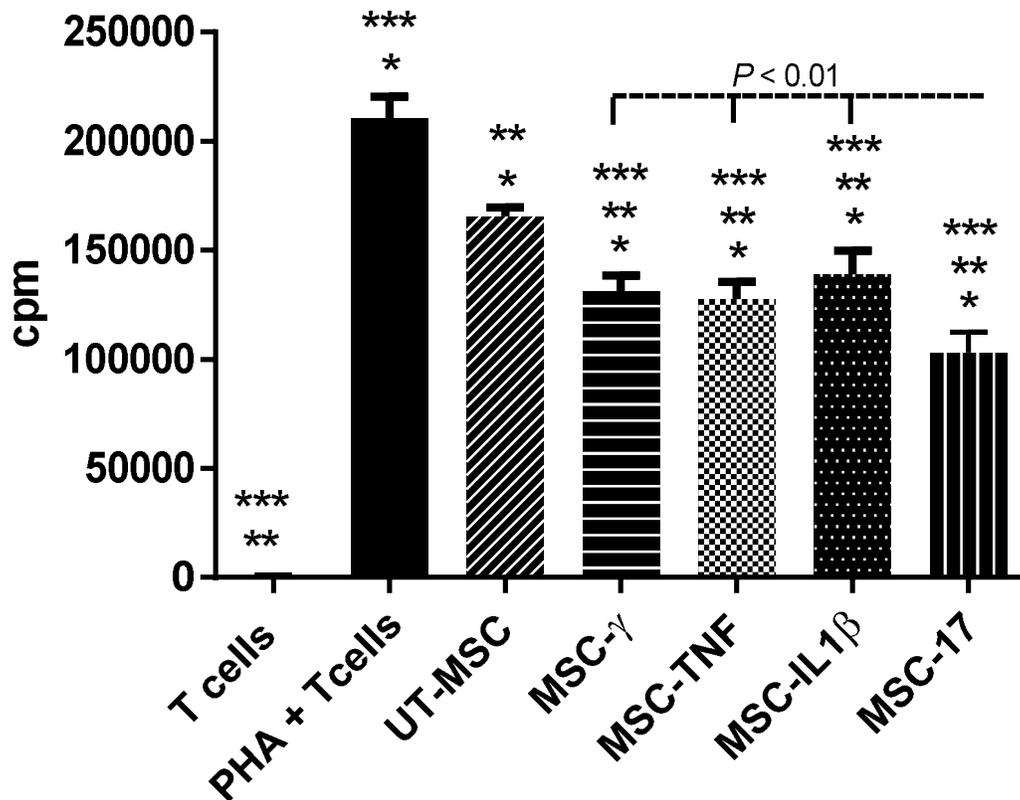


Figure 3.3.3 Preliminary findings: MSC-17 enhance inhibition of T cell proliferation.

1% UT-MSC, MSC- γ , MSC-TNF, MSC-IL1 β or MSC-17 treated for 5 days with IFN- γ , TNF- α (20 ng/ml), IL-1 β (50 ng/ml) or IL-17A respectively were co-cultured with PHA activated CD3⁺ T cells. T cell proliferation was measured in a [³H]-Thymidine assay. Data are one representative of 4 independent experiments. *p < 0.05 vs. T cells alone, **p < 0.05 vs. PHA + T cells and ***p < 0.05 vs. UT-MSC determined by one-way ANOVA with post-Sidak multiple comparison test. Error bars depict mean of triplicates \pm SD. Adapted from supplemental online Figure S2 Sivanathan KN, et. al., (2015)².

3.3.3 MSC-17 conforms to UT-MSC phenotype and function

We assessed the effect of 5 day IL-17A or IFN- γ treatment on MSC phenotype and differentiation potential. Firstly, IL-17A receptors (IL-17RA and IL-17RC) as well as the IFN- γ receptors (IFN- γ R1 and IFN- γ R2) gene expression on MSC were confirmed (**Figure 3.3.4**). Immunophenotypic analysis (**Figure 3.3.5 A**) of IL-17A treated MSC (MSC-17) was similar to untreated MSC (UT-MSC). MSC-17 expressed standard MSC markers CD44, CD73, CD90, CD105, CD146, CD166 and STRO-4 and were negative for monocyte markers (CD11a and CD14) CD34 (haematopoietic cells), and CD45 (lymphocytes). Both UT-MSC and MSC-17 expressed similar levels of MHC class I but lacked MHC class II. IFN- γ treated MSC (MSC- γ) expressed higher levels of MHC class I (4.5-fold; $p < 0.0001$) and induced MHC class II compared to UT-MSC. None of the MSC groups expressed T cell co-stimulatory molecules CD80, CD83, CD86, while only MSC- γ expressed CD40. The programmed death ligand-1 (PD-L1) / PD-1 pathway regulates T cell activation and promotes tolerance induction^{317,363}. UT-MSC and MSC-17 generally exhibited low levels of PD-L1 surface expression but MSC- γ showed high PD-L1 expression ($p < 0.0001$). Similar to plastic-adherent MSC, STRO-1 purified MSC were positive for the standard MSC markers CD44, CD73, CD90, CD105, CD166 and were negative for CD11a, CD14, CD34, CD80, and CD83. Upregulation or induction of MHC class I and MHC class II, respectively, were only evident in IFN- γ treated STRO-1 MSC (**Figure 3.3.6**). Extended MSC cultures of plastic-adherent (**Figure 3.3.5 A**) and STRO-1 purified MSC (**Figure 3.3.6**) reduced STRO-1 expression, an observation also reported by others^{364,365}.

MSC-17 showed no apparent changes to morphology and size compared to UT-MSC (**Figure 3.3.5 B**). Advantageously, MSC-17 exhibited enhanced proliferation, increasing by 4-fold ($p < 0.005$) relative to UT-MSC (**Figure 3.3.5 C**). In contrast, MSC- γ exhibited altered MSC morphology, changing their fibroblastic-like appearance to a hypertrophic flattened

irregular shape and decreased MSC growth potential by 2-fold ($p < 0.005$), when compared to UT-MSC (**Figure 3.3.5 B, C**). All MSC groups retained functional capacity and could differentiate into adipocytes, osteoblasts and chondroblasts (**Figure 3.3.5 D**). Together, these data suggest that MSC-17 conform to all UT-MSC characteristics.

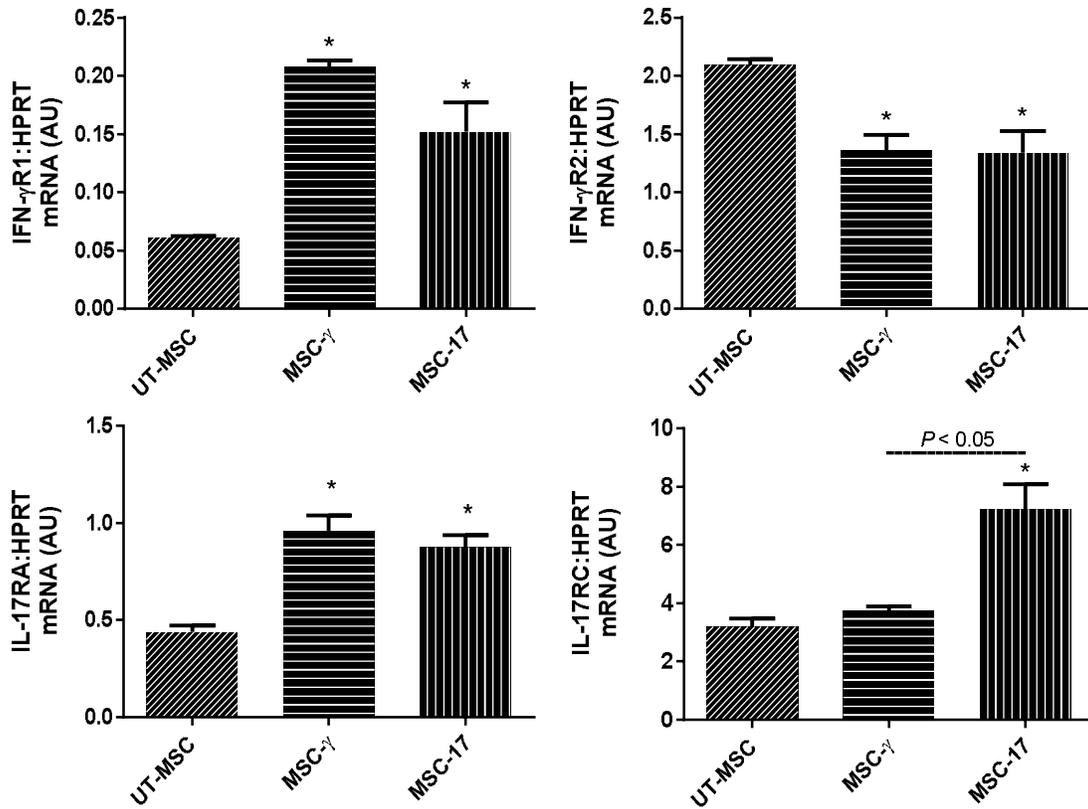


Figure 3.3.4 IFN- γ and IL-17A receptor gene expression on MSC.

IFN- γ receptors IFN- γ R1 and IFN- γ R2 as well as the IL-17A receptors IL-17RA and IL-17RC gene expression on UT-MSC, MSC- γ and MSC-17 were evaluated at day 5 of cytokine treatment by real-time PCR. Data are one representative of 3 MSC donors. *p < 0.05 vs. UT-MSC were determined by one-way ANOVA with post-Sidak multiple comparison test. Error bars depict means of triplicates \pm SD. Adapted from supplemental online Figure S1 Sivanathan KN, et. al., (2015)².

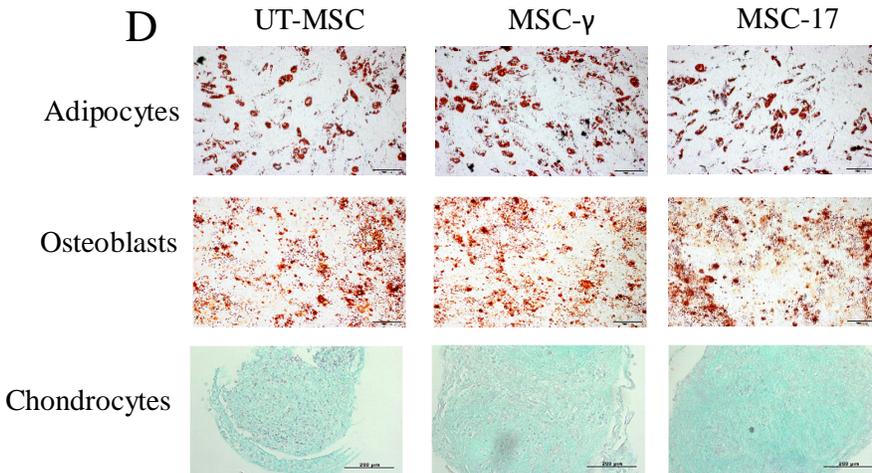
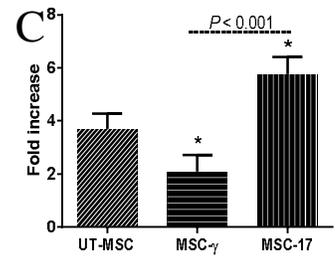
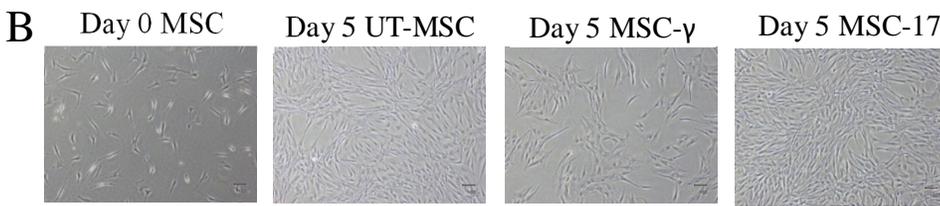
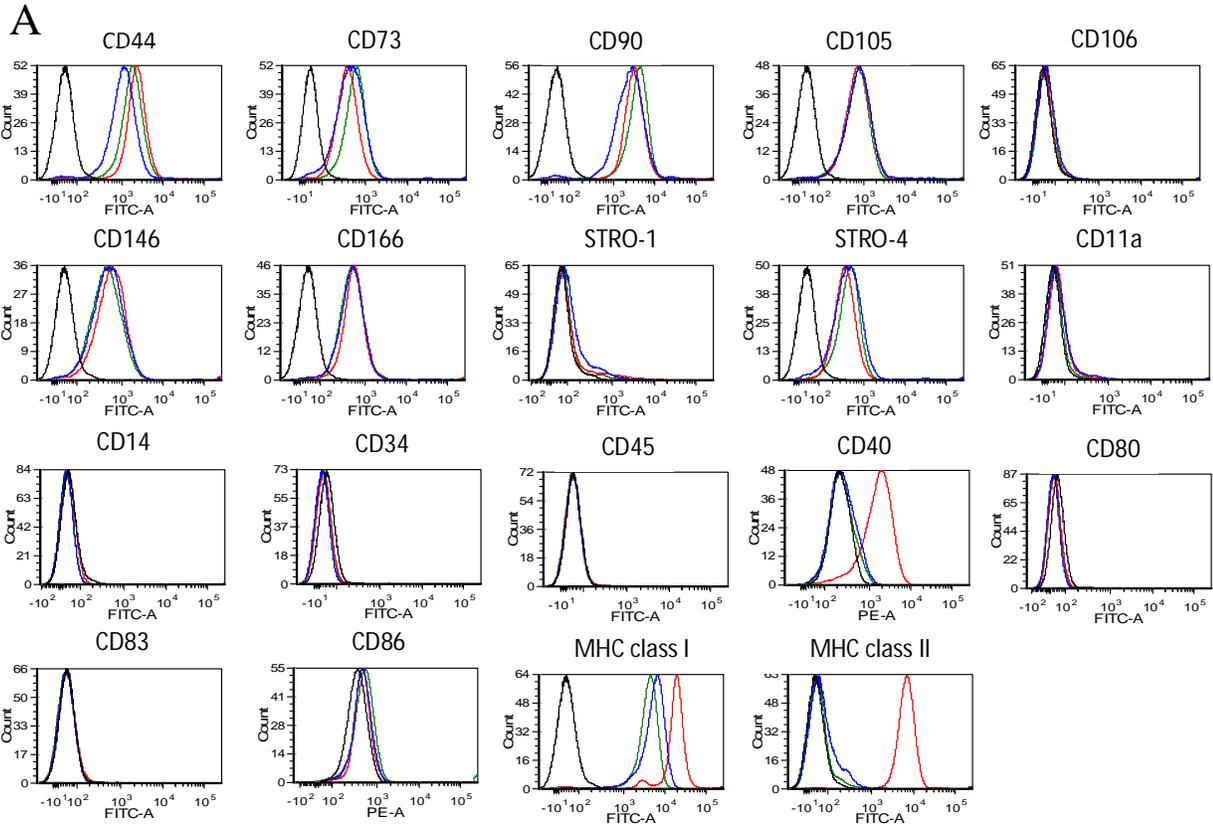


Figure 3.3.5 Characterisation of MSC-17 and MSC- γ .

Human bone marrow-derived MSC were either untreated (UT-MSC) or treated with IFN- γ (MSC- γ) or IL-17A (MSC-17) for 5 days. (A) The expression of surface markers on MSC was assessed by flow cytometry. Histograms show level of surface marker expression on MSC and are depicted by the isotype control (black), **UT-MSC** (green), **MSC- γ** (red) and **MSC-17** (blue). Data are one representative of 3 MSC donors. (B) Morphology of day 0 MSC and day 5 UT-MSC, MSC- γ and MSC-17 (original magnification x40). (C) Fold increase in MSC numbers calculated as harvest cell number divided by the initial seeding number. Cell numbers were determined by trypan blue exclusion assay. Data are pooled from 3 independent experiments with 3 MSC donors. * $p < 0.05$ vs. UT-MSC was determined by two-way ANOVA with post-Sidak multiple comparison test. Error bars depict mean \pm SEM. (D) Functional differentiation of MSC into adipocytes (Oil red stained fat; original magnification x100), osteoblasts (Alizarin red stained mineral; original magnification x100), and chondrocytes (Alician blue stained glycosaminoglycans; original magnification x200) for 5 weeks. The Olympus IX51 inverted microscope was used to capture images. *Adapted from Figure 1 Sivanathan KN, et. al., (2015)².*

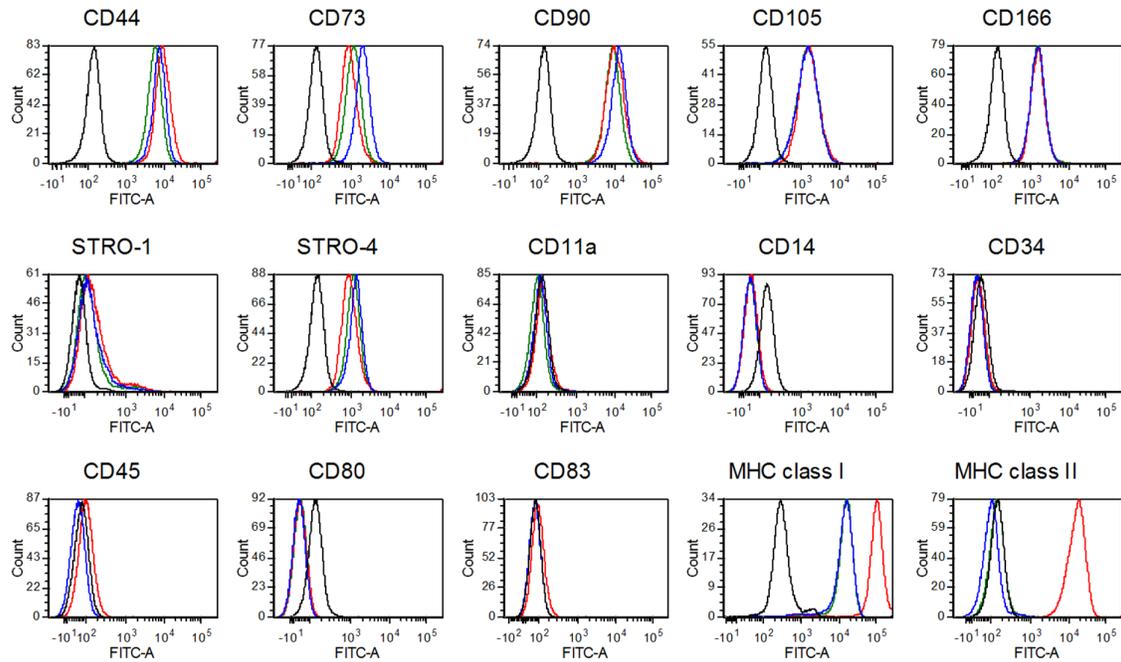


Figure 3.3.6 Characterisation of STRO-1 purified MSC-17 and MSC- γ .

Human STRO-1 purified bone marrow-derived MSC were either untreated (UT-MSC) or treated with IFN- γ (MSC- γ) or IL-17A (MSC-17) for 5 days. (A) The expression of surface markers on MSC was assessed by flow cytometry. Histograms show level of surface marker expression on MSC and are depicted by the isotype control (black), **UT-MSC** (green), **MSC- γ** (red) and **MSC-17** (blue). Data are one representative of 2 MSC donors. (*unpublished*).

3.3.4 MSC-17 are superior suppressors of T cells proliferation

Changes to MSC-mediated immunosuppression on T cells were assessed at day 2 and day 5 of IFN- γ or IL-17A treatment of MSC. Day 2 MSC- γ and MSC-17 inhibited PHA-induced T cell proliferation with no further enhancement of MSC immunosuppressive function when compared to UT-MSC (**Figure 3.3.7** and **Table 3.3.4.1**). Significant changes were observed at day 5 cytokine treated MSC, with MSC-17 exhibiting superior T cell suppression compared to UT-MSC (**Figure 3.3.8**). MSC-17 enhanced T cell suppression by 21.9% while MSC- γ only showed 12.1% compared to UT-MSC (cumulative data n=4, **Table 3.3.4.2**). The extent of human T cell immunosuppression varied between MSC donors and responder T cells, however MSC-17 consistently showed greater inhibition of T cells relative to MSC- γ (**Table 3.3.4.2**). Additionally, MSC-17 showed highest T cell suppression compared to 5 day IL-1 β and TNF- α treated MSC (**Figure 3.3.3**). Day 5 cytokine treated MSC were subsequently used in all experiments. We have also shown *in vitro* that the increased numbers of MSC-17 have a similar dose-dependent effect on T cell proliferation as compared to UT-MSC under three different experimental conditions (**Figure 3.3.9**). In studies using STRO-1 purified MSC, the combination of IFN- γ and IL-17A treatment of MSC (MSC- γ /17) decreased MSC growth potential (data not shown). Additionally, we failed to show that these cytokines have an additive effect on MSC- γ /17 mediated T cell suppression in a PHA-dependent T cell proliferation assay as well as in a MLR (**Figure 3.3.10**).

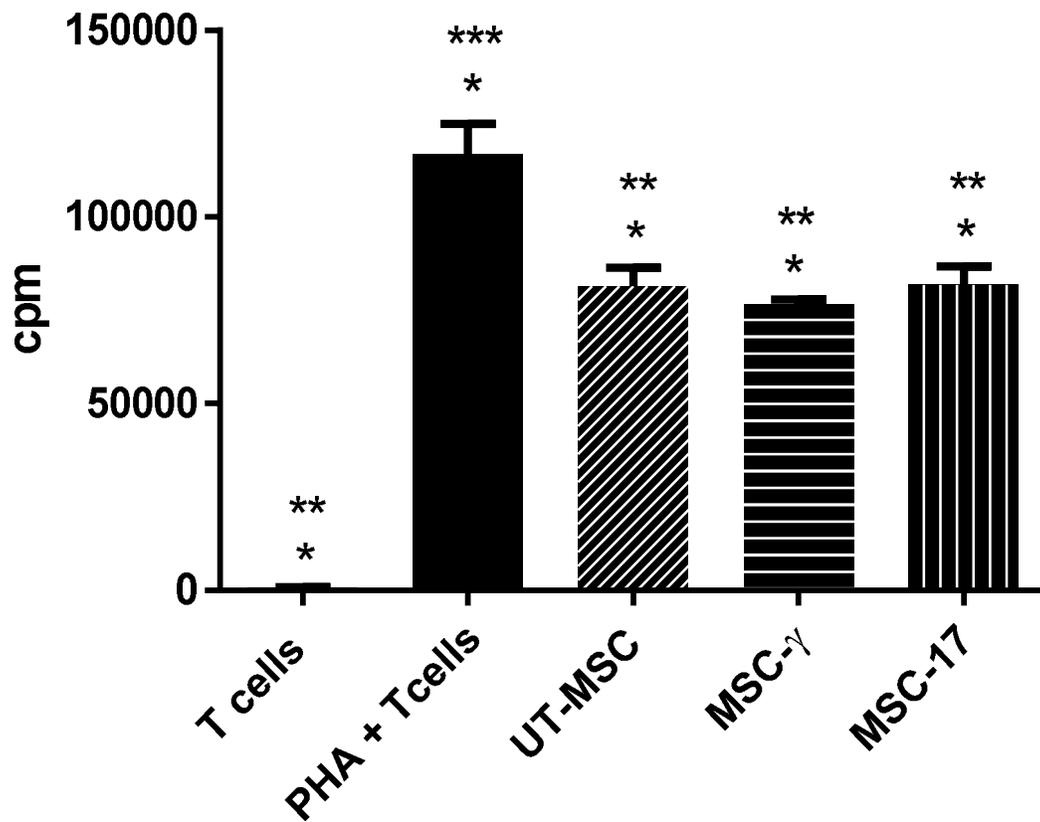


Figure 3.3.7 Day 2 cytokine treated MSC-17 and MSC- γ enhance inhibition of T cell proliferation.

1% UT-MSC, MSC- γ or MSC-17 treated for 2 days with IFN- γ or IL-17A respectively were co-cultured with PHA activated CD3⁺ T cells. T cell proliferation was measured in a [³H]-Thymidine assay. Data are one representative of 5 independent experiments. *p < 0.05 vs. T cells alone, **p < 0.05 vs. PHA + T cells and ***p < 0.05 vs. UT-MSC determined by one-way ANOVA with post-Sidak multiple comparison test. Error bars depict means of triplicates \pm SD. (*unpublished*).

Table 3.3.4.1 Individual experiment data on MSC suppression of T cell proliferation.

DAY 2	% proliferation relative to PHA and T cells (100%) \pm SD		
MSC donor	UT-MSc	MSC- γ	MSC-17
MSC 1	102.5 \pm 8.871	80.58 \pm 3.335*	82.4 \pm 6.011*
MSC 2	96.91 \pm 2.040	85.37 \pm 1.196*	71.58 \pm 1.194*
MSC 3	90.25 \pm 4.076	84.23 \pm 14.860	90.19 \pm 10.230
MSC 1	88.56 \pm 9.574	90.61 \pm 14.430	79.49 \pm 6.752
MSC 1	70.03 \pm 8.017	65.79 \pm 5.482	70.37 \pm 8.964
Mean \pm SD	89.65 \pm 12.291	81.32 \pm 9.393	78.81 \pm 8.160

1% UT-MSc or **2 day** treated MSC- γ , MSC-17 were co-cultured with PHA activated CD3⁺ T cells. T cell proliferation was measured in a [³H]-Thymidine assay. Data are presented as percentage proliferation relative to PHA + T cells (100%). Bold fonts indicate p < 0.05 vs. PHA + T cells; *p < 0.05 vs. UT-MSc determined by one-way ANOVA with post-Sidak multiple comparison tests. (*unpublished*).

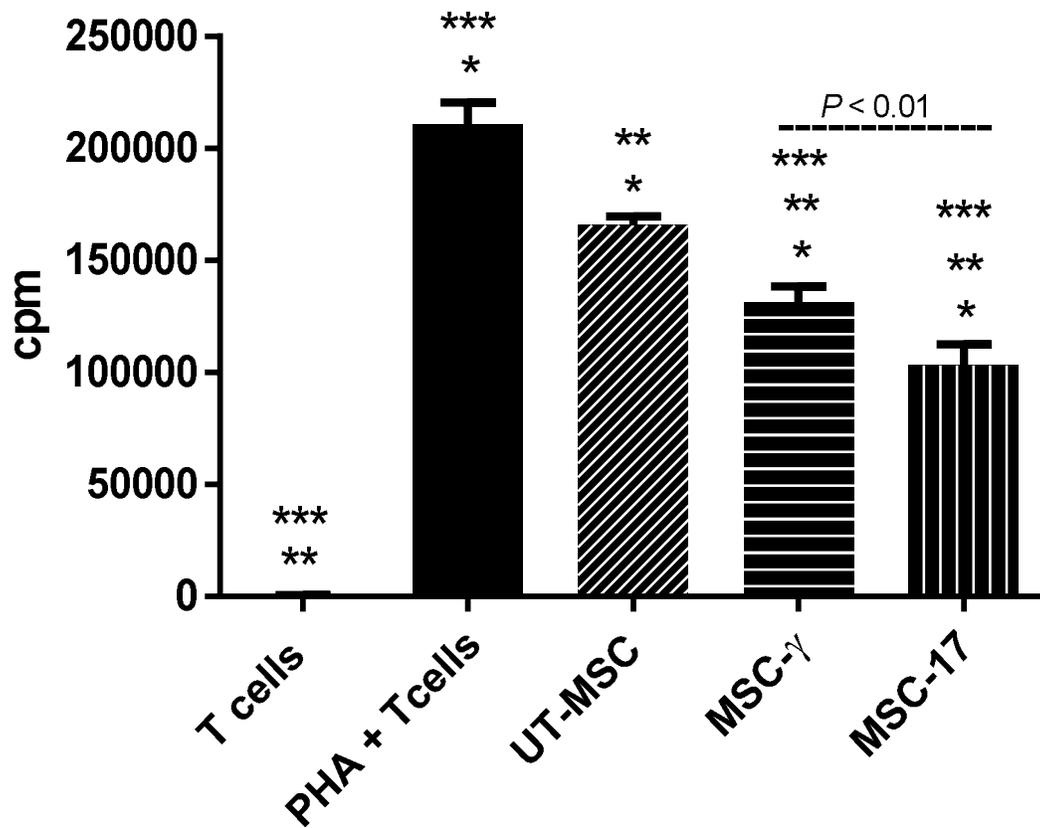


Figure 3.3.8 Day 5 cytokine treated MSC-17 and MSC- γ enhance inhibition of T cell proliferation.

1% UT-MSC, MSC- γ or MSC-17 treated for 2 days with IFN- γ or IL-17A respectively were co-cultured with PHA activated CD3⁺ T cells. T cell proliferation was measured in a [³H]-Thymidine assay. Data are one representative of 4 of independent experiments. *p < 0.05 vs. T cells alone, **p < 0.05 vs. PHA + T cells and ***p < 0.05 vs. UT-MSC determined by one-way ANOVA with post-Sidak multiple comparison test. Error bars depict means of triplicates \pm SD.

Adapted from Figure 2B Sivanathan KN, et. al., (2015)².

Table 3.3.4.2 Individual experimental data on MSC suppression of T cell proliferation.

DAY 5	% proliferation relative to PHA and T cells (100%) \pm SD		
MSC donor	UT-MSC	MSC- γ	MSC-17
MSC 1	78.63 \pm 1.688	62.51 \pm 5.224*	49.02 \pm 3.912*
MSC 2	87.55 \pm 3.279	73.03 \pm 2.051*	65.81 \pm 2.526*
MSC 3	89.14 \pm 4.758	73.65 \pm 9.514*	69.48 \pm 4.577*
MSC 1	69.01 \pm 1.615	66.73 \pm 11.420	52.58 \pm 5.804*
Mean \pm SD	81.08 \pm 9.282	69.98 \pm 5.327	59.22 \pm 9.947

1% UT-MSC or **5 day** treated MSC- γ , MSC-17 were co-cultured with PHA activated CD3⁺ T cells. T cell proliferation was measured in a [³H]-Thymidine assay. Data are presented as percentage proliferation relative to PHA + T cells (100%). Bold fonts indicate p < 0.05 vs. PHA + T cells; *p < 0.05 vs. UT-MSC determined by one-way ANOVA with post-Sidak multiple comparison tests. *Adapted from supplemental online Table S1 Sivanathan KN, et. al., (2015)².*

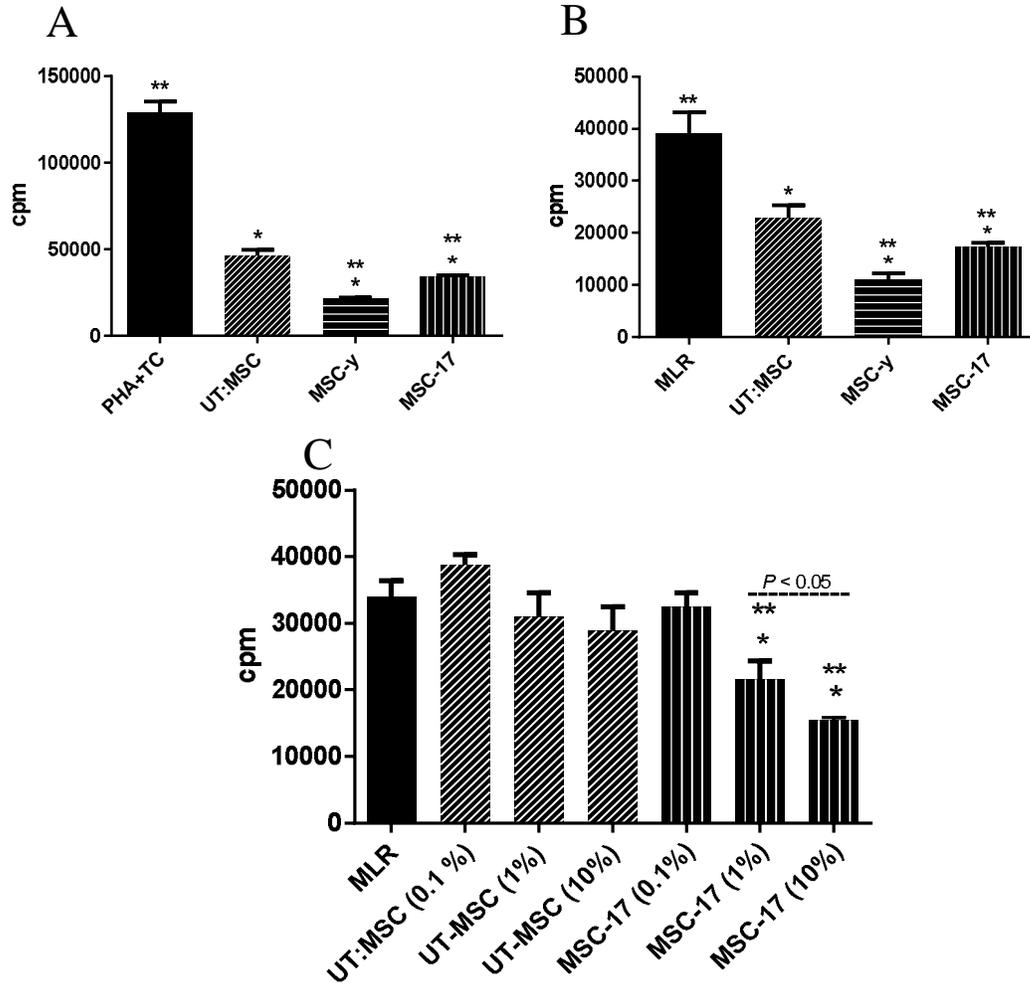


Figure 3.3.9 Dose-dependent inhibition of T cell proliferation by MSC-17.

(A) 10% UT-MSC, MSC- γ or MSC-17 were co-cultured with either (A) PHA-activated CD3⁺ T cells or (B) responder PBMC and stimulator PBMC (30 Grays irradiated) from two different buffy coat donors in a one-way mixed lymphocyte reaction (MLR). (C) 0.1%, 1% and 10% UT-MSC or MSC-17 were co-cultured with PBMC from two different buffy coats in a two-way MLR. T cell proliferation was measured in a [³H]-Thymidine assay. Data is (A) one experiment, (B) one representative of 3 independent experiments or (C) one representative of 4 independent experiments. * $p < 0.05$ vs. PHA + T cells or MLR and ** $p < 0.05$ vs. UT-MSC determined by one-way ANOVA with post-Sidak multiple comparison test. Error bars depict means of triplicates \pm SD. Adapted from supplemental online Figure S3 Sivanathan KN, et al., (2015)².

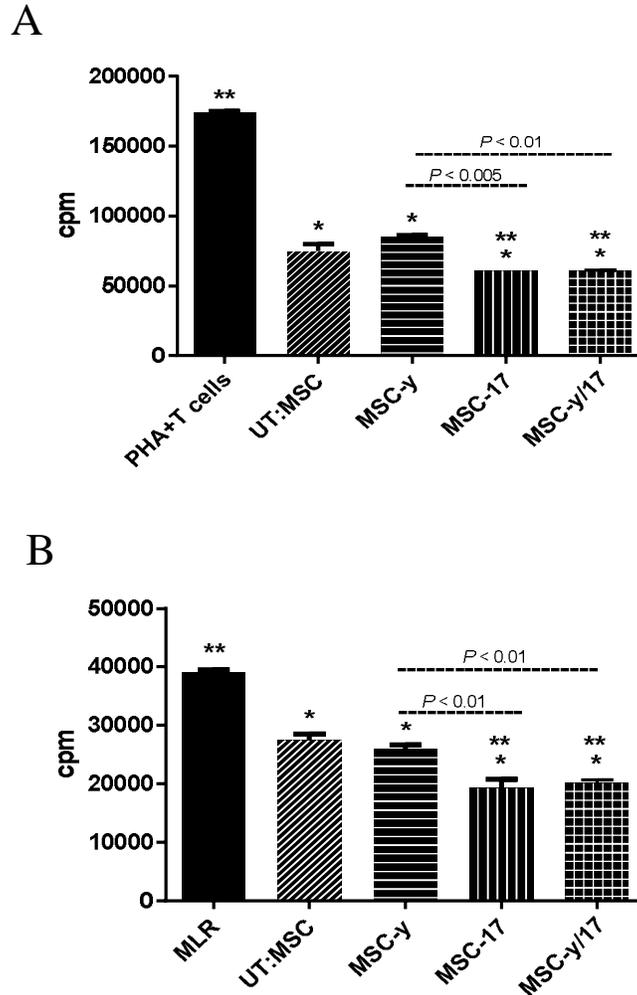


Figure 3.3.10 MSC- γ /17 show no additive effect on T cell immunosuppression.

Human bone marrow-derived STRO-1 purified MSC were either untreated (UT-MSC) or treated with IFN- γ alone (MSC- γ), IL-17A alone (MSC-17) or with the combination of IFN- γ (500 U/ml) and IL-17A (50 ng/ml) (MSC- γ /17) for 5 days. 1% MSC were co-cultured with either **(A)** PHA-activated CD3⁺ T cells or **(B)** responder PBMC and stimulator PBMC (30 Grays, irradiated) from two different buffy coat donors in a one-way MLR. T cell proliferation was measured in a [³H]-Thymidine assay. Data is one representative of 2 **(A)** or 3 **(B)** independent experiments. *p< 0.05 vs. PHA + T cells or MLR and **p< 0.05 vs. UT-MSC determined by one-way ANOVA with post-Sidak multiple comparison test. Error bars depict means of triplicates \pm SD. Adapted from supplemental online Figure S5 Sivanathan KN, et al., (2015)².

3.3.5 MSC-17 suppress T cell activation

Next, we investigated whether MSC- γ and MSC-17 could further inhibit T cell activation markers CD25 and CD69. UT-MSCs inhibited CD25 expression of PHA-activated CD4⁺ and CD8⁺ effector T cells by 23.4% and 55.6% ($p < 0.05$) respectively (**Figure 3.3.11**). MSC- γ and MSC-17 further downregulated CD25 by approximately 50% on CD4⁺ effector T cells relative to PHA-activated T cell positive control. However, suppression of CD25 on CD8⁺ effector T cells was comparable in all MSC groups (**Figure 3.3.11**). Presence of MSC had no effect on CD69 expression (**Figure 3.3.12**).

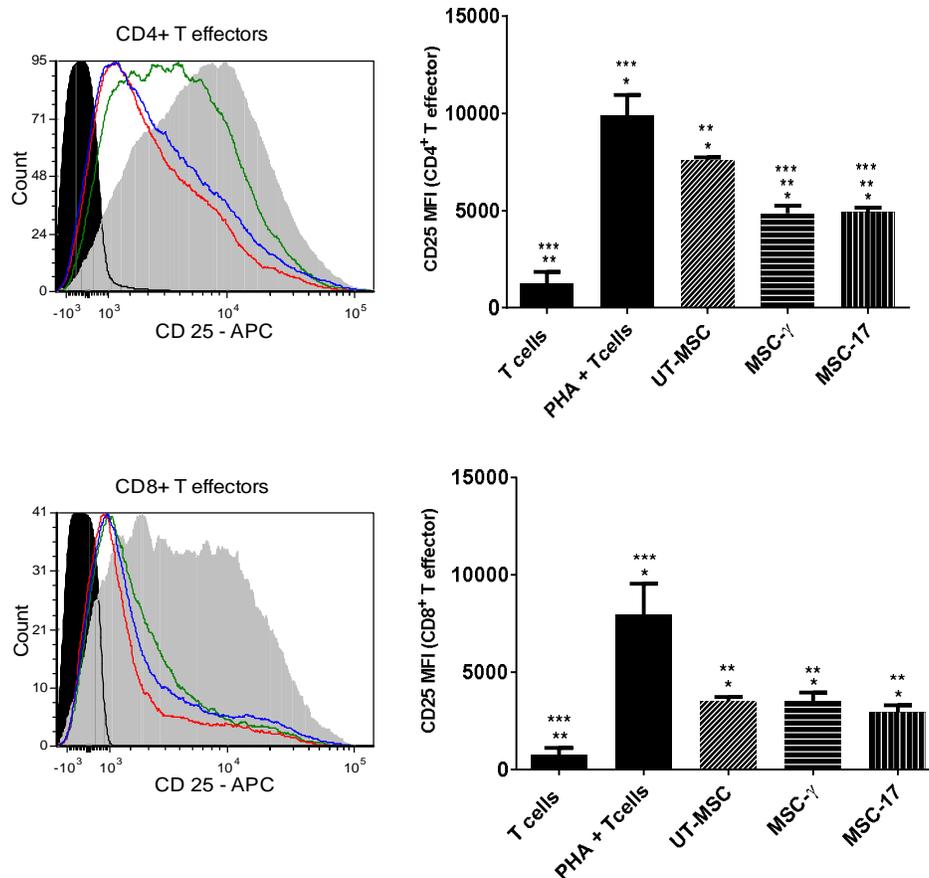


Figure 3.3.11 MSC-17 and MSC- γ enhance inhibition of T cell activation (CD25).

1% UT-MSC, MSC- γ or MSC-17 treated for 5 days with IFN- γ or IL-17A respectively were co-cultured with PHA activated CD3⁺ T cells. The expression of CD25 on CD4⁺ and CD8⁺ T effector cells co-cultured with MSC were assessed by flow cytometry. Histograms show level of CD25 expression and are depicted by the co-culture conditions: **T cell alone** (negative control; solid black), **PHA+T cells** (positive control; solid grey), **UT-MSC** (green), **MSC- γ** (red) and **MSC-17** (blue). Graphs depicts CD25 MFI (mean fluorescence intensity). Data are one representative of 3 independent experiments. * $p < 0.05$ vs. T cells alone, ** $p < 0.05$ vs. PHA + T cells and *** $p < 0.05$ vs. UT-MSC determined by one-way ANOVA with post-Sidak multiple comparison test. Error bars depict means of triplicates \pm SD. Adapted from Figure 2C Sivanathan KN, et. al., (2015)².

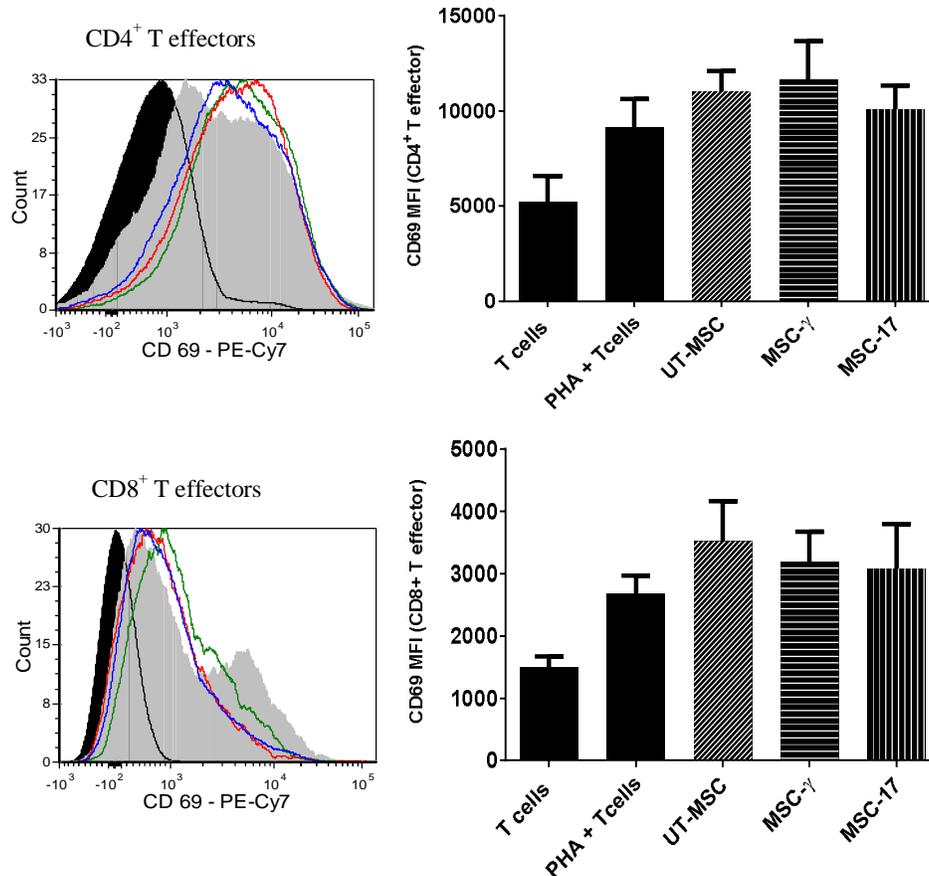


Figure 3.3.12 MSC do not inhibit the expression of the early T cell activation (CD69).

1% UT-MSC, MSC- γ or MSC-17 treated for 5 days with IFN- γ or IL-17A respectively were co-cultured with PHA activated CD3⁺ T cells. The expression of CD69 on CD4⁺ and CD8⁺ T effector cells co-cultured with MSC were assessed by flow cytometry. Histograms show level of CD69 expression and are depicted by the co-culture conditions: T cell alone (negative control; solid black), PHA+T cells (positive control; solid grey), **UT-MSC** (green), **MSC- γ** (red) and **MSC-17** (blue). Graphs depicts CD69 MFI. Data are one representative of 3 independent experiments. * $p < 0.05$ vs. T cells alone, ** $p < 0.05$ vs. PHA + T cells and *** $p < 0.05$ vs. UT-MSC determined by one-way ANOVA with post-Sidak multiple comparison test. Error bars depict means of triplicates \pm SD. Adapted from Figure 2D Sivanathan KN, et al., (2015)².

3.3.6 MSC-17 potently suppress T cell effector function

MSC- γ and MSC-17 potently suppressed T cell effector function, evident by the decrease in the secretion of Th1 driving cytokines when compared to UT-MSC (**Figure 3.3.13**). MSC-17 showed greater inhibition of IFN- γ , TNF- α and IL-2 by 2.0-, 1.9- and 5.5-fold, respectively ($p < 0.05$) compared to UT-MSC. MSC- γ co-cultures showed a slightly higher inhibition of secreted IFN- γ , TNF- α and IL-2 by 4.3-, 3.4- and 7.5-fold respectively, relative to UT-MSC ($p < 0.05$). A significant decrease of Th2 cytokine IL-10 was observed in all MSC cultures, while only MSC- γ co-cultures exhibited a 2-fold increase in IL-6 compared to UT-MSC and MSC-17 ($p < 0.01$). Thus, human MSC-17 augmented suppression of T cell proliferation and exerted similar inhibition of T cell activation and effector function as to MSC- γ , while conforming to UT-MSC phenotype.

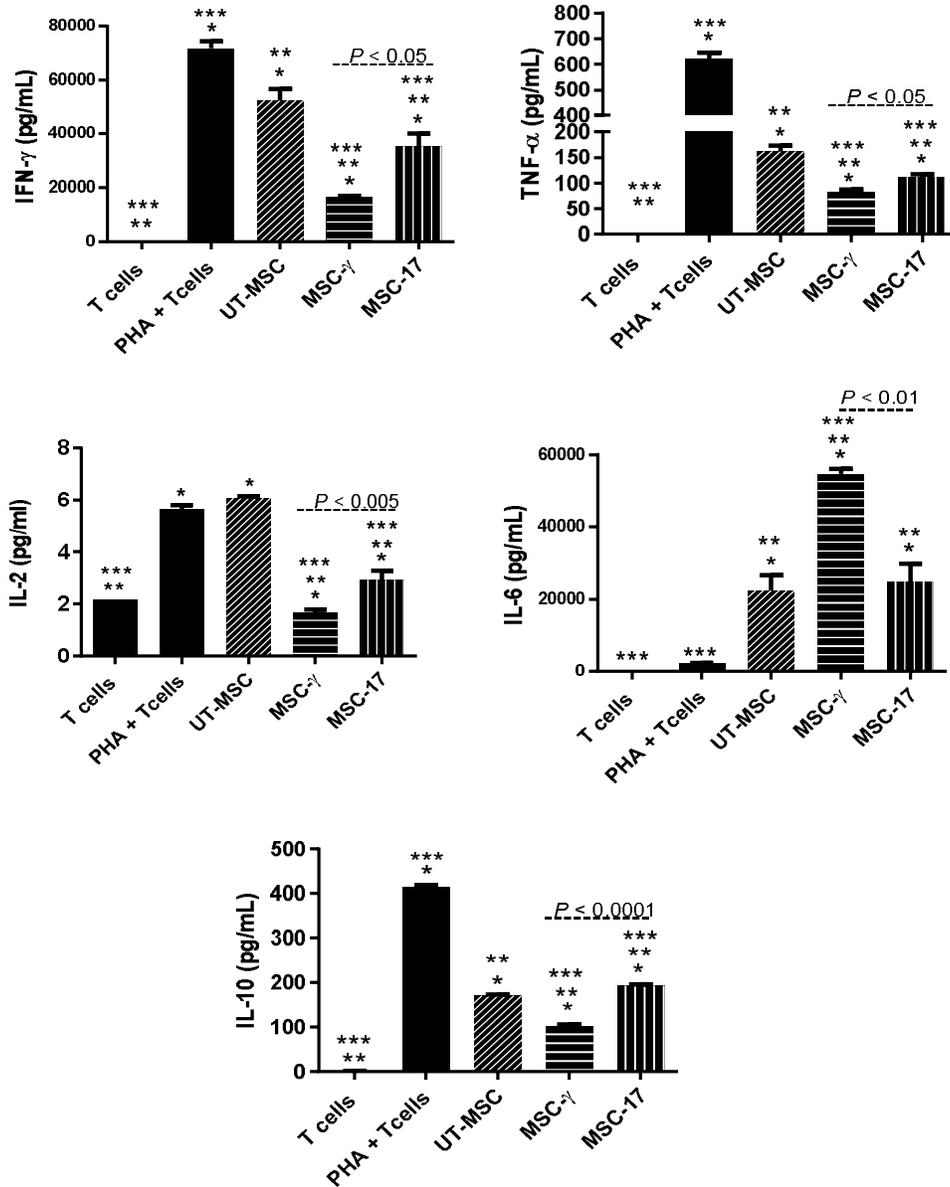


Figure 3.3.13 MSC-17 and MSC- γ inhibit Th1 cytokine secretion.

1% UT-MSC, MSC- γ or MSC-17 treated for 5 days with IFN- γ or IL-17A respectively were co-cultured with PHA activated CD3⁺ T cells. Co-culture supernatants were evaluated for the presence of IFN- γ , TNF- α , IL-2, IL-4 (undetected), IL-6 and IL-10 using a CBA assay. Data are one representative of 3 independent experiments. *p < 0.05 vs. T cells alone, **p < 0.05 vs. PHA + T cells and ***p < 0.05 vs. UT-MSC determined by one-way ANOVA with post-Sidak multiple comparison test. Error bars depict means of duplicates \pm SD. Adapted from Figure 2E Sivanathan KN, et. al., (2015)².

3.3.7 MSC- γ suppression of T cell proliferation was independent of PD-L1

UT-MSC constitutively expressed MSC immunoregulatory genes IL-6, Cox-1 and TGF- β 1 transcripts (**Figure 3.3.14**). IL-6 increased in a time-dependent manner following exposure to IFN- γ and IL-17A, peaking at day 5 with IL-17A treatment. IFN- γ treatment increased Cox-1 relative to UT-MSC and levels were similar at all-time points. IL-17A treatment showed no effect on Cox-1 expression. Only IFN- γ exposure induced IDO1 expression in a time-dependent fashion. TGF- β 1 was unaffected in MSC- γ and MSC-17.

The programmed death ligand-1 (PD-L1) / PD-1 pathway regulates T cell activation and promotes tolerance induction^{317,363}. As MSC- γ show high PD-L1 expression (**Figure 3.3.15**), we investigated its regulatory role by blockade of PD-L1 with a neutralising antibody at a pre-optimised concentration (20 μ g/ml). Antibody blockade failed to restore PHA activated T cell proliferation in UT-MSC and MSC- γ indicating that the suppression of T cells is independent of PD-L1 (**Figure 3.3.15**).

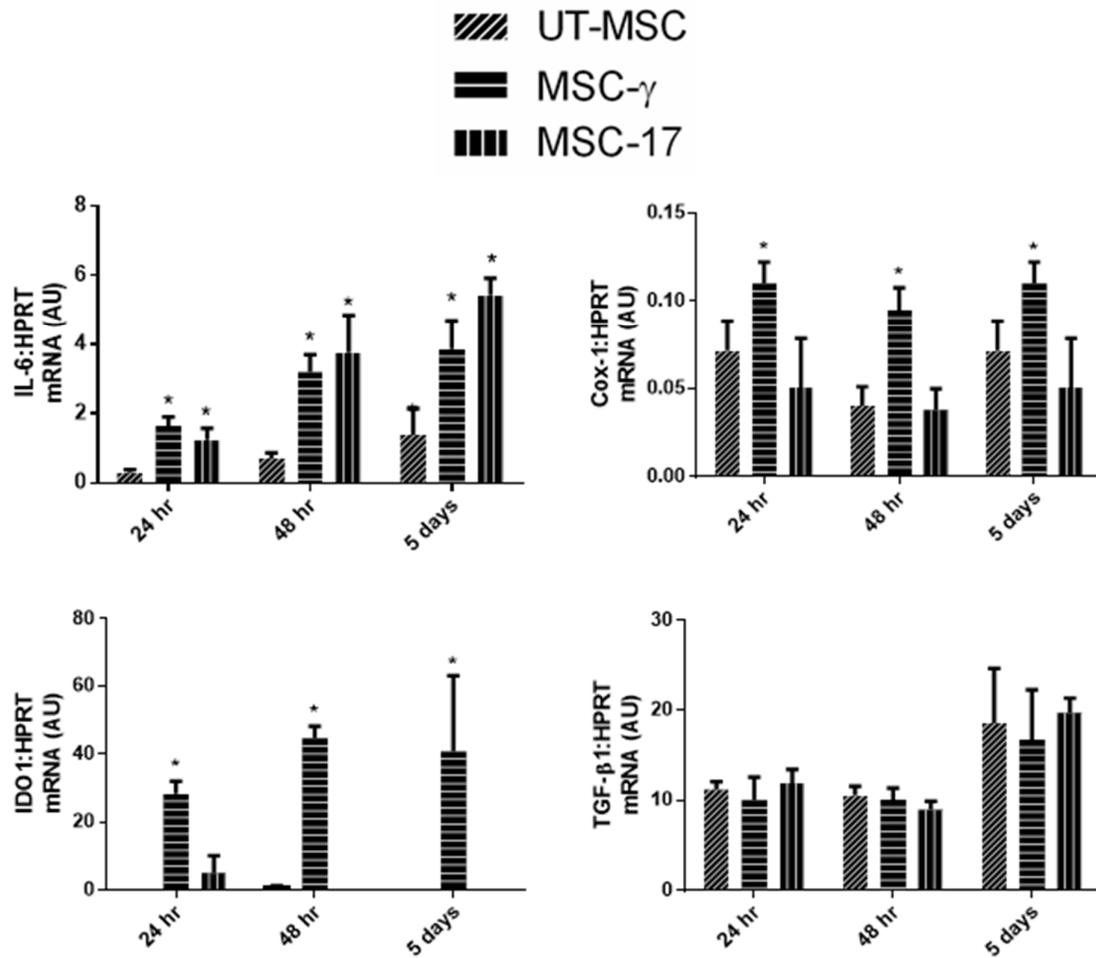


Figure 3.3.14 Mechanisms of T cell immunomodulation by MSC-17 and MSC- γ .

IL-6, Cox-1, IDO1 and TGF- β 1 gene expression following 1,2 or 5 days IFN- γ or IL-17A treatment of MSC assessed by real-time PCR. Data of 3 independent experiments. * $p < 0.05$ vs. UT-MSC were determined by one-way ANOVA with post-Sidak multiple comparison test. Error bars depict mean \pm SEM. Adapted from Figure 3A Sivanathan KN, et al., (2015)².

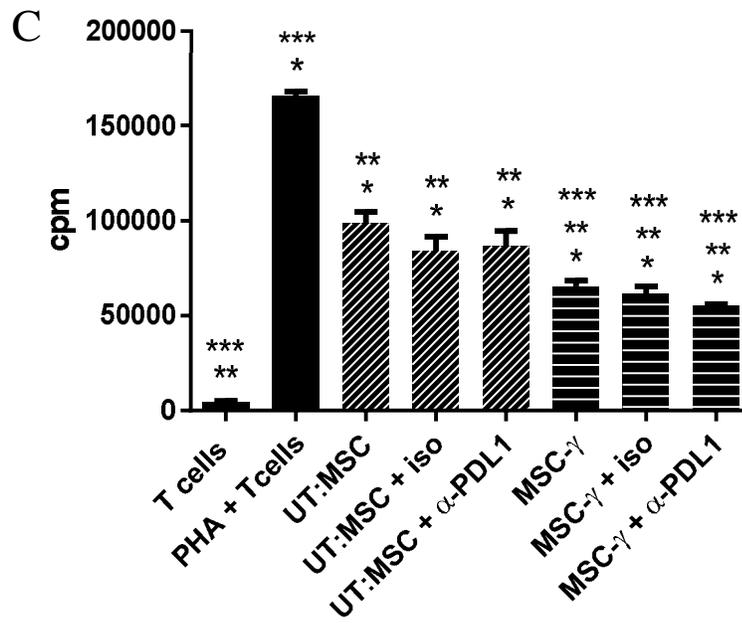
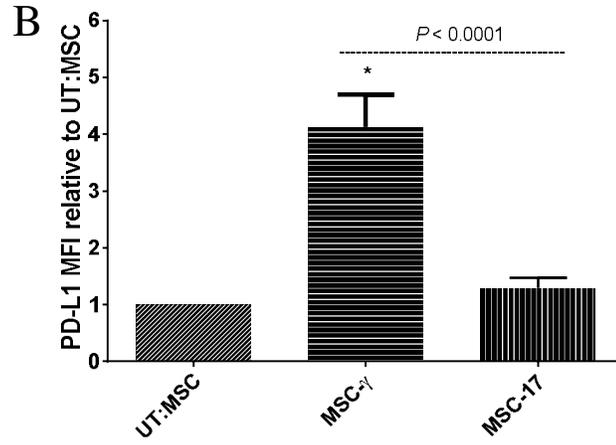
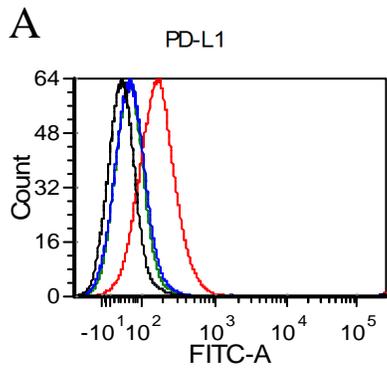


Figure 3.3.15 MSC- γ suppression of T cell proliferation is independent of PD-L1.

(A,B) PD-L1 expression was assessed on UT-MSC or 5 day cytokine treated MSC- γ and MSC-17 by flow cytometry. **(A)** Histograms show level of surface marker expression on MSC and are depicted by the isotype control (black), **UT-MSC** (green), **MSC- γ** (red) and **MSC-17** (blue). Data are one representative of 6 MSC donors. **(B)** IFN- γ upregulates PD-L1 expression on MSC as defined by the mean fluorescence intensity (MFI). Graph depicts PD-L1 MFI relative to UT-MSC (UT-MSC is indicated as 1). Data are pooled from 6 independent experiments with 6 MSC donors. * $p < 0.05$ vs. UT-MSC was determined by one-way ANOVA with post-Sidak multiple comparison test. Error bars depict mean \pm SEM. **(C)** UT-MSC or MSC- γ were co-cultured with CD3⁺ T cells at 1% MSC dose in the presence or absence of anti-PD-L1 (20 μ g/ml) (α -PDL1) or mouse IgG1 isotype control (20 μ g/ml) (iso). T cell proliferation was measured in a [³H]-Thymidine assay. Data are one representative of 3 independent experiments. * $p < 0.05$ vs. T cells alone, ** $p < 0.05$ vs. PHA + T cells and *** $p < 0.05$ vs. UT-MSC were determined by one-way ANOVA with post-Sidak multiple comparison test. Error bars depict means of duplicates \pm SD. *Adapted from supplemental online Figure S4 Sivanathan KN, et. al., (2015)².*

3.4 DISCUSSION

MSC-17 represent a novel alternative to IFN- γ treated MSC for enhanced T cell immunosuppressive properties. IL-17A functions as a MSC growth factor through the activation of signaling pathways including AKT, ERK, MEK and p38³⁴⁰⁻³⁴², consistent with our observation of increased MSC proliferation upon IL-17A exposure. IFN- γ in contrast induced growth arrest in MSC^{174,303} and this represents a significant impediment to the generation of sufficient number of cells for *in vivo* infusion. Immune responses to the presence of MSC is mediated by the recognition of allogeneic MHC molecules by recipient CD4⁺ and CD8⁺ memory T cells^{223,303,327}. Although MSC- γ are more immunosuppressive than UT-MSC, the high expression of MHC molecules may negatively impact the fate of MSC- γ *in vivo*⁴. Some studies show that allogeneic MSC- γ therapy is ineffective at ameliorating inflammation as MSC- γ are more immunogenic than UT-MSC^{217,299,300}. In addition, the expression of MHC class II on allogeneic MSC has been previously shown to induce alloimmune responses in co-culture with MHC mismatched responder cells in mixed lymphocyte reactions (MLR)^{303,327}. The absence of morphological changes in MSC-17, lack surface MHC molecule induction and CD40 expression on MSC-17 indicated that MSC-17 are less likely to be immunogenic compared to MSC- γ .

A recent study showed that IL-17 acts synergistically with IFN- γ and TNF- α to enhance immunosuppression by MSC in mouse models of hepatitis. This study however failed to show that the treatment of MSC with IL-17 alone is effective at enhancing the ability of MSC to suppress T cell proliferation³⁶². This thesis demonstrated that human MSC-17 were more potent suppressors of T cells proliferation relative to UT-MSC and MSC- γ . MSC-17 suppressed T cell Th1 cytokine responses more strongly than UT-MSC, thereby emphasising their potential benefit in treating inflammatory diseases. In studies using STRO-1 purified MSC, the combination of IFN- γ and IL-17A treatment of MSC (MSC- γ /17) decreased MSC growth

potential. Additionally, we failed to show that these cytokines have an additive effect on MSC- $\gamma/17$ mediated T cell suppression in a PHA-dependent T cell proliferation assay as well as in a MLR.

In conclusion, this thesis demonstrates for the first time that human IL-17A modified MSC (MSC-17) exhibit superior immunosuppressive properties compared to unmodified MSC (UT-MSC). IL-17A treatment of MSC (MSC-17) represents a novel immunomodulatory strategy and an alternative to IFN- γ in enhancing the immunosuppressive properties of MSC on T cells. MSC-17 suppression of T cell proliferation correlated with the increase in IL-6 immunoregulatory gene expression, downregulation of T cell activation, and inhibition of Th1 cytokines.

CHAPTER 4:

MSC INDUCTION OF REGULATORY T CELLS

This chapter is adapted and modified from the published article:

Sivanathan KN, Rojas-Canales DM, Hope CM, et al. Interleukin-17A-Induced Human Mesenchymal Stem Cells Are Superior Modulators of Immunological Function. *Stem Cells*. 2015;33(9):2850-2863.

(APPENDIX Published Paper 3)

4.1 INTRODUCTION

Several studies also report that MSC modulation of T cell responses occur indirectly through MSC mediated induction of regulatory T cells (Treg)^{224,249,283,284}. Tregs have a role in maintenance of self-tolerance and immune homeostasis. Human Tregs are defined as CD4⁺CD25⁺CD127^{low}FoxP3⁺ T cells and can be categorised into 2 subtypes: (1) thymus-derived naturally occurring Tregs (nTreg) and (2) peripheral-derived inducible Tregs (iTreg) that are generated from CD4⁺CD25⁻ T cells. Both Treg subtypes confer suppressive activities due to their expression of regulatory cytokines and suppression of immune cell activation and proliferation⁷⁹. Similar to MSC, Tregs have potential therapeutic use in transplantation, autoimmune diseases and GvHD³⁶⁶⁻³⁶⁸. For this reason, strategies to expand or induce Treg as well as enhancing Treg function *in vivo* or *ex vivo* have gained much interest in recent years.

In this chapter, we aimed to identify if MSC-17 can indirectly mediate T cell immunosuppression through the generation of functionally suppressive Tregs. MSC-17, similar to MSC- γ are capable of increasing or inducing Tregs. MSC-17 indirectly mediated the

inhibition of effector T cells through the induction of functionally suppressive iTregs. Taken together, these data suggest that MSC-17 can function as superior cells for the modulation of T cell mediated inflammatory responses or as accessory cells in *ex vivo* expansion of iTregs.

4.2 MATERIALS AND METHODS

The general materials and methods of this chapter have been described in **CHAPTER 2: MATERIALS AND METHODS**. In this section, additional chapter specific materials and methods, flow cytometry gating strategies and Treg assay optimization experiments are shown.

4.2.1 Treg gating strategy

Table 4.2.1.1 Human Treg antibody panel

Antibody	Clone	Fluorochrome	Company
<i>Treg phenotyping:</i>			
Viability dye	-	V500	Life Technologies
CD3	UCHT1	APC-eFluor	eBioscience
CD4	OKT4	PerCp-Cy5.5 / APC-eFluor	eBioscience
CD25	VT-072	APC	eBioscience
CD127	eBioRDR5	PE / FITC	eBioscience
FoxP3	PCH101	FITC / PE	eBioscience
<i>Treg functional markers:</i>			
CD39	eBioA1	PE-Cy7	eBioscience
CD73	AD2	APC	eBioscience
CD69	L78	PE-Cy7	BD Biosciences
CD103	Ber-ACT8	APC	eBioscience
OX40	ACT35	PerCp-Cy5.5	BD Pharmigen
CTLA-4	L3D10	PE-Cy7	Biologend
HLA-DR	L243	Per-Cp-Cy5.5	BD Pharmigen
GITR	eBioAITR	APC	eBioscience
GARP	7B11	APC	BD Pharmigen
LAP	TW4-2F8	PerCp-Cy5.5	BD Pharmigen

FoxP3, Forkhead box P3; CTLA-4, cytotoxic T-lymphocyte associated antigen-4; HLA-DR, human leukocyte antigen-DR; GITR, glucocorticoid-induced TNFR-related protein; GARP, glycoprotein A repetitions predominant; LAP, latency-associated peptide.

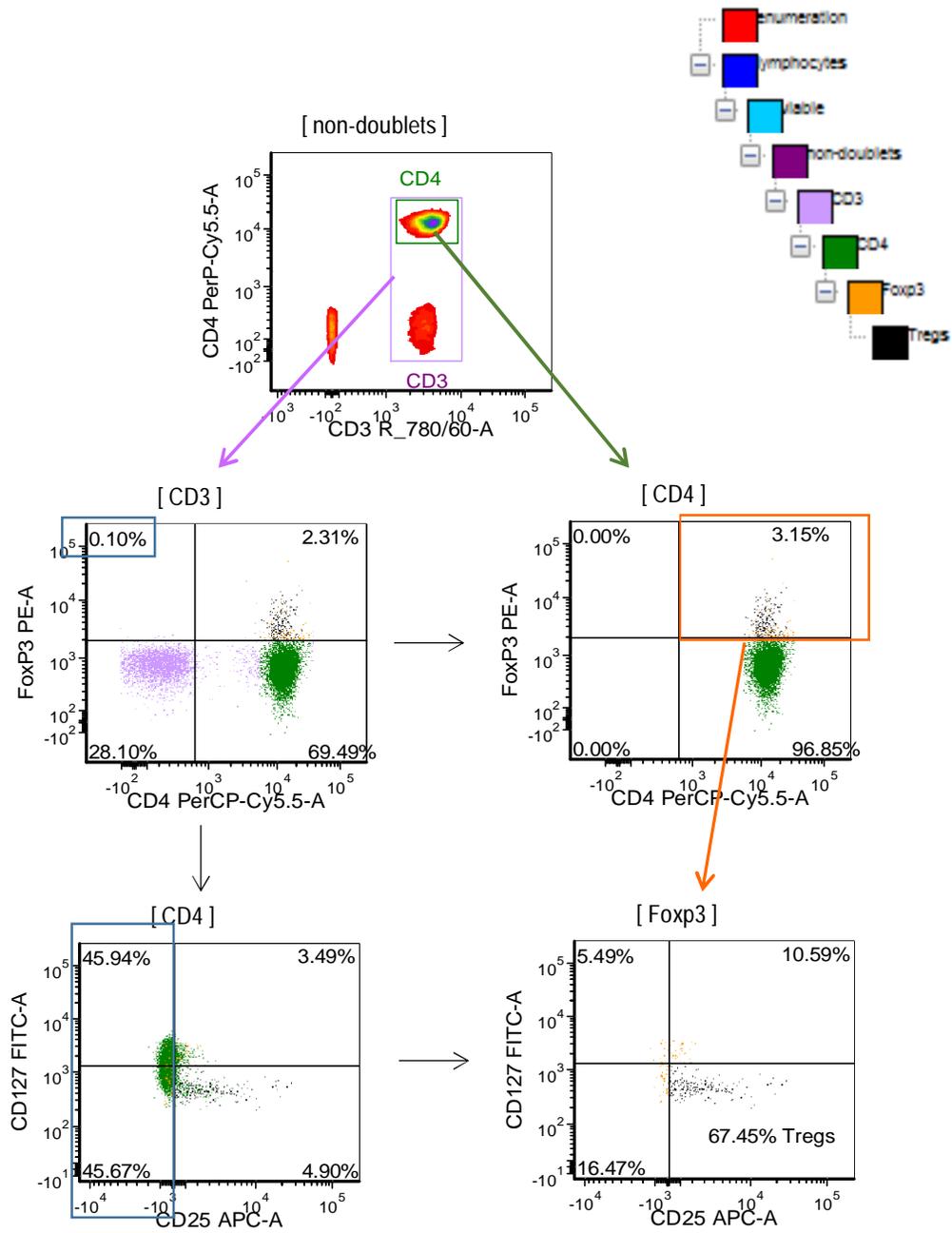


Figure 4.2.1 Regulatory T cell gating strategy set on “T cell alone” culture.

Tregs were defined as CD3⁺CD4⁺CD25^{high}CD127^{low}FoxP3⁺ T cells. The gating strategy is defined as follows: T cells were gated according to the forward scatter area (FSC-area) and side scatter (SSC-area). Dead cells were excluded by the V500 viability dye. Non-doublet cells were gated according to FSC-area and FSC-height (not shown). T cells were cell surface stained with a combination of anti-CD3, anti-CD4, anti-CD25, anti-CD127 and intracellular stained for anti-FoxP3. The CD3⁺ T cell gate was defined and CD4⁺FoxP3⁺ T cells were determined by adjusting the quadrants to CD8⁻ T cells while the FoxP3⁺ cells were set to be less than 0.1% of CD8⁺ T cells. CD127 (50th percentile) and CD25 (90th percentile) quadrants were set on the T cells alone (negative control) and left unchanged for other co-culture conditions. The CD127 and CD25 plot was used to define the quadrants for the Treg colour dot plot. The gate was set on FoxP3⁺ cells, indicated as [FoxP3] above the Treg plot and Treg percentages is indicated in the lower right quadrant of this plot. These plots are representative of Treg gating strategy set on the “T cell alone” culture.

4.2.2 Inducible regulatory T cells (iTregs) gating strategy

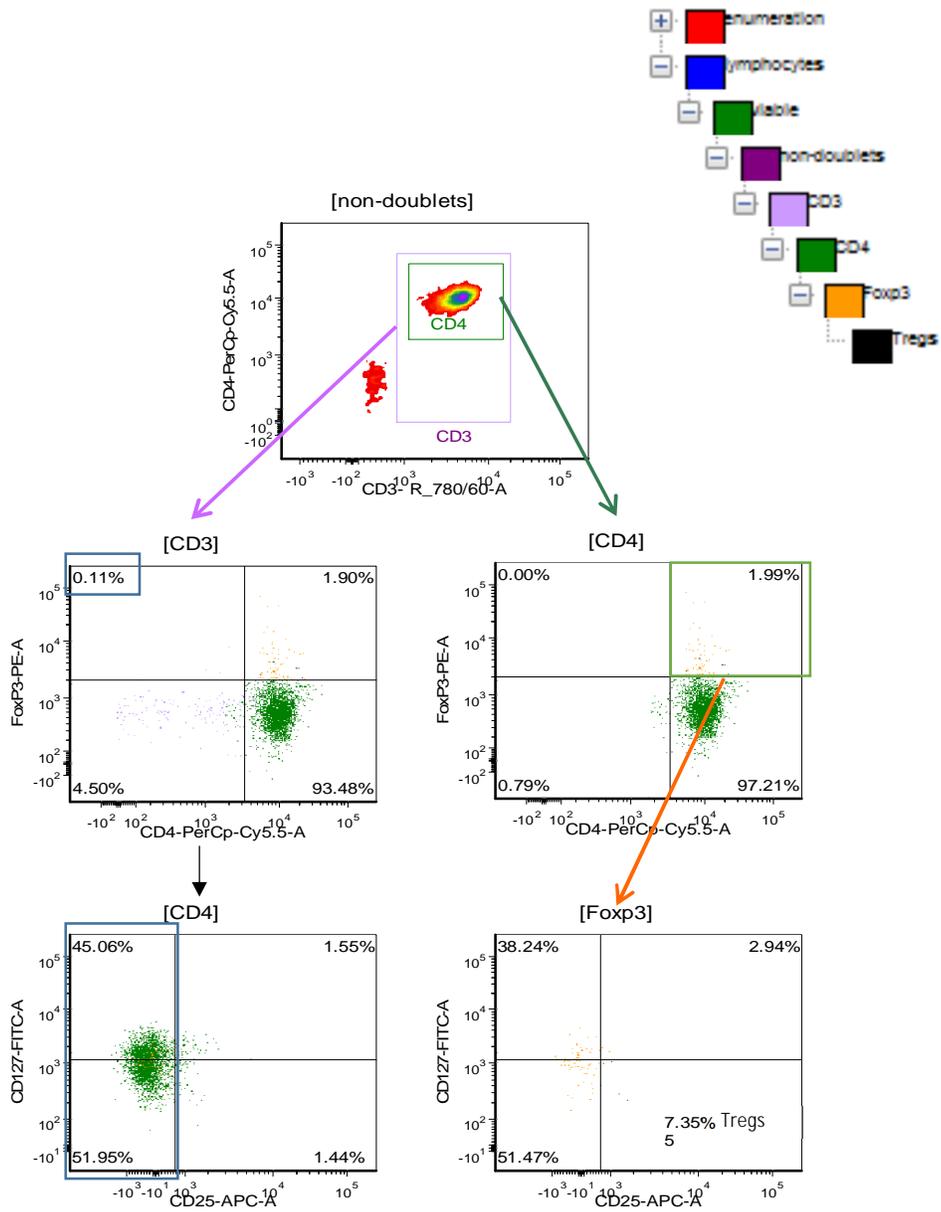


Figure 4.2.2 Inducible regulatory T cell gating strategy set on “T cells alone” culture.

Tregs were defined as $CD3^+CD4^+CD25^{high}CD127^{low}FoxP3^+$ T cells. The gating strategy is defined as follows: T cells were gated according to the forward scatter area (FSC-area) and side scatter (SSC-area). Dead cells were excluded by the V500 viability dye. Non-doublet cells were gated according to FSC-area and FSC-height (not shown). T cells were cell surface stained with a combination of anti-CD3, anti-CD4, anti-CD25, anti-CD127 and intracellular stained for anti-FoxP3. The $CD3^+$ T cell gate was defined and $CD4^+ FoxP3^+$ T cells were determined by adjusting the quadrant to $CD4^-$ T cells while $FoxP3^+$ was set based on the negative control, T cells alone. $CD127$ (50th percentile) and $CD25$ (99th percentile) quadrants were set on the T cells alone tube and left unchanged for the other co-culture conditions. The $CD127$ and $CD25$ plot was used to define the quadrants for the Treg colour dot plot. The gate was set on $FoxP3^+$ cells, indicated as [FoxP3] above the Treg plot and Treg percentage is indicated in the lower right quadrant of this plot. These plots are representative of Treg gating strategy set on the negative control, T cells alone culture.

4.2.3 FACS sorting optimization and gating strategy

Table 4.2.3.1 Treg sorting and CD154 suppression assay antibody panel

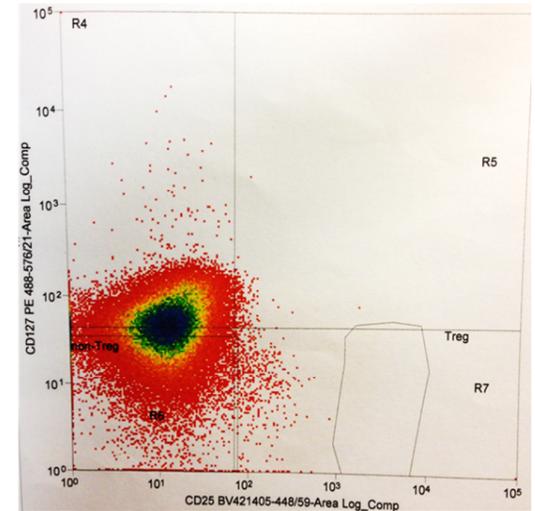
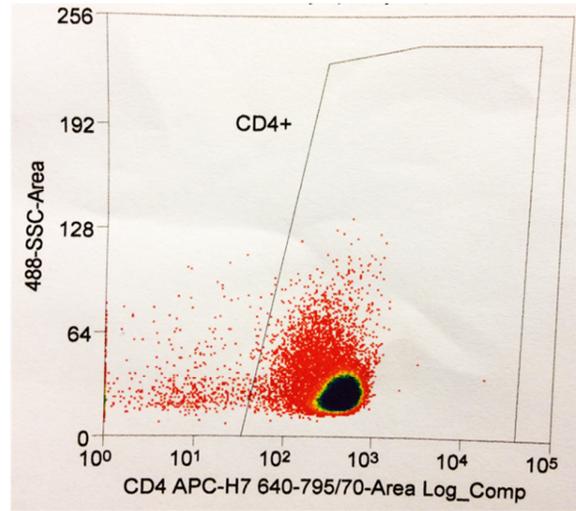
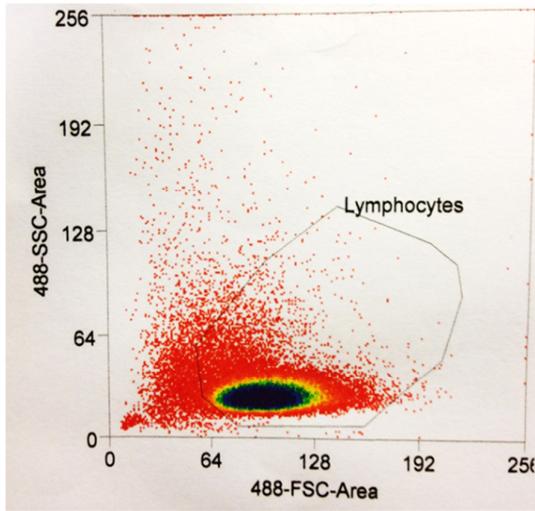
Antibody	Clone	Fluorochrome	Company
<i>Treg sorting:</i>			
CD4	OKT4	APC-eFluor	eBioscience
CD25	BC96	BV421	Biolegend
CD127	eBioRDR5	PE / FITC	eBioscience
<i>CD154 suppression assay:</i>			
CD154	89-76	APC	BD Biosciences

Table 4.2.3.2 MSC-derived iTreg sorting optimization

FACS sorting gating strategy	Sample	FoxP3 purity
Top 2% CD25 T cells CD127 fixed on 50 th percentile on T cells alone sample	MSC-17 iTregs	69.42%
Manual Gate: Not fixed on CD127 50 th percentile Gating based on distinct population of each sample tube	MSC-17 iTregs	31.83%
CD127 fixed on 50 th percentile on T cells alone sample Manual gating for CD25 population – adjustable gate of each sample tube (>10 ³ intensity of CD25)	MSC-17 iTregs	88.24%

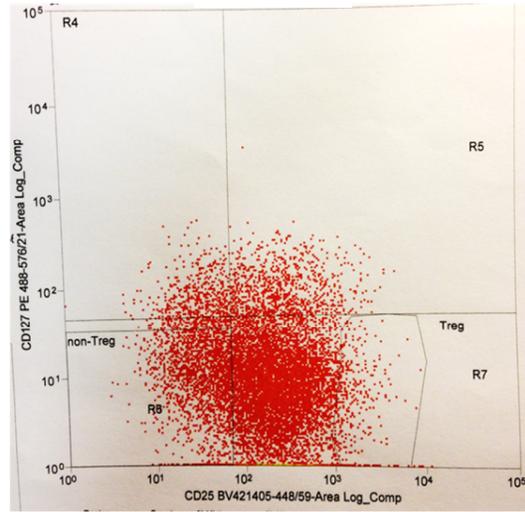
MSC-17 derived iTregs (MSC-17 iTregs) were isolated and stained using the Treg sorting antibody panel as described in section **2.13.1**. Three different iTreg gating strategies were analysed. The gating strategy showing highest FoxP3 purity (88.24%) was used in all subsequent MSC-derived iTreg sorting experiments.

A



B

UT-MSc



MSC-17

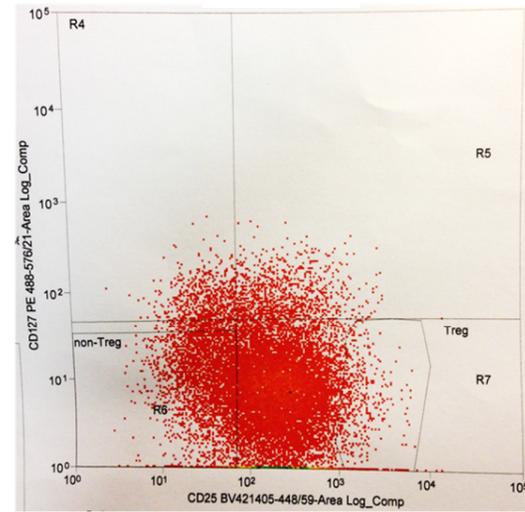


Figure 4.2.3 Flow sorting gating strategy (Treg sorting data).

CD4⁺CD25⁻ T cells that were co-cultured with or without MSC were cell surface stained with anti-CD4, anti-CD25 and anti-CD127 and sorted for CD4⁺CD25^{high}CD127^{low} iTregs. **(A)** MSC-derive iTreg gating strategy set on the negative control, T cells alone culture: T cells (lymphocytes) were gated according to the forward scatter area (FSC-area) and side scatter (SSC-area). Non-doublets cells were gated according to FSC-area and FSC-height (not shown). The CD4⁺ T cell gate was defined and the CD127 (50th percentile) quadrant was set on T cells alone (negative control) and left unchanged for the other co-culture conditions. CD25^{high} iTregs were defined based on manual gating of CD4⁺CD127^{low} T cells with fluorescence intensity >10³ of CD25. **(B)** Sorted iTregs from CD4⁺CD25⁻ T cells that were co-cultured with UT-MSC or MSC-17. These plots are representative of MSC-derived iTreg gating strategy (Hard copy and scanned Treg sorting data provided by the Detmold Sort Facility, SA Pathology).

4.2.4 CD154 suppression assay optimization

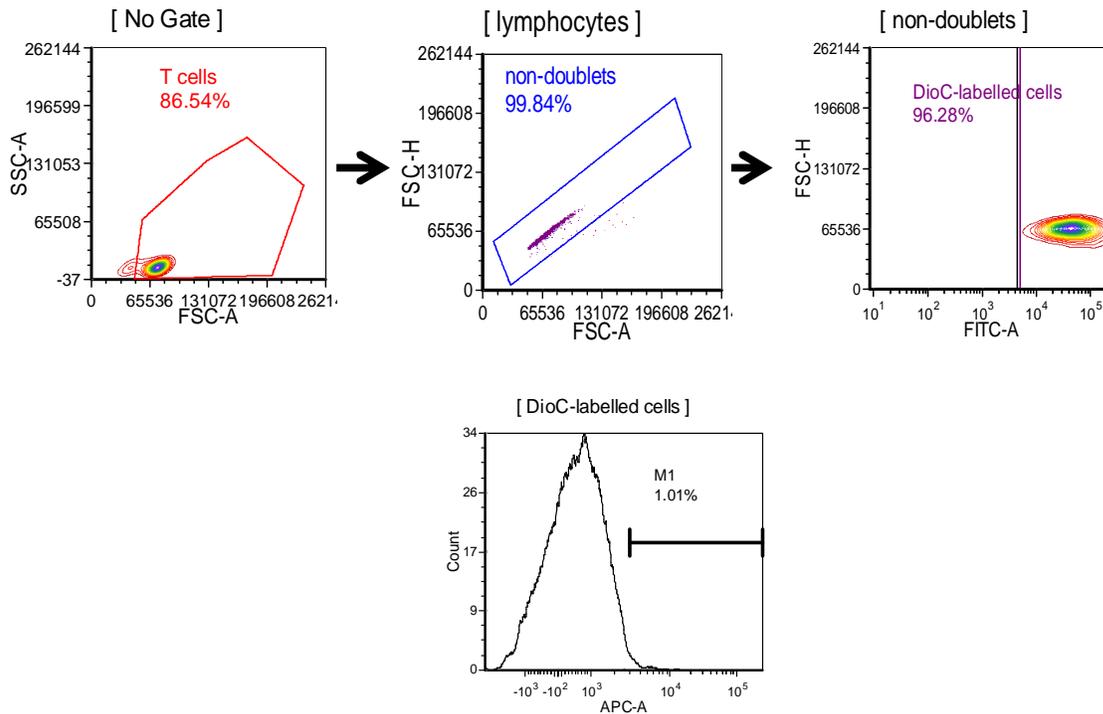


Figure 4.2.4 CD154 suppression assay gating strategy set on T cells alone culture.

Unstimulated DiOC₁₈(3)-labelled CD4⁺ CD25⁻ T effector cells (donor T cells) labelled with anti-CD154 were used as a negative control. The gating strategy to define CD154 expression on effector T cells is as follows: T cells were gated based on FSC-area and SSC-area. Non-doublet cells were gated according to FSC-area and FSC-height. DiOC-positive cells were defined as FITC positive cells. Percentage DiOC-labelled T effector cells expressing CD154 are shown in the histogram. These plots are representative of CD154 gating strategy set on the negative control, T cells alone culture.

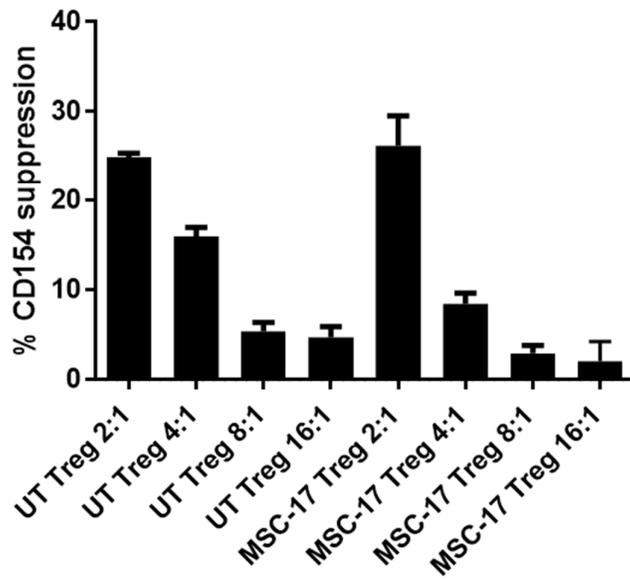
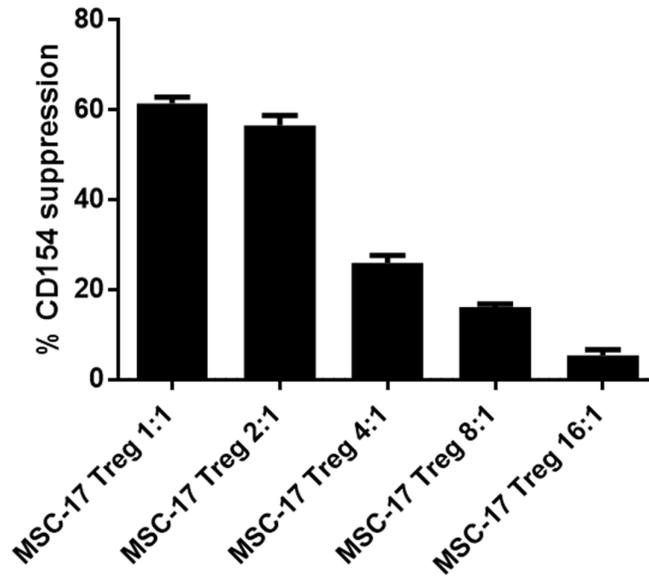


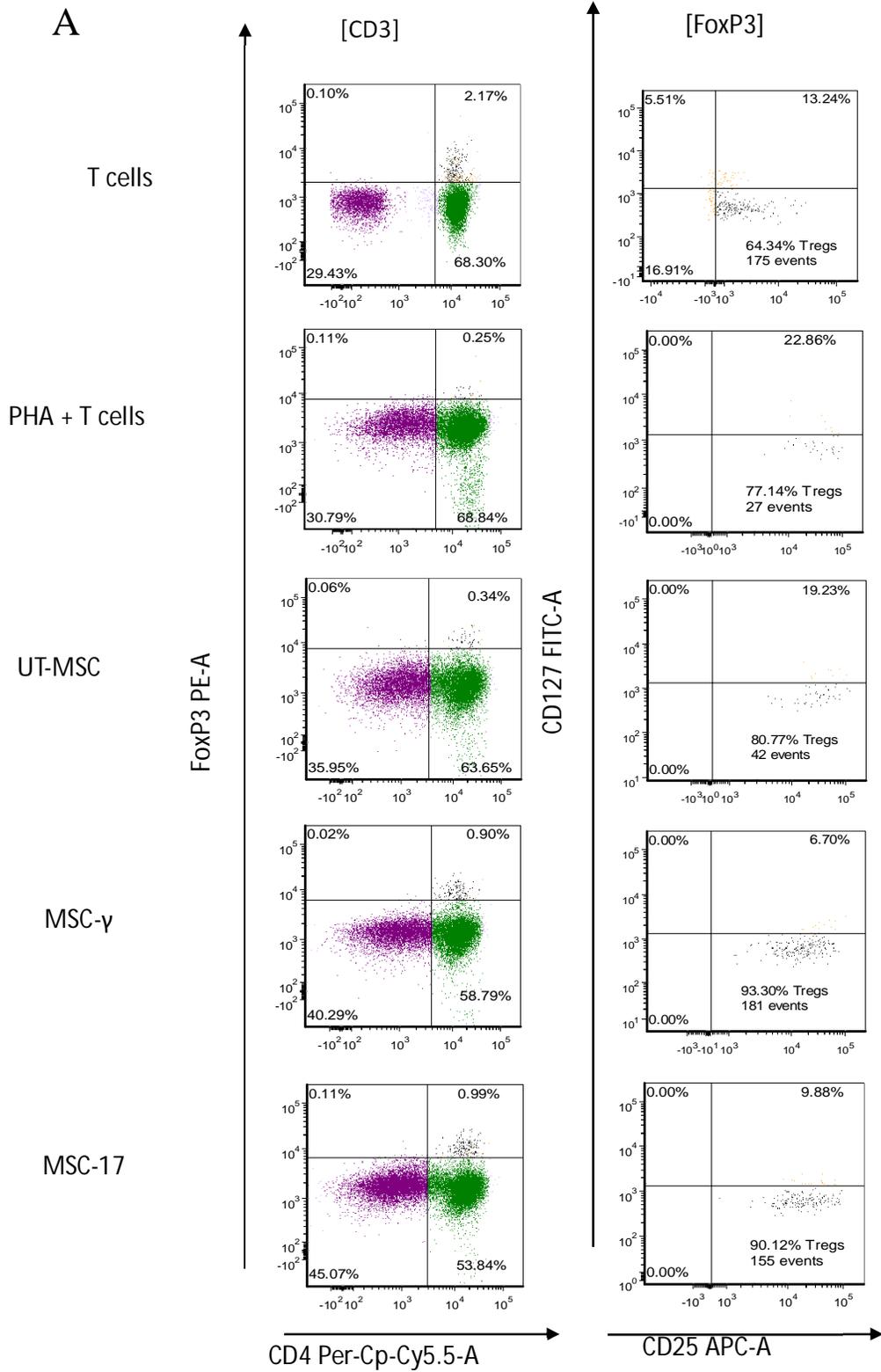
Figure 4.2.5 Dose-dependent suppression of CD154 by sorted MSC-derived iTregs.

CD154 suppression assay optimization on the functional ability of MSC-derived sorted iTregs to suppress effector T cells. UT-MSC and MSC-17 derived sorted iTregs were cultured with a DiOC₁₈(3)-labelled CD4⁺CD25⁻ effector T cells at ratios of 1:1 to 16:1 T effector: Tregs. Effector T cells were stimulated with anti-CD3/CD28 T cell expander beads to induce CD154 expression. Unstimulated T cells were used as negative controls. CD154 gating strategy is defined in **Figure 4.2.4**. UT-MSC and MSC-17 derived iTregs suppressed CD154 expression on donor reactive T effector cells in a dose-dependent manner. Percentage CD154 suppression calculated for T effector to Treg ratios 1:1 to 16:1 with error bars depicting means of triplicates \pm SD. Data are one representative of 3 independent experiments for MSC-17 iTregs and one data for UT-MSC iTregs. T effector: Treg ratio of 2:1 was used in subsequent experiments.

4.3 RESULTS

4.3.1 MSC-17 and MSC- γ engender CD4⁺CD25^{high}CD127^{low}FoxP3⁺ Treg

Previous studies have shown that MSC can indirectly modulate T cell responses through the generation of regulatory T cells^{224,249,284}. In the present study, we investigated the ability of UT-MSC, MSC- γ and MSC-17 to increase CD4⁺CD25^{high}CD127^{low}FoxP3⁺Treg numbers in co-cultures of PHA-activated CD3⁺ T cells. Both MSC- γ and MSC-17 equally increased Treg percentages and absolute numbers compared to UT-MSC ($p < 0.04$) (**Figure 4.3.1**). T cells co-cultured with MSC- γ and MSC-17 produce conditions conducive for Treg generation, with the production of TGF- β and PGE₂ (**Figure 4.3.2**). Collectively, these results indicate that MSC-17 and MSC- γ can engender Tregs.



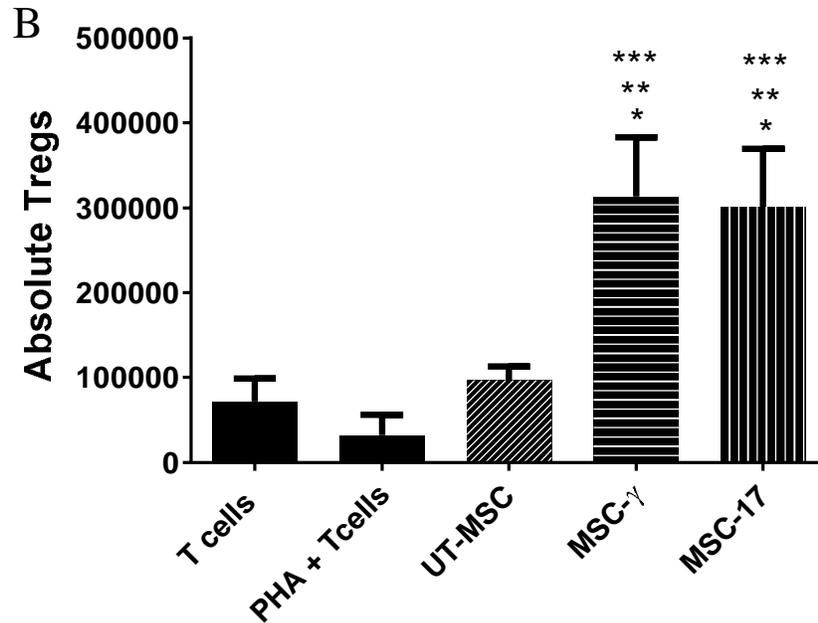


Figure 4.3.1 MSC-17 and MSC-γ engender regulatory T cells.

Absolute $CD3^+CD4^+CD25^{high}CD127^{low}FoxP3^+$ Treg numbers were determined by flow cytometry following co-culture of MSC with PHA activated $CD3^+$ T cells. (A) Dot plots show percentage of $FoxP3^+$ cells (upper right quadrant, left panel) and Tregs (lower left quadrant, right panel). (B) Graph depicts absolute number of Tregs quantified using enumeration beads. Data are (B) pooled or (A) one representative of 3 independent experiments. (B) * $p < 0.05$ vs. T cells alone, ** $p < 0.05$ vs. PHA + T cells and *** $p < 0.05$ vs. UT-MSC were determined by one-way ANOVA with post-Sidak multiple comparison test. Error bars depict (B) mean \pm SEM. Adapted from Figure 3B,C Sivanathan KN, et. al., (2015)².

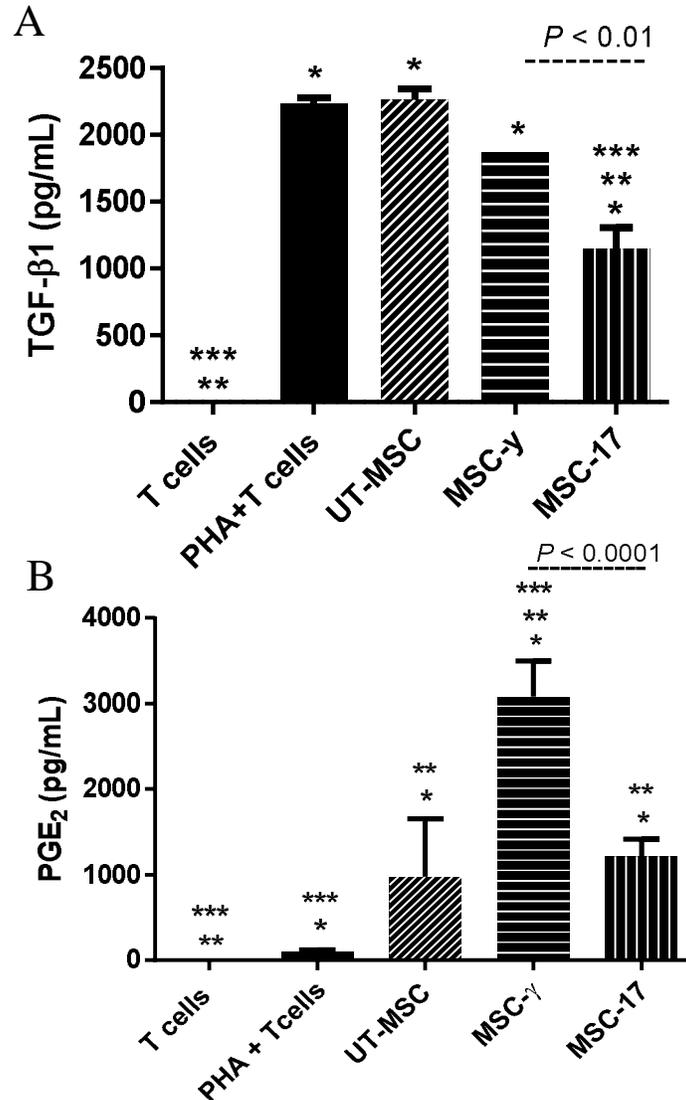


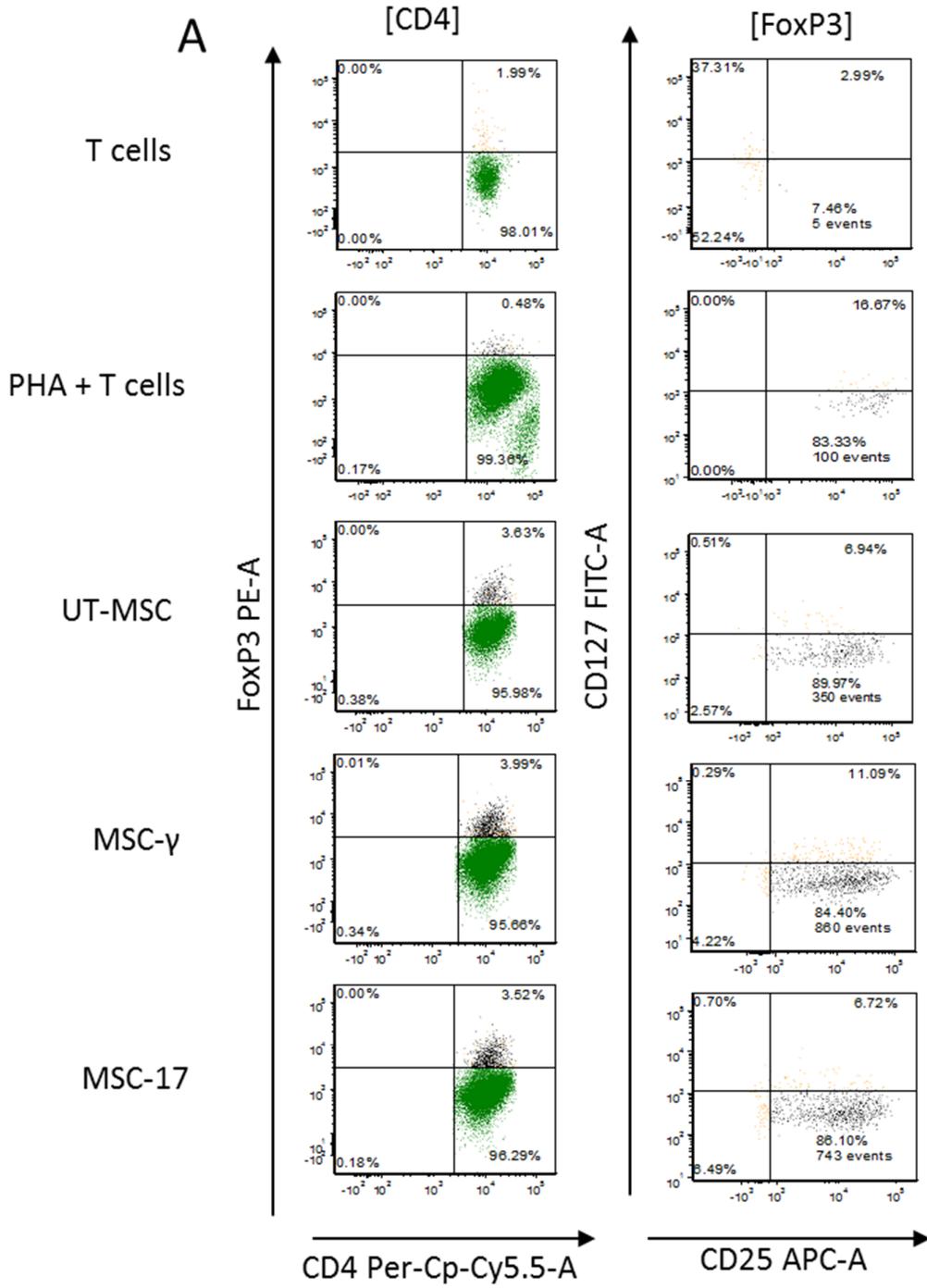
Figure 4.3.2 MSC-17 and MSC- γ engender Tregs in environments enriched of TGF- β 1 and PGE₂.

(A) TGF- β 1 and (B) PGE₂ concentrations in the cell culture supernatants were evaluated by ELISA in co-cultures of MSC with PHA activated CD3⁺ T cells. RPMI culture media alone showed no detectable levels of TGF- β 1 and PGE₂ (data not shown). Data are one representative of 3 independent experiments. * $p < 0.05$ vs. T cells alone, ** $p < 0.05$ vs. PHA + T cells and *** $p < 0.05$ vs. UT-MSC were determined by one-way ANOVA with post-Sidak multiple comparison test. Error bars depict means of duplicates \pm SD. Adapted from Figure 3D,E Sivanathan KN, et. al., (2015)².

4.3.2 Cell-contact dependent Treg induction by MSC-17 and MSC- γ

Because MSC can indirectly modulate T cell responses through the generation of Tregs^{224,249,283,284}, we investigated the ability of UT-MSC, MSC- γ and MSC-17 to induce CD4⁺CD25^{high}CD127^{low}FoxP3⁺Treg in co-cultures of PHA activated CD4⁺CD25⁻ T cells. Both MSC- γ and MSC-17 induced iTregs from PHA activated CD4⁺ CD25⁻ T cells (**Figure 4.3.3**), but only MSC-17 showed consistent iTreg induction (1.47- to 7.18-fold compared to UT-MSC) (**Table 4.3.2.1**). MSC- γ induction of iTreg ranged between 1.47 to 34.13-fold relative to UT-MSC but was less consistent (**Table 4.3.2.1**). Highest PGE₂ concentration was detected in MSC-17 and T cell co-cultures while the presence of MSC- γ showed similar levels to UT-MSC (**Figure 4.3.4**). Consistent with our data using bulk human CD3⁺ T cells (**Figure 4.3.2**), high concentrations of iTreg pro-survival factor TGF- β 1 was detected in co-cultures of T cells with MSC- γ and MSC-17 (**Figure 4.3.4**).

To further investigate the mechanisms by which modified MSC promote iTregs, we used a transwell system to determine contact-dependency. Physical separation of MSC from T cells significantly reduced iTreg numbers in co-cultures with either MSC- γ or MSC-17, in comparison to normal experimental MSC-T cell contact conditions (**Figure 4.3.5; Table 4.3.2.1**). Furthermore, the observed UT-MSC, MSC- γ and MSC-17 mediated downregulation of CD25 expression by CD4⁺ effector T cells was partially reversed in the transwell assay (**Figure 4.3.6**).



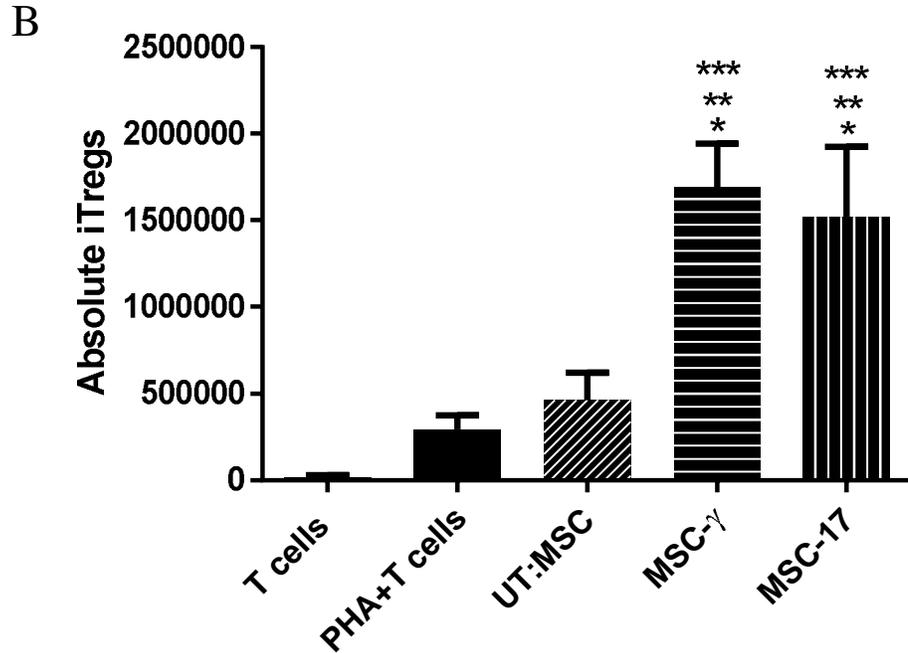


Figure 4.3.3 MSC-17 and MSC- γ induce the generation of Tregs from naïve T cells.

MSC were co-cultured with PHA activated CD4⁺CD25⁻ T cells and absolute CD3⁺CD4⁺CD25^{high}CD127^{low}FoxP3⁺ iTreg numbers were determined by flow cytometry. (A) Dot plots show percentage of FoxP3⁺ cells (upper right quadrant; left panel) and iTregs (lower right quadrant; right panel). (B) Absolute number of iTregs quantified using enumeration beads. Data are one representative of 5 independent experiments. *p < 0.05 vs. T cells alone, **p < 0.05 vs. PHA + T cells and ***p < 0.05 vs. UT-MSC were determined by one-way ANOVA with post-Sidak multiple comparison test. Error bars depict means of triplicates \pm SD.

Adapted from Figure 4A,B Sivanathan KN, et. al., (2015)².

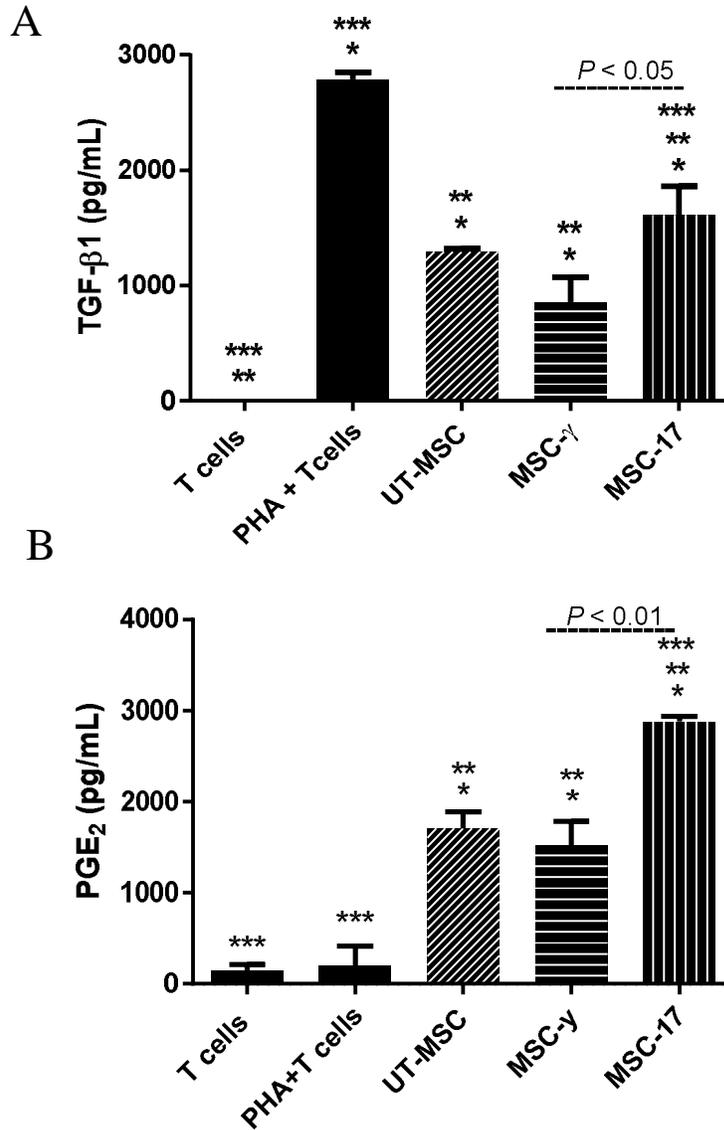


Figure 4.3.4 MSC-17 induce the generation of iTregs in environments enriched of TGF-β1 and PGE₂.

MSC were co-cultured with PHA activated CD4⁺CD25⁻ T cells. **(A)** TGF-β1 and **(B)** PGE₂ concentrations in cell culture supernatants were evaluated by ELISA. Data are one representative of 3 independent experiments. *p < 0.05 vs. T cells alone, **p < 0.05 vs. PHA + T cells and ***p < 0.05 vs. UT-MSC were determined by one-way ANOVA with post-Sidak multiple comparison test. Error bars depict means of duplicates ± SD. Adapted from Figure 4C,D Sivanathan KN, et. al., (2015)².

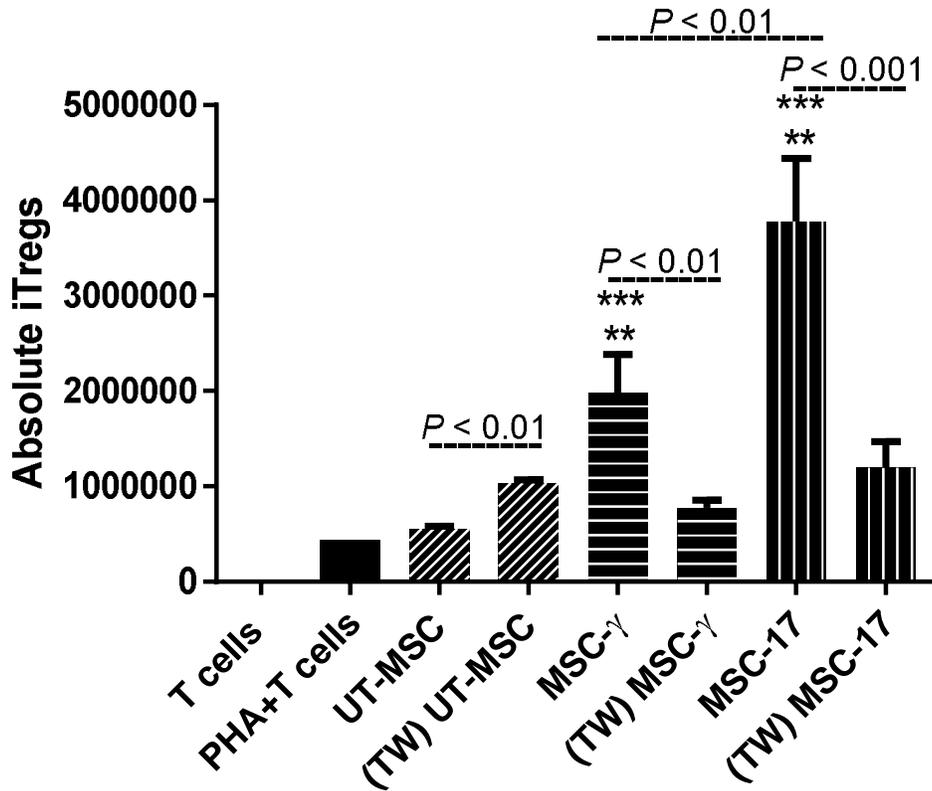


Figure 4.3.5 Cell-contact dependent induction of iTreg by MSC-17 and MSC- γ .

MSC were co-cultured with PHA activated CD4⁺CD25⁻ T cells and absolute number of iTregs in transwell assay were determined by flow cytometry. PHA-activated CD4⁺CD25⁻ T cells were co-cultured with MSC in a transwell system (TW). Numbers of iTreg in PHA activated T cells in absence of MSC with or without the transwell insert were similar (data not shown). Data are one representative of 3 independent experiments. ** $p < 0.05$ vs. PHA + T cells and *** $p < 0.05$ vs. UT-MSC were determined by one-way ANOVA with post-Sidak multiple comparison test. Error bars depict means of triplicates \pm SD. Adapted from Figure 4E Sivanathan KN, et. al., (2015)².

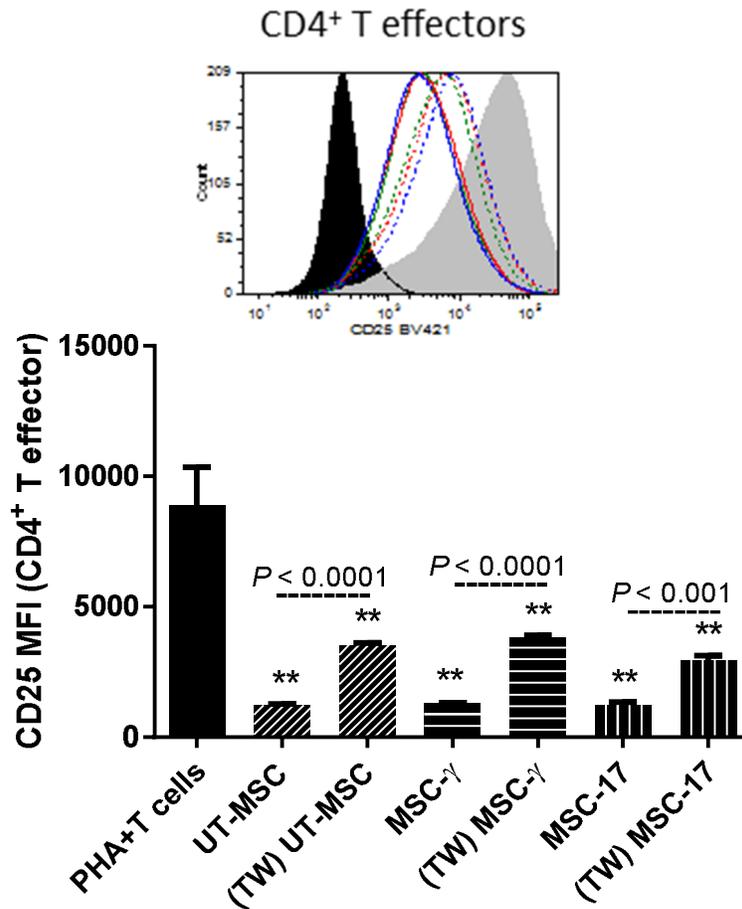


Figure 4.3.6 MSC downregulation of CD25 on CD4⁺ effector T cells was cell-contact dependent.

MSC were co-cultured with PHA activated CD4⁺CD25⁻ T cells and CD25 downregulation on CD4⁺ effector T cells was determined. Histograms show level of CD25 depicted by the proliferation assay culture conditions: T cell alone (negative control; solid black), PHA + T cells (positive control; solid grey), **UT-MSC** (green), **MSC- γ** (red), **MSC-17** (blue). Dotted lined histograms indicate co-cultures in a transwell system and lined histograms are under normal culture conditions. Graph depict CD25 MFI on CD4⁺ effector T cells. Data are one representative of 5 independent experiments. **p < 0.05 vs. PHA + T cells and ***p < 0.05 vs. UT-MSC were determined by one-way ANOVA with post-Sidak multiple comparison test. Error bars depict means of triplicates \pm SD. Adapted from Figure 4F Sivanathan KN, et al., (2015)².

Table 4.3.2.1 Individual experiment summary data on MSC induction of iTregs

MSC donor	iTreg fold-induction vs. PHA+TC			iTreg induction		
	UT-MSC	MSC- γ	MSC-17	MSC- γ > UT-MSC (fold-induction vs. UT-MSC)	MSC-17 > UT-MSC (fold-induction vs. UT-MSC)	MSC- γ vs. MSC-17
MSC 1	ns	3.72	4.81	Yes (4.40)	Yes (5.66)	MSC-17 > MSC- γ
MSC 2	30.96	27.45	96.31	No	Yes (3.11)	MSC-17 > MSC- γ
MSC 3	13.00	443.51	93.27	Yes (34.13)	Yes (7.18)	MSC- γ > MSC-17
MSC 4	12.51	12.71	30.64	No	Yes (2.45)	MSC-17 > MSC- γ
MSC 4	ns	5.75	5.17	Yes (3.69)	Yes (3.31)	ns
MSC 2	ns	4.46	8.53			
(transwell) MSC 2	(not cell contact)	(contact)	(contact)	Yes (3.54)	Yes (6.77)	MSC-17 > MSC- γ
MSC 2	7.90	20.67	17.41			
(transwell) MSC 3	(contact)	(contact)	(contact)	Yes (2.62)	Yes (2.20)	MSC- γ > MSC-17
MSC 3	2.48	2.22	4.57			
(transwell) MSC 3	(not cell contact)	(contact)	(not cell contact)	No	Yes (1.84)	MSC-17 > MSC- γ
MSC 3	1.50	1.90	2.20			
(transwell) MSC 3	(not cell contact)	(contact)	(contact)	No	Yes (1.47)	ns
MSC 3	ns	1.31	1.90			
(transwell) MSC 3		(contact)	(contact)	Yes (1.47)	Yes (2.13)	MSC-17 > MSC- γ

UT-MSC, MSC- γ or MSC-17 induction of iTreg from CD4⁺CD25⁻ T cells relative to PHA⁺ T cells alone.

(Transwell) indicate experiments conducted under transwell conditions; (no cell contact), iTreg induction is not cell contact dependent, (cell-contact), iTreg induction is cell contact dependent; ns, not significant. *Adapted from supplemental online Table S2 Sivanathan KN, et. al., (2015)².*

4.3.3 MSC induce highly heterogeneous iTregs progeny

MSC-induced iTregs were heterogeneous with respect to iTreg markers with no significant differences between all MSC groups other than the absolute iTreg numbers (**Figure 4.3.7**). Approximately 20-25% of MSC derived iTregs were CD39⁺ or CD73⁺, 85-95% CD69⁺, 70-75% OX40⁺, 10-20% cytotoxic T-lymphocyte associated antigen-4 (CTLA-4⁺) and 25-35% glucocorticoid-induced TNFR-related protein (GITR⁺). Only <1% MSC-induced iTregs were CD103⁺ or glycoprotein A repetitions predominant (GARP⁺) and <5% were latency-associated peptide (LAP⁺). Of note, the proportion of iTreg expressing CD73, CD69 and GITR were significantly higher in all MSC groups compared to PHA activated T cells alone and only MSC- γ derived iTregs expressed human leukocyte antigen-DR (HLA-DR) (**Figure 4.3.7**).

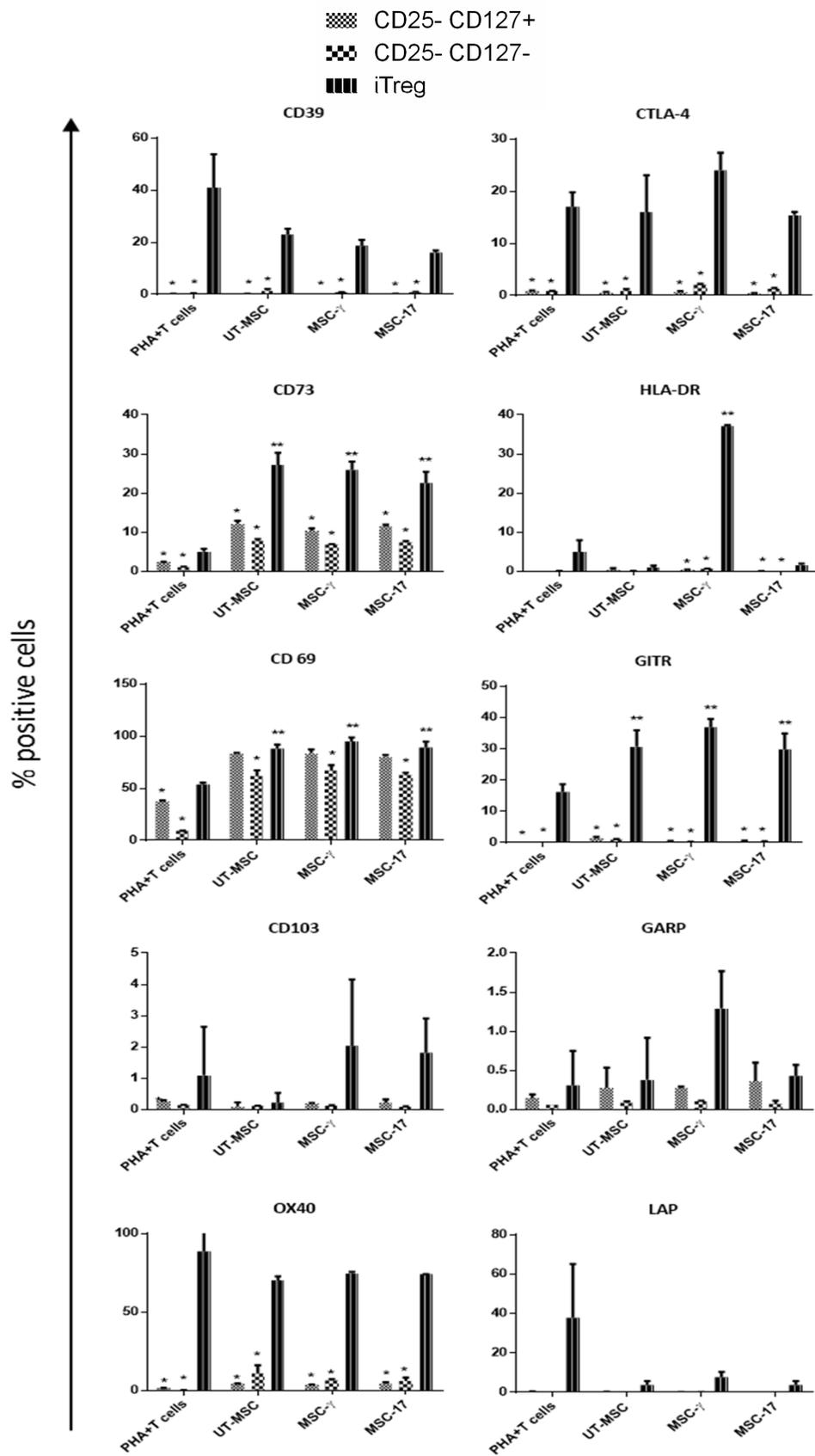


Figure 4.3.7 MSC induce a heterogeneous iTreg progeny.

MSC were co-cultured with PHA activated CD4⁺CD25⁻ T cells. (A) Percentages of CD4⁺CD25^{high}CD127^{low}FoxP3⁺ iTreg expressing CD39, CD73, CD69, CD103, OX40, CTLA-4, HLA-DR, GITR, GARP and LAP were determined by flow cytometry. T cell alone (negative control) showed no iTreg induction (data not shown). *p< 0.05 vs. iTreg, **p< 0.05 between. MSC and PHA + T cells iTreg groups were determined by one-way ANOVA with post-Sidak multiple comparison test. Error bars depict means of duplicates ± SD. *Adapted from Figure 5A Sivanathan KN, et. al., (2015)².*

4.3.4 FoxP3 purity and immunophenotype of flow sorted MSC-derived iTregs

The FoxP3 purity of freshly sorted UT-MSC, MSC- γ and MSC-17 derived iTregs was >90%, conforming that the flow sorted CD4⁺CD25^{high}CD127^{low} cells were iTregs (**Figure 4.3.8**). To further characterise the immunophenotype of the flow sorted MSC-derived iTregs, we evaluated the expression of activation and suppressive associated iTreg cell surface markers. Similar to our previous observation (**Figure 4.3.7**), these flow sorted MSC-induced iTregs were heterogeneous with respect to iTreg markers with no significant differences between all MSC groups. Approximately 53-65% of flow sorted MSC-derived iTregs were CD39⁺ or CD73⁺, >90% CD45RA⁺ or CD62L⁺ and 36-50% HLA-DR⁺ (**Figure 4.3.9**). There was also no difference in the expression levels of the cell surface markers on the sorted iTreg progeny (**Figure 4.3.9**).

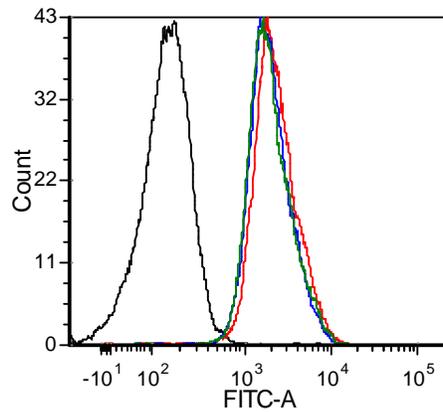


Figure 4.3.8 FoxP3 purity of flow sorted MSC-derived iTregs.

CD4⁺CD25⁻ T cells co-cultured with either UT-MS, MSC- γ or MSC-17 were cell surface stained with anti-CD4, anti-CD25 and anti-CD127 and sorted for CD4⁺CD25^{high}CD127^{low} iTregs. Freshly sorted iTregs were stained intracellularly with anti-FoxP3 and purity of FoxP3 in sorted iTreg cells was shown in the representative histograms: FMO control (black), **UT-MS** (green), **MSC- γ** (red), **MSC-17** (blue). Data are one representative of 3 independent experiments. (*unpublished*).

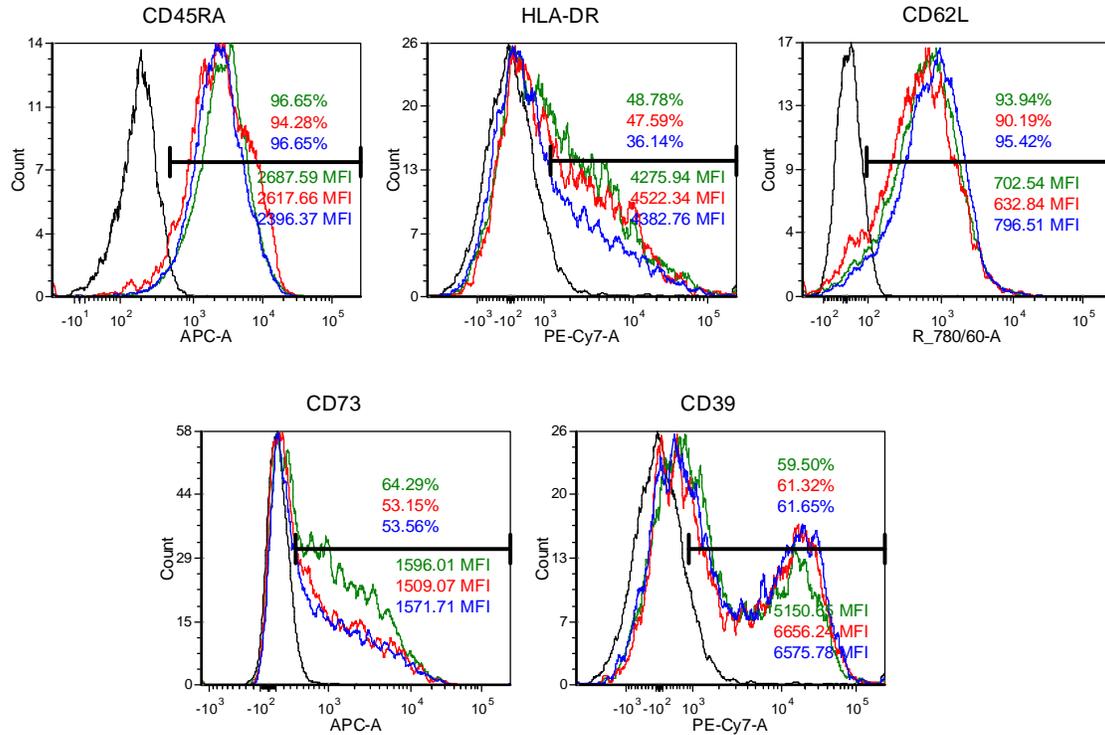


Figure 4.3.9 Immunophenotype of flow sorted MSC-derived iTregs.

CD4⁺CD25⁻ T cells co-cultured with either UT-MSC, MSC-γ or MSC-17 were cell surface stained with anti-CD4, anti-CD25 and anti-CD127 and sorted for CD4⁺CD25^{high}CD127^{low} iTregs. Sorted iTregs were cell surface stained with CD45RA, HLA-DR, CD62L, CD73 and CD39. The percentages and MFI of sorted iTregs expressing these markers were shown in representative histograms: FMO control (black), **UT-MSC** (green), **MSC-γ** (red), **MSC-17** (blue). Freshly sorted iTregs had > 90% FoxP3 purity (as shown in **Figure 4.3.8**). Data are one representative of 3 independent experiments. (*unpublished*).

4.3.5 Flow sorted MSC-derived iTregs are functionally suppressive

CD154 is transiently expressed on activated CD4⁺ T cells. The inhibition of activation induced CD154 of CD4⁺CD25⁻ T cells is a robust measure of Treg suppressive function^{354,369}. We therefore assessed the functional capacity of UT-MSC and MSC-17 derived iTreg to suppress CD154 expression on donor effector CD4⁺CD25⁻ T cells. Sorted human and 48hr rested iTregs used in the CD154 suppression assay were >80% FoxP3⁺ (**Figure 4.3.10 A**). Anti-CD3/CD28 activated T cells showed 57.65% CD154 positivity (**Figure 4.3.10 B**). Both UT-MSC and MSC-17 generated iTregs equivalently suppressed CD154 expression on effector T cells by 60% (**Figure 4.3.10 B, C**). The suppression of CD154 ranged from 15% to 60% depending on the MSC and T cell donor. This data suggest that MSC-17 induce high numbers of functionally suppressive iTregs to partially mediate T cell immunomodulation.

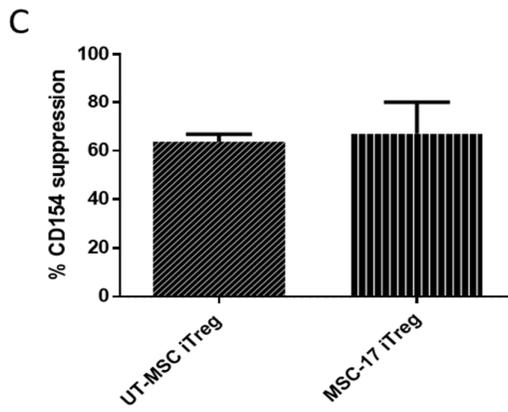
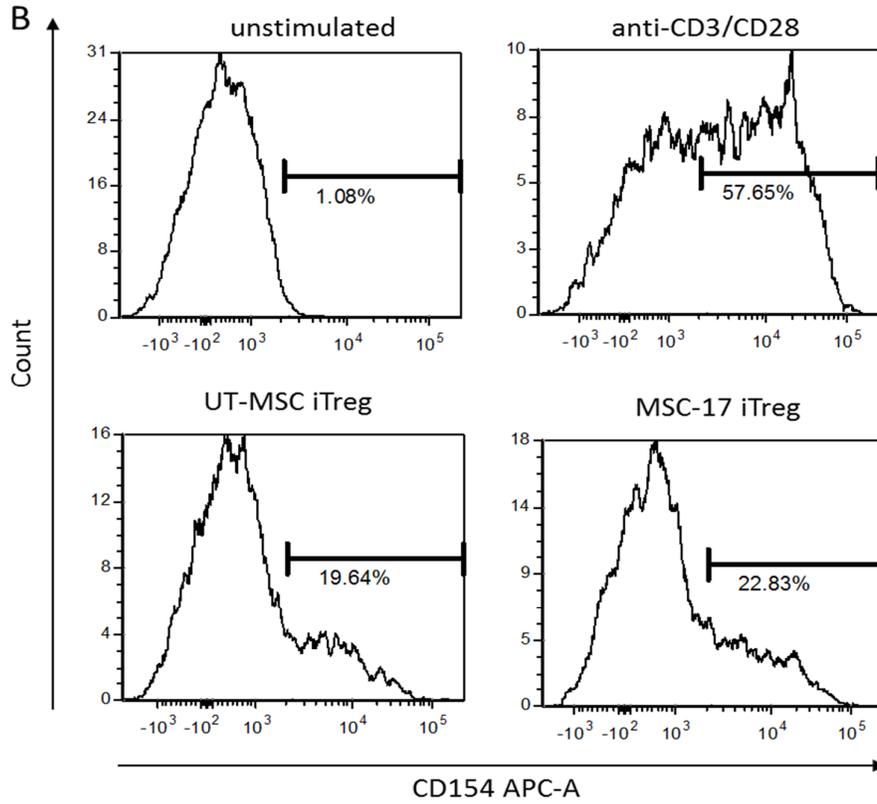
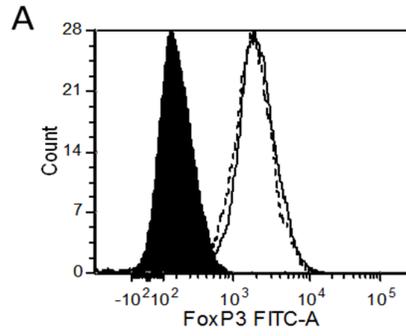


Figure 4.3.10 MSC induce a functionally suppressive iTreg progeny.

CD4⁺CD25⁻ T cells co-cultured with either UT-MSC or MSC-17 were cell surface stained with anti-CD4, anti-CD25 and anti-CD127 and sorted for CD4⁺CD25^{high}CD127^{low} iTregs. **(A)** 48hr rested iTregs were stained intracellularly with anti-FoxP3 and purity of FoxP3 in sorted iTreg cells was shown in the representative histograms: FMO controls (solid black), UT-MSC induced iTreg (black) and MSC-17 induced iTreg (black dotted line). **(B,C)** Function of MSC-derived sorted iTregs was determined in a CD154 suppression assay. CD154 gating strategy is defined in **Figure 4.2.4**. **(B)** T effector cells upregulate CD154 upon stimulation with anti-CD3/CD28 beads. UT-MSC and MSC-17 derived iTreg suppress CD154 expression on donor reactive T effector cells at T effector: Treg ratio of 2:1. **(B)** Representative histograms showing percentage of CD154 expression. **(C)** Percentage CD154 suppression calculated for T effector to Treg ratios 2:1 with error bars depicting means of triplicates \pm SD. Data are one representative of 3 independent experiments. *Adapted from Figure 5B,C,D Sivanathan KN, et. al., (2015)².*

4.4 DISCUSSION

Following our findings from **CHAPTER 3**, we also report that MSC-17 increase Tregs from activated CD3⁺ T cells, similar to MSC- γ . Tregs can be generated in environments enriched for TGF- β 1 and PGE₂^{107,283,370-372}. MSC-17 immunomodulation correlated with increased gene expression of IL-6 but not IDO1, Cox-1, TGF- β 1 in MSC-17. IL-6 gene levels in MSC-17 however were similar to MSC- γ thereby suggesting that other novel mechanisms are involved in MSC-17 immunosuppression. Indeed, high concentrations of TGF- β 1 and PGE₂ in MSC-17 co-cultures with CD3⁺ T cells may have prompted Treg expansion or induction.

Consistent with an increase in Tregs from CD3⁺ T cells, we observed a decrease of IL-2 in both MSC-17 and MSC- γ co-cultures. Tregs do not produce IL-2 but express higher levels of CD25 (IL-2R) compared to effector cells for their survival^{84,373,374}. Our data provides evidence that MSC-17 and MSC- γ may indirectly inhibit T cell responses via Treg mediated IL-2 deprivation.

In iTreg induction experiments, MSC-17 more consistently induced iTregs from CD4⁺CD25⁻ T cells compared to MSC- γ . PGE₂ levels were highest in MSC-17 and CD4⁺CD25⁻ T cell cultures and iTreg induction could be PGE₂ dependent^{283,284,370}. Nevertheless, the transwell assays show that MSC-17 function in a cell-contact dependent manner to induce iTreg similar to MSC- γ mediated iTreg induction. Although we did not evaluate PGE₂ concentrations in the transwell assay supernatant, it is known from a previous study that PGE₂ production in MSC induction of Tregs from CD4⁺T cells is initiated through cell-contact and followed by PGE₂ and TGF- β expression²⁸³.

The activation, function and homeostasis of Tregs can be attributed by the expression of cell-surface markers such as CD39, CD73, CD69, CD103, OX40, CTLA-4, HLA-DR, GITR, GARP and LAP^{109,111,149,151,157,168,375-377}. A heterogeneous population of iTregs with an activated and functional immunophenotype were induced in the presence of all MSC groups.

Interestingly, iTregs numbers that were CD73⁺, CD69⁺ and GITR⁺ were significantly higher in all MSC groups suggesting that these iTregs have enhanced activation and functional properties compared to iTregs induced in the absence of MSC. Although there were no significant differences in the percentages of iTreg populations expressing the Treg markers between UT-MSC and modified MSC, MSC-17 consistently showed higher numbers of iTreg.

Given that MSC-17 represented a more reliable source for iTreg induction, we then isolated MSC-17 derived iTregs and showed that these cells were functionally immunosuppressive. The extent of MSC-17 induced iTreg suppressive function was not different to UT-MSC derived iTreg and varied between experiments. This data clearly suggests potential differences in the mechanisms by which MSC mediate suppression of T cells through induction of iTregs in different donors. Indirect T cell immunoregulation by high numbers of MSC-17 derived iTregs, therefore, represents one mechanism amongst others involved in the enhancement of MSC-17 mediated suppression.

MSC-17 markedly suppress T cell proliferation and activation over co-culture with unmodified MSC. In these MSC-17 co-cultures, we observed induction of high numbers of iTreg suggesting a potential direct role of iTregs in the suppression of conventional T cells. However, there is the possibility that MSC also directly dampen effector T cell activation. MSC can directly modulate T cell proliferation and activation through cell-contact (EphB / Ephrin B, CD200 / CD200R, TLR, B7-H1) or soluble factor (TGF- β , PGE₂, MMP, IL-10, IL-6, IDO, HO-1) mediated mechanisms⁴. Some studies also suggest that MSC indirectly modulate T cells via the induction of Tregs. MSC can favour the generation of Tregs *in vitro* from activated T cells^{238,283,378}. Our study provides an extension on the functional role of MSC-17 derived iTreg isolated by FACS sorting from co-cultures of MSC with PHA activated T cells. Beyond the direct T cell immunosuppressive role of MSC expressed IL-10, TGF- β , PGE₂, HO-1, these factors can also directly promote the generation of Tregs in co-cultures of MSC and T

cells^{239,249,283-285,379}. The protective effect of MSC on cancer cell immune invasion is mediated by MSC-secreted TGF- β dependent iTreg increase. iTreg induced by MSC then suppresses PBMC proliferation and effector function thereby reducing lysis of cancer cells²⁸⁵. Treg depletion using anti-CD25 or TGF- β neutralizing antibodies however only partially reversed PBMC proliferation and effector function suggesting that other regulatory mechanisms are involved in MSC immunosuppression²⁸⁵. Similarly, other studies have shown that the blockade of Treg induction results in partial restoration of T cell proliferation^{239,284}. Together, these studies suggest that the expression of MSC immunoregulatory factors as well as the induction of Tregs are important in the potent immunosuppressive function of MSC. Therefore, an interesting possibility is that IL-17 treatment enhanced MSC-dependent suppression of T cell activation by the recruitment of Tregs, which together with other direct mechanisms resulted in superior function. Future studies to elucidate the mechanism by which MSC promote iTreg induction are warranted. This would enable specific blockade of iTreg generation by MSC to determine if MSC-17 act directly or indirectly via Tregs to promote enhancement of T cell immunosuppression.

In summary, MSC-17 can indirectly suppress T cell proliferation through the induction of iTregs. MSC-17 in direct contact with T cells and in environments enriched of TGF- β 1 and PGE₂ favours the induction of functionally suppressive iTregs. This study also highlights that MSC-17 may be optimal accessory cells to generate high yields of phenotypically and functionally active iTregs. MSC-17 are superior modulators of T cells however mechanisms exclusive to MSC-17 mediated immunomodulation warrants further investigation. MSC-17 are similar to UT-MSC in terms of MSC morphology and immunophenotype, yet these cells are highly immunoregulatory. In addition, the enhanced proliferative feature of MSC-17 is beneficial as high yields of cells can be generated for *in vivo* MSC-17 infusion. More importantly, unlike MSC- γ , MSC-17 show no upregulation or induction of MHC molecules

suggesting that they are less likely to be immunogenic *in vivo* and can function as superior modulators of immunological function.

CHAPTER 5:

GENE MICROARRAY COMPARATIVE ANALYSIS OF IFN- γ AND IL-17A PRECONDITIONED MSC

5.1 INTRODUCTION

It was previously established in **CHAPTER 3** and **CHAPTER 4** that human bone marrow derived mesenchymal stem cells (MSC) pre-treated with IL-17A represent a novel immunomodulatory strategy and an alternative to IFN- γ treatment of MSC in enhancing MSC immunosuppression on T cells². The present study showed that human MSC-17 potently suppressed PHA-induced human T cell proliferation and activation (reduced surface CD25 expression, and decreased IFN- γ , TNF- α and IL-2 production). In co-cultures of MSC with purified human CD4⁺CD25⁻ T cells, MSC-17 consistently induced high numbers of functionally suppressive iTregs². Whilst MSC-17 appeared to be superior modulators of T cells, mechanisms exclusive to MSC-17 mediated immunomodulation warranted further investigation. This chapter, aimed to identify key candidate molecules and molecular mechanisms by which MSC-17 mediate their superior modulation of immunological functions. The effects of IFN- γ and IL-17A on the gene expression profile and biological functions of human MSC was investigated using microarray and bioinformatics analysis.

Multiplex gene microarray chips, are a powerful tool used to identify changes in gene expression profiles between different environmental conditions and to determine a large variety of biological mechanisms that can be altered in a cell-type under these conditions. DNA hybridization microarrays are fabricated on silicon, plastic substrates or on glass, and comprise of many thousands of gene test sites. DNA probes that include amplicons, synthetic oligonucleotides or larger DNA/RNA fragments are selectively spotted to each test site. These

DNA probes are attached to the supporting material by covalent or noncovalent bonds. Based on the type of array, probes can be the target DNA or RNA sequences that hybridise with other reporter probes³⁸⁰. The concept of microarray array was first introduced by Tse Wen Chang (1983) in antibody microarrays (also known as antibody matrix)³⁸¹. Following this discovery, Ron Davis and Pat Brown (1995) designed a quantitative monitoring of gene expression patterns with complementary DNA microarrays³⁸². This high-throughput technology was designed to monitor expression of a large numbers of genes in parallel using very small sample hybridization volumes.

Affymetrix Inc. is one company that manufactures high density arrays by photolithographic process for gene expression and genotyping³⁸⁰. The Affymetrix Human GeneChip 2.0 ST Array is a high-throughput microarray gene chip that enables gene-level view of coding and non-coding transcripts. This gene chip can provide whole-transcriptome gene expression analysis at both the gene and exon level, as well as to study transcript variants and alternative splicing events. The mRNA molecule of interest is represented by a probe set (11-20 probe pairs) with each probe pair comprising of a **(1)** perfect match (pm) – section of the mRNA molecule of interest and **(2)** mismatch (mm) – change in the 13th base pair with the intention of measuring non-specific binding events. The GeneChip Human Gene 2.0 ST Array Design is summarised in **Table 5.2.2.1**. This array has >48 000 gene-level probe sets that will permit the study of changes in the gene expression profile of human MSC when pre-treated with either IL-17A or IFN- γ , in this thesis.

Interleukin-17A (IL-17A) is a member of the family of IL-17 cytokines (IL-17A, IL-17B, IL-17C, IL-17D, IL-17E and IL-17F) secreted predominantly by the T helper 17 (Th17) subset of CD4⁺ T cells. IL-17A is also expressed by other T cell subsets (cytotoxic CD8⁺ T cells, NKT, $\gamma\delta$ T cells), innate lymphoid populations (neutrophils, NKT, activated monocytes) and nonlymphoid cells (Paneth cells)³⁸³. IL-17A, the most investigated cytokine in the IL-17

family, is a potent proinflammatory mediator and is involved in the pathogenesis of autoimmune diseases (eg. rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease, psoriasis, T1D), allergic responses (asthma, atopic dermatitis), and in other immune cell mediated diseases including allograft rejection, sepsis and GvHD^{383,384}. Apart from the pathogenic roles of IL-17A, this cytokine is important for host defence response against fungal and bacterial infections^{3,383}.

Human IL-17A is a glycoprotein that consists of 155 amino acid residues with a molecular weight of 155kDa. Murine IL-17A has a 63% amino acid homology with human IL-17A. IL-17A can either be secreted as a disulfide-linked homodimer of 33kDa or as a heterodimer (ie. IL-17A complexed with IL-17F, IL-17A/F)³⁸³. The IL-17 receptor family are comprised of five homologous type I transmembrane protein receptors, IL-17RA, IL-17RB, IL-17RC, IL-17RD and IL-17RE³⁸³. The IL-17A homodimer signals through the IL-17RA and IL-17RC dimeric receptor complex, where binding of IL-17A homodimer to the IL-17RA/RC complex recruits the key cytosolic adaptor molecule Act1, that is known to be the master mediator of downstream IL-17 signaling^{383,385}. Act1 binds to the IL-17RA/RC complex via its SEFIR (SEF/ IL-17R) domains and this complex then recruits TRAF6 (TNF receptor-associated factor 6), leading to the activation of several downstream signaling pathways including the MAPKs-AP-1 (mitogen-activated protein kinases, MAPKs; activator protein 1, AP-1), C/EBPs (CCAAT/enhancer-binding proteins) and NFκB (nuclear factor kappa B). Activation of these signaling cascades induce the gene expression of antimicrobial peptides, chemokines, MMPs and proinflammatory cytokines as shown in other cell types such as endothelial cells, epithelial cells and fibroblasts^{3,383}. Act1 also mediates mRNA stabilization of genes via TRAF5/TRAF2 dependent but TRAF6 independent pathways. IL-17 receptor activation recruits SF2 (splicing factor) to the Act1/TRAF5/TRAF2 complex, after which the

mRNA is released and stabilized. The IL-17A signaling pathway is summarized in **Figure 5.1.1**.

IL-17A has emerged to be a growth factor for MSC by activating the Akt-Erk-MEK-p38 transduction molecules involved in MAPK signaling cascades³⁴⁰⁻³⁴². Previous studies have also shown that IL-17A can affect differentiation potential of MSC. Long-term culture of human MSC with IL-17A inhibits adipocyte differentiation of MSC^{386,387}. In human bone-marrow derived MSC, this process is partially mediated by Cox-2 upregulation and subsequent production of the anti-adipogenic factor, PGE₂³⁸⁶. Chondrogenic induction of human MSC is also inhibited in the presence of IL-17A³⁸⁸. On the contrary, IL-17A has been shown to stimulate osteoblastic differentiation of MSC, thereby suggesting a potential role for IL-17A in bone remodelling and homeostasis^{341,389}.

In addition, IL-17A has been shown to increase MSC motility and chemotaxis. Huang and colleagues demonstrated that IL-17A increased human MSC migration in an *in vitro* wound healing assay³⁴¹. Similarly, IL-17A has been shown to enhance peripheral blood-derived MSC migration in a wound healing assay by inducing the expression of urokinase type plasminogen activator (uPA) through the activation of ERK1,2-MAPK signaling pathway. Increased uPA has been reported to facilitate MSC transendothelial migration, potentially contributing to MSC motility to sites of inflammation for tissue regeneration or immunosuppression³⁹⁰. However, the precise mechanisms and chemotactic factors involved in driving human bone-marrow MSC migration in response to IL-17A is as yet unknown. Published work from our laboratory, presented in this thesis demonstrated for the first time that IL-17A enhances the immunomodulatory capacity of human MSC². This chapter investigated the gene expression profile of human bone marrow-derived MSC when exposed to IL-17A for 5 days using a microarray approach. Furthermore, the data sets were used to identify biological processes that are enriched in the IL-17A treated MSC (MSC-17) using bioinformatics analysis.

Interferons are cytokines with anti-proliferative, anti-viral and immunomodulatory properties³⁹¹. Interferon-gamma (IFN- γ), originally known as a macrophage-activating factor, is a type II interferon that is produced predominantly by CD8⁺ T cells and NK cells and at lower levels by CD4⁺ T cells³⁹². IFN- γ is also expressed by other cell types including NKT, B cells and professional APC (monocytes, dendritic cells, macrophages)³⁹². IFN- γ binds to a heterodimeric cell surface receptor complex consisting of the interferon-gamma receptor 1 (IFNGR1) and IFGR2, activating the classical JAK-STAT (signal transducer and activator of transcription) signaling pathways³⁹¹. Activation of this pathway regulates several downstream cascades and induces expression of many genes, thereby contributing to the diverse biological effects of IFN- γ in different cell types^{391,393,394}. IFN- γ activates macrophages to induce anti-tumor³⁹⁵ and anti-microbial activities³⁹⁶. It is also well established that IFN- γ induces antigen processing and presentation pathways in different cell types for MHC antigen presentation to T cells^{392,397-399}. In B cells, IFN- γ regulates immunoglobulin production and class switching^{398,400}. IFN- γ also attracts leukocytes and favours the growth, differentiation and maturation of many cells types^{393,398}. IFN- γ is classically known as a cytokine that favours Th1 cell development^{398,401}. In an allotransplantation setting, IFN- γ promotes antigen-specific Th1 differentiation that drives cell-mediated allograft rejection⁴⁰². Together, these findings suggest the potent proinflammatory role of IFN- γ .

IFN- γ is generally termed as a proinflammatory cytokine, however, there is growing body of evidence to suggest that IFN- γ has a paradoxical function as a regulatory cytokine. Studies of autoimmune disease mouse models deficient of IFN- γ have suggested that this cytokine exhibits a protective effect during the onset and progression of collagen-induced arthritis and in experimental autoimmune encephalomyelitis⁴⁰³⁻⁴⁰⁵. In models of cardiac and skin allotransplantation, IFN- γ facilitated prolonged allograft survival and induced tolerance by co-stimulatory blockade⁴⁰⁶⁻⁴⁰⁸. Unexpectedly, IFN- γ has also been shown to induce

regulatory phenotypes in different cell types. The exogenous addition of IFN- γ to DC-allogeneic T cell co-cultures promoted the generation of Tregs with anti-rejection properties⁴⁰⁹. Another study reported that IFN- γ also regulated CD4⁺ regulatory T cell function on donor alloantigens *in vivo*⁴¹⁰. Moreover, IFN- γ has been shown to induce tolerogenic DC, with reduced T cell allostimulatory capacity, from monocyte-derived DC cultures⁴¹¹. IFN- γ also modulates tolerogenic DC to express inhibitory molecules⁴¹²⁻⁴¹⁴.

The role of IFN- γ in MSC immunomodulation, reparative properties and homing potential has been extensively reviewed in **CHAPTER 1**. IFN- γ treated MSC (MSC- γ) as shown in **CHAPTER 3** and **CHAPTER 4**, have enhanced immunomodulatory properties but are potentially immunogenic when administered in allogeneic or third-party hosts. In this chapter, microarray and bioinformatics approaches were used to further identify novel candidate molecules expressed by MSC- γ that enhance the immunomodulatory properties of MSC. Genes and biological processes that may contribute to MSC- γ immunogenicity in allogeneic or third-party hosts were also explored in this chapter. In addition, the gene expression profile of human MSC-17 and MSC- γ were compared to UT-MSC.

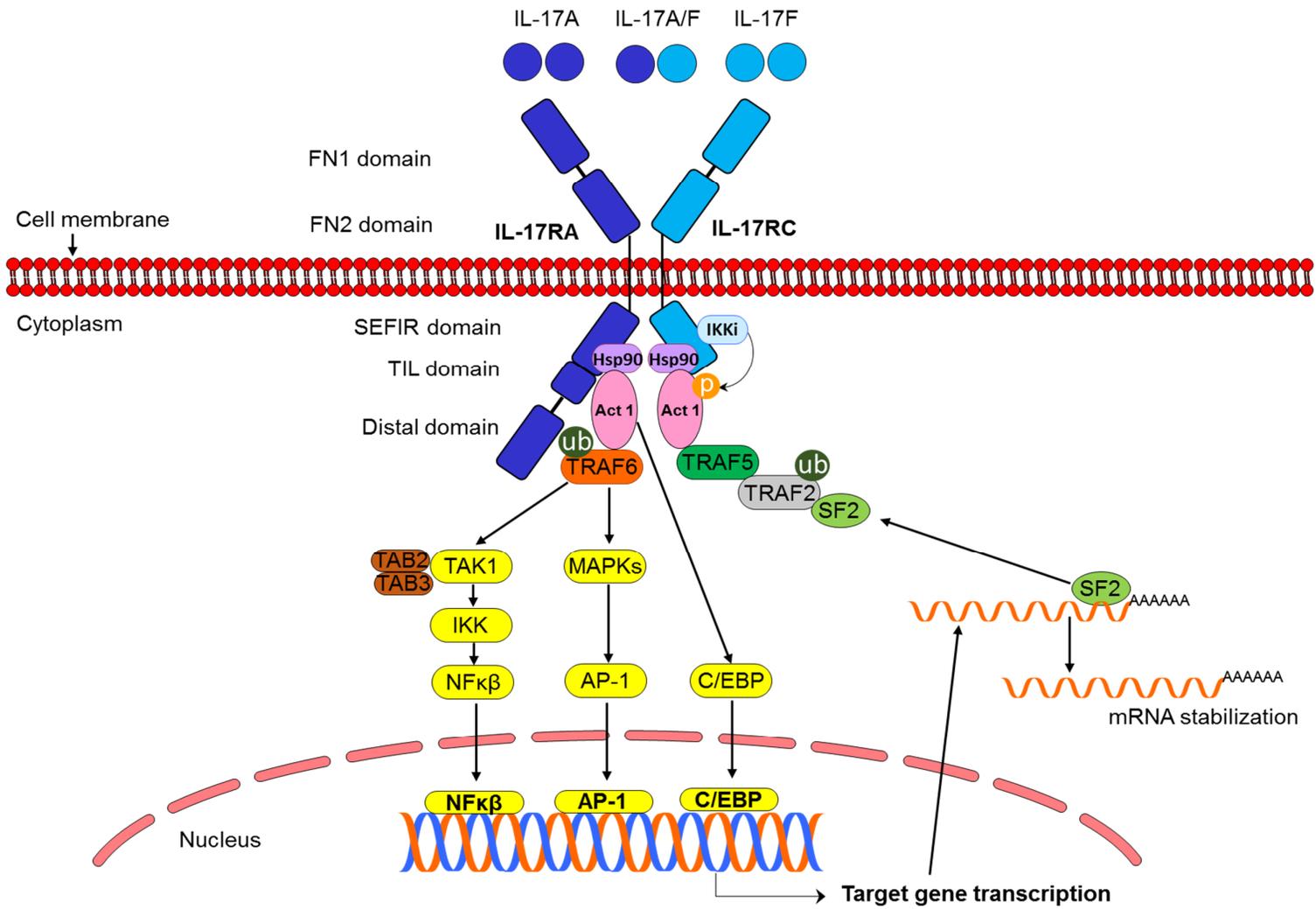


Figure 5.1.1 IL-17A signaling pathway.

IL-17A, IL-17F or IL-17A/F cytokine binding to the IL-17RA/IL-17RC heterodimeric receptor complex recruits the cytosolic adaptor molecule Act1. Act1 binds to the IL-17R receptor complex via the SEFIR domain and by interacting with the molecular chaperone, Hsp90. TRAF6 is recruited to the IL-17R complex through Act1. Act1 functions as an E3 ubiquitin ligase to poly-ubiquinate TRAF6. Poly-ubiquinated TRAF6 then associates with the TAK1-TAB2-TAB3 complex, activating IKK for subsequent NF- κ B activation. Act1 is also required for IL-17 mediated MAPK-AP-1 and C/EBP activation. mRNA stabilization of genes is also mediated by Act1 via TRAF6 independent but TRAF5 and TRAF2 dependent pathways. In absence of IL-17 stimulation, SF2 binds to mRNA to mediate its degradation. Upon IL-17 stimulation, IKKi associates Act1 with TRAF5 or TRAF2 by directly phosphorylating Act1. SF2 is then recruited to the Act1-TRAF5-TRAF2 complex and the mRNA is released and stabilized. **Abbreviations:** Act1, NF-kappa B activator 1; Hsp90, heat shock protein; TRAF, TNF receptor-associated factor; SF2, splicing factor; TAK1, TGF- β activated kinase 1; TAB, TAK-binding protein; IKK, I kappa β kinase; NF- κ B, nuclear factor kappa B; AP-1, activator protein 1; C/EBP CCAAT-enhancer-binding proteins; ub, ubiquinated; p, phosphorylated. *Adapted from Song et. al., (2013)³.*

5.2 MATERIALS AND METHODS

The general materials and methods of this chapter have been described in **CHAPTER 2: MATERIALS AND METHODS**. In this section, additional chapter specific materials and methods for microarray and bioinformatics analysis are described.

5.2.1 Human MSC RNA isolation

Human MSC were either untreated MSC (UT-MSC) or treated with 500U/ml IFN- γ (MSC- γ) or 50ng/ml IL-17A (MSC-17) for 5 days as described in section 2.5. MSC were harvested using 0.25% trypsin/EDTA (Sigma) for 4min, 37°C, rinsed with HFF and RNA was extracted according to the protocol established by the Adelaide Microarray Centre (<http://www.microarray.adelaide.edu.au/protocols/>). Briefly, total RNA was extracted by dissolving the cell pellet in 500 μ L TRIzol reagent (Invitrogen) and 100 μ L chloroform was added to the mixture. The mixture was kept on ice for 15min followed by centrifugation at 6500xg for 30min, 4°C. The upper aqueous phase was retained and mixed with an equal volume of 70% ethanol in diethylpyrocarbonate (DEPC) H₂O. Total RNA was further purified using the RNeasy mini kit (Qiagen) with the following modification: DNA was digested using the DNase I from the RNase-free DNase set (Qiagen). The quantity of total RNA was measured using NanoDrop 1000 (Thermo Scientific). Samples were adjusted to a concentration of 100ng/ μ L for microarray and were sent to the Adelaide Microarray Centre, University of Adelaide for microarray gene expression profiling. The RNA sample was determined using the Agilent RNA Bioanalyzer (**Figure 5.2.1**). Only RNA samples with RNA integrity number (RIN) of ≥ 8 were used for microarray analysis.

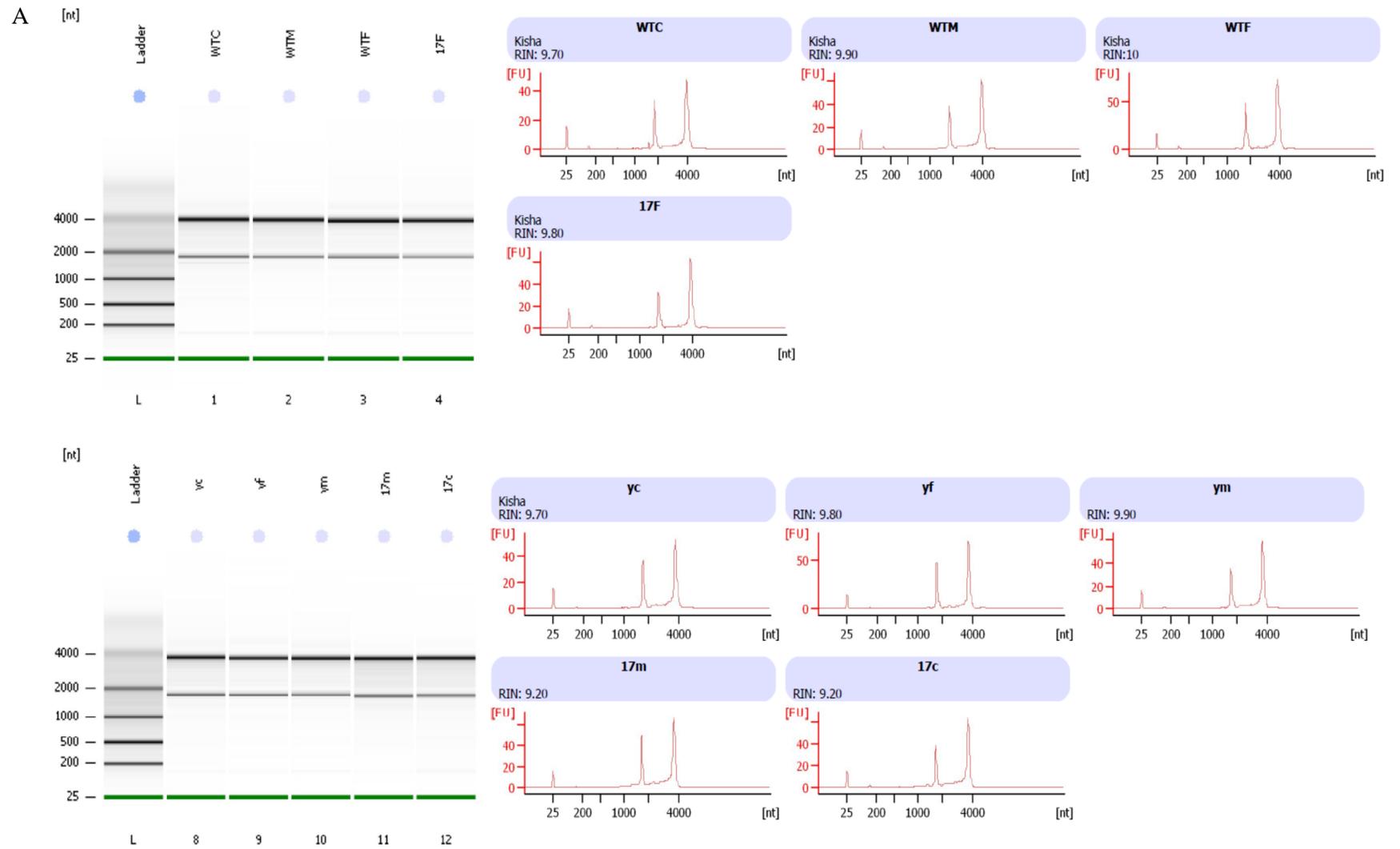


Figure 5.2.1 RNA quality analysis of MSC samples.

(A) Electrophoresis file run summary. (B) Chromatogram for each sample with main peaks corresponding to the 18S and 28S ribosomal RNA. The quality of the RNA samples was calculated and the RNA integrity number (RIN) score is provided on each chromatogram. RIN score from 10 (highest RNA quality) and 1 (degraded RNA) indicate the quality of the RNA samples. The RNA samples for UT-MSC, MSC- γ and MSC-17 are indicated as WT, γ and 17 respectively. The 3 different MSC donors were abbreviated as donor “c”, “m” and “f”. All RNA samples had high RNA quality evident by the RIN score of >9 and were subsequently used for the microarray experiments and analysis. These Agilent RNA Bioanalyzer analysis and results were provided by the Adelaide Microarray Centre, University of Adelaide.

5.2.2 Microarray analysis

The standard gene microarray experiment work flow is illustrated in **Figure 5.2.2**. RNA extracted from human MSC samples were analysed using the Affymetrix Human Gene 2.0 ST Array (Affymetrix Inc., High Wycombe, UK) for gene expression profiling. Details of the array design are summarized in **Table 5.2.2.1**. Microarray gene expression profiling was performed on UT-MSC, MSC- γ and MSC-17 from 3 human MSC donor biological replicates (passage 6). Microarray experiments were conducted by the Adelaide Microarray Centre, University of Adelaide.

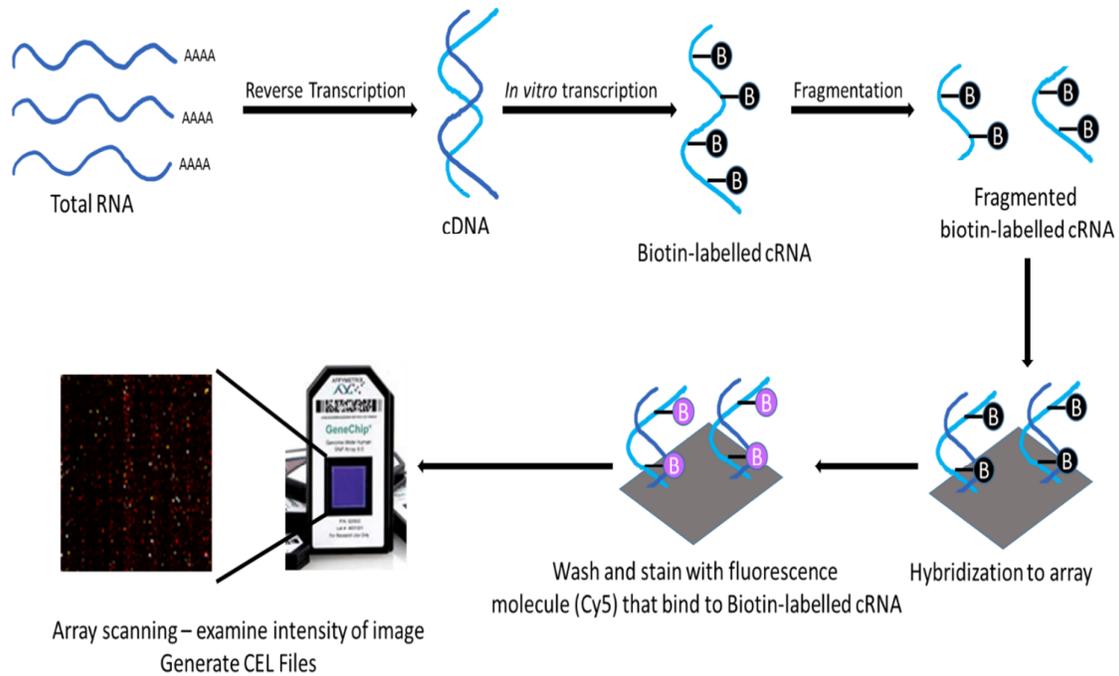


Figure 5.2.2 A standard gene microarray experiment workflow.

Total cytoplasmic RNA was isolated from MSC samples. The RNA was converted into double standard complementary DNA (cDNA) through reverse transcription. Samples were stored until ready to be run on the Affymetrix GeneChip Human Gene 2.0 ST Array. The cDNA was converted back to cRNA labelled with biotin. The biotin-labelled cRNA was randomly fragmented to 30 to 400 base pairs in length and added to the array. The biotin-labelled fragmented cRNA was then hybridized to the complementary probe and the array was washed to remove any unbound cRNA. The array was then stained with a fluorescent molecule (streptavidin-conjugated Cy5) that binds to biotin. Finally, the arrays were scanned with a laser and the probe cell intensities (CEL) files were generated for each sample to examine the intensity of each image. This procedure was conducted by the Adelaide Microarray Centre and the CEL files were obtained for further analysis (normalization, data quality control and summarization of genes) using the Expression Console Software and the Transcriptome Analysis Console (TAC) software.

Table 5.2.2.1 GeneChip Human Gene 2.0 ST Array Design

Array Design	
Total Probes	> 1.35 million
Exon-level probe sets	> 418 000
Gene-level probe sets	> 48 000
ERC C (eukaryotic control) probe sets	92
Background probes	Antigenomic set
Poly-A controls	<i>dap, lys, phe, thr</i>
Hybridisation controls	<i>bioB, bioC, bioD, creX</i>
Total RNA input required	50 – 500ng
Probe feature size	5µm
Probe length	25-mer
Probe per gene (median)	21
Target RNA orientation	Sense target

5.2.3 Microarray quality control and gene expression analysis

The workflow for microarray gene expression analysis is summarized in **Figure 5.2.3**. The probe cell intensity (CEL) files were obtained from the Adelaide Microarray Centre. The Expression Console Software (Affymetrix) was used for data quality control, normalization and differential gene level analysis. CEL files of each array showed no major issues or damage with the images (**Figure 5.2.4A**). No outlier samples were identified based on the configurable QA/QC metrics (**Figure 5.2.4 B, C** and **Figure 5.2.5**). The RMA (robust multi-array analysis) algorithm was used to perform background subtraction, normalization and summarization of probe sets (**Figure 5.2.5**). Following gene-level normalization, CHP files were generated from the Expression Console software for further Principal Component Analysis (PCA) and gene-level summarization using the Transcriptome Analysis Console (TAC) software (Affymetrix). After normalization, UT-MSC, MSC- γ and MSC-17 from 3 donor samples of each treatment group were averaged and an unpaired one-way ANOVA was performed with significantly regulated genes identified by $p < 0.05$ and fold-change < -2 and > 2 . Gene lists for comparison of MSC-17 and UT-MSC as well as MSC- γ and UT-MSC were generated for subsequent bioinformatics analysis.

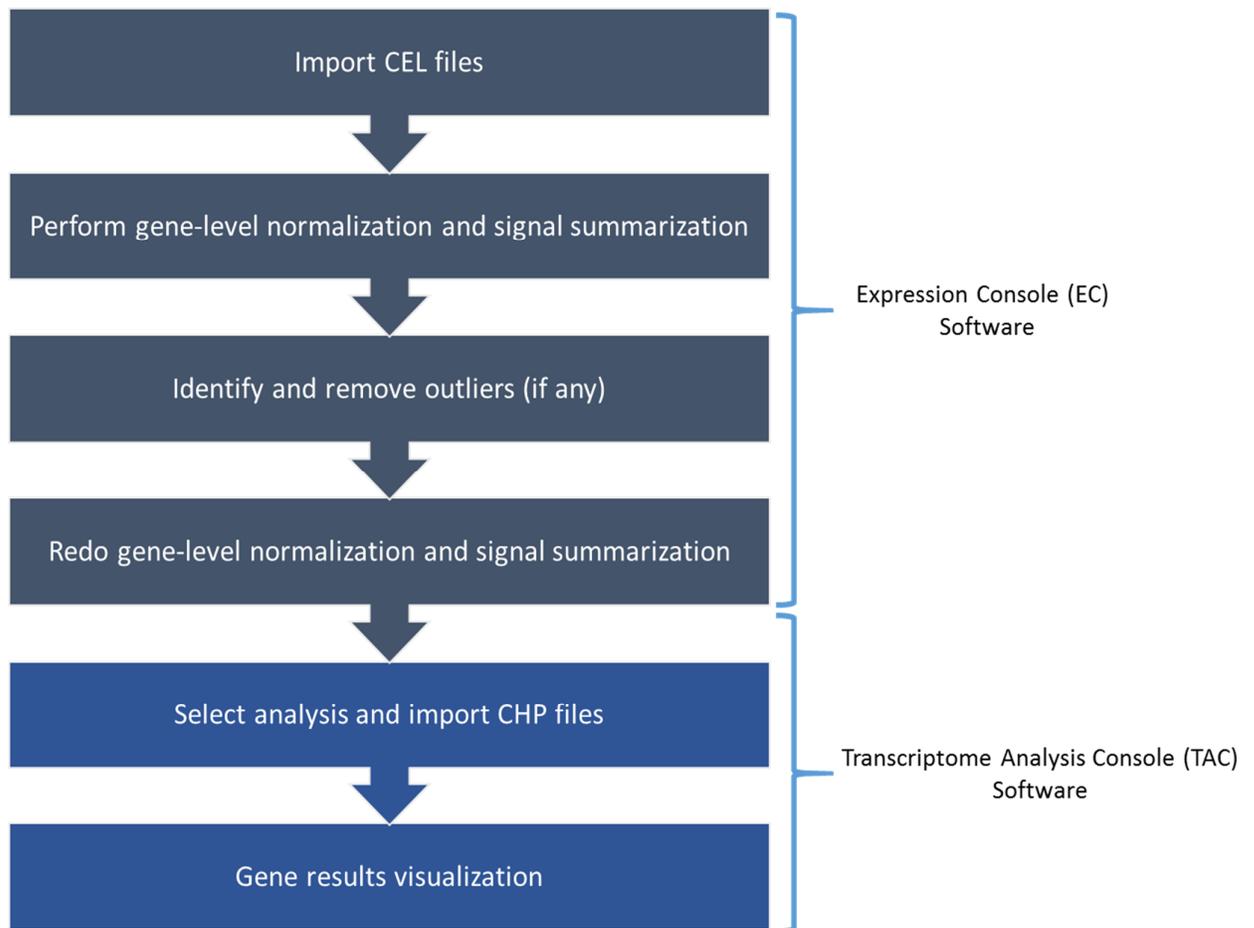


Figure 5.2.3 Microarray data analysis workflow.

Adapted from Affymetrix Expression Console Software data sheet.

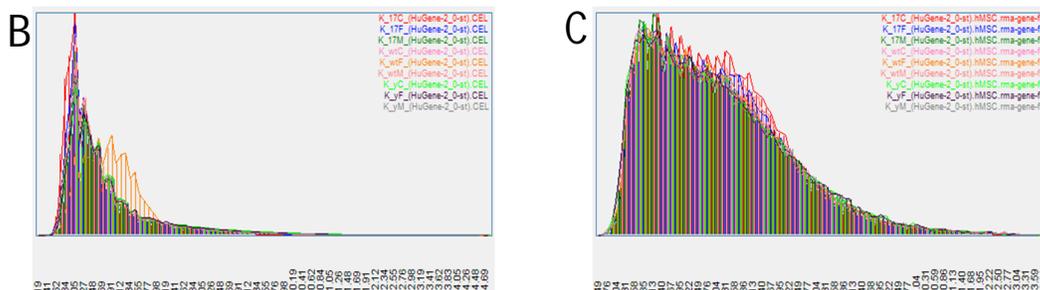
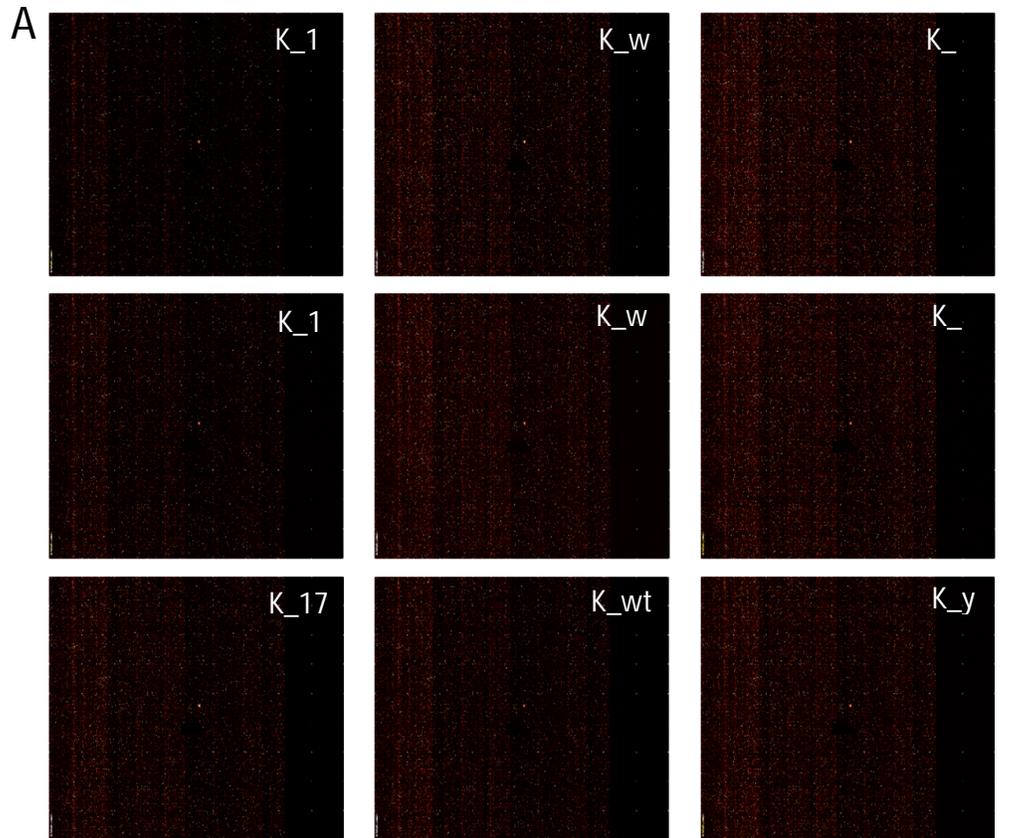


Figure 5.2.4 Probe level QA/QC analysis of arrays.

(A) Pseudo-image of each array chip. Probe cell intensity (CEL files) of each array showing no major issues or damage with the images (not normalised). (B-C) Histogram representation of (B) not normalised and (C) normalised probe cell intensity (CEL) of each array. Some differences in distribution can be expected from array-to-array in not normalised probe cell intensity (A) images or (B) histogram.

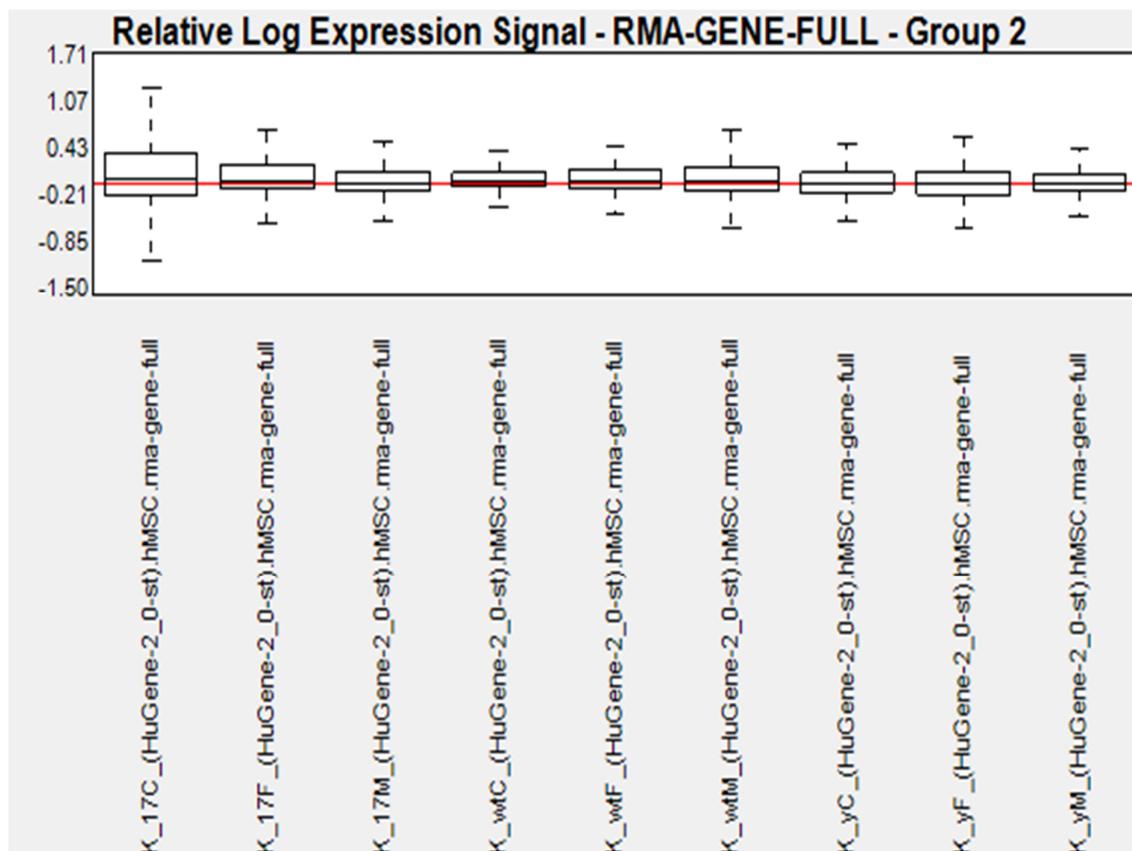
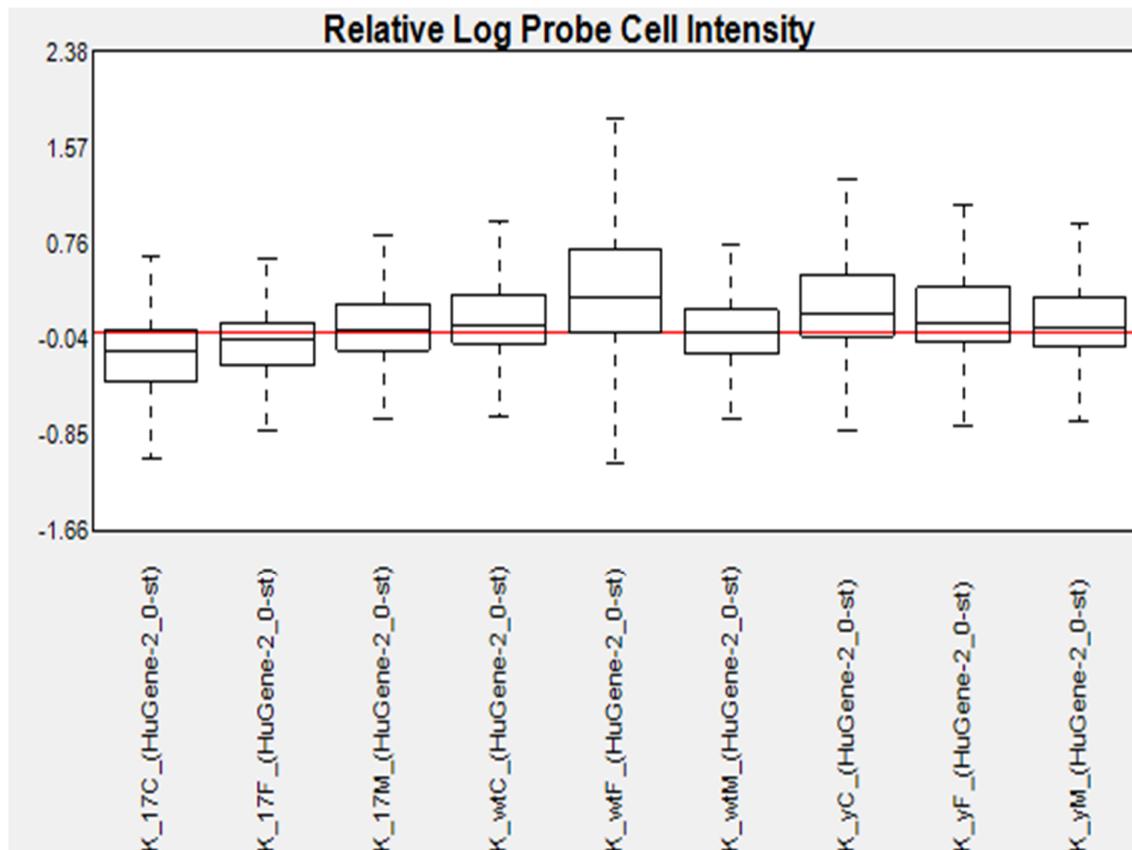


Figure 5.2.5 QA/QC analysis of arrays.

(A) The relative log probe cell intensity (not normalised) shows the distribution of the ratio intensity of each probe to the median probe intensity (red line) across all the arrays. There was no major difference in relative probe set intensities present between arrays. (B) The relative log expression signal (RMA normalised) summarises the distribution ratio of signal for each probe set to the median probe set signal (red line) across all arrays. There were no arrays with divergent signal distribution relative to other arrays in this study.

5.2.4 Functional enrichment analysis by DAVID

Gene lists for comparison of MSC-17 vs. UT-MSC and MSC- γ vs. UT-MSC were analysed for their biological functions using the Database for Annotation, Visualisation and Integrated Discovery (DAVID; <http://david.abcc.ncifcrf.gov/>). The gene list was uploaded using the Official gene symbol onto DAVID for functional annotation clustering analysis with medium classification stringency and $p < 0.05$ ⁴¹⁵. Briefly, “**Functional Annotation**” was selected (**Figure 5.2.6**). The gene list was uploaded at “**A. Paste A List**”, the “**OFFICIAL_GENE_SYMBOL**”, “**Gene list**” and the “**Submit List**” to upload the gene list were selected (**Figure 5.2.7**). Functional annotation clustering analysis based on DAVID’s default settings was performed as shown in **Figure 5.2.8**. An example layout of cluster of genes that show significant functional annotation clustering enrichment (medium classification stringency and $p < 0.05$ was considered statistically significant) were demonstrated in **Figure 5.2.9** with enrichment scores > 1.5 were considered to be significant. The gene sets were also sub-categorised based on functional annotation of interest such as biological process (GOTERM_BP_FAT), molecular function (GOTERM_MF_FAT) and cellular component (GOTERM_CC_FAT) (**Figure 5.2.10**). All data were summarized and presented in Results (sections **5.3.2** and **5.3.3**).


DAVID Bioinformatics Resources 6.7
 National Institute of Allergy and Infectious Diseases (NIAID), NIH

[Home](#) | [Start Analysis](#) | [Shortcut to DAVID Tools](#) | [Technical Center](#) | [Downloads & APIs](#) | [Term of Service](#) | [Why DAVID?](#) | [About Us](#)

Shortcut to DAVID Tools

▶ **Functional Annotation**

Gene-annotation enrichment analysis, functional annotation clustering, BioCarta & KEGG pathway mapping, gene-disease association, homologue match, ID translation, literature match and [more](#)

▶ **Gene Functional Classification**

Provide a rapid means to reduce large lists of genes into functionally related groups of genes to help unravel the biological content captured by high throughput technologies. [More](#)

▶ **Gene ID Conversion**

Convert list of gene ID/accessions to others of your choice with the most comprehensive gene ID mapping repository. The ambiguous accessions in the list can also be determined semi-automatically. [More](#)

▶ **Gene Name Batch Viewer**

Display gene names for a given gene list; Search functionally related genes within your list or not in your list; Deep links to enriched detailed information. [More](#)

Recommending: A paper published in *Nature Protocols* describes step-by-step procedure to use DAVID!

Select

Welcome to DAVID 6.7

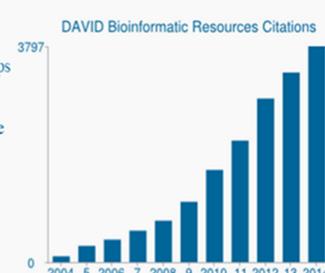
2003 - 2015

What's Important in DAVID?

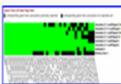
- [Current \(v 6.7\) release note](#)
- [New requirement to cite DAVID](#)
- [IDs of Affy Exon and Gene arrays supported](#)
- [Novel Classification Algorithms](#)
- [Pre-built Affymetrix and Illumina backgrounds](#)
- [User's customized gene background](#)
- [Enhanced calculating speed](#)

Statistics of DAVID

Identify enriched biological themes, particularly GO terms
 Discover enriched functional-related gene groups
 Cluster redundant annotation terms
 Visualize genes on BioCarta & KEGG pathway maps
 Display related many-genes-to-many-terms on 2-D view.
 Search for other functionally related genes not in the list
 List interacting proteins
 Explore gene names in batch
 Link gene-disease associations
 Highlight protein functional domains and motifs
 Redirect to related literatures
 Convert gene identifiers from one type to another.
 And more



• [≥ 17,000 Citations](#)
 • Daily Usage: ~1200 gene lists/sublists from ~400 unique researchers.
 • Total Usage: ~800,000 gene lists/sublists from >5,000 research institutes world-wide



Screen Shot 1



Screen Shot 2



Screen Shot 3

Figure 5.2.6 An example layout to perform DAVID Functional Annotation analysis.

The image shows two screenshots of the DAVID Functional Annotation Tool interface. The top screenshot displays the 'Upload Gene List' section with four steps highlighted by red boxes and arrows: Step 1: Enter Gene List (A. Paste a list), Step 2: Select Identifier (OFFICIAL_GENE_SYMBOL), Step 3: List Type (Gene List), and Step 4: Submit List (Submit List). The bottom screenshot shows the 'Annotation Summary Results' section with a red box highlighting the 'Select Species' dropdown menu, which is set to 'Homo sapiens(33)'. The interface includes a navigation bar at the top with links like Home, Start Analysis, and Technical Center, and a sidebar on the left with tabs for Upload, List, and Background.

Figure 5.2.7 An example layout of gene list entry for DAVID functional annotation analysis.

Functional Annotation Tool
DAVID Bioinformatics Resources 6.7, NIAID/NIH

Home Start Analysis Shortcut to DAVID Tools Technical Center Downloads & APIs Term of Service Why DAVID? About Us

Upload List Background

Gene List Manager

Select to limit annotations by one or more species [Help](#)

- Use All Species -
Homo sapiens(33)
Pan troglodytes(23)
Mus musculus(21)
Select Species

List Manager [Help](#)

List_1

Select List to:
Use Rename
Remove Combine
Show Gene List

Annotation Summary Results [Help and Tool Manual](#)

Current Gene List: List_1
Current Background: Homo sapiens
33 DAVID IDs
Check Defaults Clear All

- Disease (1 selected)
- Functional_Categories (3 selected)
- Gene_Ontology (3 selected)
- General Annotations (0 selected)
- Literature (0 selected)
- Main_Accessions (0 selected)
- Pathways (3 selected)
- Protein_Domains (3 selected)
- Protein_Interactions (0 selected)
- Tissue_Expression (0 selected)

Red annotation categories denote DAVID defined defaults

Combined View for Selected Annotation

Functional Annotation Clustering
Functional Annotation Chart
Functional Annotation Table

1. Run Functional annotation analysis on DAVID's Default settings

2. Select to view Functional Annotation Clustering Results

Figure 5.2.8 An example layout to perform functional annotation clustering analysis based on DAVID's default settings.

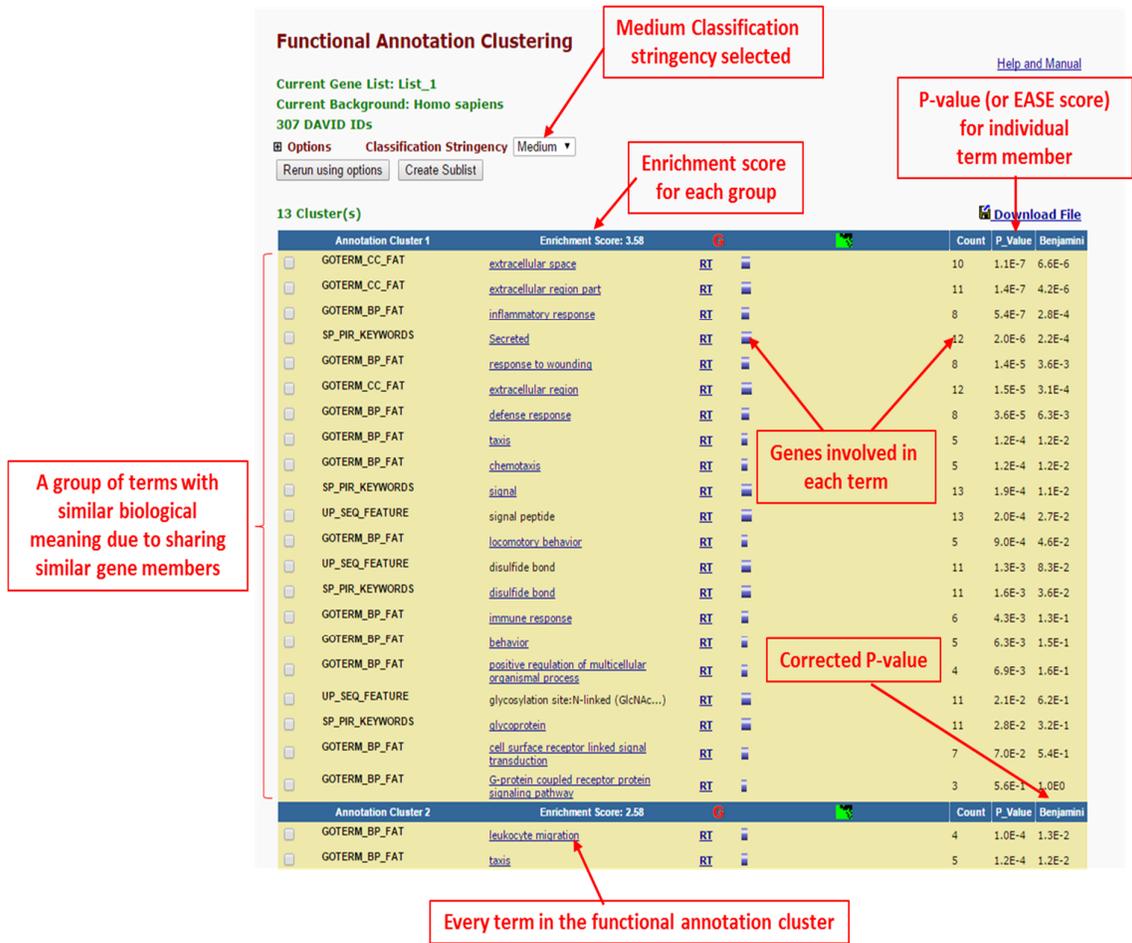


Figure 5.2.9 An example layout of the functional annotation clustering results of enriched gene sets.

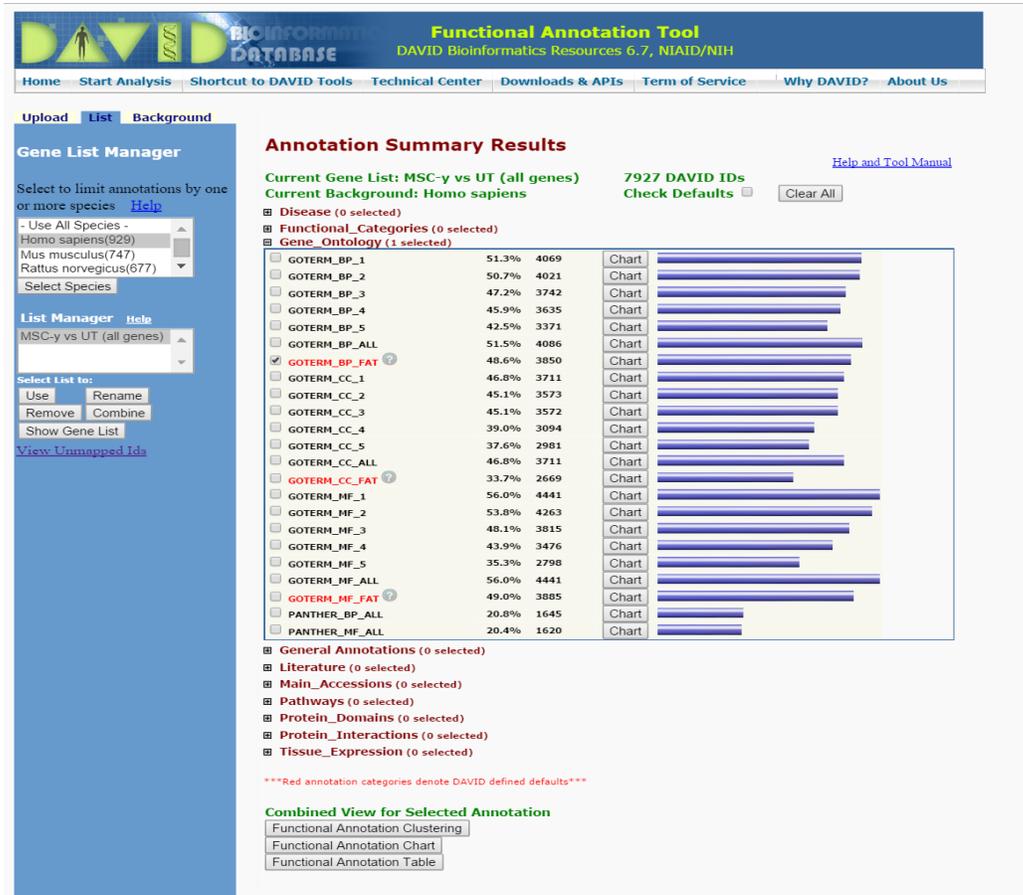


Figure 5.2.10 Screen shot of the selected functional annotation of interest.

This screen shot is an example for the analysis of Biological Processed (BP) in the Gene Ontology (GO), the “GOTERM_BP_FAT”.

5.2.6 Real-time PCR gene validation

Genes of interest identified by microarray were validated by real-time PCR (RT-PCR) as described in section **2.14**. Gene expression (10-100ng cDNA per reaction) was measured in real-time PCR with the Taqman[®] gene expression master mix and using the following gene specific human Taqman[®] primers MMP13 (Hs00233992_ml), C3 (Hs00163811_ml), LBP (Hs01084621_ml) and CH25H (Hs02379634_s1), purchased from Applied Biosystems. Samples were run in triplicate and data were presented and normalized to the house keeping gene hypoxanthine phosphoribosyltransferase-1 (HPRT1) (Hs99999909_ml). Mean normalized expression was calculated using the Qgene Module software as previously described³⁵⁵.

5.3 RESULTS

5.3.1 Transcriptome profiling of UT-MSC, MSC- γ and MSC-17

The transcriptome differences between UT-MSC, MSC- γ and MSC-17 from 3 different human MSC donors were compared in this study. Principal component analysis (PCA) was performed to visualize variances between the 3 donors and treatment groups. PCA analysis revealed that the 3 donor replicates of MSC- γ “clustered” together. The gene expression pattern in the MSC- γ groups were clearly distinct from the UT-MSC and MSC-17 (**Figure 5.3.1**). Microarray analysis revealed that 1278 genes (902 upregulated; 376 downregulated) were differentially regulated between MSC- γ and UT-MSC (**Table 5.3.1.1**).

There were however donor variances that exist between MSC-17 and UT-MSC. Among the 3 MSC donor samples evaluated, 2 MSC donors (ie. donor C and F) “clustered” together and were distinct from UT-MSC (**Figure 5.3.1**). It should also be noted that in donor C and F MSC-17 “clusters”, there were less variability in the gene expression profile in MSC-17 vs. UT-MSC compared to the MSC- γ vs. UT-MSC groups (**Figure 5.3.1**). Donor M on the contrary, had a different gene expression pattern in both UT-MSC and MSC-17 (**Figure 5.3.1**). Based on these 3 MSC donors, microarray analysis identified that only 67 genes (39 upregulated; 28 downregulated) were differentially regulated between MSC-17 and UT-MSC (**Table 5.3.1.1**).

The chromosome location of the differentially expressed genes between MSC-17 vs. UT-MSC and MSC- γ vs. UT-MSC comparison groups were shown in **Figure 5.3.2**. Volcano plots (**Figure 5.3.3**) and supervised hierarchical clustering of the differentially expressed genes (**Figure 5.3.4**) provides a global visualization of genes regulated by IL-17 or IFN- γ treatment of MSC compared to UT-MSC.

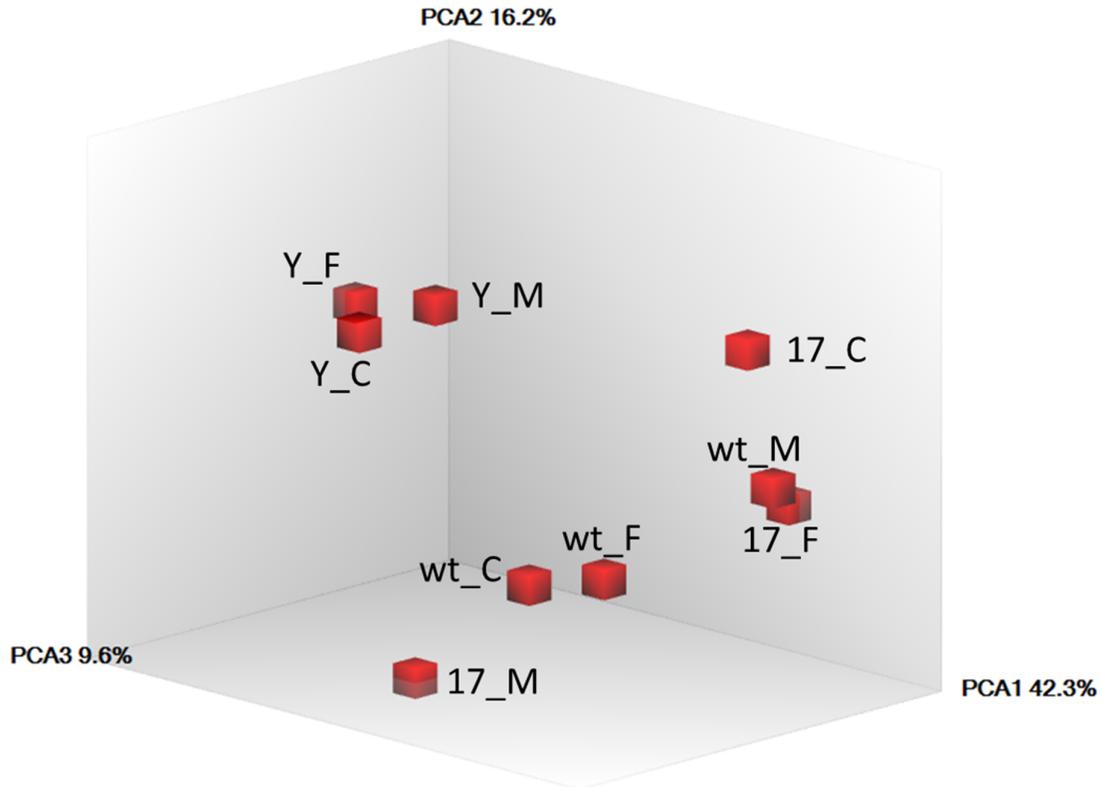


Figure 5.3.1 Principal Component Analysis (PCA) of IL-17 (17_), IFN- γ (Y_) or untreated (wt_) MSC from 3 different donors as indicated by C, M and F.

This 3-dimensional PCA graph identifies a new set of variables (PCA1, PCA2, PCA3) that account for majority of the variability among the samples. PCA1 captures as much variability in the data as possible, PCA2 captures as much variability of the remaining variability not accounted by PCA1 and PCA3 captures as much of the remaining variability not accounted by PCA2.

Table 5.3.1.1 Gene Level Differential Expression Analysis

MSC-17 vs. UT-MSC vs. MSC-y**Analysis Type: Gene Level Differential Expression Analysis****Array Type: HuGene-2_0-st****Genome Version: hg19****Annotation File: HuGene-2_0-st-v1.na34.hg19.transcript.csv**

Summary:

- 1.) Total number of genes: 48226
- 2.) 2150 genes are differentially expressed

3.) MSC-17 vs. UT-MSC:

- a. 39 genes are up-regulated
- b. 28 genes are down-regulated

4.) MSC-17 vs. MSC-y:

- a. 391 genes are up-regulated
- b. 1415 genes are down-regulated

5.) MSC-y vs. UT-MSC:

- a. 902 genes are up-regulated
 - b. 376 genes are down-regulated
-

Algorithm Options:

- 1.) One-Way Between-Subject ANOVA (Unpaired)
-

Default Filter Criteria:

- 1.) Fold Change (linear) < -2 or Fold Change (linear) > 2
 - 2.) ANOVA p-value (Condition pair) < 0.05
-

Conditions:**MSC-17 (3)**

- 1.) K_17C_(HuGene-2_0-st).hMSC.rma-gene-full.chp
- 2.) K_17F_(HuGene-2_0-st).hMSC.rma-gene-full.chp
- 3.) K_17M_(HuGene-2_0-st).hMSC.rma-gene-full.chp

UT-MSC (3)

- 1.) K_wtC_(HuGene-2_0-st).hMSC.rma-gene-full.chp
- 2.) K_wtF_(HuGene-2_0-st).hMSC.rma-gene-full.chp
- 3.) K_wtM_(HuGene-2_0-st).hMSC.rma-gene-full.chp

MSC-y (3)

- 1.) K_yC_(HuGene-2_0-st).hMSC.rma-gene-full.chp
 - 2.) K_yF_(HuGene-2_0-st).hMSC.rma-gene-full.chp
 - 3.) K_yM_(HuGene-2_0-st).hMSC.rma-gene-full.chp
-

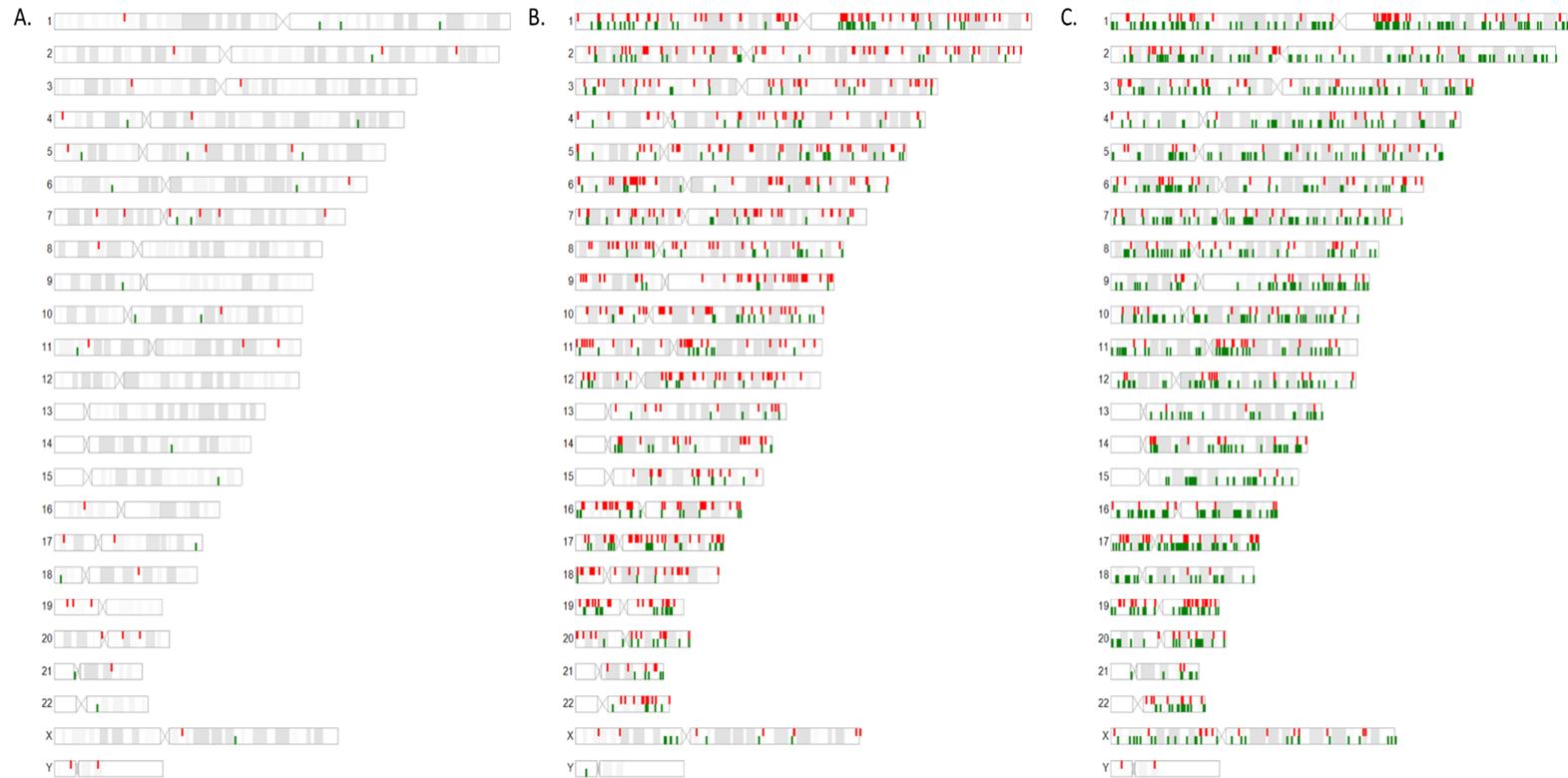


Figure 5.3.2 . Chromosome overview showing locations of the differentially expressed genes between (A) MSC-17 vs. UT-MSC, (B) MSC- γ vs. UT-MSC, and (C) MSC-17 vs. MSC- γ .

Red colour indicates upregulated genes and the green represents downregulated genes. The grey region indicates genes that were not differentially expressed and not statistically significant.

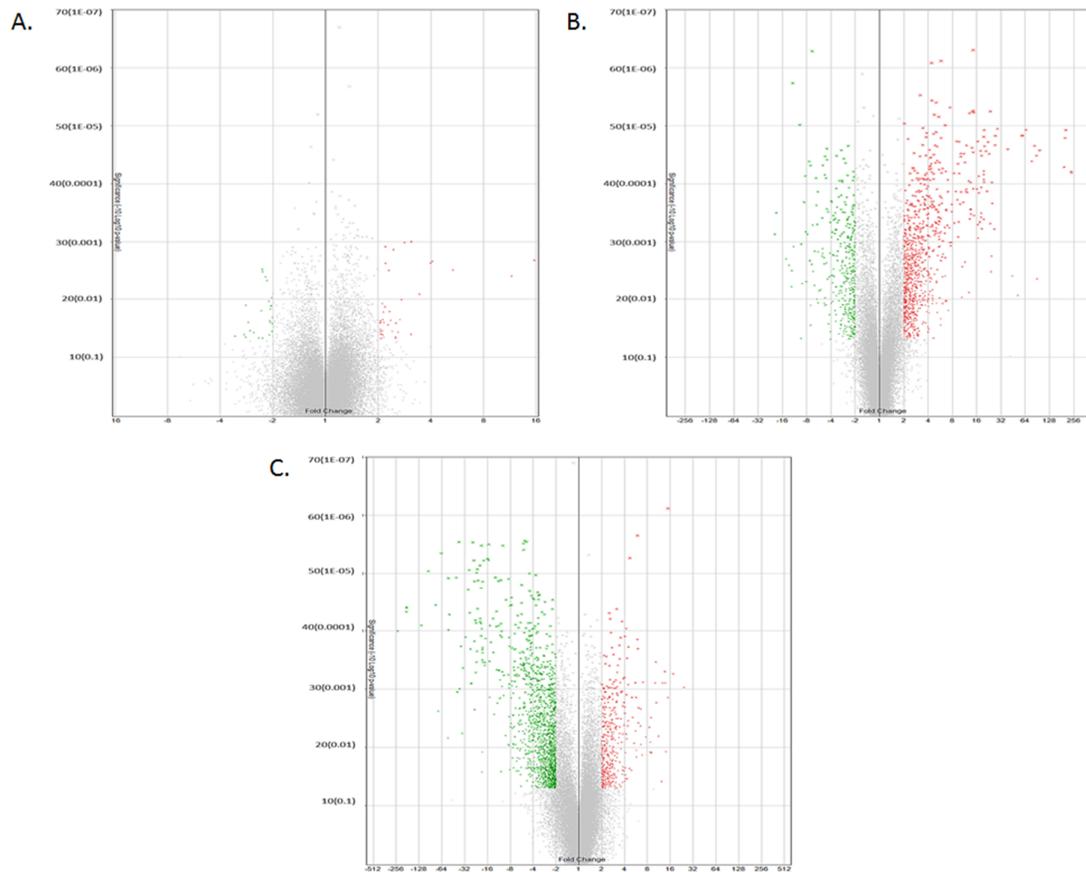


Figure 5.3.3 Volcano plots to identify changes in gene expression between (A) MSC-17 vs. UT-MSC, (B) MSC- γ vs. UT-MSC, and (C) MSC-17 vs. MSC- γ .

Axes of these plots represent: significance ($-10 \log_{10}$ p-value of the ANOVA p-values; y-axes) vs. fold-changes (linear fold change from condition pairing; x-axes) Red colour indicates upregulated genes and the green represents downregulated genes. The grey region indicates genes that were not differentially expressed and not statistically significant.

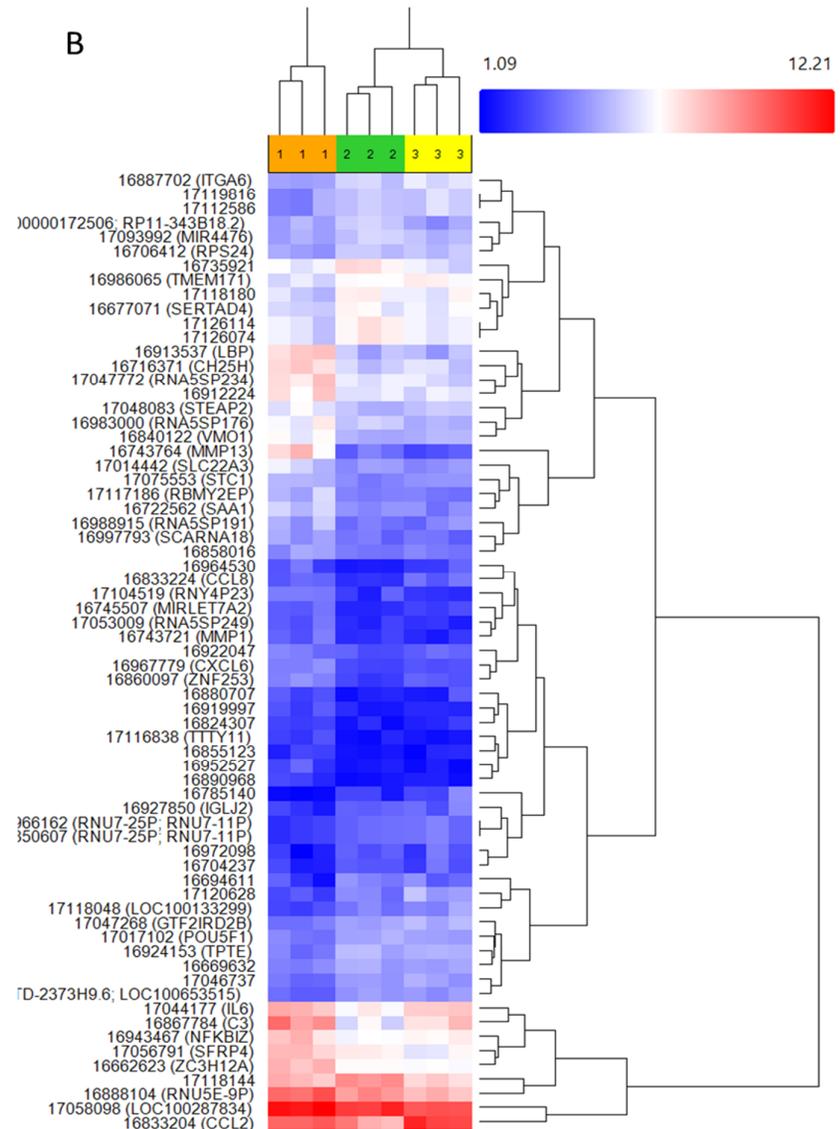
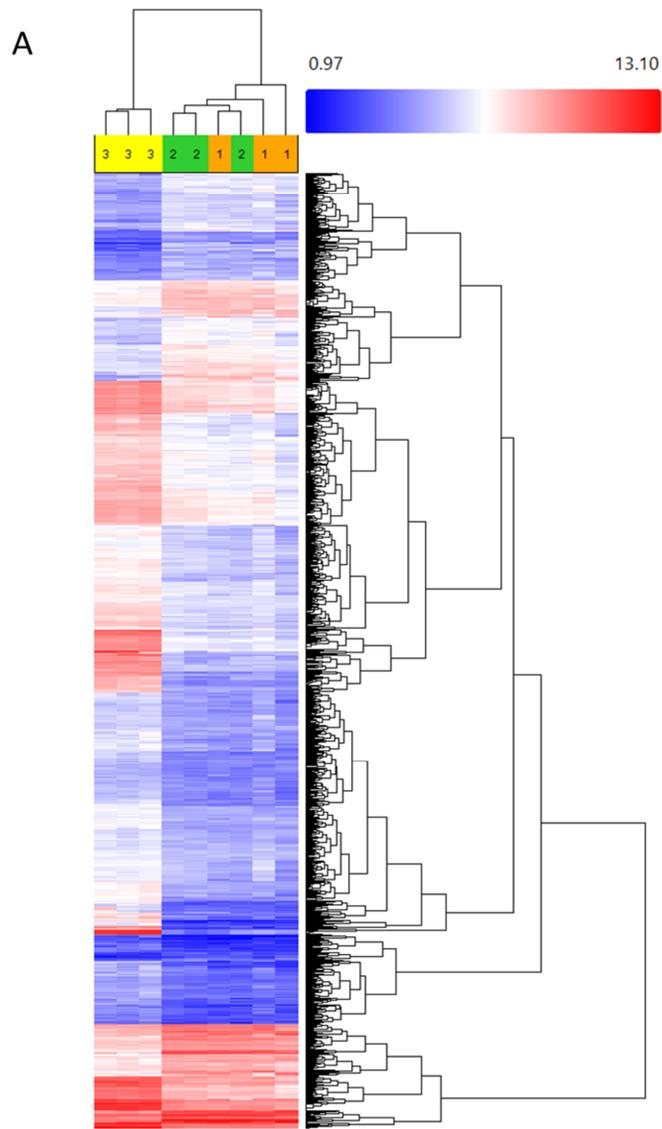


Figure 5.3.4 Gene expression profile of MSC-17 (1), UT-MSC (2) and MSC- γ (3) from 3 MSC donors determined with Affymetrix Human Gene ST 2.0 microarrays.

Supervised hierarchical clustering of genes differentially expressed between (A) MSC- γ vs. UT-MSC and (B) MSC-17 vs. UT-MSC determined by ANOVA p-value (condition pair) $p < 0.05$ and fold change (linear) < -2 or > 2 . (A) 1278 and (B) 67 genes were differentially regulated between the treatment groups. The normalized expression value for each gene is visualised by a colour gradient: blue represents low gene expression; red represents high gene expression.

5.3.2 Functional enrichment analysis: MSC- γ vs. UT-MSC

A total of 1278 genes (902 upregulated; 376 downregulated) were differentially regulated in the MSC- γ vs. UT-MSC comparison group. The top 30 upregulated and downregulated genes in the MSC- γ were shown in **Table 5.3.2.1**. Upregulated and downregulated gene lists were submitted to DAVID for functional annotation clustering analysis to identify gene sets that were enriched following IFN- γ treatment of human MSC. There were 90 and 62 official gene symbols from the upregulated (**Table 5.3.2.2**) and downregulated (**Table 5.3.2.3**) gene entry lists, respectively, that were unmapped by DAVID. These were mainly non-coding genes including microRNA (miRNA), long non-coding RNA (lncRNA) and small nucleolar RNA (snoRNA). Gene ontology analysis by DAVID functional annotation clustering was performed on the upregulated and downregulated MSC- γ vs. UT-MSC gene lists to identify enriched gene sets (enrichment score > 1.5) for biological processes (**Table 5.3.2.4**; **Table 5.3.2.5**) molecular functions (**Table 5.3.2.6** and **Table 5.3.2.7**) and cellular components (**Table 5.3.2.8**; **Table 5.3.2.9**).

Gene ontology analysis for biological processes of **upregulated MSC- γ genes** (**Table 5.3.2.4**) uncovered highest enrichment of genes associated with antigen processing and presentation via MHC class I (annotation cluster 1, enrichment score 8.03). These genes were mainly HLA type genes and have roles in antigen presentation. Enriched genes in annotation cluster 1 also include aminopeptidases that process (or hydrolyses) antigenic peptides for MHC class I peptide binding and antigen presentation (eg. endoplasmic reticulum aminopeptidase ERAP1 and ERAP2), peptide transporter genes (eg. transported associated with antigen processing, TAP2) and other genes involved in the antigen processing and presentation pathway (eg. TAP binding protein, TAPBPL; β_2 microglobulin, B2M; CD74). Gene sets involved with antigen processing and presentation via MHC class II were also upregulated in the MSC- γ groups (annotation cluster 4, enrichment score 4.45). In annotation cluster 2

(enrichment score 6.06) there were also enriched gene sets involved in immune response activation (innate, adaptive and lymphocytes mediated immunity), humoral response (immunoglobulin mediated immune response, B cell mediated immunity, humoral immune response mediated by circulating immunoglobulin) and complement pathways (classical and alternative) activation.

Apart from genes that are involved in increased MSC- γ immunogenicity, there were genes with regulatory roles upregulated following IFN- γ treatment (**Table 5.3.2.4**). For example, these gene sets were involved in the regulation of programmed cell death, apoptosis, translation regulation, protein modification, transcription regulation and DNA binding activity, cell-cell communication and signal transduction as well as the regulation of cytokine production. Moreover, genes upregulated in the MSC- γ group were enriched for the TGF- β receptor signaling pathway (annotation cluster 19, enrichment score 1.74 eg. FMOD, CCL2, MAPK3K1, SMAD6, GDF15, TGFB2). Other genes of interest upregulated in MSC- γ include IL-6, TLR3, TLR4 and IDO, with the gene ontology term for positive regulation of defense response. Regulatory genes with nucleotide binding activity and transcription (corepressor, repressor, cofactor) activity as well as transcription factor binding genes were also enriched and upregulated in MSC- γ as identified by DAVID gene ontology analysis for molecular function (**Table 5.3.2.6**).

MSC- γ have enhanced migratory potential to sites of inflammation⁴. Based on DAVID analysis for biological processes, we have identified gene sets in annotation cluster 10 (enrichment score 2.78) that were enriched for the gene ontology terms regulation of cell motion, cell migration and locomotion (**Table 5.3.2.4**). These upregulated MSC- γ genes include chemokines (CXCL10, CXCL16), intracellular adhesion molecule 1 (ICAM1), IL-6 and VEGFA. The upregulation of chemotactic factors that may increase MSC- γ homing potential were also identified when gene ontology analysis for molecular function was

performed on DAVID. In annotation cluster 3 (enrichment score 3.10; **Table 5.3.2.6**), genes were enriched for chemokine receptor binding and chemokine activity. These chemokines include CCL13, CCL2, CXCL16, CXCL9, CCL8, CXCL11 and CXCL10.

Based on the **downregulated MSC- γ vs. UT-MSC gene list**, we identified that there were genes highly enriched for the gene ontology terms for biological processes involving extracellular matrix or structure organisation (annotation cluster 1, enrichment score 11.10; **Table 5.3.2.5**), consistent with our previous observation of changes in MSC- γ morphology (**Figure 3.3.5B**). These were mainly collagen type genes (collagenase I, III, IV, V, XI, XII, XIV). Interestingly, the downregulated gene sets also have enriched terms for biological processes involved in the cell division cycle (annotation cluster 2, enrichment score 8.80; **Table 5.3.2.5**) These downregulated genes were essential for M phase, nuclear division, mitosis and cell division. Genes essential for regulation of cell-cycle division were also downregulated in MSC- γ (annotation cluster 7, enrichment score 2.03), in coherence with the observation of decreased MSC- γ growth kinetics compared to UT-MSC (**Figure 3.3.5C**).

Gene ontology analysis for cellular components (**Table 5.3.2.8**; **Table 5.3.2.9**) of the differentially regulated genes in the MSC- γ vs. UT-MSC groups uncovered that these genes were located in the extracellular space (or) region (annotation cluster 1, enrichment score 2.69 **Table 5.3.2.8** and enrichment score 12.76 **Table 5.3.2.9**). Many downregulated genes were located in collagen, the main structural protein in the extracellular region.

Table 5.3.2.1 Top 30 differentially expressed genes: MSC- γ vs. UT-MSC

Gene Symbol	Gene name	mRNA Accession	Fold Change	p-value
Upregulated genes:				
HLA-DRA	major histocompatibility complex, class II, DR alpha	NM_019111	387.78	0.00049
GBP4	guanylate binding protein 4	NM_052941	199.41	0.00002
IDO1	indoleamine 2,3-dioxygenase 1	NM_002164	96.72	0.00003
HLA-DRB	major histocompatibility complex, class II, DR beta	ENST00000307137	89.67	0.00435
GBP5	guanylate binding protein 5	NM_052942	88.07	0.00003
CXCL9	chemokine (C-X-C motif) ligand 9	NM_002416	83.6	0.00002
GBP2	guanylate binding protein 2, interferon-inducible	ENST00000464839	77	0.00004
SECTM1	secreted and transmembrane 1; NULL	NM_003004	57.59	0.00002
HLA-DRB3	major histocompatibility complex, class II, DR beta 3	ENST00000426847	51.65	0.00868
CIITA	class II, major histocompatibility complex, transactivator	NM_00024	38.84	0.00003
GBP1	guanylate binding protein 1, interferon-inducible	NM_002053	29.09	0.00001
RP11-44K6.2	NULL	ENST00000520185	26.39	0.00060
GCH1	GTP cyclohydrolase 1	NM_000161	24.93	0.00022
USP30-AS1	USP30 antisense RNA 1	ENST00000478808	24.75	0.00009
GBP2	guanylate binding protein 2, interferon-inducible; NULL	NM_004120	23.6	0.00001
HLA-DOA	major histocompatibility complex, class II, DO alpha; NULL	NM_002119	22.9	0.00003
IFIT3	interferon-induced protein with tetratricopeptide repeats 3	NM_001031683	21.49	0.00013
FAM129A	family with sequence similarity 129, member A	NM_052966	20.89	0.00003
CTSS	cathepsin S	NM_004079	20.1	0.00002
SLC7A11	solute carrier family 7 (anionic amino acid transporter light chain, xc- system), member 11	NM_014331	19.7	0.00009
IRF1	interferon regulatory factor 1	NM_002198	19.55	0.00002
CD74	CD74 molecule, major histocompatibility complex, class II invariant chain; NULL	NM_001025159	18.49	0.00060
ICAM1	intercellular adhesion molecule 1	NM_000201	18.43	0.00003
HCP5	HLA complex P5 (non-protein coding); NULL	ENST00000457127	18.15	0.00032
LGALS17A	Charcot-Leyden crystal protein pseudogene	ENST00000412609	18.12	0.00038

PARP14	poly (ADP-ribose) polymerase family, member 14	NM_017554;	17.05	0.00014
RARRES3	retinoic acid receptor responder (tazarotene induced) 3	NM_004585	17	0.00007
WARS	tryptophanyl-tRNA synthetase; NULL	NM_004184	16.49	0.00002
IFIT2	interferon-induced protein with tetratricopeptide repeats 2	NM_001547	16.43	0.00051
TMEM140	transmembrane protein 140	NM_018295	16.08	0.00012

Downregulated genes:

LRRC15	leucine rich repeat containing 15	NM_001135057	-19.91	0.0007
KIAA1199	KIAA1199; NULL	NM_018689	-13.26	0.0025
RNU5A-8P	RNA, U5A small nuclear 8, pseudogene	ENST00000364102	-12.36	0.0061
COL10A1	collagen, type X, alpha 1	NM_000493;	-12.25	0.0031
COL3A1	collagen, type III, alpha 1; microRNA 3606	NM_000090	-11.88	0.0000
HIST1H2A	histone cluster 1, H2ai; histone cluster 1, H2ah; histone cluster 1, H2ag; histone cluster 1, H2am; histone cluster 1, H2al; histone cluster 1, H2ak; histone cluster 1, H3f	NM_003509	-11.67	0.0012
SCD	stearoyl-CoA desaturase (delta-9-desaturase)	NM_005063	-9.75	0.0000
U2	U2 spliceosomal RNA	ENST00000410792	-9.41	0.0476
HIST1H3	histone cluster 1, H3b; histone cluster 1, H3f; histone cluster 1, H3h; histone cluster 1, H3j; histone cluster 1, H3g; histone cluster 1, H3i; histone cluster 1, H3e; histone cluster 1, H3c; histone cluster 1, H3d; histone cluster 1, H3a	NM_003537	-9.06	0.0053
HIST1H1B	histone cluster 1, H1b	NM_00532	-8.53	0.0002
-	-	ENST00000408768	-8.25	0.0001
KDEL3	KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 3	NM_016657	-8.22	0.0007
-	-	BC091525	-7.87	0.0007
SNORD114-11	small nucleolar RNA, C/D box 114-11	NR_003204	-7.33	0.0021
WISP1	WNT1 inducible signaling pathway protein 1	NM_003882	-7.18	0.0000
U3	Small nucleolar RNA U3	ENST00000390893	-7.14	0.0128
HIST1H2BM	histone cluster 1, H2bm	NM_003521	-6.92	0.0024
COL1A1	collagen, type I, alpha 1; NULL	NM_000088	-6.84	0.0000
HIST1H3	histone cluster 1, H3g; histone cluster 1, H3f; histone cluster 1, H3b; histone cluster 1, H3h; histone cluster 1, H3j; histone cluster 1, H3i; histone cluster 1, H3e; histone cluster 1, H3c; histone cluster 1, H3d; histone cluster 1, H3a	NM_003534	-6.68	0.0032

HIST1H3	histone cluster 1, H3f; histone cluster 1, H3b; histone cluster 1, H3h; histone cluster 1, H3j; histone cluster 1, H3g; histone cluster 1, H3i; histone cluster 1, H3e; histone cluster 1, H3c; histone cluster 1, H3d; histone cluster 1, H3a	NM_021018	-6.41	0.0058
AL732479.1	-	ENST00000459197	-6.38	0.0015
ADAM12	ADAM metalloproteinase domain 12; NULL	NM_003474;	-6.13	0.0001
ENPP1	ectonucleotide pyrophosphatase/phosphodiesterase 1	NM_006208	-6.06	0.0002
NDNF	neuron-derived neurotrophic factor	NM_024574	-6	0.0100
DHCR7	7-dehydrocholesterol reductase; NULL	NM_001360	-5.88	0.0005
DHCR24	24-dehydrocholesterol reductase	NM_014762	-5.84	0.0001
RGS4	regulator of G-protein signaling 4; NULL	NM_001102445	-5.78	0.0116
CRABP2	cellular retinoic acid binding protein 2	NM_001878	-5.76	0.0015
KIF20A	kinesin family member 20A; NULL	NM_005733	-5.6	0.0072
U1	U1 spliceosomal RNA	-	-5.38	0.0069

Table 5.3.2.2 Upregulated genes (unmapped by DAVID): MSC- γ vs. UT-MSC

Upregulated	Upregulated (continued)	Upregulated (continued)
P4-620F22.2	MIR2909	LOC100505573
MIR4442	LOC100653057	HIF1A-AS2
OTTHUMG00000164188	ZFAS1	Y_RNA.512-201
LOC101060720	AJUBA	LOC101060449
ARL14EP	LOC100505633	DLGAP1-AS2
RP11-44K6.2	RNF185-AS1	FAM86JP
HLA-DM	snoU13	AC089987.2
MIR4751	LRIF1	lnc-EXT1-2
RP1-102E24.8	RP4-791M13.3	LOC100505794
C2CD5	OTTHUMG00000169784	LOC100507419
LY75-CD302	LOC100507449	
AK123300	NPIPA5	
GBP1P1	LINC00265	
SLC35F6	CERS6	
MIR3614	CCP110	
ARHGEF28	MIR3689F	
XXbac-BPG116M5.17	LINC00965	
RNU7-40P	MIR3189	
ZFAND4	FOPNL	
OTTHUMG00000168357	LURAP1L	
NPIP3	RP11-274J7.2	
LOC100509976	RP11-638I2.8	
lnc-GLI3-1	RP11-468E2.5	
NPIPA1	NPIP5	
TMEM9B-AS1	LOC101060503	
lnc-ADCY9-1	PS1TP5	
RNU4-9P	AC108676.1	
RNU6-336P-201	RP11-44K6.4	
Y_RNA	HLA-DRB	
SMG1P1	SUZ12P1	
NPIP11	USP30-AS1	
LOC100507516	linc-SLC9A3-2	
AC023818.2	PSMG3-AS1	
LOC100506544	EPB41L4A-AS1	
OTTHUMG00000159600	lnc-TEKT1-1	
FOXN3-AS1	LGALS17A	
PLCE1-AS1	RP11-841O20.2	
OTTHUMG00000168751	AC005161.1	
GSAP	CTA-384D8.31	
RNU6-620P-201	HLA-DQA	

Table 5.3.2.3 Downregulated genes (unmapped by DAVID): MSC- γ vs. UT-MSC

Downregulated	Downregulated (continued)
RP11-94A24.1	RNU5E-1
AC093850.2	RNU5A-8P
U3	OSTCP2
NDNF	OTTHUMG00000165130
RNA5SP418	RP13-631K18.5
RP11-283C24.1	AC003968.1
LEPREL4	RP11-160E2.6
RN5S475	7SK
MIR323A	AL596092.1
ANKRD18CP	RP13-582L3.4
SNORD3	MIR4649
OTTHUMG00000154838	AC092143.2
Y_RNA	OTTHUMG00000175982
MMP24-AS1	AL732479.1
MIR4532	RN5S203
OTTHUMG00000165727	AC116154.1
U2	HIST1H3
U6	OTTHUMG00000170901
ADIRF	U1
U3	MSMO1
SNORA35	LOC100506188
KRTAP2	IFITM10
HIST1H4	OTTHUMG00000074741
AL121652.2	HIST1H2A
OTTHUMG00000178158	
AC108865.1	
RP11-939C17.2	
MBNL1-AS1	
LOC100505502	
AP001422.1	
AC125238.4	
snoU13	
PRADC1	
MIR4327	
RP4-541C22.4	
HIST2H3	
U1	
HIST1H2B	

Table 5.3.2.4 Functional annotation clustering - Gene Ontology Terms for Biological Processes: MSC- γ vs. UT-MSC (upregulated genes)

Term	Count	Genes	P-Value	Fold Enrichment
		Annotation Cluster 1 Enrichment Score: 8.03		
GO:0019882~antigen processing and presentation	25	HLA-DRB3, IFI30, HLA-DMB, CD74, B2M, TAP2, ERAP1, ERAP2, HLA-DPB1, HLA-DOA, ICAM1, HLA-L, HLA-A, HLA-C, HLA-B, HLA-E, HLA-DQA2, HLA-G, HLA-DQA1, PSMB9, HLA-F, PSME1, ULBP1, HLA-DPA1, TAPBPL, HLA-DRA	5.59E-17	9.37
GO:0048002~antigen processing and presentation of peptide antigen	14	HLA-A, IFI30, HLA-C, HLA-B, HLA-E, HLA-G, CD74, B2M, HLA-F, TAP2, ERAP1, ERAP2, HLA-DOA, TAPBPL, HLA-DRA	7.71E-13	15.55
GO:0002474~antigen processing and presentation of peptide antigen via MHC class I	10	TAP2, HLA-A, ERAP1, HLA-C, ERAP2, HLA-B, HLA-E, TAPBPL, HLA-G, B2M, HLA-F	6.42E-10	18.29
GO:0019883~antigen processing and presentation of endogenous antigen	5	TAP2, ERAP1, ERAP2, TAPBPL, CD74	6.60E-05	19.44
GO:0002483~antigen processing and presentation of endogenous peptide antigen	4	TAP2, ERAP1, ERAP2, TAPBPL	6.10E-04	20.73
GO:0019885~antigen processing and presentation of endogenous peptide antigen via MHC class I	4	TAP2, ERAP1, ERAP2, TAPBPL	6.10E-04	20.73
		Annotation Cluster 2 Enrichment Score: 6.06		
GO:0006952~defense response	55	TLR3, TLR4, CXCL11, CXCL10, HCP5, MX1, MX2, CIITA, SP100, C4A, LY96, C4B, SCUBE1, HLA-C, SERPING1, HLA-B, HLA-G, RIPK2, HLA-DRA, IFIH1, CCL2, NMI, C3, CLU, CXCL9, RSAD2, CCL8, CALCOCO2, C1R, IL32, C1S, CD74, GCH1, LGALS3BP, TAP2, BCL2, TAP1, C2, PTX3, ITK, IL18R1, IL6, CEBPB, CFB, CEBPG, SAMHD1, MALT1, IDO1, APOL2, DDX58, APOL3, CCL13, APOL1, NUPR1, CXCL16, C1RL	1.32E-11	2.78
GO:0045087~innate immune response	24	CIITA, IL18R1, IFIH1, SP100, C4A, CFB, C4B, C3, CEBPG, CLU, TLR3, CALCOCO2, SAMHD1, MALT1, SERPING1, C1R, TLR4, C1S, GCH1, DDX58, APOL1, CXCL16, C1RL, C2	7.52E-11	5.41
GO:0002252~immune effector process	21	ICAM1, IL6, NBN, C4A, CFB, C4B, C3, CEBPG, CLU, RSAD2, SAMHD1, SERPING1, C1R, C1S, CD74, TNFSF13B, BCL2, C1RL, C2, PTX3, HLA-DRA	9.55E-09	4.87
GO:0048584~positive regulation of response to stimulus	28	C3, IL6ST, CLU, TLR3, C1R, TLR4, C1S, TGFB2, B2M, EDNRA, TAP2, C2, IL6, C4A, CFB, C4B, CEBPG, EDA2R, MALT1, SERPING1, IDO1, TNFSF13B, HIPK2, VEGFA, C1RL, RIPK2, JAK2, HLA-DRA	9.91E-09	3.69
GO:0002684~positive regulation of immune system process	28	C3, IL6ST, CLU, TLR3, C1R, TLR4, C1S, CD74, B2M, TGFB2, CD47, TAP2, BCL6, C2, ICAM1, IL6, C4A, CFB, C4B, MALT1, SERPING1, IDO1, CBLB, TNFSF13B, VEGFA, C1RL, RIPK2, HLA-DRA	1.19E-08	3.66
GO:0002250~adaptive immune response	16	ICAM1, NBN, C4A, C3, C4B, CLU, SERPING1, C1R, TLR4, C1S, CD74, IL18BP, VEGFA, C1RL, C2, HLA-DRA	1.76E-08	6.46
GO:0002460~adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains	16	ICAM1, NBN, C4A, C3, C4B, CLU, SERPING1, C1R, TLR4, C1S, CD74, IL18BP, VEGFA, C1RL, C2, HLA-DRA	1.76E-08	6.46

GO:0050778~positive regulation of immune response	21	C4A, CFB, C3, IL6ST, C4B, CLU, TLR3, MALT1, SERPING1, C1R, TLR4, IDO1, C1S, B2M, TGFB2, TNFSF13B, TAP2, C1RL, RIPK2, C2, HLA-DRA	3.76E-08	4.50
GO:0006959~humoral immune response	15	PSMB10, IL6, CCL2, BST2, C4A, C3, C4B, CFB, CLU, SERPING1, C1R, C1S, BCL2, C1RL, C2	1.84E-07	5.90
GO:0002449~lymphocyte mediated immunity	14	ICAM1, NBN, C4A, C4B, C3, CEBPG, CLU, C1RL, C1R, SERPING1, C1S, C2, CD74, HLA-DRA	2.86E-07	6.22
GO:0002526~acute inflammatory response	16	IL6, CEBPB, C4A, C3, C4B, CFB, CLU, SERPING1, C1R, TLR4, IDO1, C1S, APOL2, NUPR1, C1RL, C2	4.92E-07	5.08
GO:0002443~leukocyte mediated immunity	15	ICAM1, NBN, IL6, C4A, C4B, C3, CEBPG, CLU, SERPING1, C1R, C1S, CD74, C1RL, C2, HLA-DRA	5.46E-07	5.42
GO:0006954~inflammatory response	30	NMI, CCL2, C3, CLU, CXCL9, TLR3, CCL8, C1R, TLR4, C1S, CXCL11, CXCL10, C2, PTX3, CIITA, IL6, CEBPB, C4A, C4B, LY96, CFB, SCUBE1, SERPING1, IDO1, APOL2, APOL3, CCL13, NUPR1, C1RL, RIPK2	6.59E-07	2.87
GO:0016064~immunoglobulin mediated immune response	12	NBN, C4A, C3, C4B, CLU, C1RL, C1R, SERPING1, C1S, C2, CD74, HLA-DRA	9.00E-07	6.91
GO:0019724~B cell mediated immunity	12	NBN, C4A, C3, C4B, CLU, C1RL, C1R, SERPING1, C1S, C2, CD74, HLA-DRA	1.32E-06	6.66
GO:0006958~complement activation, classical pathway	9	C4A, C3, C4B, CLU, C1RL, C1R, SERPING1, C1S, C2	2.50E-06	9.65
GO:0002455~humoral immune response mediated by circulating immunoglobulin	9	C4A, C3, C4B, CLU, C1RL, C1R, SERPING1, C1S, C2	4.33E-06	9.03
GO:0006956~complement activation	10	C4A, C3, CFB, C4B, CLU, C1RL, C1R, SERPING1, C1S, C2	5.76E-06	7.40
GO:0002541~activation of plasma proteins involved in acute inflammatory response	10	C4A, C3, CFB, C4B, CLU, C1RL, C1R, SERPING1, C1S, C2	7.08E-06	7.23
GO:0002253~activation of immune response	14	C4A, C3, C4B, CFB, CLU, TLR3, MALT1, SERPING1, C1R, TLR4, C1S, C1RL, RIPK2, C2	9.03E-06	4.63
GO:0009611~response to wounding	37	CCL2, NMI, C3, CLU, CXCL9, TLR3, CCL8, TLR4, C1R, C1S, CXCL11, MDK, CXCL10, TGFB2, MAP3K1, BCL2, C2, PTX3, FGF2, CIITA, IL6, CEBPB, C4A, LY96, C4B, CFB, SCUBE1, SERPING1, IDO1, APOL2, APOL3, PLSCR1, CCL13, NUPR1, C1RL, RIPK2, JAK2	1.78E-05	2.17
GO:0051605~protein maturation by peptide bond cleavage	10	C4A, C3, CFB, C4B, CLU, C1RL, C1R, SERPING1, C1S, C2	0.0017	3.62
GO:0016485~protein processing	10	C4A, C3, CFB, C4B, CLU, C1RL, C1R, SERPING1, C1S, C2	0.0099	2.78
GO:0006957~complement activation, alternative pathway	4	C4A, C3, CFB, C2	0.0112	8.29
GO:0051604~protein maturation	10	C4A, C3, CFB, C4B, CLU, C1RL, C1R, SERPING1, C1S, C2	0.0167	2.55
Annotation Cluster 3 Enrichment Score: 4.97				
GO:0002237~response to molecule of bacterial origin	16	IL6, CCL2, PTGS2, LY96, SOCS1, ADH5, MALT1, TIMP4, TLR4, IDO1, STAT1, B2M, GCH1, TAP2, RIPK2, VLDLR	8.34E-08	5.79
GO:0010033~response to organic substance	45	ADCY3, CYP1B1, CCL2, PTGS2, IL6ST, ADH5, TLR3, CALCOCO2, TLR4, ASNS, TIMP4, TRIM16, PMAIP1, C1S, GCH1, TGFB2, B2M, EDNRA, EIF4EBP1, GOT1, TAP2, BCL2, DNAJA1, IDH1, TXNIP, CIITA, IRS2, IL6, SPI00, LY96, CFB, SOCS1,	3.03E-05	1.94

GO:0032496~response to lipopolysaccharide	12	MALT1, IDO1, STAT1, DDIT3, ID1, CXCL16, ERN1, RIPK2, JAK2, NFE2L2, EIF2AK2, PPP1R15A, VLDLR	3.24E-05	4.85
GO:0009617~response to bacterium	18	CCL2, PTGS2, LY96, SOCS1, ADH5, RIPK2, TIMP4, TLR4, IDO1, STAT1, VLDLR, GCH1	1.62E-04	2.90
Annotation Cluster 4 Enrichment Score: 4.45				
GO:0002504~antigen processing and presentation of peptide or polysaccharide antigen via MHC class II	10	HLA-DRB3, IFI30, HLA-DPA1, HLA-DPB1, HLA-DMB, HLA-DOA, HLA-DQA2, CD74, HLA-DQA1, HLA-DRA	6.44E-07	9.42
GO:0019884~antigen processing and presentation of exogenous antigen	7	PSME1, TAP2, IFI30, HLA-DOA, CD74, HLA-DRA, B2M	2.54E-06	15.55
GO:0002478~antigen processing and presentation of exogenous peptide antigen	6	TAP2, IFI30, HLA-DOA, CD74, HLA-DRA, B2M	1.31E-05	16.96
GO:0002495~antigen processing and presentation of peptide antigen via MHC class II	4	IFI30, HLA-DOA, CD74, HLA-DRA	0.0016	15.55
GO:0019886~antigen processing and presentation of exogenous peptide antigen via MHC class II	4	IFI30, HLA-DOA, CD74, HLA-DRA	0.0016	15.55
Annotation Cluster 5 Enrichment Score: 3.78				
GO:0043067~regulation of programmed cell death	50	HTATIP2, PTGS2, CBX4, TLR4, PMAIP1, TGFB2, NQO1, MX1, FGF2, ALX3, ARHGEF2, AIFM2, IFI16, DDIT3, JMY, DAPK1, TNFRSF10B, TNFSF13B, HIPK2, VEGFA, ERN1, RIPK2, FOXC1, IFIH1, CCL2, CLU, ASNS, RRM2B, CD74, GCH1, SQSTM1, BCL2, MAP3K1, BCL6, HSPA9, TXNIP, CFLAR, IL6, CEBPB, CEBPG, SMAD6, MALT1, STRADB, IDO1, STAT1, TNFSF10, NUPR1, JAK2, IFI6, TP53INP1	1.40E-05	1.91
GO:0010941~regulation of cell death	50	HTATIP2, PTGS2, CBX4, TLR4, PMAIP1, TGFB2, NQO1, MX1, FGF2, ALX3, ARHGEF2, AIFM2, IFI16, DDIT3, JMY, DAPK1, TNFRSF10B, TNFSF13B, HIPK2, VEGFA, ERN1, RIPK2, FOXC1, IFIH1, CCL2, CLU, ASNS, RRM2B, CD74, GCH1, SQSTM1, BCL2, MAP3K1, BCL6, HSPA9, TXNIP, CFLAR, IL6, CEBPB, CEBPG, SMAD6, MALT1, STRADB, IDO1, STAT1, TNFSF10, NUPR1, JAK2, IFI6, TP53INP1	1.55E-05	1.91
GO:0043065~positive regulation of apoptosis	32	HTATIP2, PTGS2, TLR4, RRM2B, PMAIP1, GCH1, TGFB2, SQSTM1, MAP3K1, BCL2, BCL6, MX1, NQO1, TXNIP, CFLAR, ARHGEF2, CEBPB, AIFM2, CEBPG, IFI16, STAT1, DDIT3, JMY, DAPK1, TNFSF10, TNFRSF10B, NUPR1, HIPK2, ERN1, RIPK2, JAK2, TP53INP1	2.26E-05	2.31
GO:0042981~regulation of apoptosis	49	HTATIP2, PTGS2, CBX4, TLR4, PMAIP1, TGFB2, NQO1, MX1, ALX3, ARHGEF2, AIFM2, IFI16, DDIT3, JMY, DAPK1, TNFRSF10B, TNFSF13B, HIPK2, VEGFA, ERN1, RIPK2, FOXC1, IFIH1, CCL2, CLU, ASNS, RRM2B, CD74, GCH1, SQSTM1, BCL2, MAP3K1, BCL6, HSPA9, TXNIP, CFLAR, IL6, CEBPB, CEBPG, SMAD6, MALT1, STRADB, IDO1, STAT1, TNFSF10, NUPR1, JAK2, IFI6, TP53INP1	2.29E-05	1.90
GO:0043068~positive regulation of programmed cell death	32	HTATIP2, PTGS2, TLR4, RRM2B, PMAIP1, GCH1, TGFB2, SQSTM1, MAP3K1, BCL2, BCL6, MX1, NQO1, TXNIP, CFLAR, ARHGEF2, CEBPB, AIFM2, CEBPG, IFI16, STAT1, DDIT3, JMY, DAPK1, TNFSF10, TNFRSF10B, NUPR1, HIPK2, ERN1, RIPK2, JAK2, TP53INP1	2.59E-05	2.30

GO:0010942~positive regulation of cell death	32	HTATIP2, PTGS2, TLR4, RRM2B, PMAIP1, GCH1, TGFB2, SQSTM1, MAP3K1, BCL2, BCL6, MX1, NQO1, TXNIP, CFLAR, ARHGEF2, CEBPB, AIFM2, CEBPG, IFI16, STAT1, DDIT3, JMY, DAPK1, TNFSF10, TNFRSF10B, NUPR1, HIPK2, ERN1, RIPK2, JAK2, TP53INP1	2.82E-05	2.29
GO:0006916~anti-apoptosis	17	CFLAR, HTATIP2, CCL2, CEBPB, CLU, CBX4, MALT1, STRADB, DAPK1, TNFSF13B, SQSTM1, BCL2, VEGFA, RIPK2, FOXC1, IFI6, HSPA9	0.0010	2.57
GO:0043066~negative regulation of apoptosis	24	CFLAR, IL6, HTATIP2, CCL2, CEBPB, SMAD6, CLU, CBX4, MALT1, ASNS, IDO1, STRADB, CD74, DAPK1, TNFSF13B, SQSTM1, BCL2, HIPK2, VEGFA, RIPK2, FOXC1, BCL6, IFI6, HSPA9	0.0011	2.11
GO:0043069~negative regulation of programmed cell death	24	CFLAR, IL6, HTATIP2, CCL2, CEBPB, SMAD6, CLU, CBX4, MALT1, ASNS, IDO1, STRADB, CD74, DAPK1, TNFSF13B, SQSTM1, BCL2, HIPK2, VEGFA, RIPK2, FOXC1, BCL6, IFI6, HSPA9	0.0013	2.08
GO:0060548~negative regulation of cell death	24	CFLAR, IL6, HTATIP2, CCL2, CEBPB, SMAD6, CLU, CBX4, MALT1, ASNS, IDO1, STRADB, CD74, DAPK1, TNFSF13B, SQSTM1, BCL2, HIPK2, VEGFA, RIPK2, FOXC1, BCL6, IFI6, HSPA9	0.0014	2.07
GO:0006917~induction of apoptosis	22	CFLAR, ARHGEF2, HTATIP2, CEBPB, AIFM2, CEBPG, RRM2B, IFI16, PMAIP1, STAT1, GCH1, JMY, DAPK1, TNFSF10, TNFRSF10B, NUPR1, SQSTM1, HIPK2, ERN1, JAK2, MX1, TP53INP1	0.0016	2.14
GO:0012502~induction of programmed cell death	22	CFLAR, ARHGEF2, HTATIP2, CEBPB, AIFM2, CEBPG, RRM2B, IFI16, PMAIP1, STAT1, GCH1, JMY, DAPK1, TNFSF10, TNFRSF10B, NUPR1, SQSTM1, HIPK2, ERN1, JAK2, MX1, TP53INP1	0.0016	2.13
Annotation Cluster 6 Enrichment Score: 3.73				
GO:0043039~tRNA aminoacylation	11	IARS, WARS, YARS, CARS, NARS, ZNFX1, SARS, LARS, GARS, EPRS, MARS	1.51E-06	7.44
GO:0043038~amino acid activation	11	IARS, WARS, YARS, CARS, NARS, ZNFX1, SARS, LARS, GARS, EPRS, MARS	1.51E-06	7.44
GO:0006418~tRNA aminoacylation for protein translation	11	IARS, WARS, YARS, CARS, NARS, ZNFX1, SARS, LARS, GARS, EPRS, MARS	1.51E-06	7.44
GO:0006399~tRNA metabolic process	11	IARS, WARS, YARS, CARS, NARS, ZNFX1, SARS, LARS, GARS, EPRS, MARS	0.0046	2.90
GO:0006412~translation	18	YARS, CARS, NARS, ZNFX1, SARS, GARS, EPRS, RPL37, EIF1B, RPLP0P2, MRRF, IARS, WARS, RPL23AP32, EIF2S2, LARS, EIF2AK2, MARS	0.0387	1.69
Annotation Cluster 7 Enrichment Score: 3.62				
GO:0043388~positive regulation of DNA binding	13	ICAM1, IL6, SP100, NCOA3, CEBPG, HIPK2, TLR3, RIPK2, EDA2R, MALT1, JAK2, TLR4, JMY	2.01E-06	5.78
GO:0051091~positive regulation of transcription factor activity	12	ICAM1, IL6, SP100, NCOA3, CEBPG, TLR3, RIPK2, EDA2R, MALT1, JAK2, TLR4, JMY	2.71E-06	6.22
GO:0051090~regulation of transcription factor activity	15	ICAM1, IL6, SP100, CEBPG, EDA2R, TLR3, MALT1, EGLN1, TLR4, DDIT3, JMY, NCOA3, ID1, RIPK2, JAK2	5.04E-06	4.53
GO:0051099~positive regulation of binding	13	ICAM1, IL6, SP100, NCOA3, CEBPG, HIPK2, TLR3, RIPK2, EDA2R, MALT1, JAK2, TLR4, JMY	6.51E-06	5.18
GO:0051101~regulation of DNA binding	16	ICAM1, IL6, SP100, CEBPG, EDA2R, TLR3, MALT1, EGLN1, TLR4, DDIT3, JMY, NCOA3, ID1, HIPK2, RIPK2, JAK2	7.41E-06	4.11

GO:0051098~regulation of binding	17	ICAM1, IL6, SP100, CEBPG, TLR3, EDA2R, MALT1, EGLN1, TLR4, DDIT3, JMY, NCOA3, ID1, BCL2, HIPK2, RIPK2, JAK2	3.25E-05	3.46
GO:0051092~positive regulation of NF-kappaB transcription factor activity	7	ICAM1, IL6, TLR3, RIPK2, EDA2R, MALT1, TLR4	0.0018	5.31
GO:0001819~positive regulation of cytokine production	10	DDX58, IL6, IL6ST, TLR3, RIPK2, MALT1, JAK2, TRIM16, TLR4, IDO1	0.0024	3.46
GO:0043410~positive regulation of MAPKKK cascade	7	IL6, HIPK2, TLR3, RIPK2, EDA2R, TLR4, TGFB2	0.0041	4.54
GO:0043392~negative regulation of DNA binding	6	SP100, ID1, CEBPG, JAK2, EGLN1, DDIT3	0.0233	3.66
GO:0051100~negative regulation of binding	6	SP100, ID1, CEBPG, JAK2, EGLN1, DDIT3	0.0404	3.16
Annotation Cluster 8 Enrichment Score: 3.49				
GO:0010740~positive regulation of protein kinase cascade	18	CFLAR, SECTM1, IL6, BST2, IL6ST, TLR3, EDA2R, MALT1, TLR4, LGALS9, TGFB2, TRIM38, APOL3, TNFSF10, TNFRSF10B, HIPK2, RIPK2, JAK2	2.63E-05	3.35
GO:0010627~regulation of protein kinase cascade	21	SECTM1, CFLAR, IL6, BST2, IL6ST, SOCS1, TLR3, EDA2R, MALT1, TLR4, LGALS9, TGFB2, TRIM38, APOL3, TNFSF10, TNFRSF10B, SQSTM1, MAP3K1, HIPK2, RIPK2, JAK2	1.57E-04	2.62
GO:0010647~positive regulation of cell communication	25	CCL2, PTGS2, IL6ST, CSF1, TLR3, TRIM16, TLR4, JAG1, TGFB2, CFLAR, SECTM1, IL6, BST2, EDA2R, MALT1, LGALS9, TRIM38, APOL3, TNFSF10, TNFRSF10B, NCOA3, VEGFA, HIPK2, RIPK2, JAK2	1.60E-04	2.36
GO:0043122~regulation of I-kappaB kinase/NF-kappaB cascade	13	CFLAR, SECTM1, BST2, TLR3, TLR4, MALT1, LGALS9, TRIM38, APOL3, TNFSF10, TNFRSF10B, SQSTM1, RIPK2	1.61E-04	3.78
GO:0009967~positive regulation of signal transduction	23	SECTM1, CFLAR, IL6, BST2, IL6ST, CSF1, TLR3, EDA2R, MALT1, TRIM16, TLR4, JAG1, LGALS9, TGFB2, TRIM38, APOL3, TNFSF10, TNFRSF10B, NCOA3, HIPK2, VEGFA, RIPK2, JAK2	2.18E-04	2.42
GO:0043123~positive regulation of I-kappaB kinase/NF-kappaB cascade	12	TRIM38, APOL3, SECTM1, CFLAR, TNFSF10, TNFRSF10B, BST2, TLR3, RIPK2, MALT1, TLR4, LGALS9	2.71E-04	3.85
Annotation Cluster 9 Enrichment Score: 3.27				
GO:0008219~cell death	41	HTATIP2, CLU, TRIB3, PMAIP1, TGFB2, RRAGC, UNC5B, TRIM69, SQSTM1, MAP3K1, BCL2, XAF1, FGF2, RNF144B, CFLAR, ARHGEF2, YARS, IL6, AIFM2, KLF11, RYBP, GARS, OPTN, STAT1, ITPR1, DDIT4, DAPK1, TNFSF10, APOL1, TNFRSF10B, HIPK2, CYFIP2, ERN1, RIPK2, JAK2, EIF2AK2, DRAM1, PPP1R15A, GADD45A, IFI6, TP53INP1	4.86E-04	1.77
GO:0006915~apoptosis	36	HTATIP2, CLU, TRIB3, PMAIP1, RRAGC, UNC5B, TRIM69, SQSTM1, MAP3K1, BCL2, XAF1, FGF2, RNF144B, CFLAR, ARHGEF2, YARS, IL6, AIFM2, KLF11, RYBP, STAT1, DDIT4, DAPK1, TNFSF10, TNFRSF10B, HIPK2, CYFIP2, ERN1, RIPK2, JAK2, EIF2AK2, DRAM1, PPP1R15A, GADD45A, IFI6, TP53INP1	5.02E-04	1.86
GO:0016265~death	41	HTATIP2, CLU, TRIB3, PMAIP1, TGFB2, RRAGC, UNC5B, TRIM69, SQSTM1, MAP3K1, BCL2, XAF1, FGF2, RNF144B, CFLAR, ARHGEF2, YARS, IL6, AIFM2, KLF11, RYBP, GARS, OPTN, STAT1, ITPR1, DDIT4, DAPK1, TNFSF10, APOL1, TNFRSF10B, HIPK2, CYFIP2, ERN1, RIPK2, JAK2, EIF2AK2, DRAM1, PPP1R15A, GADD45A, IFI6, TP53INP1	5.45E-04	1.76

GO:0012501~programmed cell death	36	HTATIP2, CLU, TRIB3, PMAIP1, RRAGC, UNC5B, TRIM69, SQSTM1, MAP3K1, BCL2, XAF1, FGF2, RNF144B, CFLAR, ARHGEF2, YARS, IL6, AIFM2, KLF11, RYBP, STAT1, DDIT4, DAPK1, TNFSF10, TNFRSF10B, HIPK2, CYFIP2, ERN1, RIPK2, JAK2, EIF2AK2, DRAM1, PPP1R15A, GADD45A, IFI6, TP53INP1	6.53E-04	1.83
Annotation Cluster 10 Enrichment Score: 2.78				
GO:0051270~regulation of cell motion	19	ICAM1, PARD3, IL6, IRS2, SPI100, PTPRM, IL6ST, CSF1, JAG1, TGFB2, CXCL10, CXCL16, BCL2, MAP3K1, VEGFA, JAK2, BCL6, FGF2, IGFBP5	4.98E-05	3.06
GO:0051272~positive regulation of cell motion	13	ICAM1, IRS2, IL6, IL6ST, CSF1, TGFB2, CXCL10, CXCL16, BCL2, VEGFA, BCL6, JAK2, FGF2	6.83E-05	4.13
GO:0030335~positive regulation of cell migration	12	ICAM1, IRS2, IL6, IL6ST, CXCL16, BCL2, CSF1, VEGFA, JAK2, FGF2, TGFB2, CXCL10	1.25E-04	4.19
GO:0040017~positive regulation of locomotion	12	ICAM1, IRS2, IL6, IL6ST, CXCL16, BCL2, CSF1, VEGFA, JAK2, FGF2, TGFB2, CXCL10	2.97E-04	3.81
GO:0030334~regulation of cell migration	16	ICAM1, IRS2, IL6, PTPRM, IL6ST, CSF1, JAG1, TGFB2, CXCL10, CXCL16, BCL2, MAP3K1, VEGFA, JAK2, FGF2, IGFBP5	3.57E-04	2.94
GO:0040012~regulation of locomotion	16	ICAM1, IRS2, IL6, PTPRM, IL6ST, CSF1, JAG1, TGFB2, CXCL10, CXCL16, BCL2, MAP3K1, VEGFA, JAK2, FGF2, IGFBP5	0.0013	2.59
Annotation Cluster 11 Enrichment Score: 2.51				
GO:0051092~positive regulation of NF-kappaB transcription factor activity	7	ICAM1, IL6, TLR3, RIPK2, EDA2R, MALT1, TLR4	0.0018	5.31
GO:0001817~regulation of cytokine production	15	IL6, CEBPB, IL6ST, CEBPG, TLR3, MALT1, TLR4, TRIM16, IDO1, TGFB2, DDX58, IRF1, RIPK2, JAK2, BCL6	0.0021	2.58
GO:0031349~positive regulation of defense response	9	EDNRA, IL6, C3, IL6ST, TLR3, RIPK2, JAK2, TLR4, IDO1	0.0023	3.83
GO:0001819~positive regulation of cytokine production	10	DDX58, IL6, IL6ST, TLR3, RIPK2, MALT1, JAK2, TRIM16, TLR4, IDO1	0.0024	3.46
GO:0050865~regulation of cell activation	14	CD47, IL6, CBLB, TNFSF13B, IL6ST, CD274, RIPK2, MALT1, JAK2, TLR4, BCL6, IDO1, HLA-DOA, CD74	0.0042	2.49
GO:0050867~positive regulation of cell activation	10	CD47, IL6, TNFSF13B, IL6ST, RIPK2, MALT1, BCL6, JAK2, TLR4, CD74	0.0094	2.80
Annotation Cluster 12 Enrichment Score: 2.38				
GO:0009069~serine family amino acid metabolic process	7	CTH, SEPHS2, SHMT2, PHGDH, DMGDH, PSAT1, PSPH	1.40E-04	8.37
GO:0009070~serine family amino acid biosynthetic process	5	CTH, SEPHS2, PHGDH, PSAT1, PSPH	2.88E-04	14.14
GO:0009309~amine biosynthetic process	10	CTH, SEPHS2, GOT1, ASS1, PHGDH, ASNS, PSAT1, PSPH, GCH1, TGFB2	0.0011	3.84
GO:0008652~cellular amino acid biosynthetic process	8	CTH, SEPHS2, GOT1, ASS1, PHGDH, ASNS, PSAT1, PSPH	0.0011	4.88
GO:0006563~L-serine metabolic process	4	SHMT2, PHGDH, PSAT1, PSPH	0.0016	15.55
GO:0006564~L-serine biosynthetic process	3	PHGDH, PSAT1, PSPH	0.0030	31.10

GO:0016053~organic acid biosynthetic process	13	SEPHS2, CTH, GOT1, PTGS2, PTGDS, ASS1, PHGDH, LIAS, ASNS, IDO1, PSAT1, PSPH, CD74	0.0042	2.61
GO:0046394~carboxylic acid biosynthetic process	13	SEPHS2, CTH, GOT1, PTGS2, PTGDS, ASS1, PHGDH, LIAS, ASNS, IDO1, PSAT1, PSPH, CD74	0.0042	2.61
GO:0044271~nitrogen compound biosynthetic process	18	ADCY3, MOCOS, NAMPT, SEPHS2, PRTFDC1, ASS1, ASNS, RRM2B, ATP6V1B2, PSPH, GCH1, CMPK2, TGFB2, CTH, GOT1, PHGDH, PSAT1, NQO1	0.0334	1.72
Annotation Cluster 13 Enrichment Score: 2.00				
GO:0034620~cellular response to unfolded protein	5	ERN1, NFE2L2, EIF2AK2, PPP1R15A, DDIT3	0.0041	7.40
GO:0030968~endoplasmic reticulum unfolded protein response	5	ERN1, NFE2L2, EIF2AK2, PPP1R15A, DDIT3	0.0041	7.40
GO:0034976~response to endoplasmic reticulum stress	6	ERN1, NFE2L2, EIF2AK2, FAM129A, PPP1R15A, DDIT3	0.0043	5.49
GO:0051789~response to protein stimulus	9	ID1, CFB, BCL2, DNAJA1, ERN1, NFE2L2, EIF2AK2, PPP1R15A, DDIT3	0.0217	2.62
GO:0006984~ER-nuclear signaling pathway	5	ERN1, NFE2L2, EIF2AK2, PPP1R15A, DDIT3	0.0249	4.44
GO:0006986~response to unfolded protein	7	CFB, DNAJA1, ERN1, NFE2L2, EIF2AK2, PPP1R15A, DDIT3	0.0261	3.07
Annotation Cluster 14 Enrichment Score: 1.99				
GO:0008637~apoptotic mitochondrial changes	6	AIFM2, MAP3K1, BCL2, CLU, PMAIP1, IFI6	0.0028	6.02
GO:0007005~mitochondrion organization	11	GRPEL2, LONP1, AIFM2, EPAS1, MTX3, MAP3K1, BCL2, CLU, RRM2B, PMAIP1, IFI6	0.0134	2.48
GO:0001836~release of cytochrome c from mitochondria	4	BCL2, CLU, PMAIP1, IFI6	0.0284	5.92
Annotation Cluster 15 Enrichment Score: 1.94				
GO:0044093~positive regulation of molecular function	35	PSMB10, ADCY3, PARD3, CSF1, TLR3, KITLG, TLR4, PMAIP1, CD74, GCH1, TGFB2, EDNRA, MAP3K1, BCL2, FGF2, ICAM1, IL6, SP100, CEBPG, EDA2R, MALT1, STRADB, STAT1, JMY, PSMB9, TNFRSF10B, PSME1, NCOA3, PSME2, PSMA4, HIPK2, ERN1, RIPK2, JAK2, VLDLR	6.22E-04	1.86
GO:0032147~activation of protein kinase activity	11	EDNRA, ADCY3, PARD3, TNFRSF10B, MAP3K1, TLR3, MALT1, JAK2, STRADB, FGF2, TGFB2	0.0036	3.00
GO:0045860~positive regulation of protein kinase activity	16	ADCY3, PARD3, CSF1, TLR3, KITLG, MALT1, STRADB, CD74, TGFB2, EDNRA, TNFRSF10B, MAP3K1, ERN1, JAK2, FGF2, VLDLR	0.0056	2.23
GO:0033674~positive regulation of kinase activity	16	ADCY3, PARD3, CSF1, TLR3, KITLG, MALT1, STRADB, CD74, TGFB2, EDNRA, TNFRSF10B, MAP3K1, ERN1, JAK2, FGF2, VLDLR	0.0077	2.15
GO:0042325~regulation of phosphorylation	26	ADCY3, PARD3, IL6ST, CSF1, TLR3, TRIB3, KITLG, TLR4, CD74, TGFB2, EDNRA, BCL2, MAP3K1, FAM129A, FGF2, IL6, SMAD6, SOCS1, MALT1, STRADB, TNFRSF10B, ERN1, JAK2, GADD45A, VLDLR, DUSP6	0.0085	1.74
GO:0051347~positive regulation of transferase activity	16	ADCY3, PARD3, CSF1, TLR3, KITLG, MALT1, STRADB, CD74, TGFB2, EDNRA, TNFRSF10B, MAP3K1, ERN1, JAK2, FGF2, VLDLR	0.0107	2.07

GO:0019220~regulation of phosphate metabolic process	26	ADCY3, PARD3, IL6ST, CSF1, TLR3, TRIB3, KITLG, TLR4, CD74, TGFB2, EDNRA, BCL2, MAP3K1, FAM129A, FGF2, IL6, SMAD6, SOCS1, MALT1, STRADB, TNFRSF10B, ERN1, JAK2, GADD45A, VLDLR, DUSP6	0.0138	1.67
GO:0051174~regulation of phosphorus metabolic process	26	ADCY3, PARD3, IL6ST, CSF1, TLR3, TRIB3, KITLG, TLR4, CD74, TGFB2, EDNRA, BCL2, MAP3K1, FAM129A, FGF2, IL6, SMAD6, SOCS1, MALT1, STRADB, TNFRSF10B, ERN1, JAK2, GADD45A, VLDLR, DUSP6	0.0138	1.67
GO:0045859~regulation of protein kinase activity	19	ADCY3, PARD3, CSF1, TLR3, TRIB3, KITLG, MALT1, STRADB, CD74, TGFB2, EDNRA, TNFRSF10B, MAP3K1, ERN1, JAK2, FGF2, GADD45A, VLDLR, DUSP6	0.0299	1.71
GO:0043549~regulation of kinase activity	19	ADCY3, PARD3, CSF1, TLR3, TRIB3, KITLG, MALT1, STRADB, CD74, TGFB2, EDNRA, TNFRSF10B, MAP3K1, ERN1, JAK2, FGF2, GADD45A, VLDLR, DUSP6	0.0398	1.66
GO:0043085~positive regulation of catalytic activity	25	PSMB10, ADCY3, PARD3, CSF1, TLR3, KITLG, MALT1, PMAIP1, STRADB, STAT1, CD74, GCH1, TGFB2, PSMB9, EDNRA, TNFRSF10B, PSME1, PSME2, BCL2, PSMA4, MAP3K1, ERN1, JAK2, FGF2, VLDLR	0.0485	1.50
Annotation Cluster 16 Enrichment Score: 1.91				
GO:0048872~homeostasis of number of cells	11	IL6, TNFSF13B, EPAS1, BCL2, CSF1, CEBPG, VPS54, VEGFA, DYRK3, BCL6, TCEA1	0.0014	3.42
GO:0030097~hemopoiesis	17	EPAS1, CEBPG, CSF1, KITLG, MALT1, IFI16, JAG1, CD74, TGFB2, FLT3LG, BCL2, VEGFA, IRF1, JAK2, TCEA1, BCL6, DYRK3	0.0040	2.24
GO:0002520~immune system development	18	NBN, EPAS1, CEBPG, CSF1, KITLG, MALT1, IFI16, JAG1, CD74, TGFB2, FLT3LG, BCL2, VEGFA, IRF1, JAK2, TCEA1, BCL6, DYRK3	0.0079	2.03
GO:0048534~hemopoietic or lymphoid organ development	17	EPAS1, CEBPG, CSF1, KITLG, MALT1, IFI16, JAG1, CD74, TGFB2, FLT3LG, BCL2, VEGFA, IRF1, JAK2, TCEA1, BCL6, DYRK3	0.0099	2.03
GO:0030099~myeloid cell differentiation	9	EPAS1, CSF1, CEBPG, VEGFA, DYRK3, BCL6, JAK2, TCEA1, IFI16	0.0099	3.01
GO:0030218~erythrocyte differentiation	6	EPAS1, CEBPG, VEGFA, DYRK3, BCL6, TCEA1	0.0117	4.34
GO:0034101~erythrocyte homeostasis	6	EPAS1, CEBPG, VEGFA, DYRK3, BCL6, TCEA1	0.0199	3.81
Annotation Cluster 17 Enrichment Score: 1.82				
GO:0043388~positive regulation of DNA binding	13	ICAM1, IL6, SP100, NCOA3, CEBPG, HIPK2, TLR3, RIPK2, EDA2R, MALT1, JAK2, TLR4, JMY	2.01E-06	5.78
GO:0051091~positive regulation of transcription factor activity	12	ICAM1, IL6, SP100, NCOA3, CEBPG, TLR3, RIPK2, EDA2R, MALT1, JAK2, TLR4, JMY	2.71E-06	6.22
GO:0051099~positive regulation of binding	13	ICAM1, IL6, SP100, NCOA3, CEBPG, HIPK2, TLR3, RIPK2, EDA2R, MALT1, JAK2, TLR4, JMY	6.51E-06	5.18
GO:0070304~positive regulation of stress-activated protein kinase signaling pathway	6	HIPK2, TLR3, RIPK2, EDA2R, TLR4, TGFB2	4.42E-04	8.89
GO:0046330~positive regulation of JNK cascade	5	HIPK2, TLR3, RIPK2, EDA2R, TLR4	0.0018	9.15
GO:0051092~positive regulation of NF-kappaB transcription factor activity	7	ICAM1, IL6, TLR3, RIPK2, EDA2R, MALT1, TLR4	0.0018	5.31
GO:0031349~positive regulation of defense response	9	EDNRA, IL6, C3, IL6ST, TLR3, RIPK2, JAK2, TLR4, IDO1	0.0023	3.83

GO:0043410~positive regulation of MAPKKK cascade	7	IL6, HIPK2, TLR3, RIPK2, EDA2R, TLR4, TGFB2	0.0041	4.54
GO:0032755~positive regulation of interleukin-6 production	4	IL6, TLR3, RIPK2, TLR4	0.0217	6.55
GO:0070302~regulation of stress-activated protein kinase signaling pathway	7	MAP3K1, HIPK2, TLR3, RIPK2, EDA2R, TLR4, TGFB2	0.0231	3.15
GO:0032675~regulation of interleukin-6 production	5	IL6, CEBPB, TLR3, RIPK2, TLR4	0.0274	4.32
GO:0045088~regulation of innate immune response	6	SAMHD1, ERAP1, TLR3, RIPK2, SERPING1, TLR4	0.0290	3.46
GO:0032874~positive regulation of stress-activated MAPK cascade	3	RIPK2, TLR4, TGFB2	0.0318	10.37
GO:0032872~regulation of stress-activated MAPK cascade	3	RIPK2, TLR4, TGFB2	0.0390	9.33
GO:0080135~regulation of cellular response to stress	8	MAP3K1, CEBPG, HIPK2, TLR3, RIPK2, EDA2R, TLR4, TGFB2	0.0477	2.42

Annotation Cluster 18 Enrichment Score: 1.77

GO:0007259~JAK-STAT cascade	8	NMI, CCL2, IL6ST, SOCS1, PKD2, JAK2, STAT1, STAT2	2.11E-04	6.38
GO:0050729~positive regulation of inflammatory response	7	EDNRA, IL6, C3, IL6ST, JAK2, TLR4, IDO1	3.25E-04	7.26
GO:0050727~regulation of inflammatory response	10	EDNRA, IL6, PTGS2, C3, IL6ST, BCL6, SERPING1, JAK2, TLR4, IDO1	7.03E-04	4.09
GO:0031349~positive regulation of defense response	9	EDNRA, IL6, C3, IL6ST, TLR3, RIPK2, JAK2, TLR4, IDO1	0.0023	3.83
GO:0032103~positive regulation of response to external stimulus	8	EDNRA, IL6, C3, IL6ST, VEGFA, JAK2, TLR4, IDO1	0.0043	3.89
GO:0051247~positive regulation of protein metabolic process	17	PSMB10, IL6, IL6ST, CSF1, KITLG, TLR4, PSMB9, EDNRA, CBLB, PSME1, PSME2, BCL2, PSMA4, MDM2, JAK2, FAM129A, FGF2	0.0053	2.18
GO:0031401~positive regulation of protein modification process	14	PSMB10, IL6, IL6ST, KITLG, TLR4, PSMB9, EDNRA, PSME1, PSME2, PSMA4, BCL2, JAK2, FAM129A, FGF2	0.0073	2.33
GO:0001934~positive regulation of protein amino acid phosphorylation	9	EDNRA, IL6, IL6ST, BCL2, KITLG, JAK2, TLR4, FAM129A, FGF2	0.0077	3.14
GO:0032270~positive regulation of cellular protein metabolic process	16	PSMB10, IL6, IL6ST, CSF1, KITLG, TLR4, PSMB9, EDNRA, PSME1, PSME2, BCL2, PSMA4, MDM2, JAK2, FAM129A, FGF2	0.0084	2.14
GO:0042325~regulation of phosphorylation	26	ADCY3, PARD3, IL6ST, CSF1, TLR3, TRIB3, KITLG, TLR4, CD74, TGFB2, EDNRA, BCL2, MAP3K1, FAM129A, FGF2, IL6, SMAD6, SOCS1, MALT1, STRADB, TNFRSF10B, ERN1, JAK2, GADD45A, VLDLR, DUSP6	0.0085	1.74
GO:0042327~positive regulation of phosphorylation	9	EDNRA, IL6, IL6ST, BCL2, KITLG, JAK2, TLR4, FAM129A, FGF2	0.0126	2.89
GO:0019220~regulation of phosphate metabolic process	26	ADCY3, PARD3, IL6ST, CSF1, TLR3, TRIB3, KITLG, TLR4, CD74, TGFB2, EDNRA, BCL2, MAP3K1, FAM129A, FGF2, IL6, SMAD6, SOCS1, MALT1, STRADB, TNFRSF10B, ERN1, JAK2, GADD45A, VLDLR, DUSP6	0.0138	1.67

GO:0051174~regulation of phosphorus metabolic process	26	ADCY3, PARD3, IL6ST, CSF1, TLR3, TRIB3, KITLG, TLR4, CD74, TGFB2, EDNRA, BCL2, MAP3K1, FAM129A, FGF2, IL6, SMAD6, SOCS1, MALT1, STRADB, TNFRSF10B, ERN1, JAK2, GADD45A, VLDLR, DUSP6	0.0138	1.67
GO:0045937~positive regulation of phosphate metabolic process	9	EDNRA, IL6, IL6ST, BCL2, KITLG, JAK2, TLR4, FAM129A, FGF2	0.0150	2.80
GO:0010562~positive regulation of phosphorus metabolic process	9	EDNRA, IL6, IL6ST, BCL2, KITLG, JAK2, TLR4, FAM129A, FGF2	0.0150	2.80
GO:0042516~regulation of tyrosine phosphorylation of Stat3 protein	4	IL6, IL6ST, SOCS1, JAK2	0.0160	7.32
GO:0032268~regulation of cellular protein metabolic process	25	PSMB10, IL6ST, CSF1, MKNK2, KITLG, TLR4, EDNRA, EIF4EBP1, BCL2, MAP3K1, FAM129A, FGF2, IL6, SMAD6, SOCS1, SERPING1, EIF1B, PSMB9, PSME1, PSME2, PSMA4, MDM2, JAK2, PPP1R15A, IGFBP5	0.0189	1.64
GO:0001932~regulation of protein amino acid phosphorylation	12	EDNRA, IL6, IL6ST, MAP3K1, BCL2, SMAD6, SOCS1, KITLG, JAK2, TLR4, FAM129A, FGF2	0.0238	2.16
GO:0002673~regulation of acute inflammatory response	4	IL6, C3, IL6ST, SERPING1	0.0284	5.92
GO:0031399~regulation of protein modification process	17	PSMB10, IL6, IL6ST, SMAD6, SOCS1, KITLG, TLR4, PSMB9, EDNRA, PSME1, PSME2, BCL2, PSMA4, MAP3K1, JAK2, FAM129A, FGF2	0.0288	1.79
GO:0032101~regulation of response to external stimulus	11	EDNRA, IL6, PTGS2, C3, IL6ST, VEGFA, BCL6, SERPING1, JAK2, TLR4, IDO1	0.0323	2.15
GO:0042517~positive regulation of tyrosine phosphorylation of Stat3 protein	3	IL6, IL6ST, JAK2	0.0466	8.48
Annotation Cluster 19 Enrichment Score: 1.74				
GO:0007179~transforming growth factor beta receptor signaling pathway	7	FMOD, CCL2, ID1, MAP3K1, SMAD6, GDF15, TGFB2	0.0122	3.63
GO:0007178~transmembrane receptor protein serine/threonine kinase signaling pathway	9	FMOD, CCL2, ID1, MAP3K1, SMAD6, HIPK2, FST, GDF15, TGFB2	0.0177	2.72
GO:0007167~enzyme linked receptor protein signaling pathway	19	TXNIP, FMOD, IRS2, CCL2, IL6ST, SMAD6, ARID5B, FST, TGFB2, EPS15, EIF4EBP1, ID1, MAP3K1, HIPK2, VEGFA, JAK2, FOXC1, GDF15, FGF2	0.0277	1.73
Annotation Cluster 20 Enrichment Score: 1.66				
GO:0001666~response to hypoxia	12	EDNRA, LONP1, CCL2, EPAS1, BCL2, VEGFA, CAMK2D, EGLN1, ITPR1, VLDLR, DDIT4, TGFB2	0.0039	2.78
GO:0070482~response to oxygen levels	12	EDNRA, LONP1, CCL2, EPAS1, BCL2, VEGFA, CAMK2D, EGLN1, ITPR1, VLDLR, DDIT4, TGFB2	0.0057	2.65
Annotation Cluster 21 Enrichment Score: 1.61				
GO:0002821~positive regulation of adaptive immune response	7	TNFSF13B, C3, IL6ST, TAP2, MALT1, IDO1, B2M	3.92E-04	7.02
GO:0002819~regulation of adaptive immune response	8	TNFSF13B, C3, IL6ST, TAP2, MALT1, BCL6, IDO1, B2M	0.0020	4.44
GO:0002824~positive regulation of adaptive immune response based on somatic recombination	6	TNFSF13B, C3, TAP2, MALT1, IDO1, B2M	0.0024	6.22

of immune receptors built from immunoglobulin superfamily domains				
GO:0002822~regulation of adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains	7	TNFSF13B, C3, TAP2, MALT1, BCL6, IDO1, B2M	0.0081	3.96
GO:0002697~regulation of immune effector process	9	DDX58, ICAM1, IL6, C3, TAP2, MALT1, BCL6, SERPING1, B2M	0.0158	2.77

Table 5.3.2.5 Functional annotation clustering-Gene Ontology Terms for Biological Processes: MSC- γ vs. UT-MSC (downregulated genes)

Term	Count	Genes	P-Value	Fold Enrichment
Annotation Cluster 1 Enrichment Score: 11.10				
GO:0030198~extracellular matrix organization	17	RECK, LUM, COL3A1, ELN, CCDC80, DCN, COL5A2, COL5A1, EMILIN1, COL14A1, P4HA1, TGFBI, COL1A2, COL12A1, COL1A1, COL11A1, ADAMTS2	4.73E-13	12.22
GO:0030199~collagen fibril organization	11	COL14A1, P4HA1, LUM, COL3A1, COL1A2, COL12A1, COL1A1, COL5A2, COL11A1, ADAMTS2, COL5A1	2.18E-12	28.35
GO:0043062~extracellular structure organization	17	RECK, LUM, COL3A1, ELN, CCDC80, DCN, COL5A2, COL5A1, EMILIN1, COL14A1, P4HA1, TGFBI, COL1A2, COL12A1, COL1A1, COL11A1, ADAMTS2	5.00E-10	7.80
Annotation Cluster 2 Enrichment Score: 8.80				
GO:0000279~M phase	25	KIFC1, PRC1, TUBB2A, ANLN, AURKB, SPC24, CDCA8, NCAPH, HSPA2, CCNA2, TRIP13, CDC6, KIF11, MKI67, DLGAP5, CCNF, TPX2, BIRC5, CDC20, PBK, UBE2C, CDK2, RAD51, CCNB1, PLK1	1.11E-11	5.68
GO:0000280~nuclear division	20	CDC6, KIFC1, KIF11, TUBB2A, DLGAP5, CCNF, TPX2, CDC20, ANLN, BIRC5, AURKB, PBK, UBE2C, CDK2, SPC24, CCNB1, NCAPH, CDCA8, PLK1, CCNA2	1.02E-10	6.79
GO:0007067~mitosis	20	CDC6, KIFC1, KIF11, TUBB2A, DLGAP5, CCNF, TPX2, CDC20, ANLN, BIRC5, AURKB, PBK, UBE2C, CDK2, SPC24, CCNB1, NCAPH, CDCA8, PLK1, CCNA2	1.02E-10	6.79
GO:0000087~M phase of mitotic cell cycle	20	CDC6, KIFC1, KIF11, TUBB2A, DLGAP5, CCNF, TPX2, CDC20, ANLN, BIRC5, AURKB, PBK, UBE2C, CDK2, SPC24, CCNB1, NCAPH, CDCA8, PLK1, CCNA2	1.40E-10	6.67
GO:0048285~organelle fission	20	CDC6, KIFC1, KIF11, TUBB2A, DLGAP5, CCNF, TPX2, CDC20, ANLN, BIRC5, AURKB, PBK, UBE2C, CDK2, SPC24, CCNB1, NCAPH, CDCA8, PLK1, CCNA2	2.04E-10	6.53
GO:0022403~cell cycle phase	26	KIFC1, PRC1, TUBB2A, ANLN, AURKB, GTSE1, SPC24, NCAPH, CDCA8, HSPA2, CCNA2, TRIP13, CDC6, KIF11, MKI67, DLGAP5, CCNF, TPX2, BIRC5, CDC20, PBK, UBE2C, CDK2, RAD51, CCNB1, PLK1	2.35E-10	4.69
GO:0022402~cell cycle process	28	KIFC1, PRC1, TUBB2A, ANLN, AURKB, CALR, GTSE1, SPC24, CDCA8, NCAPH, HSPA2, CCNA2, TRIP13, DHCR24, CDC6, KIF11, MKI67, DLGAP5, CCNF, TPX2, BIRC5, CDC20, PBK, UBE2C, CDK2, RAD51, CCNB1, PLK1	7.19E-09	3.70
GO:0000278~mitotic cell cycle	22	CDC6, KIFC1, KIF11, PRC1, TUBB2A, DLGAP5, CCNF, TPX2, CDC20, ANLN, BIRC5, AURKB, PBK, UBE2C, CDK2, GTSE1, SPC24, CCNB1, NCAPH, CDCA8, PLK1, CCNA2	2.14E-08	4.44
GO:0007049~cell cycle	30	KIFC1, PRC1, TUBB2A, FOXM1, ANLN, AURKB, CALR, GTSE1, SPC24, CDCA8, NCAPH, HSPA2, HJURP, CCNA2, DHCR24, TRIP13, CDC6, KIF11, MKI67, DLGAP5, CCNF, TPX2, BIRC5, CDC20, PBK, UBE2C, CDK2, RAD51, CCNB1, PLK1	3.98E-07	2.89
GO:0051301~cell division	17	KIFC1, CDC6, KIF11, PRC1, CCNF, CDC20, ANLN, BIRC5, AURKB, UBE2C, CDK2, SPC24, CCNB1, NCAPH, CDCA8, PLK1, CCNA2	2.04E-06	4.31
Annotation Cluster 3 Enrichment Score: 4.20				
GO:0030199~collagen fibril organization	11	COL14A1, P4HA1, LUM, COL3A1, COL1A2, COL12A1, COL1A1, COL5A2, COL11A1, ADAMTS2, COL5A1	2.18E-12	28.35
GO:0043588~skin development	7	COL3A1, COL1A2, COL1A1, COL5A2, ADAMTS2, COL5A1, DHCR24	1.88E-06	18.04
GO:0008544~epidermis development	11	FLG, CRABP2, KRT14, COL3A1, COL1A2, COL1A1, COL5A2, SNAI1, ADAMTS2, COL5A1, DHCR24	1.75E-04	4.47

GO:0007398~ectoderm development	11	FLG, CRABP2, KRT14, COL3A1, COL1A2, COL1A1, COL5A2, SNAI1, ADAMTS2, COL5A1, DHCR24	3.29E-04	4.13
GO:0032964~collagen biosynthetic process	3	COL3A1, COL1A1, COL5A1	0.001715	44.84
GO:0032963~collagen metabolic process	4	COL3A1, COL1A1, ADAMTS2, COL5A1	0.005943	10.68
GO:0044259~multicellular organismal macromolecule metabolic process	4	COL3A1, COL1A1, ADAMTS2, COL5A1	0.00792	9.64
GO:0044236~multicellular organismal metabolic process	4	COL3A1, COL1A1, ADAMTS2, COL5A1	0.012918	8.08
Annotation Cluster 4 Enrichment Score: 3.46				
GO:0016126~sterol biosynthetic process	7	EBP, MVD, HMGCR, SQLE, DHCR7, HMGCS1, DHCR24	6.01E-06	14.95
GO:0006695~cholesterol biosynthetic process	6	EBP, MVD, HMGCR, DHCR7, HMGCS1, DHCR24	2.07E-05	17.25
GO:0008203~cholesterol metabolic process	9	SREBF1, EBP, LDLR, MVD, HMGCR, SQLE, DHCR7, HMGCS1, DHCR24	3.02E-05	7.31
GO:0016125~sterol metabolic process	9	SREBF1, EBP, LDLR, MVD, HMGCR, SQLE, DHCR7, HMGCS1, DHCR24	5.93E-05	6.66
GO:0008610~lipid biosynthetic process	15	EBP, MVD, HMGCR, SCD, HMGCS1, FADS2, ACLY, LPCAT3, PLAUR, SQLE, ANG, DHCR7, FASN, DHCR24, PC	1.07E-04	3.47
GO:0006694~steroid biosynthetic process	7	EBP, MVD, HMGCR, SQLE, DHCR7, HMGCS1, DHCR24	9.36E-04	6.16
GO:0008202~steroid metabolic process	9	SREBF1, EBP, LDLR, MVD, HMGCR, SQLE, DHCR7, HMGCS1, DHCR24	0.005649	3.33
GO:0006720~isoprenoid metabolic process	4	MVD, HMGCR, CRABP2, HMGCS1	0.020584	6.79
GO:0008299~isoprenoid biosynthetic process	3	MVD, HMGCR, HMGCS1	0.028588	11.21
Annotation Cluster 5 Enrichment Score: 2.76				
GO:0006323~DNA packaging	10	HIST1H2BO, HIST2H2AB, NCAPH, HIST1H2BM, HIST1H1C, HJURP, HIST1H1B, HIST1H2AJ, ASF1B, TOP2A	2.58E-05	6.39
GO:0006334~nucleosome assembly	8	HIST1H2BO, HIST2H2AB, HIST1H2BM, HIST1H1C, HJURP, HIST1H1B, HIST1H2AJ, ASF1B	1.26E-04	7.12
GO:0031497~chromatin assembly	8	HIST1H2BO, HIST2H2AB, HIST1H2BM, HIST1H1C, HJURP, HIST1H1B, HIST1H2AJ, ASF1B	1.57E-04	6.87
GO:0065004~protein-DNA complex assembly	8	HIST1H2BO, HIST2H2AB, HIST1H2BM, HIST1H1C, HJURP, HIST1H1B, HIST1H2AJ, ASF1B	2.08E-04	6.57
GO:0034728~nucleosome organization	8	HIST1H2BO, HIST2H2AB, HIST1H2BM, HIST1H1C, HJURP, HIST1H1B, HIST1H2AJ, ASF1B	2.38E-04	6.43
GO:0034622~cellular macromolecular complex assembly	14	HIST1H2BO, HIST2H2AB, HIST1H2BM, HIST1H1C, ANG, TUBB2A, HJURP, HIST1H1B, ANLN, HIST1H2AJ, CALR, ASF1B, TUBA1A, TUBA1B	3.34E-04	3.29
GO:0034621~cellular macromolecular complex subunit organization	14	HIST1H2BO, HIST2H2AB, HIST1H2BM, HIST1H1C, ANG, TUBB2A, HJURP, HIST1H1B, ANLN, HIST1H2AJ, CALR, ASF1B, TUBA1A, TUBA1B	9.85E-04	2.93

GO:0065003~macromolecular complex assembly	20	HIST1H1C, HIST1H1B, TUBB2A, ANLN, CALR, SOD2, RAD51, JUP, HIST1H2BO, HIST2H2AB, HIST1H2BM, ANG, HJURP, RRM2, CDA, QPRT, HIST1H2AJ, TUBA1A, ASF1B, TUBA1B	0.001388	2.25
GO:0006333~chromatin assembly or disassembly	8	HIST1H2BO, HIST2H2AB, HIST1H2BM, HIST1H1C, HJURP, HIST1H1B, HIST1H2AJ, ASF1B	0.001537	4.71
GO:0043933~macromolecular complex subunit organization	20	HIST1H1C, HIST1H1B, TUBB2A, ANLN, CALR, SOD2, RAD51, JUP, HIST1H2BO, HIST2H2AB, HIST1H2BM, ANG, HJURP, RRM2, CDA, QPRT, HIST1H2AJ, TUBA1A, ASF1B, TUBA1B	0.002907	2.11
GO:0051276~chromosome organization	13	KIFC1, HIST1H1C, HIST1H1B, DLGAP5, HIST1H2BO, HIST2H2AB, CDCA8, HIST1H2BM, NCAPH, HJURP, HIST1H2AJ, ASF1B, TOP2A	0.028839	2.00
Annotation Cluster 6 Enrichment Score: 2.38				
GO:0001568~blood vessel development	12	PLAT, RECK, ANG, DHCR7, ITGAV, COL3A1, ITGA7, COL1A2, RHOB, COL1A1, COL5A1, THY1	4.41E-04	3.66
GO:0001944~vasculature development	12	PLAT, RECK, ANG, DHCR7, ITGAV, COL3A1, ITGA7, COL1A2, RHOB, COL1A1, COL5A1, THY1	5.39E-04	3.57
Annotation Cluster 7 Enrichment Score: 2.03				
GO:0007346~regulation of mitotic cell cycle	8	CDC6, DLGAP5, BIRC5, ANLN, UBE2C, CCNA2, GTSE1, CDK2	0.004219	3.93
GO:0007096~regulation of exit from mitosis	3	BIRC5, ANLN, UBE2C	0.010645	18.69
GO:0010564~regulation of cell cycle process	6	DLGAP5, BIRC5, ANLN, CALR, UBE2C, GTSE1	0.018087	3.93
Annotation Cluster 8 Enrichment Score: 1.79				
GO:0031589~cell-substrate adhesion	6	CORO1A, ITGAV, COL3A1, ITGA7, ITGA10, THY1	0.009902	4.58
GO:0007229~integrin-mediated signaling pathway	5	ITGA5, ITGAV, COL3A1, ITGA7, ITGA10	0.014062	5.34
GO:0007160~cell-matrix adhesion	5	ITGAV, COL3A1, ITGA7, ITGA10, THY1	0.030887	4.20

Table 5.3.2.6 Functional annotation clustering - Gene Ontology Terms for Molecular Functions: MSC- γ vs. UT-MSC (upregulated genes)

Term	Count	Genes	PValue	Fold Enrichment
Annotation Cluster 1 Enrichment Score: 5.75				
GO:0016876~ligase activity, forming aminoacyl-tRNA and related compounds	11	IARS, WARS, YARS, CARS, NARS, ZNFX1, SARS, LARS, GARS, EPRS, MARS	1.79E-06	7.30
GO:0016875~ligase activity, forming carbon-oxygen bonds	11	IARS, WARS, YARS, CARS, NARS, ZNFX1, SARS, LARS, GARS, EPRS, MARS	1.79E-06	7.30
GO:0004812~aminoacyl-tRNA ligase activity	11	IARS, WARS, YARS, CARS, NARS, ZNFX1, SARS, LARS, GARS, EPRS, MARS	1.79E-06	7.30
Annotation Cluster 2 Enrichment Score: 3.39				
GO:0000166~nucleotide binding	106	ADCY3, GRPEL2, ACSS3, RNF213, NLRC5, RAVR2, MLKL, DHX36, RAB27B, MX1, MX2, GTPBP2, CIITA, CARS, GBP6, YARS, GBP5, NAV3, RND3, RENBP, RIPK2, RAB12, EIF2AK2, GBP4, GBP3, SRXN1, GBP2, GBP1, HLA-DRA, IFIH1, ME2, ABCA9, ASS1, OAS3, OAS1, OAS2, RRAGC, CMPK2, SGK223, MOV10, IDH1, TUBE1, SUPV3L1, DYRK3, RHOBTB3, ITK, SMCHD1, RAB8B, MYO1B, DOCK9, GARS, EPRS, YTHDC2, U2AF1L4, DDX58, SCN8A, SLC27A1, SEPHS2, NARS, ZNFX1, CPEB4, SLFN5, HLCS, HLA-DMB, GSR, WARS, LONP1, DDX60, AGAP1, AIFM2, SARS, DAPK1, RPL23AP32, DHRS3, HIPK2, ERN1, TXNRD1, REV3L, MKNK2, ADH5, HK2, TRIB3, ASNS, GCH1, IARS, PDE1C, MAP3K1, TAP2, TAP1, LARS, CAMK2D, DMGDH, GUF1, MARS, ACSL5, HSPA9, ALPK1, ALPK2, STRADB, PCK2, RAB31, ABCC3, PHGDH, SLFN12, ABCC1, JAK2	1.87E-05	1.47
GO:0017076~purine nucleotide binding	93	ADCY3, GRPEL2, SEPHS2, NARS, ZNFX1, HLCS, SLFN5, HLA-DMB, ACSS3, WARS, NLRC5, GSR, LONP1, DDX60, MLKL, DHX36, AGAP1, MX1, RAB27B, MX2, GTPBP2, CIITA, GBP6, CARS, YARS, GBP5, AIFM2, SARS, DAPK1, RND3, RENBP, HIPK2, ERN1, RIPK2, TXNRD1, RAB12, GBP4, EIF2AK2, GBP3, SRXN1, GBP2, GBP1, HLA-DRA, IFIH1, ABCA9, ASS1, MKNK2, HK2, OAS3, TRIB3, OAS1, ASNS, OAS2, CMPK2, RRAGC, GCH1, IARS, SGK223, MOV10, PDE1C, TAP2, MAP3K1, TAP1, LARS, CAMK2D, DMGDH, TUBE1, SUPV3L1, DYRK3, GUF1, MARS, RHOBTB3, ACSL5, HSPA9, ITK, ALPK1, SMCHD1, RAB8B, MYO1B, ALPK2, DOCK9, GARS, EPRS, YTHDC2, STRADB, PCK2, DDX58, RAB31, SLFN12, ABCC3, ABCC1, JAK2, SCN8A	2.78E-05	1.51
GO:0032555~purine ribonucleotide binding	88	ADCY3, SEPHS2, NARS, ZNFX1, HLCS, SLFN5, HLA-DMB, ACSS3, WARS, NLRC5, LONP1, DDX60, MLKL, DHX36, AGAP1, MX1, RAB27B, MX2, GTPBP2, CIITA, GBP6, CARS, YARS, GBP5, SARS, DAPK1, RND3, RENBP, HIPK2, ERN1, RIPK2, RAB12, GBP4, EIF2AK2, GBP3, GBP2, SRXN1, GBP1, HLA-DRA, IFIH1, ABCA9, ASS1, MKNK2, HK2, OAS3, TRIB3, OAS1, ASNS, OAS2, CMPK2, RRAGC, GCH1, IARS, SGK223, MOV10, PDE1C, TAP2, MAP3K1, TAP1, LARS, CAMK2D, TUBE1, SUPV3L1, DYRK3, GUF1, MARS, RHOBTB3, ACSL5, HSPA9, ITK, ALPK1, SMCHD1, RAB8B, MYO1B, ALPK2, DOCK9, GARS, EPRS, YTHDC2, STRADB, PCK2, DDX58, RAB31, SLFN12, ABCC3, ABCC1, JAK2, SCN8A	7.82E-05	1.50
GO:0032553~ribonucleotide binding	88	ADCY3, SEPHS2, NARS, ZNFX1, HLCS, SLFN5, HLA-DMB, ACSS3, WARS, NLRC5, LONP1, DDX60, MLKL, DHX36, AGAP1, MX1, RAB27B, MX2, GTPBP2, CIITA, GBP6, CARS, YARS, GBP5, SARS, DAPK1, RND3, RENBP, HIPK2, ERN1, RIPK2, RAB12, GBP4, EIF2AK2, GBP3, GBP2, SRXN1, GBP1, HLA-DRA, IFIH1, ABCA9, ASS1, MKNK2, HK2, OAS3, TRIB3, OAS1, ASNS, OAS2, CMPK2, RRAGC,	7.82E-05	1.50

		GCH1, IARS, SGK223, MOV10, PDE1C, TAP2, MAP3K1, TAP1, LARS, CAMK2D, TUBE1, SUPV3L1, DYRK3, GUF1, MARS, RHOBTB3, ACSL5, HSPA9, ITK, ALPK1, SMCHD1, RAB8B, MYO1B, ALPK2, DOCK9, GARS, EPRS, YTHDC2, STRADB, PCK2, DDX58, RAB31, SLFN12, ABCC3, ABCC1, JAK2, SCN8A			
GO:0001883~purine nucleoside binding	73	ADCY3, GRPEL2, SEPHS2, NARS, ZNFX1, HLCS, SLFN5, HLA-DMB, ACSS3, WARS, NLRC5, GSR, LONP1, DDX60, DHX36, MLKL, CIITA, CARS, YARS, AIFM2, SARS, DAPK1, RENBP, HIPK2, ERN1, RIPK2, TXNRD1, EIF2AK2, SRXN1, HLA-DRA, IFIH1, ABCA9, ASS1, MKNK2, HK2, OAS3, TRIB3, OAS1, ASNS, OAS2, RRAGC, CMPK2, IARS, SGK223, MOV10, PDE1C, TAP2, MAP3K1, TAP1, LARS, CAMK2D, DMGDH, SUPV3L1, DYRK3, MARS, ACSL5, HSPA9, RHOBTB3, ITK, ALPK1, SMCHD1, MYO1B, ALPK2, GARS, EPRS, YTHDC2, STRADB, DDX58, SLFN12, ABCC3, ABCC1, JAK2, SCN8A	0.001553	1.42	
GO:0030554~adenyl nucleotide binding	72	ADCY3, GRPEL2, SEPHS2, NARS, ZNFX1, HLCS, SLFN5, HLA-DMB, ACSS3, WARS, NLRC5, GSR, LONP1, DDX60, DHX36, MLKL, CIITA, CARS, YARS, AIFM2, SARS, DAPK1, RENBP, HIPK2, ERN1, RIPK2, TXNRD1, EIF2AK2, SRXN1, HLA-DRA, IFIH1, ABCA9, ASS1, MKNK2, HK2, OAS3, TRIB3, OAS1, ASNS, OAS2, CMPK2, IARS, SGK223, MOV10, PDE1C, TAP2, MAP3K1, TAP1, LARS, CAMK2D, DMGDH, SUPV3L1, DYRK3, MARS, ACSL5, HSPA9, RHOBTB3, ITK, ALPK1, SMCHD1, MYO1B, ALPK2, GARS, EPRS, YTHDC2, STRADB, DDX58, SLFN12, ABCC3, ABCC1, JAK2, SCN8A	0.001645	1.42	
GO:0001882~nucleoside binding	73	ADCY3, GRPEL2, SEPHS2, NARS, ZNFX1, HLCS, SLFN5, HLA-DMB, ACSS3, WARS, NLRC5, GSR, LONP1, DDX60, DHX36, MLKL, CIITA, CARS, YARS, AIFM2, SARS, DAPK1, RENBP, HIPK2, ERN1, RIPK2, TXNRD1, EIF2AK2, SRXN1, HLA-DRA, IFIH1, ABCA9, ASS1, MKNK2, HK2, OAS3, TRIB3, OAS1, ASNS, OAS2, RRAGC, CMPK2, IARS, SGK223, MOV10, PDE1C, TAP2, MAP3K1, TAP1, LARS, CAMK2D, DMGDH, SUPV3L1, DYRK3, MARS, ACSL5, HSPA9, RHOBTB3, ITK, ALPK1, SMCHD1, MYO1B, ALPK2, GARS, EPRS, YTHDC2, STRADB, DDX58, SLFN12, ABCC3, ABCC1, JAK2, SCN8A	0.001895	1.41	
GO:0032559~adenyl ribonucleotide binding	67	ADCY3, SEPHS2, NARS, ZNFX1, HLCS, SLFN5, HLA-DMB, ACSS3, WARS, NLRC5, LONP1, DDX60, DHX36, MLKL, CIITA, CARS, YARS, SARS, DAPK1, RENBP, HIPK2, ERN1, RIPK2, EIF2AK2, SRXN1, HLA-DRA, IFIH1, ABCA9, ASS1, MKNK2, HK2, OAS3, TRIB3, OAS1, ASNS, OAS2, CMPK2, IARS, SGK223, MOV10, PDE1C, TAP2, MAP3K1, TAP1, LARS, CAMK2D, SUPV3L1, DYRK3, MARS, ACSL5, HSPA9, RHOBTB3, ITK, ALPK1, SMCHD1, ALPK2, MYO1B, GARS, EPRS, YTHDC2, STRADB, DDX58, SLFN12, ABCC3, ABCC1, JAK2, SCN8A	0.004036	1.40	
GO:0005524~ATP binding	66	ADCY3, SEPHS2, NARS, ZNFX1, HLCS, SLFN5, HLA-DMB, ACSS3, WARS, NLRC5, LONP1, DDX60, DHX36, MLKL, CIITA, CARS, YARS, SARS, DAPK1, RENBP, HIPK2, ERN1, RIPK2, EIF2AK2, SRXN1, HLA-DRA, IFIH1, ABCA9, ASS1, MKNK2, OAS3, HK2, TRIB3, OAS1, ASNS, OAS2, CMPK2, IARS, SGK223, MOV10, TAP2, MAP3K1, TAP1, LARS, CAMK2D, SUPV3L1, DYRK3, MARS, ACSL5, HSPA9, RHOBTB3, ITK, ALPK1, SMCHD1, ALPK2, MYO1B, GARS, EPRS, YTHDC2, STRADB, DDX58, SLFN12, ABCC3, ABCC1, JAK2, SCN8A	0.004548	1.39	
Annotation Cluster 3 Enrichment Score: 3.10					
GO:0005125~cytokine activity	18	SECTM1, NAMPT, IL6, CCL2, CSF1, CXCL9, CCL8, IL32, CXCL11, TGFB2, FLT3LG, CXCL10, TNFSF10, CCL13, TNFSF13B, CXCL16, VEGFA, GDF15	1.74E-04	2.88	
GO:0042379~chemokine receptor binding	8	YARS, CCL13, CCL2, CXCL16, CXCL9, CCL8, CXCL11, CXCL10	8.73E-04	5.10	
GO:0008009~chemokine activity	7	CCL13, CCL2, CXCL16, CXCL9, CCL8, CXCL11, CXCL10	0.0033	4.75	
Annotation Cluster 4 Enrichment Score: 1.90					

GO:0003924~GTPase activity	16	GTPBP2, GBP6, GBP5, RAB8B, RRAGC, RND3, RAB31, TUBE1, GUF1, GBP4, MX1, RAB27B, MX2, GBP3, GBP2, GBP1	0.0033	2.37
GO:0005525~GTP binding	21	GTPBP2, GBP6, GBP5, RAB8B, DOCK9, PCK2, RRAGC, GCH1, RND3, RAB31, TUBE1, RAB12, GUF1, AGAP1, GBP4, MX1, RAB27B, MX2, GBP3, GBP2, GBP1	0.0166	1.76
GO:0019001~guanyl nucleotide binding	21	GTPBP2, GBP6, GBP5, RAB8B, DOCK9, PCK2, RRAGC, GCH1, RND3, RAB31, TUBE1, RAB12, GUF1, AGAP1, GBP4, MX1, RAB27B, MX2, GBP3, GBP2, GBP1	0.0214	1.72
GO:0032561~guanyl ribonucleotide binding	21	GTPBP2, GBP6, GBP5, RAB8B, DOCK9, PCK2, RRAGC, GCH1, RND3, RAB31, TUBE1, RAB12, GUF1, AGAP1, GBP4, MX1, RAB27B, MX2, GBP3, GBP2, GBP1	0.0214	1.72
Annotation Cluster 5 Enrichment Score: 1.84				
GO:0003714~transcription corepressor activity	13	SP100, CBX4, RYBP, TRIB3, MXD1, TRIM22, MSC, DDIT3, ATF3, HIPK2, CREG1, ID4, NFIL3	0.0024	2.80
GO:0016564~transcription repressor activity	20	CIITA, SP100, NACC2, ARID5B, CBX4, RYBP, TRIB3, IFI16, MXD1, MSC, TRIM22, DDIT3, ATF3, ID1, HIPK2, CREG1, MDM2, ID4, BCL6, NFIL3	0.0064	1.98
GO:0003712~transcription cofactor activity	19	CIITA, SP100, HTATIP2, NMI, EPAS1, RYBP, CBX4, TRIB3, MXD1, MSC, TRIM22, DDIT3, JMY, ATF3, NCOA3, HIPK2, CREG1, ID4, NFIL3	0.0442	1.63
GO:0008134~transcription factor binding	24	CIITA, NBN, SP100, HTATIP2, NMI, EPAS1, CFB, CEBPG, RYBP, CBX4, TRIB3, MXD1, MSC, TRIM22, DDIT3, JMY, ATF3, NCOA3, BCL2, HIPK2, CREG1, ID4, FOXC1, NFIL3	0.0625	1.46
Annotation Cluster 6 Enrichment Score: 1.67				
GO:0042803~protein homodimerization activity	20	GRPEL2, CARS, SP100, CEBPB, IL6ST, SMAD6, CSF1, ADH5, CALCOCO2, ASNS, GCH1, TGFB2, FLT3LG, GSR, RENBP, TAP2, BCL2, VEGFA, TAP1, CLIP1	0.0112	1.87
GO:0046983~protein dimerization activity	28	GRPEL2, IL6ST, CSF1, ADH5, CALCOCO2, ASNS, GCH1, RRAGC, TGFB2, FLT3LG, GSR, BCL2, TAP2, TAP1, NFIL3, CARS, SP100, CEBPB, EPAS1, SCUBE1, CEBPG, SMAD6, DDIT3, RENBP, ATF3, VEGFA, CLIP1, NFE2L2	0.0150	1.61

Table 5.3.2.7 Functional annotation clustering - Gene Ontology Terms for Molecular Functions: MSC- γ vs. UT-MSC (downregulated genes)

Term	Count	Genes	P-Value	Fold Enrichment
Annotation Cluster 1		Enrichment Score: 2.30		
GO:0019798~procollagen-proline dioxygenase activity	4	LEPRE1, LEPREL2, P4HA1, P4HA3	8.10E-05	42.15
GO:0031543~peptidyl-proline dioxygenase activity	4	LEPRE1, LEPREL2, P4HA1, P4HA3	1.28E-04	36.88
GO:0019842~vitamin binding	8	LEPRE1, LEPREL2, P4HA1, P4HA3, CRABP2, FASN, GCAT, PC	0.0019	4.54
GO:0031418~L-ascorbic acid binding	4	LEPRE1, LEPREL2, P4HA1, P4HA3	0.0027	14.05
GO:0016706~oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, 2-oxoglutarate as one donor, and incorporation of one atom each of oxygen into both donors	4	LEPRE1, LEPREL2, P4HA1, P4HA3	0.0062	10.54
GO:0031406~carboxylic acid binding	7	LEPRE1, LEPREL2, P4HA1, P4HA3, CRABP2, FASN, PC	0.0133	3.59
Annotation Cluster 2		Enrichment Score: 2.17		
GO:0030246~carbohydrate binding	14	GALNT1, ENPP1, ANG, LGALS1, CD248, CCDC80, VCAN, DCN, CALR, THBS2, FUCA1, COL5A1, CLEC11A, PRELP	0.0010	2.92
GO:0001871~pattern binding	8	ENPP1, ANG, CCDC80, VCAN, DCN, THBS2, COL5A1, PRELP	0.0048	3.83
GO:0030247~polysaccharide binding	8	ENPP1, ANG, CCDC80, VCAN, DCN, THBS2, COL5A1, PRELP	0.0048	3.83
GO:0005539~glycosaminoglycan binding	7	ANG, CCDC80, VCAN, DCN, THBS2, COL5A1, PRELP	0.0117	3.69
Annotation Cluster 3		Enrichment Score: 2.10		
GO:0003756~protein disulfide isomerase activity	3	PDIA6, PDIA5, PDIA4	0.0061	24.59
GO:0016864~intramolecular oxidoreductase activity, transposing S-S bonds	3	PDIA6, PDIA5, PDIA4	0.0061	24.59
GO:0016862~intramolecular oxidoreductase activity, interconverting keto- and enol-groups	3	PDIA6, PDIA5, PDIA4	0.0076	22.13
GO:0016860~intramolecular oxidoreductase activity	4	EBP, PDIA6, PDIA5, PDIA4	0.0144	7.76

Table 5.3.2.8 Functional annotation clustering- Gene Ontology Terms for Cellular components: MSC- γ vs. UT-MSC (upregulated genes)

Term	Count	Genes	P-Value	Fold Enrichment
Annotation Cluster 1 Enrichment Score: 2.69				
GO:0005615~extracellular space	39	CCL2, IL6ST, C3, CSF1, CLU, CXCL9, KITLG, CCL8, TIMP4, IL32, CXCL11, CXCL10, FLT3LG, TGFB2, LGALS3BP, ISG15, IL15RA, C2, FGF2, SECTM1, ICAM1, YARS, IL6, C4A, LY96, C4B, SCUBE1, SERPING1, TNFSF10, CCL13, APOL1, TNFSF13B, CXCL16, VEGFA, C1RL, GDF15, TNFAIP2, IGFBP5, VLDLR	6.27E-05	1.99
GO:0044421~extracellular region part	43	FMOD, CCL2, IL6ST, C3, CSF1, CLU, CXCL9, KITLG, CCL8, TIMP4, IL32, CXCL11, CXCL10, FLT3LG, TGFB2, LGALS3BP, ISG15, IL15RA, C2, FGF2, SECTM1, ICAM1, YARS, IL6, C4A, LY96, C4B, SCUBE1, NTN4, SERPING1, ADAMTS9, TNFSF10, CCL13, APOL1, TNFSF13B, CXCL16, VEGFA, C1RL, GDF15, TNFAIP2, ADAMTS5, IGFBP5, VLDLR	0.0035	1.57

Table 5.3.2.9 Functional annotation clustering- Gene Ontology Terms for Cellular components: MSC- γ vs. UT-MSC (downregulated genes)

Term	Count	Genes	P-Value	Fold Enrichment
Annotation Cluster 1 Enrichment Score: 12.76				
GO:0005578~proteinaceous extracellular matrix	34	CTHRC1, CD248, LUM, COL3A1, ELN, SPOCK1, DCN, CALR, HMCN1, ANG, TGFB1, COL6A1, COL12A1, ADAMTS12, COL11A1, LOXL1, COL10A1, COL4A1, LGALS1, CCDC80, SPARC, COL5A2, COL5A1, PRELP, EMILIN1, LEPRE1, COL14A1, COL1A2, VCAN, MFAP2, COL1A1, MFAP4, MFAP5, ADAMTS2	1.87E-18	6.89
GO:0031012~extracellular matrix	35	CTHRC1, LUM, CD248, COL3A1, ELN, SPOCK1, DCN, CALR, HMCN1, ANG, TGFB1, COL6A1, COL12A1, ADAMTS12, COL11A1, LOXL1, PRSS12, COL10A1, COL4A1, LGALS1, CCDC80, SPARC, COL5A2, COL5A1, PRELP, EMILIN1, LEPRE1, COL14A1, COL1A2, VCAN, MFAP2, COL1A1, MFAP4, MFAP5, ADAMTS2	2.23E-18	6.58
GO:0044420~extracellular matrix part	20	COL4A1, LUM, COL3A1, CCDC80, SPARC, COL5A2, COL5A1, COL14A1, HMCN1, ANG, COL1A2, COL12A1, COL6A1, MFAP2, COL1A1, MFAP4, COL11A1, MFAP5, PRSS12, COL10A1	1.30E-14	11.09
GO:0044421~extracellular region part	41	CTHRC1, FGF5, LDLR, ENPP1, LUM, CD248, COL3A1, ELN, SPOCK1, DCN, CALR, HMCN1, ANG, TGFB1, COL6A1, COL12A1, ADAMTS12, COL11A1, LOXL1, PRSS12, COL10A1, PLAT, COL4A1, LGALS1, CCDC80, SPARC, COL5A2, COL5A1, PRELP, SLIT3, EMILIN1, LEPRE1, COL14A1, COL1A2, GDF11, MFAP2, VCAN, COL1A1, MFAP4, MFAP5, ADAMTS2	4.56E-09	2.77
GO:0005576~extracellular region	59	CTHRC1, FGF5, LDLR, VCL, OLFML3, WISP1, HMCN1, ANG, TGFB1, COL12A1, COL11A1, LOXL1, COL10A1, OLFML2B, PLAUR, SLIT3, PRELP, COL1A2, KRTAP1-1, MFAP2, VCAN, COL1A1, MFAP4, ADAM12, MFAP5, ADAMTS2, GALNT1, ENPP1, LUM, CD248, COL3A1, ELN, SPOCK1, DCN, CALR, MANF, FNDC1, GLIPR1, CDA, COL6A1, ADAMTS12, THBS2, PRSS12, PLAT, COL4A1, LGALS1, CCDC80, SPARC, COL5A2, COL5A1, CLEC11A, EMILIN1, C19ORF10, LEPRE1, COL14A1, SRPX2, PENK, CD59, GDF11	6.18E-07	1.90
Annotation Cluster 2 Enrichment Score: 11.16				
GO:0044420~extracellular matrix part	20	COL4A1, LUM, COL3A1, CCDC80, SPARC, COL5A2, COL5A1, COL14A1, HMCN1, ANG, COL1A2, COL12A1, COL6A1, MFAP2, COL1A1, MFAP4, COL11A1, MFAP5, PRSS12, COL10A1	1.30E-14	11.09
GO:0005581~collagen	12	COL14A1, COL4A1, LUM, COL3A1, COL1A2, COL6A1, COL12A1, COL1A1, COL5A2, COL11A1, COL5A1, COL10A1	2.52E-12	22.25
GO:0005583~fibrillar collagen	7	LUM, COL3A1, COL1A2, COL1A1, COL5A2, COL11A1, COL5A1	1.03E-08	37.85
Annotation Cluster 3 Enrichment Score: 2.01				
GO:0005694~chromosome	18	MKI67, HIST1H1C, HIST1H1B, BIRC5, AURKB, CDK2, RAD51, SPC24, HIST1H2BO, HIST2H2AB, NCAPH, CDCA8, HIST1H2BM, HJURP, SEC13, HIST1H2AJ, ASF1B, TOP2A	7.23E-04	2.54
GO:0044427~chromosomal part	16	MKI67, HIST1H1C, HIST1H1B, BIRC5, AURKB, CDK2, SPC24, HIST1H2BO, HIST2H2AB, NCAPH, CDCA8, HIST1H2BM, HJURP, SEC13, HIST1H2AJ, ASF1B	9.01E-04	2.69
GO:0000786~nucleosome	6	HIST1H2BO, HIST2H2AB, HIST1H2BM, HIST1H1C, HIST1H1B, HIST1H2AJ	0.0028	6.18
GO:0032993~protein-DNA complex	6	HIST1H2BO, HIST2H2AB, HIST1H2BM, HIST1H1C, HIST1H1B, HIST1H2AJ	0.0103	4.53

GO:0000775~chromosome, centromeric region	7	SPC24, CDCA8, MKI67, HJURP, SEC13, BIRC5, AURKB	0.0121	3.66
GO:0000793~condensed chromosome	7	SPC24, NCAPH, MKI67, HJURP, AURKB, CDK2, RAD51	0.0145	3.52
Annotation Cluster 4 Enrichment Score: 1.52				
GO:0015630~microtubule cytoskeleton	18	CDC6, KIFC1, KIF11, PRC1, TUBB2A, DLGAP5, TPX2, CDC20, BIRC5, AURKB, GTSE1, CCNB1, CDCA8, PLK1, TUBA1A, TUBA1B, TOP2A, KIF20A	0.0047	2.13
GO:0044430~cytoskeletal part	25	KIFC1, PRC1, LMNB1, TUBB2A, ANLN, AURKB, GTSE1, CDCA8, TUBA1A, TOP2A, TUBA1B, CDC6, KIF11, DLGAP5, TPX2, BIRC5, CDC20, KRTAP10-5, CCNB1, CORO1A, PLK1, FLG, KRT14, KRTAP1-1, KIF20A	0.0109	1.70
GO:0005856~cytoskeleton	32	KIFC1, PRC1, LMNB1, TUBB2A, ANLN, AURKB, GTSE1, VCL, CDCA8, TUBA1A, TUBA1B, STK38L, TOP2A, CDC6, KIF11, ACTA2, DLGAP5, MICAL2, FSCN1, TPX2, KRTAP10-5, BIRC5, CDC20, CCNB1, JUP, CORO1A, PLK1, FLG, KRT14, KRTAP1-1, FHOD1, KIF20A	0.0191	1.50

5.3.3 Functional enrichment analysis: MSC-17 vs. UT-MSC

A total of 67 genes were differently regulated in the MSC-17 vs. UT-MSC comparison groups. These genes were submitted to DAVID for functional annotation clustering to identify gene sets that were enriched following IL-17A treatment of human MSC. Genes that were mapped by DAVID were shown in **Table 5.3.3.1**. There were 23 genes from the gene entry list that were unmapped by DAVID (**Table 5.3.3.2**). These include non-coding genes, lncRNA, ribosomal RNA (rRNA), snorRNA and miRNA.

Functional annotation clustering analysis was first performed using DAVID's default settings (**Table 5.3.3.3**) to identify overall gene sets that were highly enriched (enrichment score >1.5) in MSC-17 compared to UT-MSC. Annotation cluster 1 with the highest enrichment score (3.58) had enriched terms for genes residing in the extracellular region, roles in inflammatory response, response to wounding, defense responses, signaling and disulfide bonds. Interestingly, some gene sets were enriched for gene ontology terms such as glycosylation and glycoproteins, which may relate to post-translational modification processes^{416,417}. Human MSC-17 were shown to be superior at regulating T cell inflammatory responses by suppressing T cell proliferation, activation and secretion of proinflammatory cytokines (**CHAPTER 3 and 4**)². In annotation cluster 3 (enrichment score 2.48, **Table 5.3.3.3**), genes such as IL-6, C3, serum amyloid A1 (SAA1) and lipopolysaccharide binding protein (LBP) were enriched for regulation of immune responses. IL-6, SAA1 and LBP also have roles in regulation of cytokine production.

Gene ontology analysis by DAVID functional annotation clustering was also performed on the MSC-17 vs. UT-MSC gene list to specifically determine enriched gene sets for biological processes (**Table 5.3.3.4**), molecular functions (**Table 5.3.3.5**) and cellular components (**Table 5.3.3.6**) in MSC-17. MSC-17 were previously shown to mediate Treg induction via cell-cell contact dependent mechanisms (**Figure 4.3.5**). To identify potential cell-

surface candidate molecules that mediate MSC-17 induction of Tregs, the cellular compartments of genes enriched in the MSC-17 were also evaluated. Functional enrichment for biological processes identified a set of upregulated genes (IL-6, CCL8, SLC22A3, STC1, CXCL6) that were enriched for the gene ontology term “cell-cell signaling” (fold enrichment: 4.9; $p < 0.0148$; annotation cluster 2, **Table 5.3.3.4**). Chemokines CCL2, CCL8 and CXCL6 detected by DAVID’s functional enrichment for molecular function (**Table 5.3.3.5**) showed evidence that these genes sets have a different range of binding potential including chemokine receptor, heparin, glycosaminoglycan, pattern and polysaccharide binding activities. These MSC-17 enriched genes, mainly the chemokines and MMPs were located in the extracellular space (or) region (**Table 5.3.3.6**).

Biological processes (GOTERM_BP_FAT; **Table 5.3.3.4**) and molecular functions (GOTERM_MF_FAT; **Table 5.3.3.5**) of MSC-17 enriched genes were mainly associated with cell migration and chemotaxis responses. Matrix metalloproteinases (MMPs) were also highly enriched in the MSC-17 groups. Specifically, MMP13 (FC 15.6) and MMP1 (FC 2.4) were upregulated in the MSC-17 groups as detected by microarray gene expression analysis (**Table 5.3.3.1**). DAVID’s bioinformatics analysis revealed that these genes were enriched for gene ontology terms such as secreted, extracellular space, signal, disulfide bond, glycosylation, glycoproteins and response to stimulus (**Table 5.3.3.7**). The MSC-17 vs. UT-MSC gene list when analysed by DAVID functional annotation chart (default setting) showed that these MMPs were highly enriched for metal ion binding, peptidase and collagen degradation functions (**Table 5.3.3.7**).

Table 5.3.3.1 Differentially expressed genes (mapped by DAVID): MSC-17 vs. UT-MSC

Gene Symbol	Gene name	mRNA Accession	Fold Change	P-value
<u>Upregulated genes:</u>				
MMP13	matrix metalloproteinase 13 (collagenase 3)	NM_002427	15.6	0.0021
C3	complement component 3; NULL	NM_000064	11.56	0.0039
LBP	lipopolysaccharide binding protein	NM_004139	5.35	0.0031
VMO1	vitelline membrane outer layer 1 homolog (chicken)	NM_182566	4.07	0.0022
CH25H	cholesterol 25-hydroxylase	NM_003956	3.99	0.0023
IL6	interleukin 6 (interferon, beta 2); NULL	NM_000600	3.44	0.0083
ZC3H12A	zinc finger CCCH-type containing 12A	NM_025079	3.09	0.0010
CCL2	chemokine (C-C motif) ligand 2	NM_002982	3.08	0.0405
ZNF253	zinc finger protein 253	NM_021047	2.82	0.0010
SAA1	serum amyloid A1	NM_000331	2.72	0.0102
CXCL6	chemokine (C-X-C motif) ligand 6	NM_002993	2.44	0.0014
MMP1	matrix metalloproteinase 1 (interstitial collagenase)	NM_002421	2.4	0.0356
NFKBIZ	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta; NULL	NM_031419	2.36	0.0232
MIRLET7A2	microRNA let-7a-2	NR_029477	2.30	0.0031
RBMY2EP	RNA binding motif protein, Y-linked, family 2, member E pseudogene	ENST00000444169	2.27	0.0278
CCL8	chemokine (C-C motif) ligand 8	NM_005623	2.2	0.0012
STC1	stanniocalcin 1	NM_003155	2.2	0.0023
SFRP4	secreted frizzled-related protein 4	NM_003014	2.19	0.0136
SLC22A3	solute carrier family 22 (extraneuronal monoamine transporter), member 3	NM_021977	2.15	0.0452
TTY11	testis-specific transcript, Y-linked 11 (non-protein coding)	NR_001548	2.15	0.0252
STEAP2	STEAP family member 2, metalloredutase; NULL	NM_001244944	2.12	0.0225
SCARNA18	small Cajal body-specific RNA 18	NR_003139	2.06	0.0254
LOC100287834	uncharacterised LOC100287834	NR_028349	2.06	0.0328
<u>Downregulated genes:</u>				
RPS24	ribosomal protein S24; NULL	NM_001142285	-2.01	0.0209

LOC100133299	GALI1870	AY358688	-2.03	0.0095
POU5F1	POU class 5 homeobox 1	ENST00000259915	-2.04	0.0129
TMEM171	transmembrane protein 171	NM_173490	-2.05	0.0133
IGLJ2	immunoglobulin lambda joining 2	ENST00000390322	-2.07	0.0252
ITGA6	integrin, alpha 6; NULL	ENST00000264107	-2.11	0.0109
RNU7-25P	RNA, U7 small nuclear 25 pseudogene; RNA, U7 small nuclear 11 pseudogene	ENST00000516544	-2.16	0.0047
GTF2IRD2B	GTF2I repeat domain containing 2B	NM_001003795	-2.2	0.0040
SERTAD4	SERTA domain containing 4	ENST00000367012	-2.29	0.0470
TPTE	transmembrane phosphatase with tensin homology	ENST00000415664	-2.85	0.0128

Table 5.3.3.2 Unmapped genes from the gene list entry for DAVID: MSC-17 vs. UT-MS

Gene Symbol	Gene name	mRNA Accession	Fold Change	P-value	Gene type
Upregulated genes:					
lnc-SULF2-2:2, lnc-SULF2-2:1		TCONS_00028418-XLOC_013771	2.66	0.0227	lncRNA (non coding)
lnc-GINS1-2:1		ENST00000376445	2.6	0.0373	lncRNA (non coding)
AC116562.1		ENST00000408374	2.53	0.0260	miRNA
RNA5SP191	RNA, 5S ribosomal pseudogene 191	ENST00000362585	2.52	0.0447	rRNA (non coding)
RNY4P23	RNA, Ro-associated Y4 pseudogene 23	ENST00000364507	2.51	0.0470	
RNA5SP234	RNA, 5S ribosomal pseudogene 234	ENST00000363916	2.32	0.0164	
U3	Small nucleolar RNA U3	ENST00000516996	2.14	0.0168	snoRNA (non coding)
Y_RNA	Y RNA	ENST00000516177	2.12	0.0414	ncRNA (non coding)
AC007365.2		ENST00000517273	2.11	0.0120	miRNA
RNA5SP249	RNA, 5S ribosomal pseudogene 249	ENST00000516127	2.11	0.0353	
SNORA70.1-201	Small nucleolar RNA SNORA70	ENST00000363367	2.09	0.0398	snoRNA (non coding)
U6	U6 spliceosomal RNA	ENST00000384217	2.08	0.0477	snRNA
BACH1	BTB and CNC homology 1, basic leucine zipper transcription factor 1	ENST00000551628	2.06	0.0404	
RNA5SP176	RNA, 5S ribosomal pseudogene 176	ENST00000390956	2.06	0.0358	
RNU5E-9P	RNA, U5E small nuclear 9, pseudogene	ENST00000411164	2.05	0.0257	
Downregulated Genes:					
RP11-265D17.2		ENST00000527288	-2.1	0.0415	non coding
		TCONS_12_00026700-XLOC_12_013898	-2.29	0.0033	
OTTHUMG00000177628	NULL; differential display clone 8	ENST00000586713	-2.3	0.0030	
MIR4476	microRNA 4476	NR_039687	-2.33	0.0160	
RNU7-193P	U7 small nuclear RNA	ENST00000516723	-2.41	0.0466	snRNA (non coding)
U6	U6 spliceosomal RNA	ENST00000516654	-2.57	0.0377	non coding
U6	U6 spliceosomal RNA	ENST00000411105	-2.7	0.0349	non coding

AL109750.1
Y_RNA

ENST00000516621
ENST00000383924

-2.87
-3.29

0.0453
0.0432

miRNA
ncRNA (non coding)

Table 5.3.3.3 Functional annotation clustering: Gene enrichment analysis on DAVID's Default settings: MSC-17 vs. UT-MSC

Category	Term	Count	Genes	P-Value	Fold Enrichment
Annotation Cluster 1 Enrichment Score: 3.58					
GOTERM_CC_FAT	extracellular space	10	Upregulated: IL6, CCL2, C3, SAA1, SFRP4, CCL8, STC1, CXCL6, LBP, MMP13	1.09E-07	9.82
GOTERM_CC_FAT	extracellular region part	11	Upregulated: IL6, CCL2, C3, SAA1, SFRP4, CCL8, STC1, CXCL6, LBP, MMP13, MMP1	1.37E-07	7.71
GOTERM_BP_FAT	inflammatory response	8	Upregulated: NFKBIZ, IL6, CCL2, C3, SAA1, CCL8, CXCL6, LBP	5.42E-07	14.48
SP_PIR_KEYWORDS	Secreted	12	Upregulated: IL6, CCL2, C3, SAA1, SFRP4, CCL8, STC1, CXCL6, LBP, VMO1, MMP13, MMP1	1.97E-06	5.47
GOTERM_BP_FAT	response to wounding	8	Upregulated: NFKBIZ, IL6, CCL2, C3, SAA1, CCL8, CXCL6, LBP	1.39E-05	8.88
GOTERM_CC_FAT	extracellular region	12	Upregulated: IL6, CCL2, C3, SAA1, SFRP4, CCL8, STC1, CXCL6, LBP, VMO1, MMP13, MMP1	1.54E-05	4.02
GOTERM_BP_FAT	defense response	8	Upregulated: NFKBIZ, IL6, CCL2, C3, SAA1, CCL8, CXCL6, LBP	3.64E-05	7.65
GOTERM_BP_FAT	taxis	5	Upregulated: IL6, CCL2, SAA1, CCL8, CXCL6	1.17E-04	18.38
GOTERM_BP_FAT	chemotaxis	5	Upregulated: IL6, CCL2, SAA1, CCL8, CXCL6	1.17E-04	18.38
SP_PIR_KEYWORDS	signal	13	Upregulated: IL6, CCL2, SAA1, C3, SFRP4, CCL8, STC1, CXCL6, LBP, VMO1, MMP13, MMP1 Downregulated: ITGA6	1.92E-04	3.08
UP_SEQ_FEATURE	signal peptide	13	Upregulated: IL6, CCL2, SAA1, C3, SFRP4, CCL8, STC1, CXCL6, LBP, VMO1, MMP13, MMP1 Downregulated: ITGA6,	2.04E-04	3.06
GOTERM_BP_FAT	locomotory behavior	5	Upregulated: IL6, CCL2, SAA1, CCL8, CXCL6	9.03E-04	10.73
UP_SEQ_FEATURE	disulfide bond	11	Upregulated: IL6, CCL2, C3, SFRP4, CCL8, STC1, CXCL6, VMO1, MMP13, MMP1 Downregulated: ITGA6,	0.0013	2.98
SP_PIR_KEYWORDS	disulfide bond	11	Upregulated: IL6, CCL2, C3, SFRP4, CCL8, STC1, CXCL6, VMO1, MMP13, MMP1 Downregulated: ITGA6,	0.0016	2.89
GOTERM_BP_FAT	immune response	6	Upregulated: IL6, CCL2, C3, CCL8, CXCL6, LBP	0.0043	5.11
GOTERM_BP_FAT	behavior	5	Upregulated: IL6, CCL2, SAA1, CCL8, CXCL6	0.0063	6.27

GOTERM_BP_FAT	positive regulation of multicellular organismal process	4	Upregulated: IL6, CCL2, SAA1, LBP	0.0069	9.64
UP_SEQ_FEATURE	glycosylation site:N-linked (GlcNAc...)	11	Upregulated: IL6, CCL2, C3, CH25H, SFRP4, SLC22A3, STC1, LBP, MMP13, MMP1 Downregulated: ITGA6,	0.0213	2.04
SP_PIR_KEYWORDS	glycoprotein	11	Upregulated: CCL2, C3, CH25H, SFRP4, SLC22A3, STC1, LBP, MMP13, MMP1 Downregulated: ITGA6	0.0276	1.96
Annotation Cluster 2 Enrichment Score: 2.58					
GOTERM_BP_FAT	leukocyte migration	4	Upregulated: IL6, CCL2, SAA1 Downregulated: ITGA6,	1.03E-04	41.28
GOTERM_BP_FAT	taxis	5	Upregulated: IL6, CCL2, SAA1, CCL8, CXCL6	1.17E-04	18.38
GOTERM_BP_FAT	chemotaxis	5	Upregulated: IL6, CCL2, SAA1, CCL8, CXCL6	1.17E-04	18.38
GOTERM_BP_FAT	locomotory behavior	5	Upregulated: IL6, CCL2, SAA1, CCL8, CXCL6	9.03E-04	10.73
GOTERM_BP_FAT	GO:0030595~leukocyte chemotaxis	3	Upregulated: IL6, CCL2, SAA1	0.0016	47.69
GOTERM_BP_FAT	GO:0060326~cell chemotaxis	3	Upregulated: IL6, CCL2, SAA1	0.0018	45.24
GOTERM_BP_FAT	GO:0007610~behavior	5	Upregulated: IL6, CCL2, SAA1, CCL8, CXCL6	0.0063	6.27
GOTERM_BP_FAT	GO:0051240~positive regulation of multicellular organismal process	4	Upregulated: IL6, CCL2, SAA1, LBP	0.0069	9.64
GOTERM_BP_FAT	GO:0051240~cell migration	4	Upregulated: IL6, CCL2, SAA1 Downregulated: ITGA6,	0.0097	8.52
GOTERM_BP_FAT	GO:0051674~localization of cell	4	Upregulated: IL6, CCL2, SAA1 Downregulated: ITGA6,	0.0129	7.66
GOTERM_BP_FAT	GO:0048870~cell motility	4	Upregulated: IL6, CCL2, SAA1 Downregulated: ITGA6,	0.0129	7.66
GOTERM_BP_FAT	GO:0042592~homeostatic process	5	Upregulated: IL6, CCL2, SAA1, STC1 Downregulated: RPS24	0.0310	3.92
GOTERM_BP_FAT	GO:0006928~cell motion	4	Upregulated: IL6, CCL2, SAA1 Downregulated: ITGA6,	0.0404	4.95
Annotation Cluster 3 Enrichment Score: 2.48					
GOTERM_BP_FAT	GO:0002675~positive regulation of acute inflammatory response	3	Upregulated: IL6, C3, LBP	1.65E-04	147.04
GOTERM_BP_FAT	GO:0050727~regulation of inflammatory response	4	Upregulated: IL6, C3, SAA1, LBP	2.43E-04	30.96

GOTERM_BP_FAT	GO:0002526~acute inflammatory response	4	Upregulated: IL6, C3, SAA1, LBP	5.14E-04	24.01
GOTERM_BP_FAT	GO:0002673~regulation of acute inflammatory response	3	Upregulated: IL6, C3, LBP	5.20E-04	84.02
SP_PIR_KEYWORDS	acute phase	3	Upregulated: IL6, C3, SAA1	5.93E-04	79.59
GOTERM_BP_FAT	GO:0050729~positive regulation of inflammatory response	3	Upregulated: IL6, C3, LBP	0.0011	58.82
GOTERM_BP_FAT	GO:0032101~regulation of response to external stimulus	4	Upregulated: IL6, C3, SAA1, LBP	0.0021	14.80
GOTERM_BP_FAT	GO:0006955~immune response	6	Upregulated: IL6, CCL2, C3, CCL8, CXCL6, LBP	0.0043	5.11
GOTERM_BP_FAT	GO:0032103~positive regulation of response to external stimulus	3	Upregulated: IL6, C3, LBP	0.0048	27.57
GOTERM_BP_FAT	GO:0031349~positive regulation of defense response	3	Upregulated: IL6, C3, LBP	0.0062	24.17
GOTERM_BP_FAT	GO:0002697~regulation of immune effector process	3	Upregulated: IL6, C3, LBP	0.0116	17.47
GOTERM_BP_FAT	immune effector process	3	Upregulated: IL6, C3, LBP	0.0198	13.17
Annotation Cluster 4 Enrichment Score: 2.38					
GOTERM_BP_FAT	GO:0050727~regulation of inflammatory response	4	Upregulated: IL6, C3, SAA1, LBP	2.43E-04	30.96
GOTERM_BP_FAT	GO:0002526~acute inflammatory response	4	Upregulated: IL6, C3, SAA1, LBP	5.14E-04	24.01
GOTERM_BP_FAT	GO:0006953~acute-phase response	3	Upregulated: IL6, SAA1, LBP	0.0019	44.11
GOTERM_BP_FAT	GO:0032101~regulation of response to external stimulus	4	Upregulated: IL6, C3, SAA1, LBP	0.0021	14.80
GOTERM_BP_FAT	GO:0051240~positive regulation of multicellular organismal process	4	Upregulated: IL6, CCL2, SAA1, LBP	0.0069	9.64
GOTERM_BP_FAT	GO:0001819~positive regulation of cytokine production	3	Upregulated: IL6, SAA1, LBP	0.0093	19.61
GOTERM_BP_FAT	GO:0001817~regulation of cytokine production	3	Upregulated: IL6, SAA1, LBP	0.0345	9.75
Annotation Cluster 5 Enrichment Score: 2.31					
GOTERM_BP_FAT	GO:0042330~taxis	5	Upregulated: IL6, CCL2, SAA1, CCL8, CXCL6	1.17E-04	18.38
GOTERM_BP_FAT	GO:0006935~chemotaxis	5	Upregulated: IL6, CCL2, SAA1, CCL8, CXCL6	1.17E-04	18.38

GOTERM_BP_FAT	GO:0007626~locomotory behavior	5	Upregulated: IL6, CCL2, SAA1, CCL8, CXCL6	9.03E-04	10.73
INTERPRO	IPR001811:Small chemokine, interleukin-8-like	3	Upregulated: CCL2, CCL8, CXCL6	0.0013	53.00
SP_PIR_KEYWORDS	cytokine	4	Upregulated: IL6, CCL2, CCL8, CXCL6	0.0014	17.00
SMART	SM00199:SCY	3	Upregulated: CCL2, CCL8, CXCL6	0.0017	44.29
GOTERM_MF_FAT	GO:0008009~chemokine activity	3	Upregulated: CCL2, CCL8, CXCL6	0.0020	42.34
GOTERM_MF_FAT	GO:0042379~chemokine receptor binding	3	Upregulated: CCL2, CCL8, CXCL6	0.0023	39.74
GOTERM_MF_FAT	GO:0005125~cytokine activity	4	Upregulated: IL6, CCL2, CCL8, CXCL6	0.0027	13.32
SP_PIR_KEYWORDS	chemotaxis	3	Upregulated: CCL2, CCL8, CXCL6	0.0036	32.06
GOTERM_BP_FAT	GO:0006955~immune response	6	Upregulated: IL6, CCL2, C3, CCL8, CXCL6, LBP	0.0043	5.11
KEGG_PATHWAY	hsa04621:NOD-like receptor signaling pathway	3	Upregulated: IL6, CCL2, CCL8	0.0062	22.37
GOTERM_BP_FAT	GO:0007610~behavior	5	Upregulated: IL6, CCL2, SAA1, CCL8, CXCL6	0.0063	6.27
GOTERM_BP_FAT	GO:0051240~positive regulation of multicellular organismal process	4	Upregulated: IL6, CCL2, SAA1, LBP	0.0069	9.64
GOTERM_MF_FAT	GO:0008201~heparin binding	3	Upregulated: CCL2, CCL8, CXCL6	0.0098	18.91
KEGG_PATHWAY	hsa04060:Cytokine-cytokine receptor interaction	4	Upregulated: IL6, CCL2, CCL8, CXCL6	0.0124	7.06
GOTERM_BP_FAT	GO:0007267~cell-cell signaling	5	Upregulated: IL6, CCL8, SLC22A3, STC1, CXCL6	0.0148	4.90
GOTERM_MF_FAT	GO:0005539~glycosaminoglycan binding	3	Upregulated: CCL2, CCL8, CXCL6	0.0175	13.91
GOTERM_MF_FAT	GO:0001871~pattern binding	3	Upregulated: CCL2, CCL8, CXCL6	0.0209	12.65
GOTERM_MF_FAT	GO:0030247~polysaccharide binding	3	Upregulated: CCL2, CCL8, CXCL6	0.0209	12.65
BBID	109.Chemokine_families	3	Upregulated: CCL2, CCL8, CXCL6	0.0412	7.16
KEGG_PATHWAY	hsa04062:Chemokine signaling pathway	3	Upregulated: CCL2, CCL8, CXCL6	0.0498	7.42

Annotation Cluster 6 Enrichment Score: 1.76

GOTERM_BP_FAT	GO:0006955~immune response	6	Upregulated: IL6, CCL2, C3, CCL8, CXCL6, LBP	0.0043	5.11
GOTERM_BP_FAT	GO:0051240~positive regulation of multicellular organismal process	4	Upregulated: IL6, CCL2, SAA1, LBP	0.0069	9.64

GOTERM_BP_FAT	GO:0002237~response to molecule of bacterial origin	3	Upregulated: IL6, CCL2, LBP	0.0085	20.52
GOTERM_BP_FAT	GO:0010033~response to organic substance	5	Upregulated: IL6, CCL2, LBP, STEAP2, MMP13	0.0272	4.08
GOTERM_BP_FAT	GO:0009617~response to bacterium	3	Upregulated: IL6, CCL2, LBP	0.0388	9.14
Annotation Cluster 7 Enrichment Score: 1.60					
GOTERM_BP_FAT	GO:0006955~immune response	6	Upregulated: IL6, CCL2, C3, CCL8, CXCL6, LBP	0.0043	5.11
GOTERM_BP_FAT	GO:0006959~humoral immune response	3	Upregulated: IL6, CCL2, C3	0.0072	22.34
BBID	18.Cytokine_astocytes	3	Upregulated: IL6, CCL2, C3	0.0173	11.19
Annotation Cluster 8 Enrichment Score: 1.56					
GOTERM_BP_FAT	GO:0009725~response to hormone stimulus	4	Upregulated: IL6, CCL2, STEAP2, MMP13	0.0208	6.41
GOTERM_BP_FAT	GO:0009719~response to endogenous stimulus	4	Upregulated: IL6, CCL2, STEAP2, MMP13	0.0269	5.81
GOTERM_BP_FAT	GO:0010033~response to organic substance	5	Upregulated: IL6, CCL2, LBP, STEAP2, MMP13	0.0272	4.08
GOTERM_BP_FAT	GO:0048545~response to steroid hormone stimulus	3	Upregulated: IL6, CCL2, MMP13	0.0384	9.19

Table 5.3.3.4 Functional annotation clustering - Gene Ontology Terms for Biological Processes: MSC-17 vs. UT-MSC

GOTERM ID	GO Term	Count	Genes	Fold Enrichment	P-Value
Annotation Cluster 1 Enrichment Score: 3.13					
GO:0006954	inflammatory response	8	Upregulated: NFKBIZ, IL6, CCL2, C3, SAA1, CCL8, CXCL6, LBP	14.48	5.42E-07
GO:0009611	response to wounding	8	Upregulated: NFKBIZ, IL6, CCL2, C3, SAA1, CCL8, CXCL6, LBP	8.88	1.39E-05
GO:0006952	defense response	8	Upregulated: NFKBIZ, IL6, CCL2, C3, SAA1, CCL8, CXCL6, LBP	7.65	3.64E-05
GO:0006935	chemotaxis	5	Upregulated: IL6, CCL2, SAA1, CCL8, CXCL6	18.38	1.17E-04
GO:0042330	taxis	5	Upregulated: IL6, CCL2, SAA1, CCL8, CXCL6	18.38	1.17E-04
GO:0050727	regulation of inflammatory response	4	Upregulated: IL6, C3, SAA1, LBP	30.96	2.43E-04
GO:0002526	acute inflammatory response	4	Upregulated: IL6, C3, SAA1, LBP	24.01	5.14E-04
GO:0007626	locomotory behavior	5	Upregulated: IL6, CCL2, SAA1, CCL8, CXCL6	10.73	9.03E-04
GO:0006953	acute-phase response	3	Upregulated: IL6, SAA1, LBP	44.11	0.0019
GO:0032101	regulation of response to external stimulus	4	Upregulated: IL6, C3, SAA1, LBP	14.80	0.0021
GO:0006955	immune response	6	Upregulated: IL6, CCL2, C3, CCL8, CXCL6, LBP	5.11	0.0043
GO:0007610	behavior	5	Upregulated: IL6, CCL2, SAA1, CCL8, CXCL6	6.27	0.0063
GO:0051240	positive regulation of multicellular organismal process	4	Upregulated: IL6, CCL2, SAA1, LBP	9.64	0.0069
GO:0001819	positive regulation of cytokine production	3	Upregulated: IL6, SAA1, LBP	19.61	0.0093
GO:0001817	regulation of cytokine production	3	Upregulated: IL6, SAA1, LBP	9.75	0.0345
Annotation Cluster 2 Enrichment Score: 2.51					
GO:0050900	leukocyte migration	4	Upregulated: IL6, CCL2, SAA1 Downregulated: ITGA6,	41.28	1.03E-04
GO:0006935	chemotaxis	5	Upregulated: IL6, CCL2, SAA1, CCL8, CXCL6	18.38	1.17E-04
GO:0042330	taxis	5	Upregulated: IL6, CCL2, SAA1, CCL8, CXCL6	18.38	1.17E-04
GO:0007626	locomotory behavior	5	Upregulated: IL6, CCL2, SAA1, CCL8, CXCL6	10.73	9.03E-04
GO:0030595	leukocyte chemotaxis	3	Upregulated: IL6, CCL2, SAA1	47.69	0.0016
GO:0060326	cell chemotaxis	3	Upregulated: IL6, CCL2, SAA1	45.24	0.0018
GO:0060326	immune response	6	Upregulated: IL6, CCL2, C3, CCL8, CXCL6, LBP	5.11	0.0043

GO:0007610	behavior	5	Upregulated: IL6, CCL2, SAA1, CCL8, CXCL6	6.27	0.0063
GO:0051240	positive regulation of multicellular organismal process	4	Upregulated: IL6, CCL2, SAA1, LBP	9.64	0.0069
GO:0016477	cell migration	4	Upregulated: IL6, CCL2, SAA1 Downregulated: ITGA6,	8.52	0.0097
GO:004887	cell motility	4	Upregulated: IL6, CCL2, SAA1 Downregulated: ITGA6	7.66	0.0129
GO:0051674	localization of cell	4	Upregulated: IL6, CCL2, SAA1 Downregulated: ITGA6	7.66	0.0129
GO:000726	cell-cell signaling	5	Upregulated: IL6, CCL8, SLC22A3, STC1, CXCL6	4.90	0.0148
GO:0042592	homeostatic process	5	Upregulated: IL6, CCL2, SAA1, STC1 Downregulated: RPS24	3.92	0.0310
GO:0006928	cell motion	4	Upregulated: IL6, CCL2, SAA1 Downregulated: ITGA6	4.95	0.0404
Annotation Cluster 3			Enrichment Score: 2.41		
GO:0002675	positive regulation of acute inflammatory response	3	Upregulated: IL6, C3, LBP	147.04	1.65E-04
GO:0050727	regulation of inflammatory response	4	Upregulated: IL6, C3, SAA1, LBP	30.96	2.43E-04
GO:0002526	acute inflammatory response	4	Upregulated: IL6, C3, SAA1, LBP	24.01	5.14E-04
GO:0002673	regulation of acute inflammatory response	3	Upregulated: IL6, C3, LBP	84.02	5.20E-04
GO:0050729	positive regulation of inflammatory response	3	Upregulated: IL6, C3, LBP	58.82	0.0011
GO:0032101	regulation of response to external stimulus	4	Upregulated: IL6, C3, SAA1, LBP	14.80	0.0021
GO:0006955	immune response	6	Upregulated: IL6, CCL2, C3, CCL8, CXCL6, LBP	5.11	0.0043
GO:0032103	positive regulation of response to external stimulus	3	Upregulated: IL6, C3, LBP	27.57	0.0048
GO:0031349	positive regulation of defense response	3	Upregulated: IL6, C3, LBP	24.17	0.0062
GO:005124	positive regulation of multicellular organismal process	4	Upregulated: IL6, CCL2, SAA1, LBP	9.64	0.0069
GO:0002697	regulation of immune effector process	3	Upregulated: IL6, C3, LBP	17.47	0.0116
GO:0002252	immune effector process	3	Upregulated: IL6, C3, LBP	13.17	0.0198
Annotation Cluster 4			Enrichment Score: 1.76		
GO:0006955	immune response	6	Upregulated: IL6, CCL2, C3, CCL8, CXCL6, LBP	5.11	0.0043

GO:0051240	positive regulation of multicellular organismal process	4	Upregulated: IL6, CCL2, SAA1, LBP	9.64	0.0069
GO:0002237	response to molecule of bacterial origin	3	Upregulated: IL6, CCL2, LBP	20.52	0.0085
GO:0010033	response to organic substance	5	Upregulated: IL6, CCL2, LBP, STEAP2, MMP13	4.08	0.0272
GO:0009617	response to bacterium	3	Upregulated: IL6, CCL2, LBP	9.14	0.0388
Annotation Cluster 5 Enrichment Score: 1.56					
GO:0009725	response to hormone stimulus	4	Upregulated: IL6, CCL2, STEAP2, MMP13	6.41	0.0208
GO:0009719	response to endogenous stimulus	4	Upregulated: IL6, CCL2, STEAP2, MMP13	5.81	0.0269
GO:0010033	response to organic substance	5	Upregulated: IL6, CCL2, LBP, STEAP2, MMP13	4.08	0.0272
GO:0048545	response to steroid hormone stimulus	3	Upregulated: IL6, CCL2, MMP13	9.19	0.0384

Table 5.3.3.5 Functional annotation clustering - Gene Ontology Terms for Molecular Functions: MSC-17 vs. UT-MSC

GOTERM ID	Term	Count	Genes	Fold Enrichment	P-Value
Annotation Cluster 1			Enrichment Score: 2.01		
GO:0008009	chemokine activity	3	Upregulated: CCL2, CCL8, CXCL6	42.34	0.0020
GO:0042379	chemokine receptor binding	3	Upregulated: CCL2, CCL8, CXCL6	39.74	0.0023
GO:0005125~	cytokine activity	4	Upregulated: IL6, CCL2, CCL8, CXCL6	13.32	0.0027
GO:0008201	heparin binding	3	Upregulated: CCL2, CCL8, CXCL6	18.91	0.0098
GO:0005539	glycosaminoglycan binding	3	Upregulated: CCL2, CCL8, CXCL6	13.91	0.0175
GO:0001871	pattern binding	3	Upregulated: CCL2, CCL8, CXCL6	12.65	0.0209
GO:0030247	polysaccharide binding	3	Upregulated: CCL2, CCL8, CXCL6	12.65	0.0209

Table 5.3.3.6 Functional annotation clustering – Gene Ontology Terms for Cellular Components: MSC-17 vs. UT-MSC

GOTERM ID	Term	Count	Genes	Fold Enrichment	P-Value
Annotation Cluster 1			Enrichment Score: 6.21		
GO:0005615	extracellular space	10	Upregulated: IL6, CCL2, C3, SAA1, SFRP4, CCL8, STC1, CXCL6, LBP, MMP13	9.82	1.09E-07
GO:0044421	extracellular region part	11	Upregulated: IL6, CCL2, C3, SAA1, SFRP4, CCL8, STC1, CXCL6, LBP, MMP13, MMP1	7.71	1.37E-07
GO:0005576	extracellular region	12	Upregulated: IL6, CCL2, C3, SAA1, SFRP4, CCL8, STC1, CXCL6, LBP, VMO1, MMP13, MMP1	4.02	1.54E-05

Table 5.3.3.7 Functional annotation chart: Gene enrichment analysis on DAVID's Default settings: MSC-17 vs. UT-MSC

Category	Term	Count	Genes	Fold Enrichment	P-Value
GOTERM_CC_FAT	GO:0005615~extracellular space	10	Upregulated: IL6, CCL2, C3, SAA1, SFRP4, CCL8, STC1, CXCL6, LBP, MMP13	9.82	1.09E-07
GOTERM_CC_FAT	GO:0044421~extracellular region part	11	Upregulated: IL6, CCL2, C3, SAA1, SFRP4, CCL8, STC1, CXCL6, LBP, MMP13, MMP1	7.71	1.37E-07
GOTERM_BP_FAT	GO:0006954~inflammatory response	8	Upregulated: NFKBIZ, IL6, CCL2, C3, SAA1, CCL8, CXCL6, LBP	14.48	5.42E-07
SP_PIR_KEYWORDS	Secreted	12	Upregulated: IL6, CCL2, C3, SAA1, SFRP4, CCL8, STC1, CXCL6, LBP, VMO1, MMP13, MMP1	5.47	1.97E-06
GOTERM_BP_FAT	GO:0009611~response to wounding	8	Upregulated: NFKBIZ, IL6, CCL2, C3, SAA1, CCL8, CXCL6, LBP	8.88	1.39E-05
GOTERM_CC_FAT	GO:0005576~extracellular region	12	Upregulated: IL6, CCL2, C3, SAA1, SFRP4, CCL8, STC1, CXCL6, LBP, VMO1, MMP13, MMP1	4.02	1.54E-05
GOTERM_BP_FAT	GO:0006952~defense response	8	NFKBIZ, IL6, CCL2, C3, SAA1, CCL8, CXCL6, LBP	7.65	3.64E-05
GOTERM_BP_FAT	GO:0050900~leukocyte migration	4	Upregulated: IL6, CCL2, , SAA1 Downregulated: ITGA6	41.28	1.03E-04
GOTERM_BP_FAT	GO:0042330~taxis	5	Upregulated: IL6, CCL2, SAA1, CCL8, CXCL6	18.38	1.17E-04
GOTERM_BP_FAT	GO:0006935~chemotaxis	5	Upregulated: IL6, CCL2, SAA1, CCL8, CXCL6	18.38	1.17E-04
GOTERM_BP_FAT	GO:0002675~positive regulation of acute inflammatory response	3	Upregulated: IL6, C3, LBP	147.04	1.65E-04
SP_PIR_KEYWORDS	inflammatory response signal	13	Upregulated: IL6, CCL2, , SAA1, C3, SFRP4, CCL8, STC1, CXCL6, LBP, VMO1, MMP13, MMP1 Downregulated: ITGA6	3.08	1.92E-04
UP_SEQ_FEATURE	signal peptide	13	Upregulated: IL6, CCL2, , SAA1, C3, SFRP4, CCL8, STC1, CXCL6, LBP, VMO1, MMP13, MMP1 Downregulated: ITGA6	3.06	2.04E-04
GOTERM_BP_FAT	GO:0050727~regulation of inflammatory response	4	Upregulated: IL6, C3, SAA1, LBP	30.96	2.43E-04
GOTERM_BP_FAT	GO:0002526~acute inflammatory response	4	Upregulated: IL6, C3, SAA1, LBP	24.01	5.14E-04
GOTERM_BP_FAT	GO:0002673~regulation of acute inflammatory response	3	Upregulated: IL6, C3, LBP	84.02	5.20E-04
SP_PIR_KEYWORDS	acute phase	3	Upregulated: IL6, C3, SAA1	79.59	5.93E-04
GOTERM_BP_FAT	GO:0007626~locomotory behavior	5	Upregulated: IL6, CCL2, SAA1, CCL8, CXCL6	10.73	9.03E-04

GOTERM_BP_FAT	GO:0050729~positive regulation of inflammatory response	3	Upregulated: IL6, C3, LBP	58.82	0.0011
UP_SEQ_FEATURE	disulfide bond	11	Upregulated: IL6, CCL2, , C3, SFRP4, CCL8, STC1, CXCL6, VMO1, MMP13, MMP1 Downregulated: ITGA6	2.98	0.0013
INTERPRO	IPR001811:Small chemokine, interleukin-8-like	3	Upregulated: CCL2, CCL8, CXCL6	53.00	0.0013
SP_PIR_KEYWORDS	cytokine	4	Upregulated: IL6, CCL2, CCL8, CXCL6	17.00	0.0014
GOTERM_BP_FAT	GO:0030595~leukocyte chemotaxis	3	Upregulated: IL6, CCL2, SAA1	47.69	0.0016
SP_PIR_KEYWORDS	disulfide bond	11	Upregulated: IL6, CCL2, , C3, SFRP4, CCL8, STC1, CXCL6, VMO1, MMP13, MMP1 Downregulated: ITGA6	2.89	0.0016
SMART	SM00199:SCY	3	Upregulated: CCL2, CCL8, CXCL6	44.29	0.0017
GOTERM_BP_FAT	GO:0060326~cell chemotaxis	3	Upregulated: IL6, CCL2, SAA1	45.24	0.0018
GOTERM_BP_FAT	GO:0006953~acute-phase response	3	Upregulated: IL6, SAA1, LBP	44.11	0.0019
GOTERM_MF_FAT	GO:0008009~chemokine activity	3	Upregulated: CCL2, CCL8, CXCL6	42.34	0.0020
GOTERM_BP_FAT	GO:0032101~regulation of response to external stimulus	4	Upregulated: IL6, C3, SAA1, LBP	14.80	0.0021
GOTERM_MF_FAT	GO:0042379~chemokine receptor binding	3	Upregulated: CCL2, CCL8, CXCL6	39.74	0.0023
GOTERM_MF_FAT	GO:0005125~cytokine activity	4	Upregulated: IL6, CCL2, CCL8, CXCL6	13.32	0.0027
SP_PIR_KEYWORDS	chemotaxis	3	Upregulated: CCL2, CCL8, CXCL6	32.06	0.0036
SP_PIR_KEYWORDS	inflammatory response	3	Upregulated: CCL2, C3, CCL8	29.98	0.0041
GOTERM_BP_FAT	GO:0006955~immune response	6	Upregulated: IL6, CCL2, C3, CCL8, CXCL6, LBP	5.11	0.0043
GOTERM_BP_FAT	GO:0032103~positive regulation of response to external stimulus	3	Upregulated: IL6, C3, LBP	27.57	0.0048
GOTERM_BP_FAT	GO:0009991~response to extracellular stimulus	4	Upregulated: CCL2, , SLC22A3, STC1 Downregulated: ITGA6	10.69	0.0052
KEGG_PATHWAY	hsa04621:NOD-like receptor signaling pathway	3	Upregulated: IL6, CCL2, CCL8	22.37	0.0062
GOTERM_BP_FAT	GO:0031349~positive regulation of defense response	3	Upregulated: IL6, C3, LBP	24.17	0.0062
GOTERM_BP_FAT	GO:0007610~behavior	5	Upregulated: IL6, CCL2, SAA1, CCL8, CXCL6	6.27	0.0063
GOTERM_BP_FAT	GO:0002548~monocyte chemotaxis	2	Upregulated: IL6, CCL2	294.09	0.0065
PIR_SUPERFAMILY	PIRSF500572:small inducible cytokine, A2 type	2	Upregulated: CCL2, CCL8	268.95	0.0067
GOTERM_BP_FAT	GO:0051240~positive regulation of multicellular organismal process	4	Upregulated: IL6, CCL2, SAA1, LBP	9.64	0.0069

GOTERM_BP_FAT	GO:0006959~humoral immune response	3	Upregulated: IL6, CCL2, C3	22.34	0.0072
GOTERM_BP_FAT	GO:0002237~response to molecule of bacterial origin	3	Upregulated: IL6, CCL2, LBP	20.52	0.0085
UP_SEQ_FEATURE	metal ion-binding site:Calcium 4; via carbonyl oxygen	2	Upregulated: MMP13, MMP1	218.43	0.0088
GOTERM_BP_FAT	GO:0001819~positive regulation of cytokine production	3	Upregulated: IL6, SAA1, LBP	19.61	0.0093
GOTERM_BP_FAT	GO:0016477~cell migration	4	Upregulated: IL6, CCL2, , SAA1 Downregulated: ITGA6	8.52	0.0097
GOTERM_MF_FAT	GO:0008201~heparin binding	3	Upregulated: CCL2, CCL8, CXCL6	18.91	0.0098
GOTERM_BP_FAT	GO:0048247~lymphocyte chemotaxis	2	Upregulated: CCL2, SAA1	168.05	0.0113
GOTERM_BP_FAT	GO:0002697~regulation of immune effector process	3	Upregulated: IL6, C3, LBP	17.47	0.0116
KEGG_PATHWAY	hsa04060:Cytokine-cytokine receptor interaction	4	Upregulated: IL6, CCL2, CCL8, CXCL6	7.06	0.0124
BIOCARTA	h_LDLpathway:Low-density lipoprotein (LDL) pathway during atherogenesis	2	Upregulated: IL6, CCL2	119.75	0.0125
UP_SEQ_FEATURE	metal ion-binding site:Zinc 2; in inhibited form	2	Upregulated: MMP13, MMP1	152.90	0.0125
GOTERM_BP_FAT	GO:0051674~localization of cell	4	Upregulated: IL6, CCL2, , SAA1 Downregulated: ITGA6	7.66	0.0129
GOTERM_BP_FAT	GO:0048870~cell motility	4	IL6, CCL2, , SAA1 Downregulated: ITGA6	7.66	0.0129
GOTERM_BP_FAT	GO:0048246~macrophage chemotaxis	2	Upregulated: CCL2, SAA1	130.71	0.0145
GOTERM_BP_FAT	GO:0007267~cell-cell signaling	5	Upregulated: IL6, CCL8, SLC22A3, STC1, CXCL6	4.90	0.0148
GOTERM_BP_FAT	GO:0050829~defense response to Gram-negative bacterium	2	Upregulated: IL6, LBP	117.63	0.0161
BBID	18.Cytokine_astocytes	3	Upregulated: IL6, CCL2, C3	11.19	0.0173
UP_SEQ_FEATURE	metal ion-binding site:Zinc 2; catalytic	2	Upregulated: MMP13, MMP1	109.22	0.0174
GOTERM_MF_FAT	GO:0005539~glycosaminoglycan binding	3	Upregulated: CCL2, CCL8, CXCL6	13.91	0.0175
PIR_SUPERFAMILY	PIRSF001191:matrix metalloproteinase, stromelysin type	2	Upregulated: MMP13, MMP1	96.05	0.0188
GOTERM_BP_FAT	GO:0002252~immune effector process	3	Upregulated: IL6, C3, LBP	13.17	0.0198
GOTERM_BP_FAT	GO:0009725~response to hormone stimulus	4	Upregulated: IL6, CCL2, STEAP2, MMP13	6.41	0.0208
GOTERM_MF_FAT	GO:0030247~polysaccharide binding	3	Upregulated: CCL2, CCL8, CXCL6	12.65	0.0209
GOTERM_MF_FAT	GO:0001871~pattern binding	3	Upregulated: CCL2, CCL8, CXCL6	12.65	0.0209
SP_PIR_KEYWORDS	collagen degradation	2	Upregulated: MMP13, MMP1	90.52	0.0210

UP_SEQ_FEATURE	glycosylation site:N-linked (GlcNAc...)	11	Upregulated: IL6, CCL2, , C3, CH25H, SFRP4, SLC22A3, STC1, LBP, MMP13, MMP1	2.04	0.0213
			Downregulated: ITGA6		
INTERPRO	IPR018933:Netrin module, non-TIMP type	2	Upregulated: C3, SFRP4	85.21	0.0222
INTERPRO	IPR016293:Peptidase M10A, matrix metalloproteinase	2	Upregulated: MMP13, MMP1	80.48	0.0235
PIR_SUPERFAMILY	PIRSF001191:Peptidase_M10A_matrix	2	Upregulated: MMP13, MMP1	74.71	0.0241
SMART	SM00643:C345C	2	Upregulated: C3, SFRP4	71.21	0.0259
UP_SEQ_FEATURE	metal ion-binding site:Calcium 3; via carbonyl oxygen	2	Upregulated: MMP13, MMP1	72.81	0.0261
INTERPRO	IPR002477:Peptidoglycan binding-like	2	Upregulated: MMP13, MMP1	72.43	0.0261
GOTERM_BP_FAT	GO:0009719~response to endogenous stimulus	4	Upregulated: IL6, CCL2, STEAP2, MMP13	5.81	0.0269
GOTERM_BP_FAT	GO:0010033~response to organic substance	5	Upregulated: IL6, CCL2, LBP, STEAP2, MMP13	4.08	0.0272
UP_SEQ_FEATURE	domain:Hemopexin-like 4	2	Upregulated: MMP13, MMP1	69.50	0.0273
UP_SEQ_FEATURE	domain:Hemopexin-like 3	2	Upregulated: MMP13, MMP1	69.50	0.0273
UP_SEQ_FEATURE	domain:NTR	2	Upregulated: C3, SFRP4	69.50	0.0273
SP_PIR_KEYWORDS	glycoprotein	11	Upregulated: IL6, CCL2, , C3, CH25H, SFRP4, SLC22A3, STC1, LBP, MMP13, MMP1	1.96	0.0276
			Downregulated: ITGA6		
UP_SEQ_FEATURE	domain:Hemopexin-like 2	2	Upregulated: MMP13, MMP1	66.48	0.0285
UP_SEQ_FEATURE	domain:Hemopexin-like 1	2	Upregulated: MMP13, MMP1	66.48	0.0285
GOTERM_BP_FAT	GO:0030593~neutrophil chemotaxis	2	Upregulated: CCL2, SAA1	65.35	0.0289
SP_PIR_KEYWORDS	metalloproteinase	2	Upregulated: MMP13, MMP1	64.12	0.0295
INTERPRO	IPR000585:Hemopexin/matrixin	2	Upregulated: MMP13, MMP1	62.98	0.0300
INTERPRO	IPR018487:Hemopexin/matrixin, repeat	2	Upregulated: MMP13, MMP1	62.98	0.0300
INTERPRO	IPR018486:Hemopexin/matrixin, conserved site	2	Upregulated: MMP13, MMP1	62.98	0.0300
INTERPRO	IPR001134:Netrin domain	2	Upregulated: C3, SFRP4	62.98	0.0300
GOTERM_BP_FAT	GO:0032755~positive regulation of interleukin-6 production	2	Upregulated: IL6, LBP	61.91	0.0305
PIR_SUPERFAMILY	PIRSF001950:small inducible chemokine, C/CC types	2	Upregulated: CCL2, CCL8	58.47	0.0307
SP_PIR_KEYWORDS	inflammation	2	Upregulated: CCL2, C3	61.55	0.0307
GOTERM_BP_FAT	GO:0042592~homeostatic process	5	Upregulated: IL6, CCL2, SAA1, STC1, Downregulated: RPS24	3.92	0.0310

GOTERM_BP_FAT	GO:0050830~defense response to Gram-positive bacterium	2	Upregulated: IL6, LBP	58.82	0.0320
GOTERM_BP_FAT	GO:0030574~collagen catabolic process	2	Upregulated: MMP13, MMP1	58.82	0.0320
INTERPRO	IPR000827:Small chemokine, C-C group, conserved site	2	Upregulated: CCL2, CCL8	57.94	0.0325
BIOCARTA	h_LairPathway:Cells and Molecules involved in local acute inflammatory response	2	Upregulated: IL6, C3	44.91	0.0331
GOTERM_BP_FAT	GO:0001817~regulation of cytokine production	3	Upregulated: IL6, SAA1, LBP	9.75	0.0345
SMART	SM00120:HX	2	Upregulated: MMP13, MMP1	52.63	0.0349
GOTERM_BP_FAT	GO:0006874~cellular calcium ion homeostasis	3	Upregulated: CCL2, SAA1, STC1	9.64	0.0352
GOTERM_BP_FAT	GO:0055074~calcium ion homeostasis	3	Upregulated: CCL2, SAA1, STC1	9.39	0.0370
INTERPRO	IPR006026:Peptidase, metallopeptidases	2	Upregulated: MMP13, MMP1	49.95	0.0376
GOTERM_BP_FAT	GO:0048545~response to steroid hormone stimulus	3	Upregulated: IL6, CCL2, MMP13	9.19	0.0384
GOTERM_BP_FAT	GO:0009617~response to bacterium	3	Upregulated: IL6, CCL2, LBP	9.14	0.0388
GOTERM_BP_FAT	GO:0006875~cellular metal ion homeostasis	3	Upregulated: CCL2, SAA1, STC1	9.00	0.0399
GOTERM_BP_FAT	GO:0031667~response to nutrient levels	3	Upregulated: CCL2, SLC22A3, STC1	8.96	0.0403
GOTERM_BP_FAT	GO:0006928~cell motion	4	Upregulated: IL6, CCL2, , SAA1 Downregulated: ITGA6	4.95	0.0404
BBID	109.Chemokine_families	3	Upregulated: CCL2, CCL8, CXCL6	7.16	0.0412
GOTERM_BP_FAT	GO:0044243~multicellular organismal catabolic process	2	Upregulated: MMP13, MMP1	45.24	0.0415
GOTERM_BP_FAT	GO:0055065~metal ion homeostasis	3	Upregulated: CCL2, SAA1, STC1	8.61	0.0433
SMART	SM00235:ZnMc	2	Upregulated: MMP13, MMP1	41.74	0.0438
GOTERM_BP_FAT	GO:0032940~secretion by cell	3	Upregulated: IL6, CCL8, STEAP2	8.52	0.0440
GOTERM_BP_FAT	GO:0032963~collagen metabolic process	2	Upregulated: MMP13, MMP1	42.01	0.0446
UP_SEQ_FEATURE	metal ion-binding site:Calcium 2; via carbonyl oxygen	2	Upregulated: MMP13, MMP1	39.21	0.0479
GOTERM_BP_FAT	GO:0044259~multicellular organismal macromolecule metabolic process	2	Upregulated: MMP13, MMP1	37.95	0.0493
KEGG_PATHWAY	hsa04062:Chemokine signaling pathway	3	Upregulated: CCL2, CCL8, CXCL6	7.42	0.0498

5.3.4 Microarray gene validation by real-time PCR

To validate the microarray data of MSC-17, upregulated genes were evaluated for their gene expression by RT-PCR (**Figure 5.3.5**). Gene expression of MSC-17 was compared to UT-MSC and MSC- γ from the 3 human MSC donors investigated. MMP13 was highly expressed only in MSC-17 and in all 3 MSC donors as determined by microarray (FC 15.6) and validated by RT-PCR ($p < 0.01$). Other genes such as C3 (FC 11.56), LBP (FC 5.35) and CH25H (FC 3.99) were also confirmed to be upregulated only after IL-17A treatment of MSC as detected by RT-PCR. The gene expression levels of MMP13, C3, LBP and CH25H in MSC-17 varied between the 3 MSC donors.

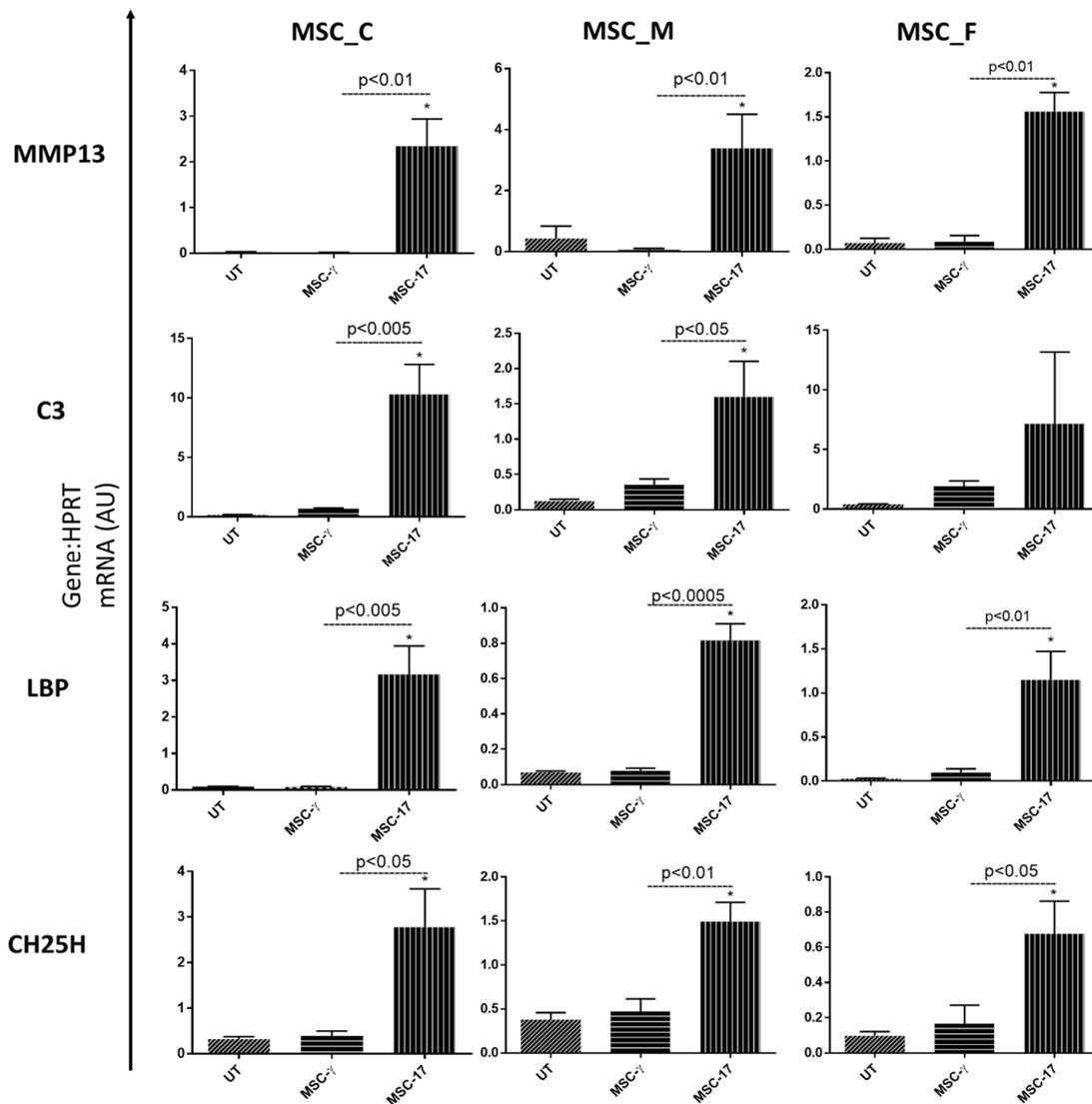


Figure 5.3.5 Microarray gene expression validation by RT-PCR.

Gene expression of MMP13, C3, LBP and CH25 in MSC detected by microarray were validated by RT-PCR following 5 days IL-17A or IFN- γ treatment of human MSC from 3 different donors (MSC_C, MSC_M and MSC_F). * $p < 0.05$ vs. UT-MSCs were determined by one-way ANOVA with post-Sidak multiple comparison test. Error bars depict mean \pm SD.

5.4 DISCUSSION

Strategies to pre-activate the immunosuppressive function of MSC prior to their infusion *in vivo* may enhance the translation of MSC as a universal cell based therapy agent to prevent allotransplantation rejection and induce immune tolerance. The *ex vivo* manipulation of MSC with proinflammatory cytokines, particularly IFN- γ modification of MSC is now widely known to enhance the immunomodulatory, reparative and homing potential of MSC⁴. This thesis describes a novel method to culture human MSC with IL-17A (MSC-17) as an alternative to IFN- γ modification of MSC (MSC- γ). In this chapter, using microarray and bioinformatics approaches, the effects of 5 days IFN- γ or IL-17A treatment on human MSC gene expression profile were investigated. The identification of novel MSC expressed immunoregulatory factors may enable the discovery of mechanisms by which MSC- γ or MSC-17 therapy may be beneficial in allotransplantation or in other inflammatory diseases.

IFN- γ pre-activation of human MSC induced the expression of various immunoregulatory molecules that may enhance the inhibitory activity of MSC- γ to directly or indirectly mediate T cell suppression. Immunoregulatory molecules including IDO, TLR3, TLR4, and IL-6 were enriched for the gene ontology term “regulation of defense response”. IDO, the tryptophan catabolising enzyme, is a well characterised immunosuppressive molecule expressed by MSC upon induction with IFN- γ ^{2,224,246,311}. Administration of IDO deficient MSC (IDO^{-/-} MSC) or inhibition of IDO activity resulted in accelerated kidney allograft rejection, decreased intragraft or circulating Tregs and showed absence of donor-specific tolerance²²⁴. IDO^{-/-}MSC were also incapable of inhibiting donor DC maturation and function, thus enabling donor DC to stimulate strong recipient T cell proliferative responses²²⁴. Consistent with previous literature^{224,311,314}, microarray analysis revealed that IDO was one of the most highly induced gene in MSC- γ (FC 96.7). IDO gene expression was validated by RT-PCR (**Figure**

3.3.14). This data suggests that IDO may be the key candidate molecule by which MSC- γ mediate enhanced immunosuppression of T cells as demonstrated in this thesis.

MSC can also mediate T cell suppression via cell-contact dependent mechanisms involving toll-like receptors (TLRs). TLRs have a critical role in the innate and adaptive immune response. TLRs sense infection and damage signals following an infection or injury. Human bone marrow-derived MSC constitutively express a range of TLRs, including TLR3 and TLR4^{255,418}. Activation of TLR3 and (or) TLR4 have been shown to amplify MSC trophic factors, anti-microbial activity and immunosuppressive potential, thereby enhancing MSC therapeutic potency^{255,419-421}. TLR3 (FC 8.42) and TLR4 (FC 2.61) were upregulated in MSC- γ compared to UT-MSC. Activation of TLR3 and TLR4 signaling with poly I:C or LPS respectively, induced IDO1 expression in MSC²⁵⁵. TLR-driven induction of IDO in MSC resulted in the degradation of tryptophan and production of immunosuppressive kynurenines²⁵⁵. TLR3 activation has also been linked to expression of IL-6 in MSC⁴¹⁹. IL-6 mediates the inhibitory effects of MSC on DC differentiation, maturation and function^{269,272}. The upregulation of IL-6 gene expression (**Table 5.3.2.1; Figure 3.3.14**) in MSC- γ and high concentrations of IL-6 in MSC- γ – T cell co-culture supernatants (**Figure 3.3.13**) suggest that MSC- γ secreted IL-6 may be involved in suppression of proinflammatory T cell responses. Nevertheless, it should be noted that some studies have demonstrated that TLR activation in MSC abrogate their immunosuppressive properties^{172,422}. Studies to determine whether TLR signaling in MSC is beneficial or detrimental for immunomodulation warrants further investigation.

A set of genes that may be involved in MSC- γ leukocyte binding as a mechanism to exert enhanced suppression on immune inflammatory cells have been identified in this chapter. TLR3/4 preactivated MSC have enhanced leukocyte binding activity mediated by the induction of the adhesion molecule ICAM-1, consistent with the observed upregulation of ICAM-1 in

MSC- γ (FC 18.43)⁴²⁰. ICAM-1 together with TLR3 and TLR4 were among the genes enriched for the gene ontology term “positive regulation of immune system process”, suggesting a potential biological role of TLR3/4 in the induction of ICAM-1 in MSC- γ ⁴²⁰. Additionally, the upregulation of chemokines such as CXCL-9, -10, -11, -16, CCL2, CCL8 and CCL13 detected by microarray may facilitate T cell recruitment to MSC- γ . Mouse MSC preactivated with proinflammatory cytokines IFN- γ and TNF- α are known to induce chemokines such as CXCL9 and CXCL10³⁰⁷. The production of these chemokines was abrogated by IFN- γ neutralization³⁰⁷. Moreover, the blockade of CXCR3, a T cell receptor for chemokines CXCL-9 and -10 eliminated T cell chemotaxis towards MSC and subsequent MSC inhibition of T cell proliferation³⁰⁷. This study concluded that cytokines induce MSC-expression of chemokines to drive T cell recruitment into close proximity with MSC, enabling MSC to suppress T cells through the secretion of immunosuppressive molecules^{307,423}. Higher expression of these chemokines in MSC- γ may also facilitate their homing potential to sites of inflammation. It was reported in one study that these chemokines potentially enhanced human MSC- γ migration to inflammatory sites to mediate their anti-inflammatory effects *in vivo* in an established mouse model of colitis³⁰⁶. Studies to validate the functional role of human MSC- γ derived chemokines and ICAM-1 in the recruitment and subsequent modulation of T cell immune responses as well as in MSC- γ homing to sites of inflammation *in vivo* are required. Taken together, this thesis data suggests that IFN- γ , not only directly induces an array of immunosuppressive molecules in MSC, but may further amplify the secretion of other MSC-inhibitory molecules such as IDO, IL-6, ICAM-1 via TLR3/4 activation. MSC- γ with higher proximity to leukocytes may serve as an additional mechanism by which MSC- γ increase their modulatory activity on T cells.

MSC- γ are also known to modulate T cell responses involving programmed cell death pathways^{194,251,252}. Based on this study, an array of genes was enriched for regulation of programmed cell death or apoptosis (**Table 5.3.2.4** annotation cluster 5). Bioinformatics

analysis also detected other novel upregulated genes in MSC- γ with cell migratory properties, with previous unidentified roles in MSC- γ and can be explored in future studies (annotation cluster 10; **Table 5.3.2.4**). In addition, there were gene sets enriched for response to wound healing in MSC- γ (annotation cluster 2; **Table 5.3.2.4**). This is in agreement with a study demonstrating increased efficacy of MSC- γ for tissue regeneration compared to UT-MSC as demonstrated in a mouse wound repair model³²². Collectively these genes can be explored in future studies for their regulatory roles in MSC- γ immunosuppression, migration and reparative properties.

A major limitation to MSC- γ therapy is their reduced proliferative capacity compared to UT-MSC (**Figure 3.3.5C**)^{174,303}. A large number of genes downregulated in MSC following IFN- γ treatment were involved in cell division processes as identified by DAVID's analysis (**Table 5.3.2.5**). Decreased MSC- γ proliferation is disadvantageous as high yields of cells are required for patient infusion. Targeting these genes may augment MSC proliferation for future studies aiming to manufacture clinical scales of MSC for *in vivo* patient administration.

Despite MSC- γ being highly immunosuppressive with enhanced homing and reparative capacities, there is a growing body of evidence suggesting that allogeneic MSC- γ are ineffective *in vivo* due to their increased immunogenicity^{217,299,300}. This represents a major limitation to MSC- γ therapy. DAVID's analysis revealed that a large number of gene sets upregulated in MSC- γ were involved in the antigen processing and presentation pathways of MHC class I and class II, with high enrichment scores, 8.03 and 4.45, respectively (**Table 5.3.2.4**).

Antigens are degraded or processed prior to being presented by MHC molecules to T cells via the direct, indirect or semi-direct pathway of allorecognition (**Figure 1.3.1**). Antigen processing and presentation occurs via 2 distinct pathways: (A) **cytosolic pathway** – endogenous antigens are presented via MHC class I molecules⁴²⁴⁻⁴²⁸; or (B) **endocytic**

pathway - exogenous antigens are uptaken by APC through endocytosis and are presented on MHC class II molecules^{428,429}.

(A) Cytosolic pathway: Intracellular proteins are degraded within the proteasome in the cytosol. Following proteolysis of intracellular proteins, these peptides are transported from the cytosol to the rough endoplasmic reticulum (RER) via the TAP (transported associated with antigen processing). TAP is a heterodimer that consists of TAP1 and TAP2 and has affinity for peptides of 8-16 amino acids. Since the optimal length for MHC class I peptide loading is 8-10 amino acid in length, peptides are trimmed by aminopeptidases ERAP (endoplasmic reticulum aminopeptidase) to enable optimal peptide loading onto MHC class I molecules. MHC class I components comprise of the class I MHC α chain and the β_2 microglobulin (B2M) chain. Assembly of MHC class I complex occurs in the RER and is aided by chaperone proteins (calnexin, Tapasin, calreticulin and ERp57). These chaperone molecules facilitate protein folding and ensure stabilization of the MHC class I molecules. Calnexin binds to the newly synthesised class I MHC α chain to promote its folding. B2M association with the α chain dissociates calnexin. This MHC class I molecule then associates with the chaperone molecules tapasin, calreticulin and ERp57. Tapasin (TAPBPL, TAP binding protein-like or also known as tapasin related protein) recruits the MHC I molecules into proximity to TAP, allowing efficient peptide loading onto MHC class I molecules, subsequently stabilizing the peptide-class I molecule complex. The Class I MHC-peptide complex is then transported from the RER to Golgi complex and to the plasma membrane for antigen-peptide presentation to CD8⁺ T cells⁴²⁴⁻⁴²⁸.

(B) Endocytic pathway: Exogenous protein antigens uptaken by APC through phagocytosis or endocytosis are digested into small peptides in the endosome. Assembly of MHC class II occurs in RER where the α and β chain associate and this newly synthesis class II MHC complex binds to the invariant chain (Ii, CD74). Ii occupies the MHC class II peptide

binding cleft to prevent endogenous peptide loading. This class II MHC-Ii complex is then transported from the RER to the Golgi complex, trans-Golgi network and to the endocytic vesicles containing the degraded exogenous antigen. As MHC class II-Ii complex is translocated into the endosomal compartment, the Ii chain is degraded leaving the CLIP fragment (Class II associated Ii peptide) bound to the MHC II peptide binding cleft. In the endosomal compartment, HLA-DM catalyses the exchange of CLIP with the antigenic peptide. The MHC class II-peptide complex is then transported to the plasma membrane for antigen presentation to T cells^{428,429}.

The induction of genes involved in the cytosolic and endocytic pathways of antigen processing and presentation was clearly evident by the expression of aminopeptidases (ERAP), peptide transporter genes (TAP2) and other genes including TAPBPL, B2M, CD74 and HLA-DM following IFN- γ treatment of human MSC. The expression of MHC class II on allogeneic MSC is known to induce alloimmune responses in co-cultures with MHC-mismatch responder cells in a one-way MLR³²⁸. *In vivo* studies by Zangi, 2009, showed that alloimmune responses against UT-MSC are mediated by the recognition of allogeneic MHC molecules by recipient CD4⁺ and CD8⁺ memory T cells²²³. Therefore, the amplification of the antigen processing and presentation machinery in MSC- γ clearly suggest that these cells are highly immunogenic and can prime proinflammatory T cell responses in allogeneic hosts.

The microarray analysis found that MSC- γ exhibited enriched levels of gene sets involved in amplification of the humoral immune response and complement pathways activation. This thesis chapter data is in accord with previous studies demonstrating that MSC- γ enhances the primary and secondary alloantibody responses compared to UT-MSC²¹⁷. Higher levels of circulating anti-donor IgM and IgG alloantibodies were detected in mice administered with MSC- γ , indicating an induction of a rapid AMR to MSC- γ ²¹⁷. Although MSC- γ lack the expression of co-stimulatory signals (CD80, CD83, CD86) to function as APC to mediate direct

T cell allorecognition and activation^{174,238,250,302}, it can be speculated that MSC- γ induce allogeneic T cell responses through the indirect or semi-direct pathway of allorecognition (**Figure 1.3.1**). Allogeneic MHC-peptide transfer from MSC- γ could be more rapid compared to UT-MSc due to high expression of MHC molecules. This enables allogeneic MHC-peptide to be recognised by recipient T cells through the semi-direct pathway. Moreover, the enhanced expression or induction of allogeneic proteins in MSC- γ may activate recipient T cells by the indirect pathway of allorecognition or induce B cell activation, alloantibody production and complement mediated-lysis of donor cells in allotransplantation. The involvement of these antigen processing and presentation pathway genes in MSC- γ induction of alloimmune responses *in vivo* remains to be explored. Understanding mechanism of MSC immunogenicity may enable targeting MSC through different pathways of activation (eg. TLR) to increase their immunomodulatory function whilst retaining MSC in a non immunogenic and inert state.

Human MSC pre-treated with IL-17A represent a novel immunomodulatory strategy and an alternative to IFN- γ in enhancing MSC immunosuppression of T cells (**CHAPTER 3 and 4**)². Human MSC-17 as reported in this thesis, exhibited similar immunophenotype and morphology to UT-MSc, yet these cells showed superior modulation of T cell responses². IL-17A ligation to the IL-17RA/RC receptor complex is known to induce the expression of an array of chemokines in other cell types including CCL2 in murine nonimmune cells⁴³⁰. Huang *et. al.*, (2009) previously reported that IL-17A increased MSC migration and homing to wound site in an *in vitro* wound-healing assay³⁴¹. Molecules involved in the enhanced MSC-17 recruitment to wound site however were not determined in this study. Microarray gene expression analysis identified a few chemokines that were upregulated in human MSC-17 (ie. CCL2, CCL8 and CXCL6) and were enriched for leukocyte migration and chemotaxis. A few studies have described the role of CCL2 in MSC immunoregulation *in vivo*^{256,431}. CCL2-deficient MSC were shown to be incapable of inhibiting T cell responses and autoimmune

EAE⁴³¹. MSC-derived CCL2 inhibited EAE driven T cell activation by suppressing STAT3 phosphorylation, blocking T cell infiltration to spinal cord and reducing systemic levels of IL-17A and TNF- α ; resulting in alleviation of EAE symptoms⁴³¹. In a subsequent study, murine MSC were shown to ameliorate colitis and systemic sclerosis via the FAS-L/FAS signaling pathway, leading to activated T cell apoptosis²⁵⁶. Mechanistically, FAS (on activated T cells) interaction with FAS-L (on MSC) upregulated CCL2 secretion in MSC, enabling MSC to recruit T cells for FASL-dependent apoptosis^{256,432}. Thus, MSC-17 derived chemokines may facilitate the migration of T cells into close proximity with MSC, enabling potent suppression of proinflammatory T cell responses either through the FAS-L/FAS pathways of T cell apoptosis or via expression of other immunoregulatory factors.

Moreover, MSC-derived CCL2 were able to inhibit immunoglobulin production by suppressing STAT3 phosphorylation, while activating the transcription factor PAX5 in plasma cells⁴³³. STAT3 activation in plasma cells is essential for antibody production as it induces the expression of BLIMP-1, a repressor of PAX5, thereby allowing Ig production^{434,435}. Neutralization of CCL2 eliminated MSC expression of PAX5 and reversed MSC suppression on antibody production⁴³³. Based on data from this chapter, CCL2 was upregulated in MSC-17 and was enriched for the gene ontology term “humoral responses” (annotation cluster 7, **Table 5.3.3.1**; **Table 5.3.3.7**). Enhanced expression of CCL2 in MSC-17 may therefore be beneficial in controlling antibody mediated rejection responses in allotransplantation.

Several studies have demonstrated the role of chemokines in Treg induction, trafficking, suppressor function and in tolerance induction^{163,436-438}. Tregs are known to express CCR2 and CCR4, chemokine receptors for CCL2 as well as CCR1 and CCR5, the receptors for CCL8^{163,436-440}. In another study, Tregs were shown to migrate specifically to a range of chemotactic factors including CCL2 and CCL8⁴³⁶. Tregs that migrated in response to chemokines markedly reduced allogeneic effector T cell proliferation with respect to non-

migrated Tregs⁴³⁶. A later study demonstrated that CCR4 deficient Tregs were incapable of prolonging cardiac allograft survival and inducing transplantation tolerance. This study concluded that the CCR4 chemokine receptor pathway is essential for Tregs migration and tolerance induction in an allotransplant setting⁴³⁷. Similarly, the blockade of CCR5 mitigated Treg migration and exacerbated chronic intestinal inflammation⁴³⁸. Together, these studies suggest that chemokine ligands expressed at inflammatory sites recruit chemokine-receptor expressing Tregs, enabling these cells to exert their immunoregulatory effects *in vivo*^{437,438}. The role of MSC-derived chemokines in Treg recruitment has also been previously reported in one study²⁵⁶. Administration of CCL2-deficient MSC resulted in diminished Treg numbers and TGF- β levels *in vivo* in mouse models of inflammatory diseases²⁵⁶, suggesting the role of chemokine pathways in MSC mediated generation of Tregs. However, the direct role of chemokines in MSC recruitment, induction and activation of Tregs have not been well established in the literature. The present study proposes that human MSC-17 secreted chemokines CCL2, CCL8 and CXCL6 may promote Treg induction or expansion. In **CHAPTER 4**, it was shown in transwell assays that cell-cell contact between MSC and naïve T cells was required to induce Treg (**Figure 4.3.5**). This data confirms that MSC-17 in close proximity with naïve T cells is essential to promote Treg induction, a mechanism potentially mediated by chemokine receptor-ligand interaction between T cells and MSC. Establishing the function of CCL2, CCL8 and CXCL6 in MSC-17 binding activity and subsequent modulation of T cell suppression as well as in the generation, recruitment and function of Treg need to be validated in future studies.

MSC are also known to secrete matrix metalloproteinases (MMPs), molecules with chemotaxis and immunosuppressive properties^{179,207,431,433,441,442}. MSC constitutively express a range of MMPs including MMP2, membrane type 1 MMP (MT1-MMP), tissue inhibitor of MMP1 (TIMP1) and TIMP2^{179,441,442}. These MMPs were essential for MSC invasion and

migration across the extracellular matrix (ECM) as demonstrated in *in vitro* transendothelial migration assays⁴⁴². Blocking with MMP specific inhibitors or gene specific knockdown of these MMPs impaired MSC transmigration capacity across Matrigels^{179,441,442}. In response to proinflammatory cues such as IL-1 β and TNF- α , MSC were also shown to amplify the expression of MMP2, MTI-MMP and (or) MMP9 in MSC, thereby promoting MSC invasiveness of across the basement membrane¹⁷⁹. Moreover, MSC have strong expression of the chemokine receptor CXCR4 that potentially drives MSC in response to stromal cell-derived factor-1 (SDF-1) gradient¹⁸⁰. SDF-1 is highly expressed at site of inflammation, thus enabling MSC to home to the inflammatory milieu of allograft¹⁸¹ and exerting its immunomodulatory effect locally. Data from this chapter demonstrated that IL-17A induced the expression of MMP13 and MMP1 in MSC. These MMPs were enriched for collagen degradation (enrichment score 90.52) and metabolic processes (enrichment score 42.01) (**Table 5.3.3.7**). Hence, MSC-17 secretion of MMPs may be essential to for these cells to invade the ECM. MSC-17 expression of MMPs combined with their strong expression of chemokines may promote MSC-17 ability to home specifically to sites of inflammation.

Rafei *et. al.*, 2008 and 2009 additionally demonstrated that MSC-derived MMPs have proteolytic activity on chemokines including CCL2. MMP processing of CC chemokines convert the biochemical properties of the chemokine target molecules from an agonist to an antagonist form with anti-inflammatory properties *in vivo*⁴⁴³. MSC-derived MMP1 cleaves CCL2, leading to the generation of CCL2 with suppressive properties on B cell production of immunoglobulins and in EAE-derived CD4⁺ T cell activation^{431,433}. In this chapter, MMP1 and CCL2 as well as MMP13 were upregulated in human MSC-17. This data suggests that MSC-17 derived MMPs may have an important role in modulating inflammatory and immune responses by processing chemokines. Evaluating the functional role of MMP-processed chemokine derivatives in MSC-17 immunomodulation on T cells in this study remains to be

elucidated. Apart from the potential immunosuppressive roles of MSC-17 expressed CCL2, CCL8 and CXCL6, these chemokines were enriched for biological processes in response to wounding. This finding indicates that human MSC-17 may also be more potent compared to UT-MSC in facilitating tissue repair in an allotransplant setting.

MSC-secreted MMPs have been demonstrated to have a role in the immunosuppressive properties of MSC. MSC are known to inhibit T cell responses by downregulating surface expression of early T cell activation markers CD25, CD38 and CD69^{248,249}. Consistent with the induction of MMP13 and MMP1, we showed that MSC-17 further downregulated CD25 expression on CD4⁺ effector T cells compared to UT-MSC (**Figure 3.3.11; Table 5.3.3.1; Figure 5.3.5**), a process partially mediated by cell-contact dependent mechanisms (**Figure 4.3.6**). The soluble factors MMP-2 and MMP-9 secreted by MSC were previously reported to cleave and reduce CD25 expression on T cells, thus impairing T cell activation and proliferation²⁰⁷. The administration of MMP inhibitors *in vivo* in an islet allotransplant model abrogated the suppressive effect of autologous MSC on alloreactive T cells, resulting in allograft rejection. This study concluded that MMPs are crucial for MSC immunosuppression²⁰⁷. The involvement of MSC-17 derived MMP13 and MMP1 in downmodulating CD25 activation marker on T cells has not been previously established. Blocking MMP13 activity using the MMP13 specific inhibitor, CL82198 may provide insights on its role in inhibiting T cell activation.

There were also other molecules such as LBP, VMOI, SLC22A3, ITAG6, SAA1, STEAP2, amongst others, and non-coding RNAs including micro RNAs (miRNA) that were differentially expressed in MSC-17 compared to UT-MSC. These genes have previous unidentified function in MSC. Non-coding RNA have formerly deemed unimportant in gene expression profile studies. These non-coding RNA are now recognised to have versatile functions such as in innate or adaptive immune response regulation, control gene expression

of cells, post-transcriptional regulation and in cell-cell signaling⁴⁴⁴⁻⁴⁴⁶. Moreover, miRNA, a noncoding RNA, have been shown to regulate T cell immune responses⁴⁴⁷⁻⁴⁵⁰ and have implications in Treg function and induction⁴⁵¹⁻⁴⁵⁴. miR-142 for example was shown to inhibit T cell proliferation *in vitro* and *in vivo* in murine models of GvHD⁴⁴⁷ while blockade of miR-17 or miR-19b inhibited alloreactive T cell expansion and IFN- γ production in addition to ameliorating GvHD⁴⁴⁹. In Treg biology, overexpression of miR-24 promoted Treg induction from naïve T cells^{450,453}. In MSC, it was previously demonstrated that miRNA expression can be upregulated in MSC following IFN- γ and TNF- α treatment⁴⁵⁵. This study reported that miRNA-155 negatively regulates the immunosuppressive properties of MSC by inhibiting the expression of iNOS in MSC⁴⁵⁵. The role of MSC-17 derived miRNA in regulating the expression of immunomodulatory molecules in MSC-17 remains to be elucidated. A recent study also published that long noncoding RNA can regulate adipose derived MSC function by activating the TLR3 signaling pathway⁴⁵⁶. All these molecules can be targeted in future studies dissecting mechanisms of MSC-17 modulation of T cell responses and in Treg induction.

In conclusion, MSC-17 function similar to MSC- γ in enhancing MSC immunomodulation of proinflammatory T cell responses (**CHAPTER 3 AND 4**). Both MSC-17 and MSC- γ exhibited increased expression of chemotactic molecules which may drive T cell recruitment to MSC to mediate their immunosuppressive and immunoregulatory properties. The increased expression of chemokines and MMP also suggest that human MSC-17 may have enhanced capacity to home specifically to sites of inflammation for tissue regeneration or immunosuppression *in vivo*. MSC-17 despite being highly regulatory exhibited similar morphology to UT-MSC. Human MSC-17 exhibited increased proliferative potential with no upregulation or induction of MHC molecules compared to MSC- γ , thereby making them less immunogenic if administered in allogeneic hosts. Enhanced expression of MHC in allogeneic MSC- γ increases their immunogenicity and this can negatively impact *in vivo*

allograft survival. Although this microarray study was limited by MSC-17 having less genetic variability to UT-MSC due to a small sample of biological replicates, bioinformatics analysis identified novel candidate molecules that may contribute to the potent MSC-17 regulation of immune response. These candidate molecules will be explored for their regulatory roles in MSC-17 suppression of T cell responses and in the generation of Tregs, as a future direction of this project.

CHAPTER 6:

IMMUNODEPLETION AND HYPOXIA PRE-CONDITIONING OF MOUSE COMPACT BONE CELLS AS A NOVEL PROTOCOL TO ISOLATE HIGHLY IMMUNOSUPPRESSIVE MSC

6.1 INTRODUCTION

The stromal tissue of the bone marrow (BM) comprise a heterogeneous population of cells including the haematopoietic stem cells (HSC), endothelial progenitor cells and mesenchymal stem cells (MSC)^{457,458}. HSC are most prevalent in the bone marrow and give rise to cells of myeloid (eg. monocytes, macrophages, neutrophils, dendritic cells) and lymphopoietic (T, B and NK cells) lineages⁴⁵⁷. Multipotent MSC were initially described by Friedenstein and colleagues (1970) as a rare fraction of bone marrow cells (0.001 - 0.01%) in rodents that have *in vitro* clonogenicity potential (ie. ability to form colonies defined as colony forming-unit fibroblast; CFU-F) with self-renewal capacity and able to form bones under osteogenic defined conditions⁴⁵⁹. CFU-F assays can identify MSC progenitor cells with greatest potential to propagate⁴⁶⁰. Our laboratory routinely uses this assay to determine the quality of human BM aspirates and the subsequent MSC enrichment. To date, there are no unique markers to define human BM MSC phenotypically, however, *The Mesenchymal and Tissue Stem Cell Committee* have proposed a simplistic set of generic criteria to define human BM MSC *in vitro* by their (1) adherence to plastic when maintained in standard culture conditions; (2) tri-lineage differentiation potential into osteoblasts, chondroblasts and adipocytes; and (3) cell surface expression of CD105, CD73 and CD90 in >95% of the culture and negative for CD34, CD45,

CD14 or CD11b, CD79a or CD19 and MHC-class-II⁴⁶¹. However, other properties of BM MSC such as the potential for self-renewal, support hematopoiesis in the BM niche and immunomodulatory properties need to also be addressed as potential cellular therapy candidates in various disease conditions (www.clinicaltrials.gov.au) and in allotransplantation^{4,359,462}

However, the exact mechanisms by which MSC function *in vivo*, the route, dose and timing of MSC patient infusion as well as safety concerns with regards to MSC, off target effects, tumorigenicity³³¹⁻³³⁴ and differentiation into other cell types³³⁵⁻³³⁷ remain unresolved. Together these issues limit the wide application of human BM MSC therapy into the clinic. Therefore, preclinical mouse models are widely utilized to address these issues associated with MSC therapy.

Human BM-derived MSC compared to their mouse MSC (mMSC) counterparts are easily isolated and expanded *ex vivo* with high purity¹⁷⁶. Isolation of mMSC from the BM however represents a major obstacle for research aiming to dissect mechanisms of MSC function *in vivo*, optimize therapeutic strategies and address safety concerns of BM MSC therapy. Mouse MSC have an extremely low incidence in BM compared to human MSC, and require modified growth conditions and media supplements for *ex vivo* expansion^{463,464}. The major hurdle in BM mMSC isolation protocols is to obtain a homogenous and highly purified population of mMSC, with less contaminating haematopoietic cells and other non-MSC cells or progenitor populations that co-exist in the BM niche⁴⁶³⁻⁴⁶⁵. Previous attempts to eliminate these contaminating cells in mMSC cultures included immunodepletion and (or) unique culture systems such as frequent media change, low cell density cultures, alteration to the standard MSC culture media components or cell culture surfaces to promote mMSC adherence⁴⁶⁶⁻⁴⁷². Despite these attempts, isolation of mMSC from whole BM remained ineffective due to poor yields or purity⁴⁷²⁻⁴⁷⁴. Absence of standardisation in BM mMSC isolation protocols may be a

contributing factor to the conflicting results on the *in vivo* therapeutic efficacy of mMSC in preclinical mouse models of diseases including allotransplantation.

We and several other groups have isolated mMSC from the compact bones (CB) as they represent a major reservoir of MSC in the murine system compared to the BM⁴⁷³⁻⁴⁷⁶. A few studies have described that enzymatic (collagenase) digestion of the human trabecular bones releases MSC⁴⁷⁷⁻⁴⁷⁹. These human bone fragment derived-MSC have similar proliferative and trilineage differentiation potential but higher colony forming efficiency compared to human BM derived-MSC⁴⁷⁹. Similarly in the mouse system, collagenase digestion of CB depleted of BM releases mMSC precursors^{348,475,476}. Studies directly comparing CB to BM derived-mMSC showed that CB mMSC have a higher purity, clonogenicity, proliferative potential as well as sustained growth kinetics and enhanced capacity to differentiate into MSC lineages under appropriate induction conditions^{348,473,474,480,481}. CB mMSC isolation protocols are more reproducible and have been shown to yield homogenous CB mMSC with *in vivo* immunoregulatory roles in models of GvHD⁴⁸², sepsis⁴⁸³ and asthma⁴⁸⁴.

Not only is the source of mMSC important but the optimization of mMSC culture conditions for example: the choice of FBS⁴⁸⁵⁻⁴⁸⁷, CB digestion protocol³⁴⁸ and the culture of mMSC under hypoxia (low oxygen tension – 2% or 5% O₂) or normoxia (21% O₂) are crucial for optimal isolation, expansion and function of mMSC. Various studies use different collagenase types (I or II), concentrations and CB digestive times which may impact the yield of cells released from the digested CB fragments and hence the enrichment of mMSC precursors^{348,475,476}. Low oxygen culture conditions (hypoxia) are also proven to facilitate enrichment and successful mMSC cultures^{472,481}.

Most *in vivo* studies on the immunosuppressive function of MSC in preclinical murine models of allotransplantation use the traditionally isolated plastic-adherent selected BM mMSC (**Table 1.8.1.1 and Table 1.8.1.2**), regardless of the well-established issues with

mMSC cultures from the BM. In this study, we established a protocol to enrich a highly purified and homogenous population of CB mMSC with potent *in vitro* immunosuppressive capacity that would be beneficial in an allotransplant setting. We employed collagenase type II digestion (3mg/ml, 1hr) of BM depleted CB combined with immunodepletion of CD45 (lymphocyte common antigen) and TER119 (erythroid lineages) cells and hypoxia (5% O₂) preconditioning to establish primary mMSC cell lines. Fetal-bovine serum (FBS) for CB mMSC cultures, CB collagenase digestion protocol and the establishment of mMSC culture conditions (hypoxia versus normoxia) were optimized in our study. Based on this isolation protocol, CB mMSC were able to potently suppress *in vitro* total T cells and subset (CD4⁺ and CD8⁺) T cell proliferation in a DC allostimulatory 1-way mixed lymphocyte reaction (MLR), that mimic an *in vivo* islet transplant setting.

6.2 MATERIALS AND METHODS

The general materials and methods of this chapter have been described in **CHAPTER 2: MATERIALS AND METHODS**. In this section, additional chapter specific materials and methods as well as *in vitro* and *in vivo* optimization experiments are shown.

6.2.1 Immunophenotype of mouse dendritic cells

Table 6.2.1.1 Mouse DC antibody panel

Antibody	Clone	Fluorochrome	Company
CD11b	M1/70	FITC	BD Pharmigen
CD11c	N418	PE-Cy7	Biolegend
CD40	3/23	FITC	BD Pharmigen
CD80	16-10A1	APC	Biolegend
CD83	Michel-19	FITC	Biolegend
CD86	GL1	FITC	BD Pharmigen
MHC class II	M5/114.15.2	FITC	Biolegend

CD11c⁺ mDC were generated and purified by positive selection from bone marrow of BALB/c mice as described in section **2.8.1**. These cells were confirmed for the expression of DC maturation markers prior to their use in a MLR. Greater than 90% CD11c⁺ mDC expressed CD11b, CD11c, CD40, CD80, CD83 and CD86 and MHC class II (**Table 6.2.1.1** and **Figure 6.2.1**). This confirms that the purified CD11c⁺ mDC are able to function as stimulator cells to induce potent MHC-mismatched T cell responses when used in a 1-way MLR as they express MHC class II for antigen presentation and T cell co-stimulatory molecules; CD40, CD80, CD83 and CD86. On the other hand, the negative cell fraction confirmed the presence of a heterogeneous cell population in the initial unpurified CD11c⁺ cultures. The negative fraction of cells had varying expression of DC markers. Less than 60% of the negative cell fraction were positive for CD40, CD80, CD83, CD86 and MHC class II. These cells may have reduced T cell allostimulatory responses when used in a 1-way MLR. Therefore, CD11c⁺ mDC from the initial DC culture were positively selected to ensure most potent T cell proliferation would

be obtained with these highly purified population of cells. CD11c⁺ mDC preparations of >90% positive for the mDC cell surface markers tested were used in all 1-way MLR assays.

Table 6.2.1.2 Immunophenotype of CD11c⁺ matured DC (mDC)

Marker	% negative cell fraction	% CD11c⁺ mDC (CD11c⁺ positive selection)
CD11b	70.63	96.26
CD11c	85.60	98.50
CD40	57.50	94.68
CD80	56.12	95.24
CD83	49.41	94.71
CD86	43.99	93.50
MHC class II	53.50	93.41

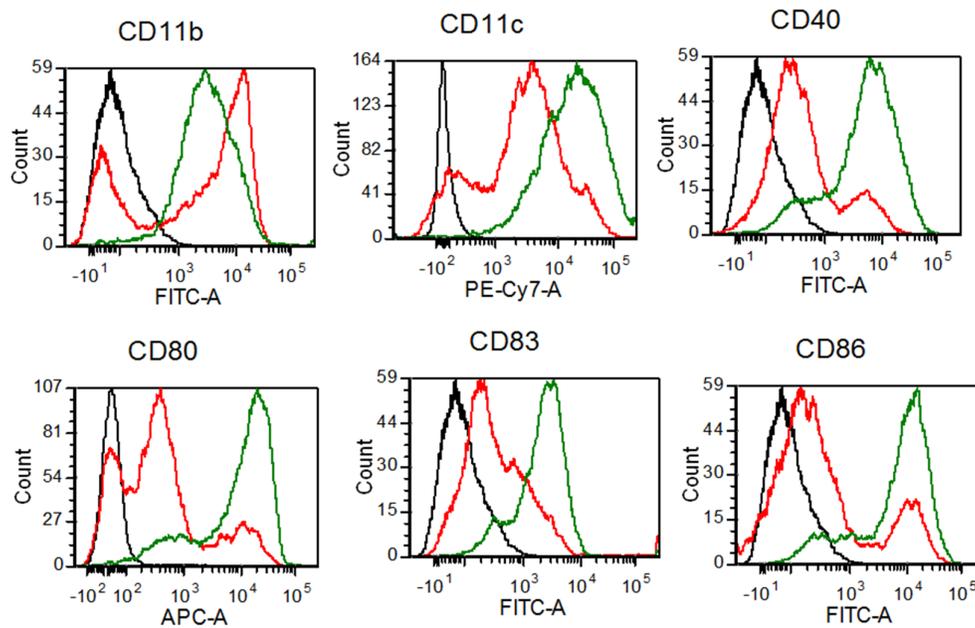


Figure 6.2.1 Immunophenotype of CD11c⁺ matured DC (mDC).

Bone marrow derived matured DC were generated from HSC *in vitro* from BALB/C mice, where CD11c⁺ mDC were purified by positive selection. The expression of mDC cell surface markers was assessed by flow cytometry. Histograms show level of surface marker expression on purified CD11c⁺ mDC (**green**) and the negative cell fraction (non-CD11c purified cells) (**red**). The black histograms depict the negative fraction unstained cell control. Data are one representative of 2 mDC cell preparations. CD11c⁺ were also 93.41% MHC class II positive while the negative cell fraction showed 53.50% positivity (data not shown).

6.2.2 Optimization of culture conditions for compact bone mMSC isolation

Fetal bovine-serum (FBS) batch testing: CB mMSC from C57BL/6 mice were isolated as described in section 2.3.1 with the following modification: 3mg/mL collagenase I digestion of compact bones for 45min with no depletion of CD45 and TER119 cells. MSC growth culture conditions were optimized using different batches of FBS. The effect of different FBS on MSC progenitor cell enrichment as well as in mMSC morphology and growth potential following mMSC sub-culturing were tested in this chapter. The three FBS batches examined included: (1) Hyclone (old) – previously used in mouse MSC isolation protocols, (2) Invitrogen (normal) – new non-MSC qualified FBS and (3) Invitrogen (MSC) –new human MSC qualified FBS.

Optimization of collagenase digestion of compact bones: Collagenase batch optimization was tested on CB mMSC precursor cells isolated from the C3H mice strain (6-7 weeks old). This protocol was conducted as described in section 2.3.1 with the following modification: compact bones were isolated from the C3H mice strain digesting the bone fragments with either collagenase I or II, at different time points and using different collagenase concentrations (**Table 6.3.1.1**). To identify the optimal collagenase enzyme type, time and duration of CB digestion, CB cell yield immediately after CB digestions and the % Sca-1⁺ cells were determined. Yield of cells was counted as the number of CB cells released immediately after CB digestion. Sca-1⁺ cells are previously known to be precursor cells of mMSC^{476,480,481}, providing an indication if the bone digestion step was sufficient to release mMSC precursors from the CB. Hence, the freshly digested CB cells were stained for Sca-1 and run on FACS to determine the % of Sca-1 positive cells. In addition, CB mMSC precursors not depleted of CD45 and TER119 cells were seeded at 1x10⁵ cells/well in a 6-well plate in triplicates and cultured for 8 days in a CFU-F assay. The colony forming efficacy of the differently digested bone fragments confirmed that digestive protocol was sufficient to release mMSC precursor cells from CB.

Primary mMSC culture condition optimization: mMSC were isolated using the optimal collagenase digestive conditions of CB cells as described in section 2.3.1 with no depletion of CD45 and TER119 cells. Digested CB cells were seeded at 1×10^5 cells/well in a 6-well plate in triplicates and cultured for 8 days in a CFU-F assay under hypoxia or normoxic conditions.

6.2.3 Mouse MSC immunophenotyping

Table 6.2.3.1 Mouse MSC antibody panel

Antibody	Clone	Fluorochrome	Company
<i>Mouse MSC phenotyping:</i>			
Sca-1	D7	PE	eBioscience
CD29	265917	PE	R&D systems
CD73	496406	PE	R&D systems
CD90 / Thy1	778053	PE	R&D systems
CD105	MJ7/18	PE	eBioscience
CD166 (ALCAM)	eBioALC48	PE	eBioscience
CD31	390	PE	eBioscience
CD34	MEC14.7	PE	Biolegend
CD44	IM7	PE	eBioscience
CD11b	M1/70	PE	eBioscience
CD45	30-F11	PE	eBioscience
IL-17RA	PAJ-17R	PE	eBioscience
IL-17RC	Leu21-Trp465	APC	R&D Systems
<i>Isotype controls</i>			
Rat IgG2a	eBR2a	PE	eBioscience
Rat IgG2b	eB149/10H5	PE	eBioscience
Rat IgG1	eBRG1	APC	eBioscience
Armenian Hamster IgG	eBio299Arm	PerCP-Cy5.5	eBioscience
Rat IgG2a	RTK2758	BV421	Biolegend

6.2.4 Optimization of mouse MSC dose for CFSE-MLR optimization

Table 6.2.4.1 Mouse T cells antibody panel

Antibody	Clone	Fluorochrome	Company
<i>Mouse T cell markers</i>			
CD3	145-2C11	PerCp-Cy5.5	eBioscience
CD4	GK1.5	APC-eFluor	eBioscience
CD8	53-6.7	Pe-Cy7	eBioscience

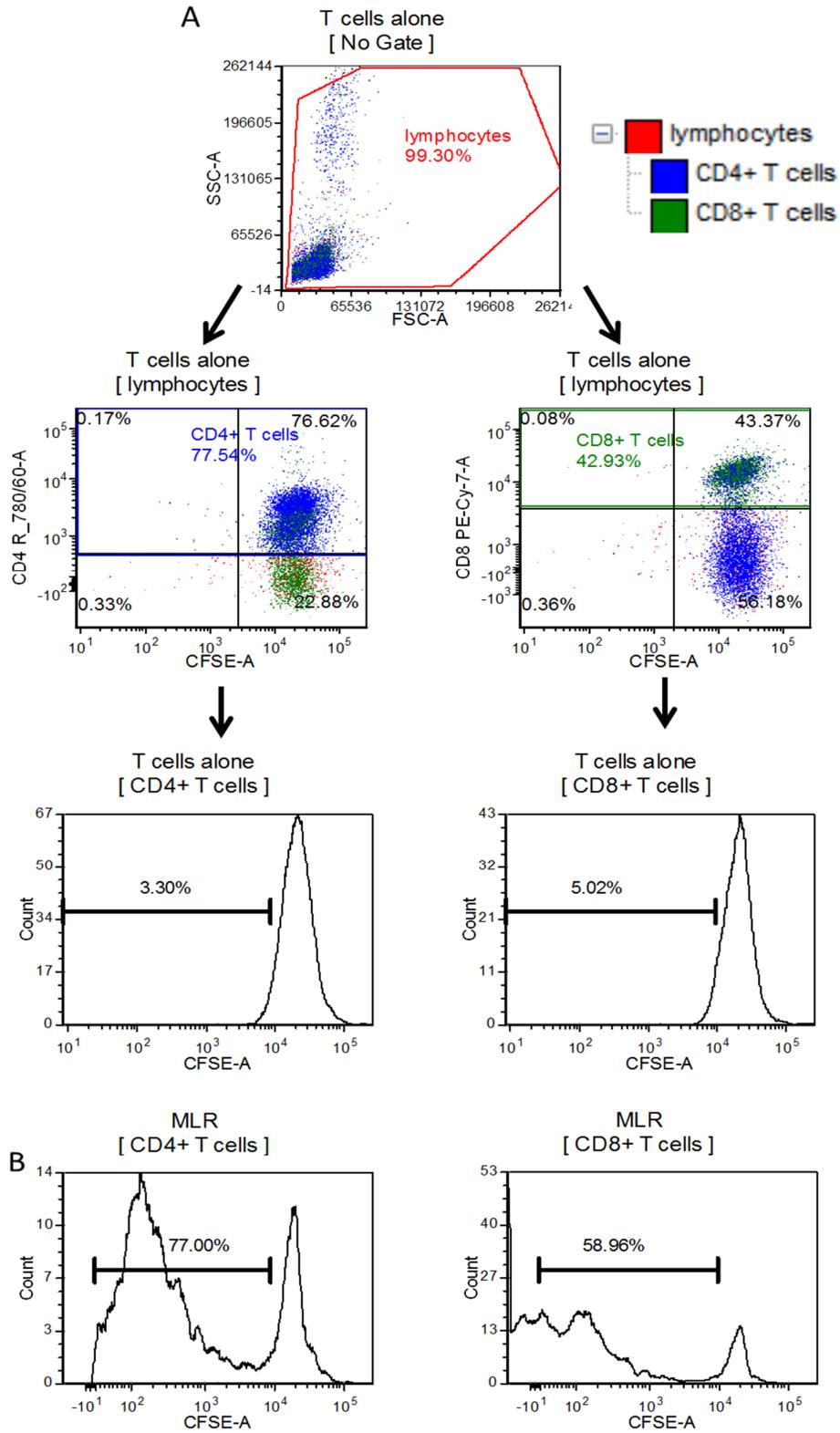


Figure 6.2.2 CFSE-suppression assay gating strategy.

CD3⁺ T cells isolated from splenocytes of C57BL/6 mice were CFSE labelled as described in section 2.7.2. **(A)** T cells alone or **(B)** T cells co-cultured with stimulator cells (CD11c⁺ mDC) in a 1-way MLR. At day 3 or 5, T cells were isolated and cell surface stained with anti-mouse CD4 and anti-mouse CD8 and acquired on BD FACSCanto II. **(A)** The gating strategy of % CD4⁺ and % CD8⁺ proliferated T cells was defined as follows: Total T cells were gated according to the FSC-area and SSC-area. The CD4⁺ and CD8⁺ T cell gates were defined based on undivided and proliferated CFSE-labelled T cells. The unstimulated CFSE-stained CD3⁺ T cells (T cells alone control) labelled with anti-CD4 and anti-CD8 were used to set the marker for the proliferated CD4⁺ and CD8⁺ T cell populations, respectively. The single peak in the T cell alone histograms represents the undivided CD4⁺ or CD8⁺ T cell populations. **(B)** The percentage of proliferated CD4⁺ or CD8⁺ T cells was shown in the histograms. T cells allostimulated by mDC showed up to 77% CD4⁺ and 58.95% CD8⁺ T cell proliferation, as depicted in the representative histograms.

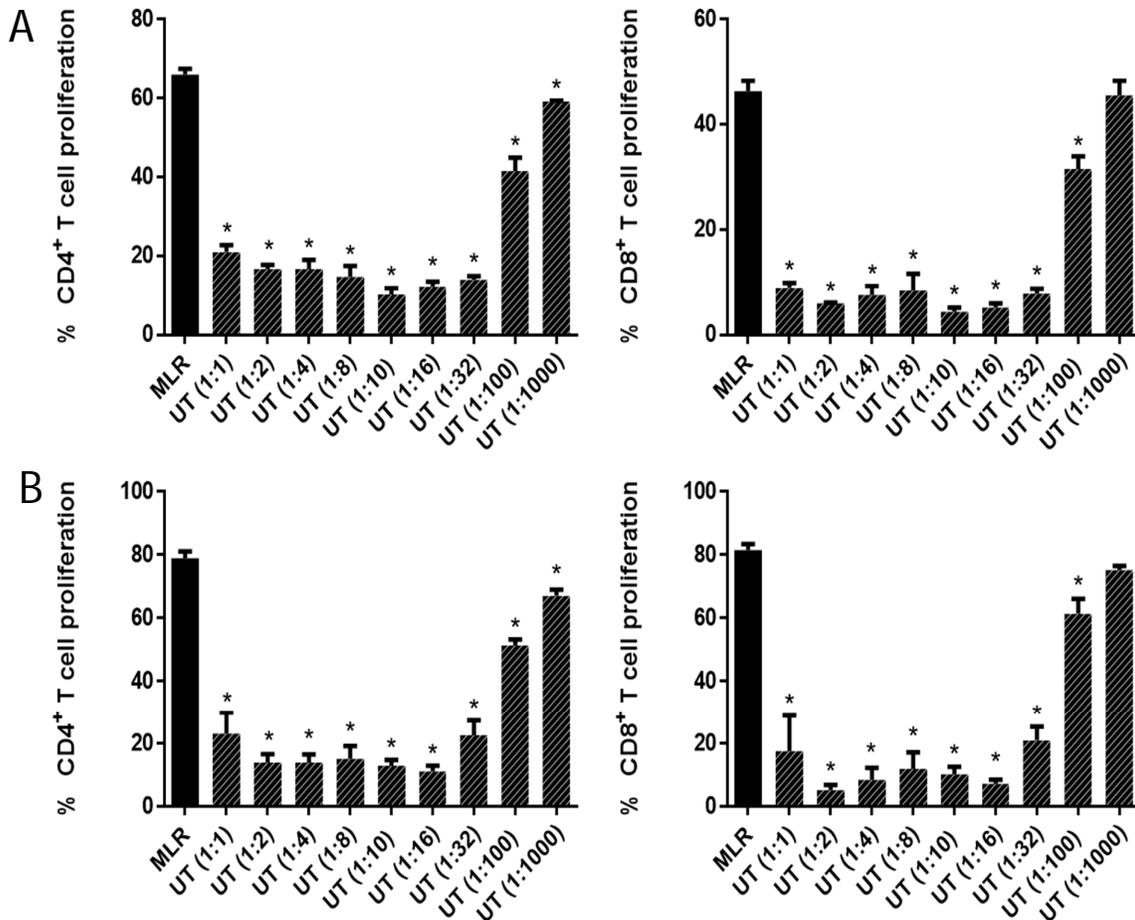


Figure 6.2.3 Optimization of mouse MSC dose for CFSE-MLR.

UT-MSC (UT) were co-cultured with CFSE-labelled CD3⁺ T cells (C57BL/6 mice) allostimulated with 30 Grays irradiated DC (BALB/c) in a 1-way MLR. UT-MSC were co-cultured with T cells at the ratios of 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:100 and 1:1000 UT-MSC: CD3⁺ T cells. At (A) 3- or (B) 5-days, T cells were isolated and cell surface stained with anti-mouse CD4 and anti-mouse CD8 and acquired on BD FACSCanto II. The percentage of proliferated CD4⁺ or CD8⁺ T cells was determined and represented in the graphs. MLR in the absence of MSC indicated maximal T cell proliferation. Data represents one experiment. *p < 0.05 vs. MLR determined by one-way ANOVA with post-Sidak multiple comparison tests. Error bars depict means of triplicates \pm SD of triplicates. MSC doses of 1:10 (10%), 1:100 (1%) and 1:1000 (0.1%) were selected for subsequent experiments.

6.2.5 *In vivo* islet allotransplant model

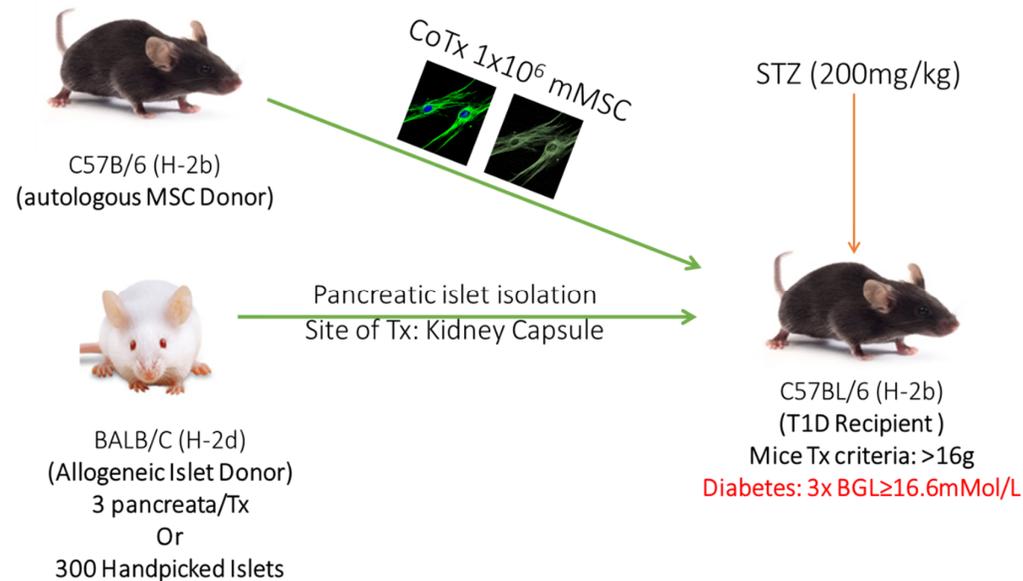


Figure 6.2.4 Illustration of islet allograft rejection model.

C57BL/6 mice were rendered diabetic by 200mg/kg STZ. C57BL/6 diabetic mice were transplanted with fully MHC-mismatched BALB/c islets (a bulk transplant of islets from 3 pancreata or 300 handpicked islets) under the kidney capsule. Mice were either transplanted with islets alone or co-transplanted with 1×10^6 mouse autologous UT-MSC derived from C57BL/6 mice. Graft survival was determined by monitoring nonfasted daily blood glucose levels (BGL). Mice with BGL > 16.6 mMol/L for 3 consecutive days were considered to be diabetic or rejected the transplanted islet allograft.

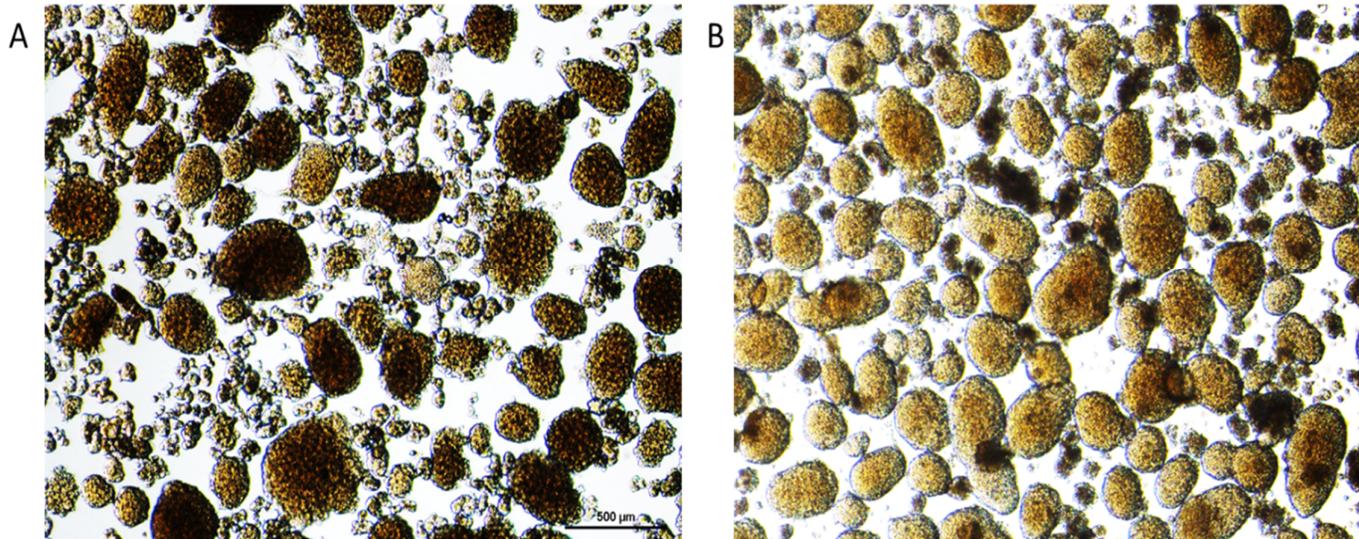


Figure 6.2.5 Morphology of isolated mouse islets.

(A) Freshly isolated islets (D0 islets). Freshly digested islets had a rougher islet surface, less defined islet membrane border, smaller islet fragments in the background and darkened due to acinar tissue attachment. (B) Day 1 cultured islets (pre-transplant islets). Islets appeared to be smooth, rounded, well defined islet membrane border and lesser acinar tissues were attached to the islets. The purity of this islet preparation was approximately 80%. Islet preparations with purity of 70-100% were used in all islet allotransplant experiments. The Nikon Eclipse Ti microscope was used to capture images.

6.2.6 Statistics

In vivo islet allograft survival and function data were reported as mean \pm SEM. Allograft survival was evaluated using the Kaplan-Meier method and compared using the log-rank test. Islet allograft function was analysed using area-under-the-curve (AUC) analysis of \log_{10} transformed data and AUC values were compared by one-way ANOVA with post Tukey multiple comparison test. A value of $p < 0.05$ was considered to be statistically significant. Analyses were performed using the GraphPad Prism software

6.3 RESULTS

6.3.1 Collagenase II for compact bone digestion to isolate mMSC precursors

Published protocols on compact bone (CB) mMSC isolation use different collagenase types (I or II), concentrations and duration of CB digestion^{348,475,476}. The collagenase digestion protocol using either collagenase I or collagenase II as well as different concentrations and durations of CB digestion to isolate mMSC progenitors from the C57BL/6 mice strain was optimized in this study. Collagenase II (3mg/mL, 1 hr) resulted in high yield of CB cells and the highest % of Sca-1⁺ cells (34.66%) immediately after CB digestion compared to all the other CB digestion conditions (**Table 6.3.1.1**). Collagenase II (3mg/mL, 1 hr) also showed highest CFU-F efficacy. Together, these data suggest that Collagenase II (3mg/mL, 1 hr) was optimal for CB digestion to release and enrich for mMSC precursor cells with low toxicity (>90% viability) and was subsequently used in all CB mMSC isolation protocols.

Table 6.3.1.1 Optimization of collagenase digestion of compact bones of C57BL/6 mice

	[Collagenase] (mg/ml)	Digestion time (hr)	Yield (x10 ⁵ cells)	% Sca-1 ⁺ cells	Mean CFU-F
Collagenase I (MSC lab)	3	1	9	2.47	59
Collagenase II	1	1	7.95	8.69	36
Collagenase II	1	2	5.2	11.09	78
Collagenase II	3	1	10.3	34.66	80
Collagenase II	3	2	13.25	2.08	55

6.3.2 Effect of different FBS on mMSC enrichment, morphology and expansion

FBS batches vary in quality and composition. This can affect adherence and mitotic expansion of primary MSC cultures as well as the retention of MSC in undifferentiated states during sub-cultivation⁴⁸⁵. All FBS batches showed no difference in the enrichment of mMSC progenitor cells evident by the similar number of CFU-F in primary mMSC cultures (**Figure 6.3.1**). There were no differences in the number of CFU-F from mouse CB cells cultured in the normal Invitrogen FBS [**Invi (normal)**] as to the Invitrogen human MSC qualified FBS [**Invi (MSC)**] (**Figure 6.3.1**). In addition, CFU-F numbers were significantly higher in mMSC progenitors cultured under hypoxia compared to normoxic conditions (**Figure 6.3.1**), suggesting that hypoxia favours the enrichment and expansion of mMSC progenitor cells. Therefore, hypoxia conditions were used in all subsequent experiments to establish the primary CB mMSC cell lines.

No aberrant changes in mMSC morphology were observed with the FBS batches at passage 0 (P0) and in subsequent mMSC passaging (P1 to P3) under normoxia (**Figure 6.3.2**). Invi (normal) FBS increased mMSC growth potential when passaged from P2 to P3, while Invi (MSC) surprisingly decreased mMSC proliferation (**Figure 6.3.3**). Since, Invi (normal) FBS favoured mMSC growth and was similar to Invi (MSC) in terms of CFU-F and mMSC morphology, Invi (normal) FBS was considered optimal for mMSC cultures. Invi (normal) was subsequently used in all mMSC isolations and culture protocols.

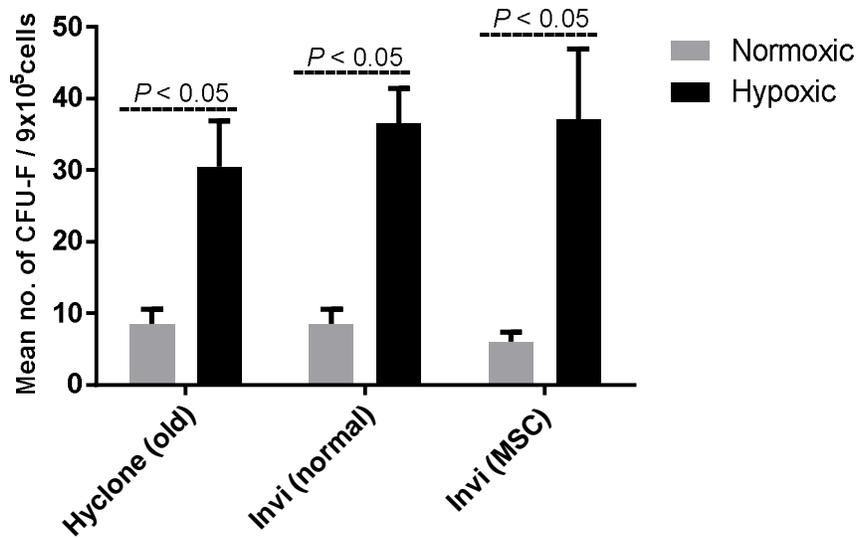


Figure 6.3.1 Effect of different FBS batches on mMSC clonogenicity and enrichment.

CB depleted of bone marrow cells from C57BL/6 mice were digested with collagenase. CB cells were cultured in a 6-well plate at 9×10^5 cells/well in 3ml primary MSC media containing 20% FBS from: (1) previously used FBS batch, **Hyclone (old)**. Two new FBS serum batches tested include: (2) normal Invitrogen FBS, **Invi (normal)** and (3) Invitrogen human MSC pre-screened FBS, **Invi (MSC)**. The 6-well plates were cultured at either normal or under hypoxic (5% O₂, 10% CO₂, 85% N₂) conditions, 37°C. The number of colony forming units fibroblast (CFU-F) between the different FBS batches were counted and compared. The figure indicates mean number of CFU-F per 9×10^5 cells. * $p < 0.05$ vs. normoxia culture conditions were determined by one-way ANOVA with post-Sidak multiple comparison test. Error bars depict mean CFU-F of triplicate wells \pm SD.

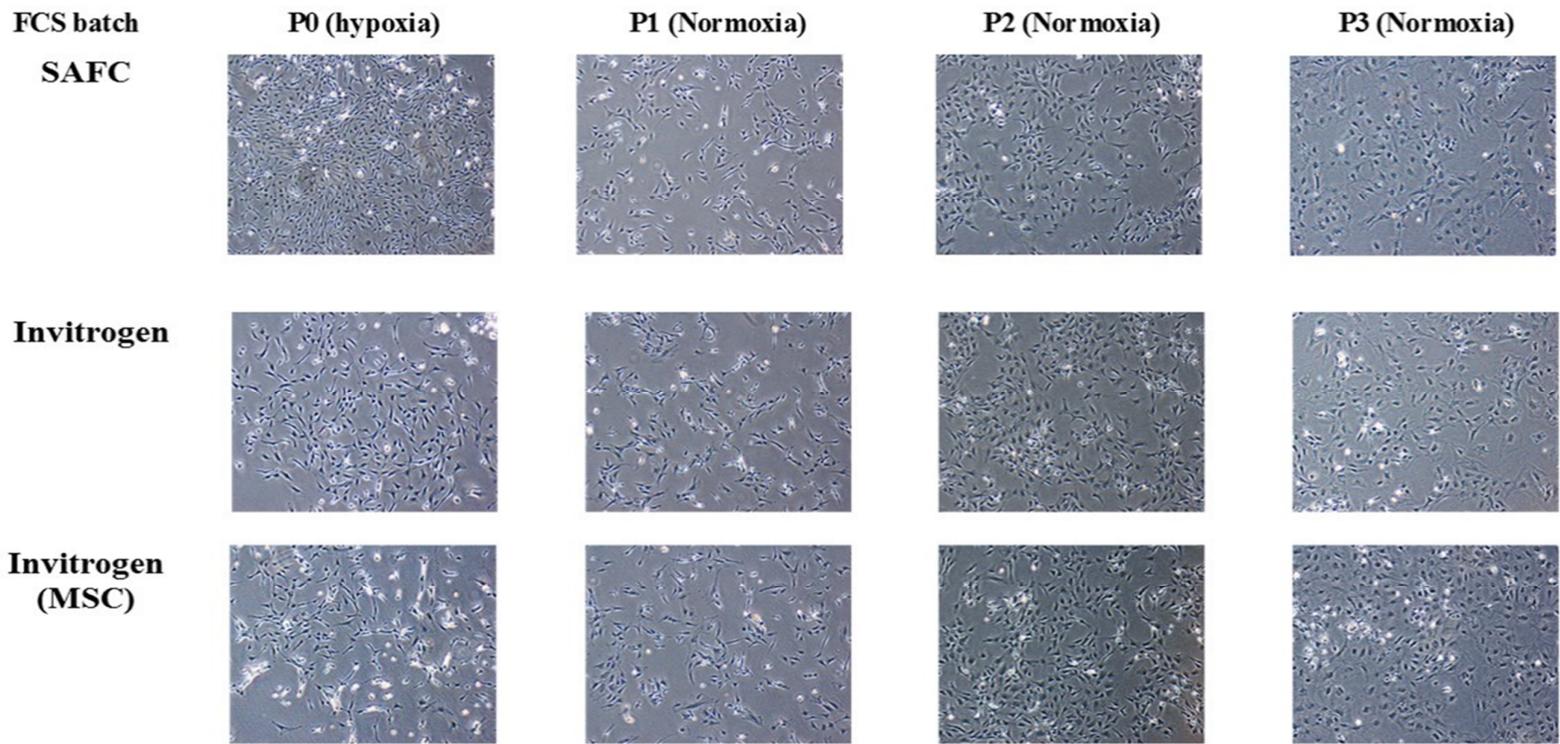


Figure 6.3.2 Effect of different FBS of mMSC morphology when sub-cultivating.

Morphology of mMSC examined in MSC media supplemented with different FBS batches: (1) Hyclone (old), (2) Invi (normal) or (3) Invi (MSC). mMSC morphology was observed at the initial establishment of mMSC cell lines under hypoxia (Passage 0, P0) and subsequent sub-culturing of cells from P1 to P3. Any aberrant changes in mMSC morphology were examined under the different FBS batches. The Olympus IX51 inverted microscope was used to capture images (original magnification x100).

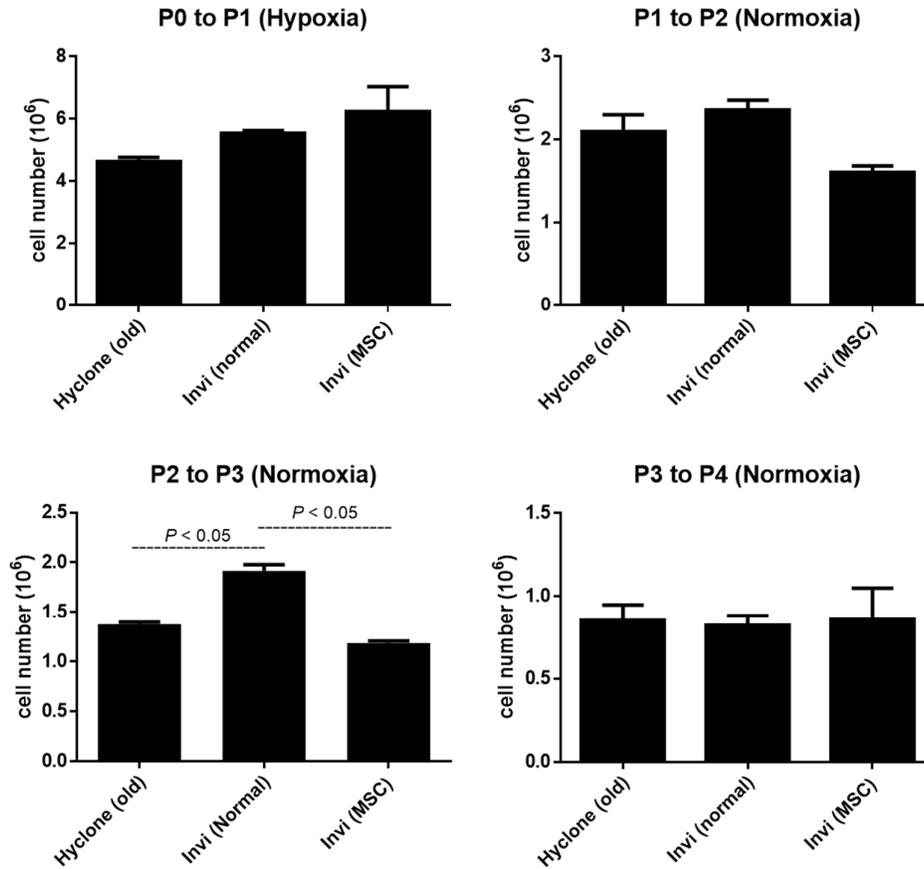


Figure 6.3.3 Effect of FBS batches on mMSC expansion: cell numbers at passaging.

C57BL/6 mice mMSC progenitors or sub-cultured mMSC were seeded at either 30 000 cells/cm² for P0 to P1 (hypoxia) or 10 000 cells/cm² for P1 to P2, P2 to P3 and P3 to P4 (normoxia), respectively. At day 4 or 5 or confluency, mMSC were detached by trypsinization and the number of mMSC was counted in a trypan blue exclusion assay. The figures indicate total mMSC yield cultured at P0 to P1 (under hypoxia) and P1 to P2, P2 to P3 and P3 to P4 (under normoxia). * $p < 0.05$ vs. Invi (normal) was determined by one-way ANOVA with post-Sidak multiple comparison test. Error bars depict mean cell numbers of duplicate cell counts \pm SD.

6.3.3 Hypoxia favours mMSC precursor cell enrichment and expansion

Hypoxic culture conditions favoured the enrichment and growth of MSC progenitor cells evident by the high numbers of CFU-F derived from these CB cells (**Figure 6.3.1, Table 6.3.3.1**). Precursors of mMSC were highly prevalent in the CB rather than in the bone marrow fraction as CB derived mMSC had higher colony forming efficiency, indicating greater enrichment of mMSC (**Table 6.3.3.1**).

Based on the optimization of culture conditions of CB mMSC isolation, we have established a protocol to maximize the enrichment and expansion of mMSC based on, one hour of 3mg/ml collagenase II digestion of the CB followed by hypoxia culture, isolated, selected and enhanced the clonogenicity of CB derived mMSC precursors. This protocol was used as a standard operating procedure for the culture and establishment of mMSC cell lines from CB precursors. In subsequent experiments, the use of these culture conditions was employed in addition to lineage depletion of CD45 (lymphocyte common antigen) and TER119 cells (erythroid lineages) of the digested CB fragments to isolate more highly purified CB mMSC precursors for immunophenotyping and T cell immunosuppressive assays. CB mMSC precursors obtained following CD45 and TER119 immunodepletion represented only a very small fraction of the digested CB cells (eg. 4.67×10^5 CB mMSC precursors from 242.5×10^5 total CB cells of 6 C57BL/6 mice). Following 5 days of culture under hypoxia with media change every 2 days, there was a significant increase in the numbers of CB mMSC (11.25×10^5 and 37.8×10^5 when subcultured from P0 to P1 and P1 to P2, respectively). Similar cell yields were obtained with the different CB mMSC batch isolations. Culture of bone marrow derived mMSC was unsuccessful under these conditions and was therefore not used for functional analysis.

Table 6.3.3.1 mMSC culture condition optimization: normoxia vs. hypoxia

	Mean CFU-F	
	Normoxia	Hypoxia
Compact bones cells	5	51
Bone marrow cells	0	1

6.3.4 Characterisation of UT-mMSC and mMSC-17

In **CHAPTER 3**, it was established that human MSC-17 conformed to UT-MSC immunophenotype (**Figure 3.3.5**). In this chapter, we aimed to determine if murine mMSC-17 have similar characteristics to human MSC-17 and retain UT-MSC phenotype. The effect of 5 days IL-17A treatment on CB derived mMSC phenotype was assessed. Immunophenotype of IL-17A treated mMSC (mMSC-17) was similar to untreated mMSC (UT-mMSC) (**Figure 6.3.4A**). Greater than 90% mMSC-17 expressed the standard mMSC markers Sca-1, CD29, CD44, CD90 and were negative for CD11b (monocytes, NK cells, granulocytes, macrophages), CD31 (endothelial cells), CD34 (haematopoietic cells) and CD45 (lymphocytes). Of note, unlike human MSC, CD73 (23.1%) and CD105 (41.7%) were only expressed in a small fraction of CB mMSC with no difference in expression levels between UT-mMSC and mMSC-17 (**Figure 6.3.4A**). The percentage of CB mMSC expressing CD73 varied between mMSC batch isolations. CD73 expression ranged from 23.1% to 67.1% CD73 positivity (**data not shown**). This finding is consistent with the variability of CD73 expression on CB mMSC reported in the literature⁴⁸¹. CD105 percentages on the other hand were low at early passages and decreased upon CB mMSC sub-culturing. Both UT-mMSC and mMSC-17 retained their functional capacity and could differentiate into adipocytes and osteoblasts (**Figure 6.3.4B**). Additionally, mMSC-17 did not exhibit enhanced proliferation compared to UT-mMSC (**Figure 6.3.4C**), inconsistent with human MSC-17 (**Figure 3.3.5C**). The protein expression of IL-17 receptors, IL-17RA and IL-17RC was also determined by flow cytometry. Surprisingly, we identified that CB mMSC had protein expression of IL-17RC but not IL-17RA (**Figure 6.3.5**).

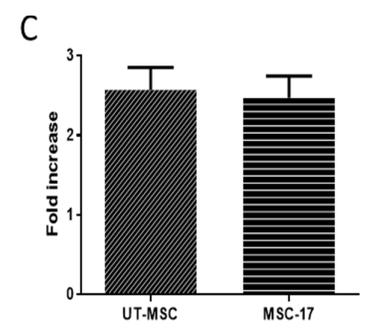
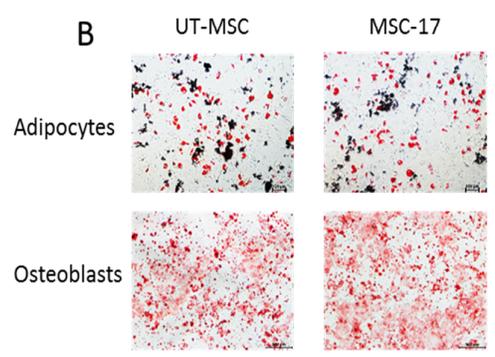
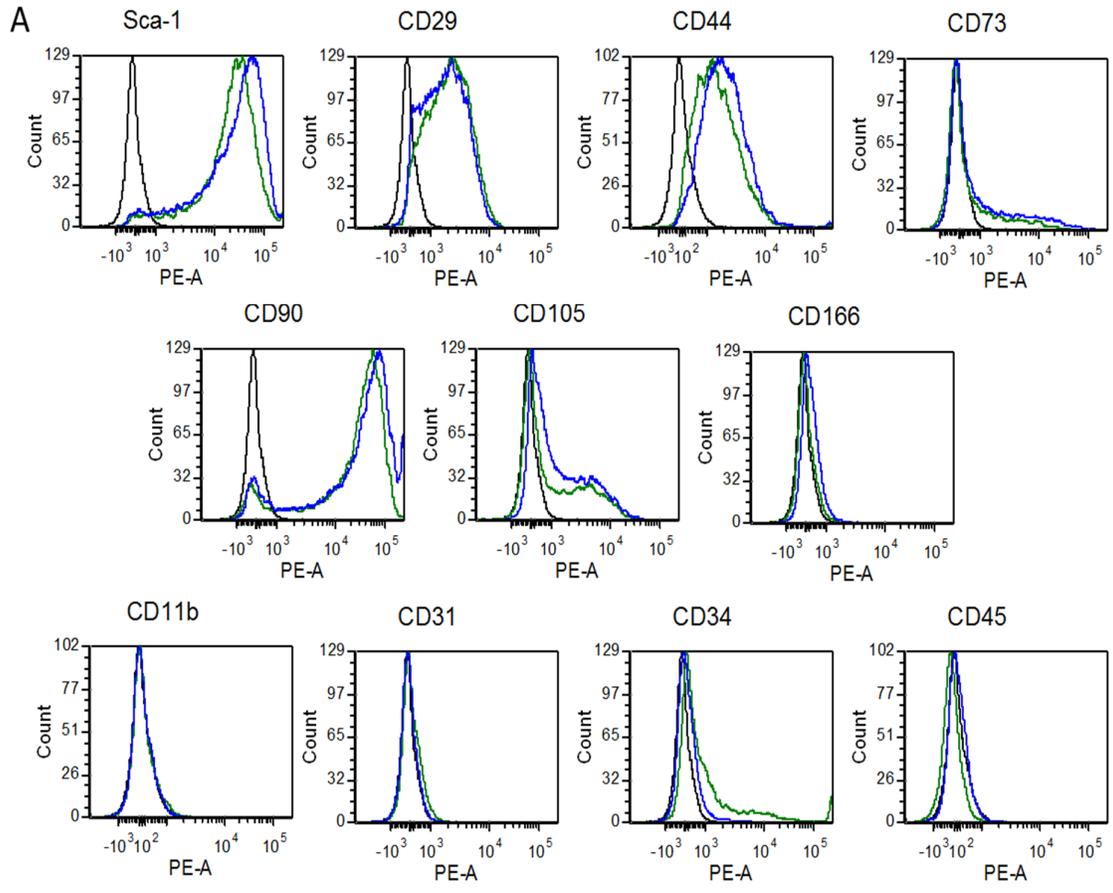


Figure 6.3.4 Characterisation of UT-mMSC and mMSC-17.

Compact bones derived MSC isolated from C57BL/6 mice were either untreated (UT-mMSC) or treated with IL-17A (mMSC-17) for 5 days. **(A)** The expression of surface markers on MSC was assessed by flow cytometry. The histograms show levels of surface marker expression on MSC and are depicted by the **isotype control** (black), **UT-MSC** (green) and **MSC-17** (blue). Data are one representative of 4 mMSC isolations. **(B)** Functional differentiation of MSC into adipocytes (Oil red stained fat; original magnification x100) and osteoblasts (Alizarin red stained mineral; original magnification x100) for 4 weeks. The Nikon Eclipse Ti inverted microscope was used to capture images. Representative of one of 3 mMSC isolations. **(C)** Fold increase in MSC numbers calculated as harvest cell number divided by the initial seeding number. Cell numbers were determined by trypan blue exclusion assay. Data are pooled from 4 independent experiments of 4 mMSC isolations. * $p < 0.05$ versus UT-mMSC was determined by two-way ANOVA with post-Sidak multiple comparison test. Error bars depict mean \pm SEM.

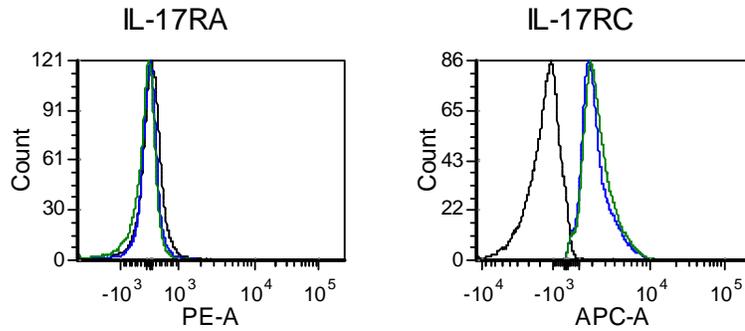


Figure 6.3.5 IL-17A receptors expression on CB mMSC.

Compact bones derived MSC isolated from C57BL/6 mice were either untreated (UT-mMSC) or treated with IL-17A (mMSC-17) for 5 days. (A) The expression of surface markers on MSC was assessed by flow cytometry. The histograms show levels of surface marker expression on MSC and are depicted by the **isotype control** (black), **UT-MSC** (green) and **MSC-17** (blue). Data are one representative of 3 mMSC isolations.

6.3.5 mMSC inhibit allogeneic T cell proliferation in a dose-dependent manner

The ability of autologous CB mMSC to inhibit *in vitro* allogeneic DC induced total, CD4⁺ or CD8⁺ T cell proliferation was evaluated in a 1-way MLR. Similar to human MSC (**Figure 3.3.1** and **Figure 3.3.2**), CB UT-mMSC inhibited allogeneically stimulated T cell proliferation in a dose-dependent manner at days 3 and 5 of the MLR assay (**Figure 6.3.6**). Maximal immunosuppression of T cells was observed at 10% mMSC dose whereby CD4⁺ T cell proliferation was suppressed by 87.1% and 87.5% at days 3 and 5 of MLR, respectively (cumulative data; **Table 6.3.5.1** and **Table 6.3.5.2**). Suppression on CD8⁺ T cell proliferation occurred at a greater extent compared to CD4⁺ T cells with the 10% UT-mMSC dose (94.1% at day 3 and 91.5% at day 5; cumulative data; **Table 6.3.5.1** and **Table 6.3.5.2**). UT-mMSC also retained their immunosuppressive capacity on CD4⁺ and CD8⁺ T cell when used at lower doses (0.1% and 1%), suggesting that the CB mMSC isolation protocol combined with hypoxia preconditioning to enrich mMSC from CB precursor cells yielded a highly suppressive population of mMSC.

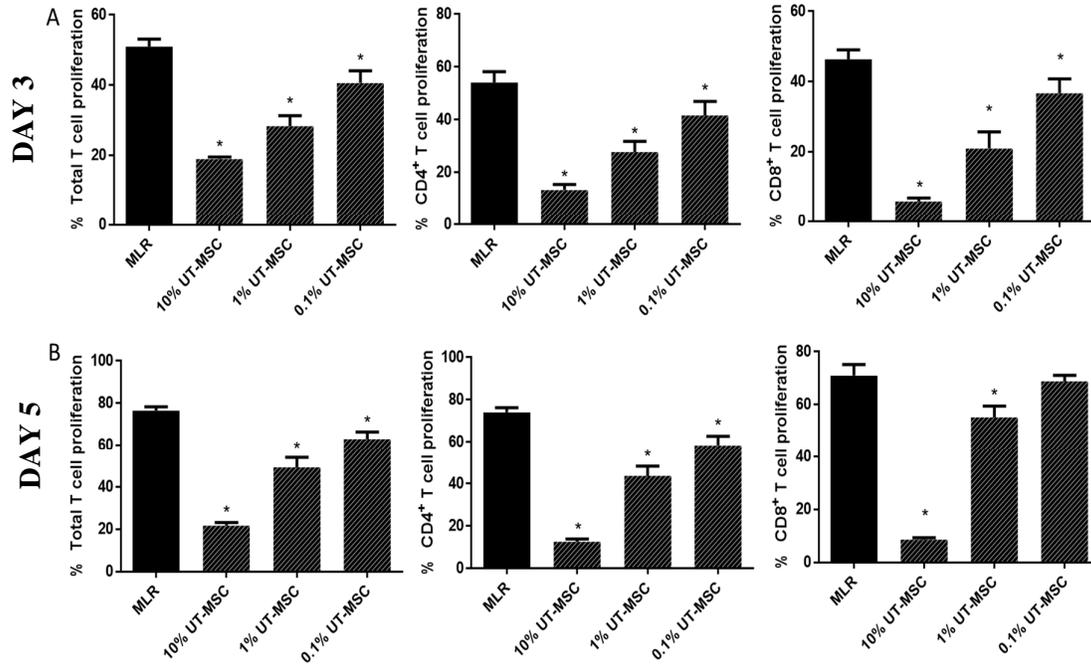


Figure 6.3.6 mMSC inhibit allogeneic T cell proliferation in a dose-dependent manner.

UT-MSC were co-cultured with CFSE-labelled CD3⁺ T cells (C57BL/6 mice) allo-stimulated by irradiated DC (BALB/c mice) at 10% UT-MSC, 1% UT-MSC and 0.1% UT-MSC. At (A) 3- or (B) 5- days, T cells were isolated and cell-surface stained with anti-mouse CD4 and anti-mouse CD8 and acquired on BD FACSCanto II. Percentage of total, CD4⁺ and CD8⁺ T cell proliferation was determined and represented in the graphs. MLR in the absence of MSC indicates maximal T cell proliferation. Data are pooled from 5 (A, B: Total T cells, CD4⁺ T cells) or 4 (B: CD8⁺ T cells) independent experiments. * $p < 0.05$ vs. MLR determined by two-way ANOVA with post-Sidak multiple comparison test. Error bars depict means of triplicates \pm SEM of triplicates.

Table 6.3.5.1 Cumulative data on mMSC suppression of T cell proliferation.

DAY 3 MLR			% proliferation \pm SEM		
T cell subsets	n	MLR	10% UT-MSC	1% UT-MSC	0.1% UT-MSC
Total T cells	n=5	50.98 \pm 2.154	18.95 \pm 0.5518	28.31 \pm 2.999	40.64 \pm 3.425
CD4 ⁺ T cells	n=5	56.70 \pm 4.236	12.88 \pm 1.785	27.86 \pm 3.340	41.88 \pm 4.3858
CD8 ⁺ T cells	n=5	46.29 \pm 3.534	5.883 \pm 1.335	23.89 \pm 4.770	39.56 \pm 3.727

0.1%, 1% or 10% UT-mMSC were co-cultured with CD11c⁺ mDC stimulated CD3⁺ T cells for 3 days. CD4⁺ and CD8⁺ T cell proliferation was measured in a 1-way MLR. Data are presented as percentage CD4⁺ and CD8⁺ T cell proliferation. Bold fonts indicate p<0.05 vs. MLR determined by two-way ANOVA with post-Sidak multiple comparison tests.

Table 6.3.5.2 Cumulative data on mMSC suppression of T cell proliferation

DAY 5 MLR			% proliferation \pm SEM		
T cell subsets	n	MLR	10% UT-MSC	1% UT-MSC	0.1% UT-MSC
Total T cells	n=5	76.47 \pm 1.85	21.74 \pm 1.46	49.33 \pm 4.702	63 \pm 3.341
CD4 ⁺ T cells	n=5	68.83 \pm 6.652	12.55 \pm 1.392	43.86 \pm 4.487	58.37 \pm 4.375
CD8 ⁺ T cells	n=4	71.00 \pm 4.141	8.535 \pm 0.7719	55.16 \pm 4.254	68.84 \pm 2.285

0.1%, 1% or 10% UT-mMSC were co-cultured with CD11c⁺ mDC stimulated CD3⁺ T cells for 5 days. CD4⁺ and CD8⁺ T cell proliferation was measured in a 1-way MLR. Data are presented as percentage CD4⁺ and CD8⁺ T cell proliferation. Bold fonts indicate p<0.05 vs. MLR determined by two-way ANOVA with post-Sidak multiple comparison tests.

6.3.6 mMSC-17 are not superior suppressors of T cells

Human MSC-17 exhibited superior suppression of T cells compared to UT-MSC and MSC- γ (**CHAPTER 3 and 4**). In this chapter, we aimed to establish the suppressive capacity of mMSC-17 using the same IL-17A concentration (50ng/ml) and 5 days cytokine pre-treatment of mMSC, similar to our human MSC-17 study. Unexpectedly, when mMSC were treated with IL-17A for 5 days prior to use in the MLR, no enhancement of T cell immunosuppression was observed in the mMSC-17 groups compared to UT-mMSC. Total T cell, CD4+ and CD8+ T cell proliferation was similar between mMSC-17 and UT-mMSC at day 3 or 5 MLR (**Figure 6.3.7**). The effect of 3 days IL-17A treatment of mMSC was also investigated. However, no significant differences in suppression of T cell proliferation was detected between UT-mMSC and mMSC-17 (**data not shown**).

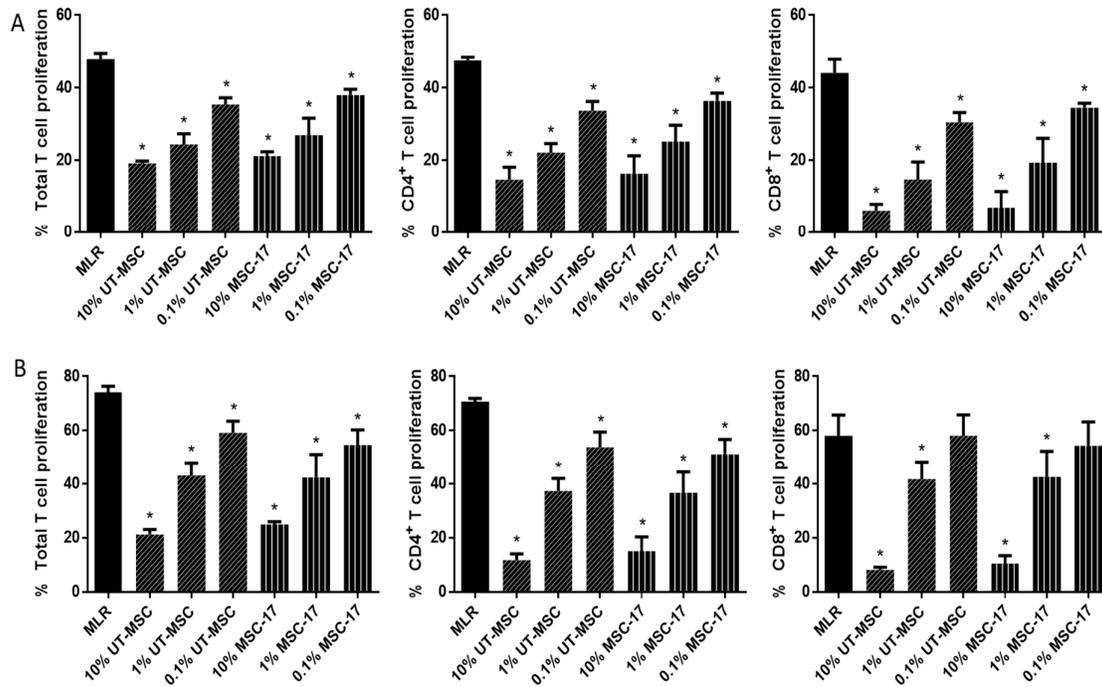


Figure 6.3.7 mMSC-17 does not enhance MSC suppression of T cell proliferation.

UT-MSC or MSC treated for 5 days with IL-17A were co-cultured with CFSE-labelled CD3⁺ T cells (C57BL/6 mice) allo-stimulated by irradiated DC (BALB/c mice) at 10%, 1% and 0.1% MSC doses. At (A) 72-hr or (D) 5 days, T cells were isolated and cell-surface stained with anti-mouse CD4 and anti-mouse CD8 and acquired on BD FACSCanto II. Percentage of total, CD4⁺ and CD8⁺ T cell proliferation was determined and represented in the graphs. MLR in the absence of MSC indicates maximal T cell proliferation. Data are pooled from 3 independent experiments. * $p < 0.05$ vs. MLR determined by two-way ANOVA with post-Sidak multiple comparison test. Error bars depict means of triplicates \pm SEM.

6.3.7 Establishing an islet allograft rejection model

Since isolated CB UT-mMSC without IL-17A treatment have potent *in vitro* immunosuppressive capacity, their ability to suppress *in vivo* islet allograft rejection was evaluated. The islet transplantation model was established to determine the duration required for islet allograft rejection to occur in absence of anti-rejection therapy. Diabetic C57BL/6 mice were transplanted with islets from 3 pancreata or 300 handpicked islets isolated from a fully MHC-mismatched BABL/c islet donor (**Figure 6.3.8**). As expected, a larger islet cell mass was observed under the kidney capsule of diabetic mice transplanted with 3 pancreata of islets compared to 300 islets (**Figure 6.3.8**). Aged-matched nondiabetic non-transplanted (nonTx) mice had daily BGL of < 11.1mMol/L with an average of 8.2mMol/L (**Figure 6.3.9A**). C56BL/6 mice induced to become diabetic with STZ, with 3 consecutive BGL readings of > 16.6mMol/L were used as recipients of islet transplantation. A successful transplant procedure was defined as the restoration of euglycemia (<11.1mMol/L) within 3 days post-transplantation. Transplantation of 3 pancreata of islets or 300 handpicked islets resulted in a 100% cure rate by day 3, with a mean time to cure of 1.1 days and 1.67 days, respectively. Mean BGL on post-operative day 3 (POD3) was not significantly changed from pre-transplant values in both islet allotransplant groups (**Figure 6.3.9A**). The weight of both islet transplanted mice groups decreased following STZ induction but gradually increased after receiving islet allotransplant until the time-point of allograft rejection. Mice weights continued to decline after the first reading to indicate allograft rejection (BGL >16.6mol/L; **Figure 6.3.9B**).

Technical failure of a transplant procedure was evident by fluctuating BGL levels, with a drop of BGL to normal and a sudden reversal of euglycemia. This could be due to the transplanted islet cell mass leaking out from the kidney capsule (mouse KS4 in the 3 pancreata/Tx group; **Figure 6.3.9**). In the 300 islets/Tx group, one mouse (KS113) showed normal BGL up to POD51. Severe fibrosis of the kidney bearing islet allograft was also evident

at the time of necropsy. The pancreas of this mouse may have undergone neogenesis following STZ-mediated damage of the pancreas, resulting in normoglycaemic levels⁴⁸⁸⁻⁴⁹¹. Partial destruction of hamster pancreas by STZ have been shown to reverse diabetes in more than 50% of the time⁴⁸⁸. Islet-associated regenerative molecules can either induce the proliferation of existing β -cells or initiate neogenesis from islet precursors^{488,491}. At time of necropsy of mice (KS4 and KS113), the pancreas appeared to be less damaged. This could suggest that STZ induction in these mice only resulted in partial pancreas destruction and subsequent β -cell regeneration *in vivo*. Hence, these mice (KS4 and KS113) were excluded for survival curve analysis.

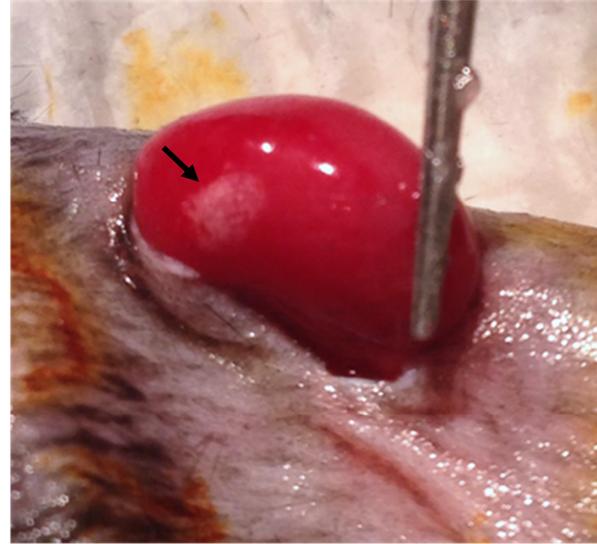
Mice receiving islet allograft transplants with no anti-rejection therapy exhibited a mean islet allograft survival time (MST) of 32 ± 3.22 and 26 ± 6.03 days in the 3 pancreata/Tx and 300 islet transplant groups, respectively, thereby conforming the robust immune response to the fully MHC-mismatched islets after transplantation under the kidney capsule (**Table 6.3.7.1, Figure 6.3.10**). Although the islet cell mass (**Figure 6.3.8**) was larger in the 3 pancreata/Tx mice group, the time-point to rejection was not significantly reduced in the 300 islet/Tx group. There was no statistical difference in the mean survival time (MST) in the 2 islet transplant groups (**Table 6.3.7.1, Figure 6.3.10**). Intra-graft infiltration of immune cells as early as POD10 was evident by H&E staining in the kidney bearing the islet allograft, thus, conforming the presence of a robust immune rejection response (**Figure 6.3.11**).

To examine the *in vivo* islet allograft function, IPGTT were performed at day 10 or 14 after transplantation (**Figure 6.3.12**). Diabetic recipients from the 3 pancreata/Tx group had normal glycaemic control at POD10 and POD14, similar to the nonTx control mice group. BGL of mice receiving 300 islets/Tx were higher at 60 and 120mins compared to nonTx group, with a larger area under the curve (AUC).

These experiments established that the transplantation of 300 islets in diabetic mice is sufficient to cure mice by POD3. This is followed by a robust rejection of the allografts by POD26 and islet graft function declining by POD14. This model was subsequently used to test the *in vivo* efficacy of UT-mMSC co-transplanted with islets under the kidney capsule as an anti-rejection therapy. Unfortunately, we experienced technical failure in this experiment as the transplantation of 300 islets alone or the co-transplantation of UT-mMSC with islets showed no restoration of euglycemia by POD3 (**Figure 6.3.13**). Weight of mice also continued to decline after transplantation (**Figure 6.3.13**). Unfortunately, these experiments halted due to time constraints to complete this PhD thesis but will be explored in future studies.

A

3 pancreata / Tx

B

300 islets / Tx

Figure 6.3.8 Islets transplanted under the kidney capsule of diabetic recipient mice.

Islets isolated from an allogeneic donor (BALB/c mice) were transplanted under the kidney capsule of a diabetic C57BL/7 mouse recipient. Islet pellet (indicated by the arrow) **(A)** isolated from 3 pancreata for each transplant or **(B)** 300 handpicked islets transplanted under the kidney capsule of one representative diabetic C57BL/7 mouse recipient per group are shown in these figures.

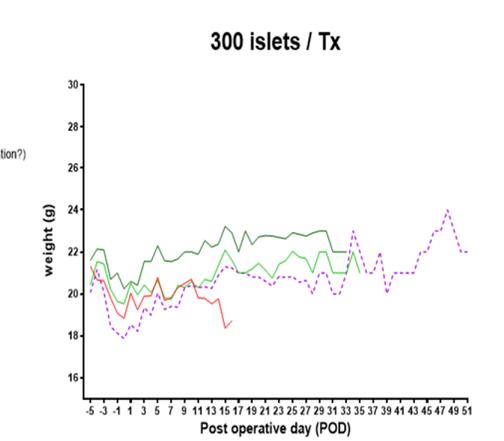
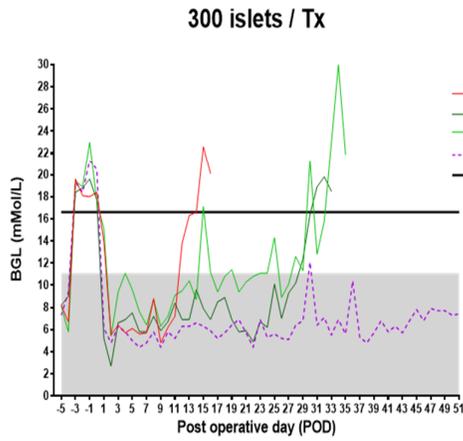
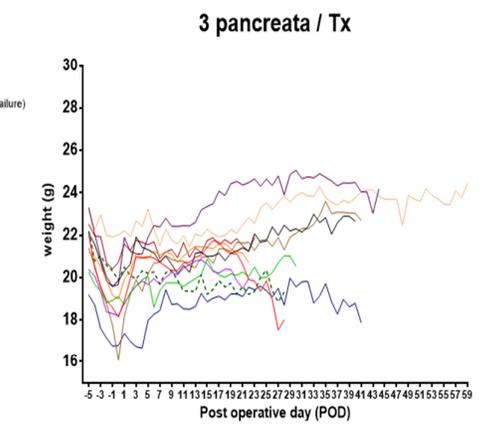
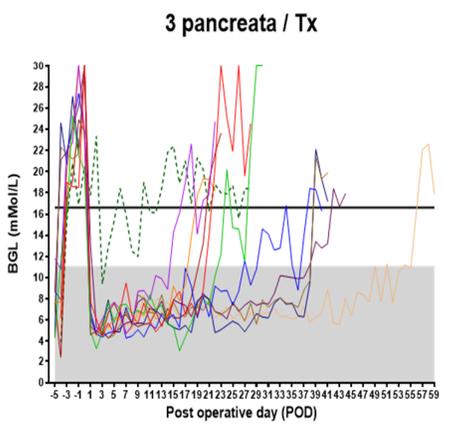
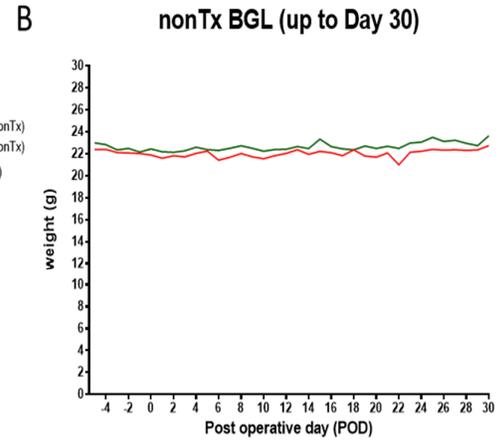
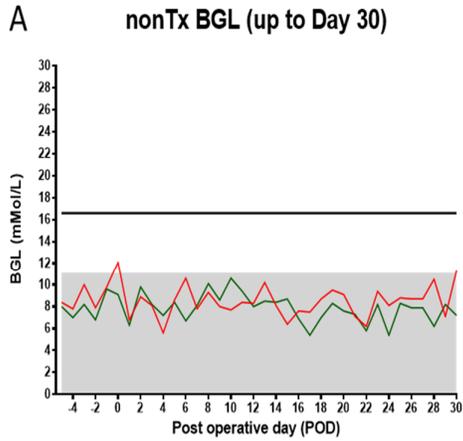


Figure 6.3.9 Islet allograft survival.

C57BL/6 diabetic mice were transplanted with fully MHC-mismatched BALB/c islets under the kidney capsule. Diabetic recipients were transplanted with 300 islets (300 islets/Tx) or a bulk transplant of islets from 3 pancreata (3 pancreata/Tx). Animals were humanely killed due to poor health or weight loss. BGL and weights of nontransplanted age-matched C57BL/6 mice (nonTx) were also tested concurrently. **(A)** Nonfasted blood glucose levels (BGL) and **(B)** weights of islet transplant recipients were measured daily until the time-point of rejection. **(A)** Graft survival was determined by monitoring nonfasted daily BGL. BGL >16.6mMol/L on 3 consecutive days was considered rejection (black line). The grey shaded area represents euglycemia (BGL < 11.1mMol/L). **(A,B)** Each line represents one recipient mouse. Dotted lines indicate mice that were excluded for survival analysis due to technical failure during the transplant procedure or potential pancreas regeneration (>50 days survival post-transplant).

Abbreviations: Tx, transplant; nonTx, non transplant.

Table 6.3.7.1 Experimental groups and islet allograft survival

Groups	Mice (n)	Graft survival (days)	MST (mean ± SEM)
3 pancreata / Tx	9	22, 28, 20, 19, 38, 39, 39, 42, 41	32 ± 3.22
300 islets / Tx	3	14, 31, 33	26 ± 6.03

Tx, transplant; n, number of mice; MST, mean survival times; SEM, standard error of mean

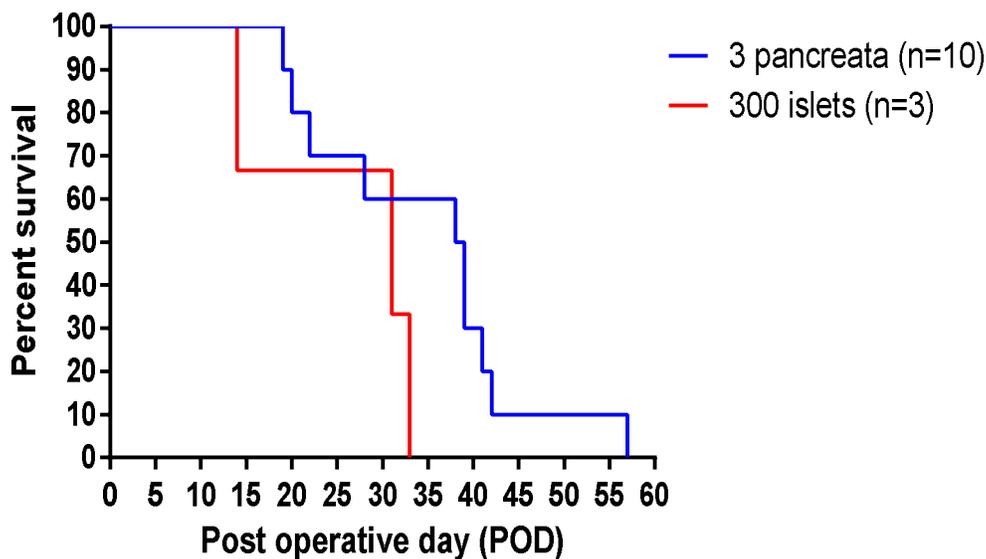


Figure 6.3.10 Islet allograft survival curves.

C57BL/6 diabetic mice were transplanted with fully MHC-mismatched BALB/c islets under the kidney capsule. Islet allograft survival was compared between mice transplanted with 300 islets (300 islets/Tx) or a bulk transplant of islets from 3 pancreata (3 pancreata/Tx). Graft survival was determined by monitoring daily blood glucose levels (BGL). BGL >16.6mMol/L on 3 consecutive days was considered rejection. The data represent the cumulative percentage of allograft survival. Differences between groups were determined using the log-rank (Mantel-Cox) test. There was no statistical significance between the two transplant groups, log-ranked chi-square of $p = 0.1271$.

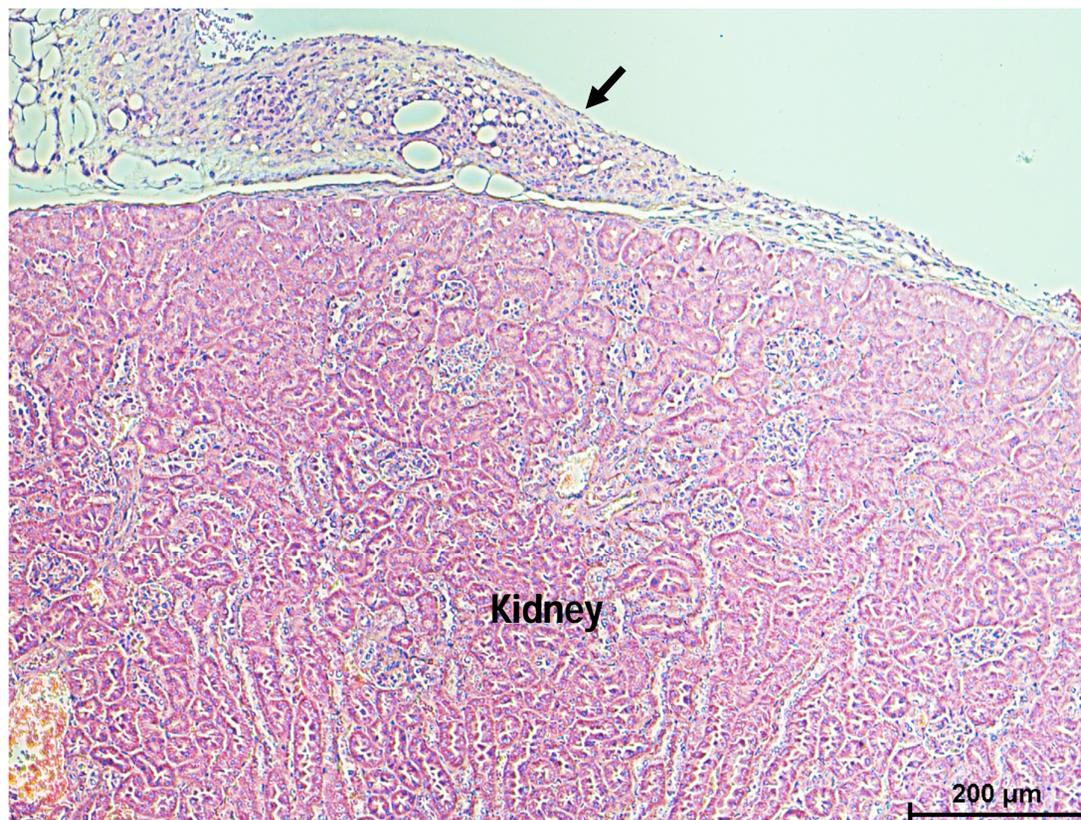


Figure 6.3.11 Histological analysis of engrafted islets at POD10.

Representative immunohistochemical examination of islet allograft under the kidney capsule (indicated by the arrow) from mice receiving islet transplants at POD10 (hematoxylin and eosin, H&E stained; original magnification x200). The Nikon Eclipse Ti inverted microscope was used to capture images.

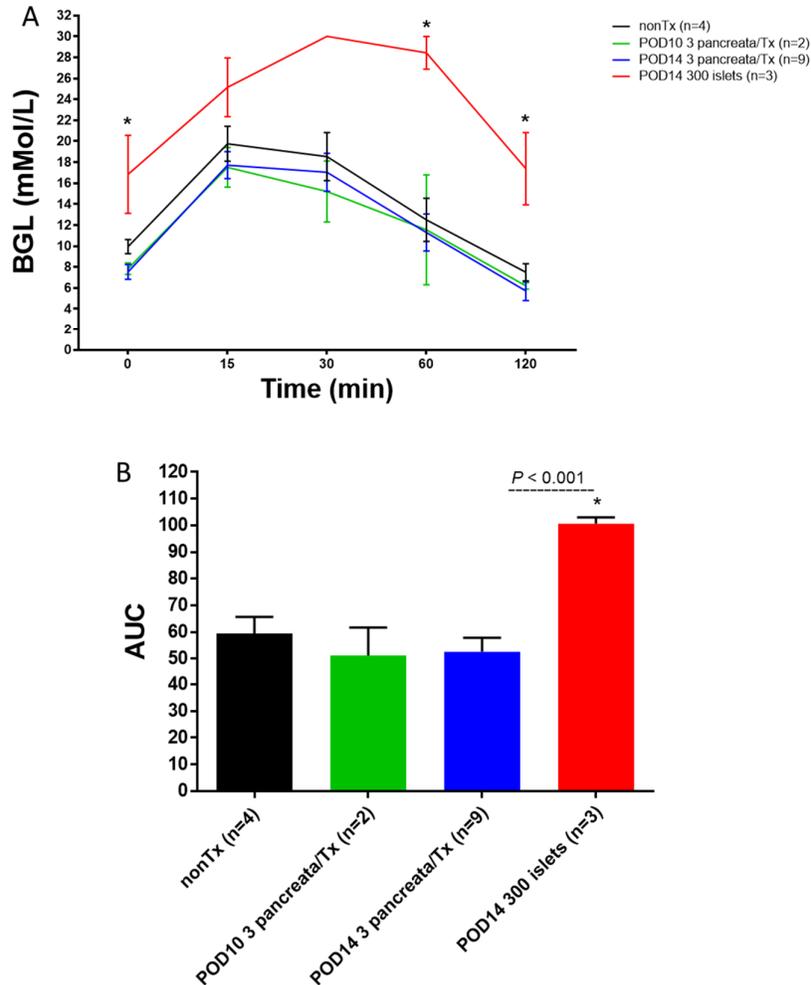


Figure 6.3.12 Islet allograft function.

Islet allograft function were compared between mice transplanted with 300 islets (300 islets/Tx) or a bulk transplant of islets from 3 pancreata (3 pancreata/Tx) in an intraperitoneal glucose tolerance test (IPGTT) at post-operative day (POD) 10 or 14 after transplantation. Four hour fasted mice were challenged with 2g/kg body weight of intraperitoneally injected glucose. **(A)** Blood glucose levels were measured at various time points post injection. Non-transplanted age-matched C57BL/6 mice (nonTx) were also tested simultaneously. **(B)** Area under the curve (AUC) as a measure of glucose tolerance. * $p < 0.05$ vs. nonTx was determined by one-way ANOVA with post Tukey multiple comparison test. Data are shown as mean **(A)** BGL or **(B)** AUC \pm SEM.

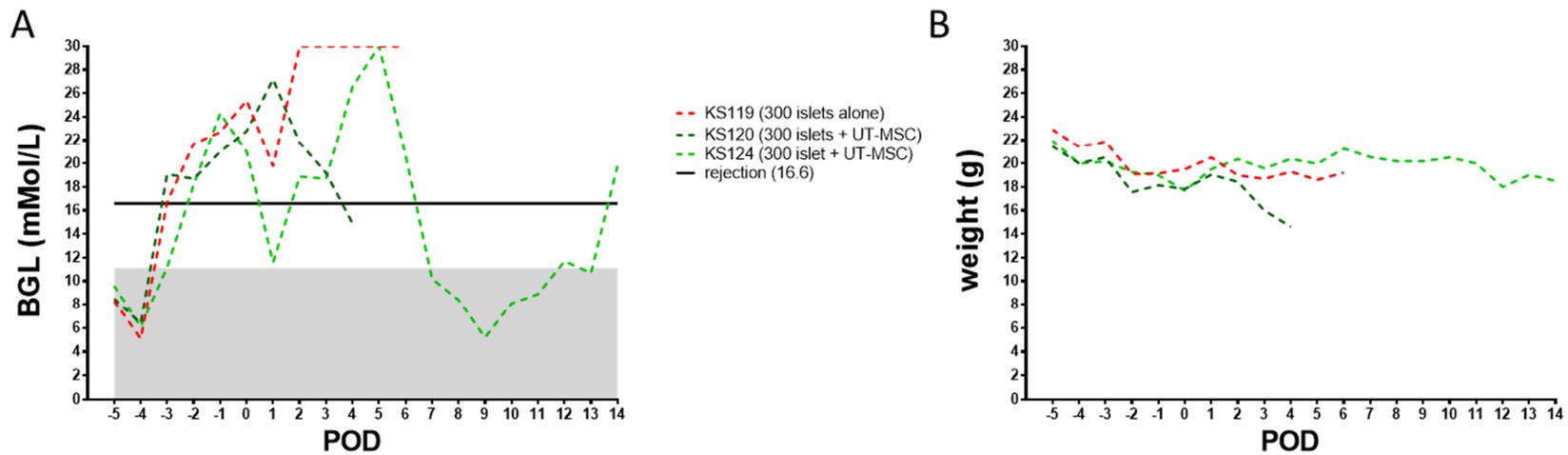


Figure 6.3.13 UT-MSC and islet co-transplantation.

C57BL/6 diabetic mice were transplanted with fully MHC-mismatched BALB/c islets (300 islets): (A) islets alone (controls) or (B) co-transplanted with 1×10^6 mouse UT-MSC (islets + UT-MSC) under the kidney capsule. Animals were humanely killed due to poor health or weight loss. (A) Nonfasted BGL and (B) weights of islet transplant recipients were measured daily until the time-point of rejection. (A) Graft survival was determined by monitoring nonfasted daily BGL. BGL >16.6 mMol/L on 3 consecutive days was considered rejection (black line). The grey shaded area represents euglycemia (BGL <11.1 mMol/L). (A,B) Each line represents one recipient mouse. Dotted lines represent mice that were excluded for survival analysis due to failure of the transplant procedure or issues with islet cell preparation. This data was excluded for further survival analysis as the islet alone controls showed no recovery from diabetes (cure <11.1 mMol/L) post-transplantation.

6.4 DISCUSSION

Compact bones (CB) have emerged to be one of the major reservoirs of MSC in the murine system⁴⁷³⁻⁴⁷⁶. CB derived mouse MSC (mMSC) represent an alternative source to bone marrow (BM) mMSC to isolate and enrich for a highly purified population of mMSC. Mouse MSC are traditionally isolated from the BM but mMSC are present at low frequencies where BM MSC preparations are highly heterogeneous with many contaminating haematopoietic cells⁴⁶³⁻⁴⁶⁵. Monocytes, granulocytes and pre-B cell progenitors were shown to exist in the whole BM fraction and co-adhere with BM mMSC in long-term cultures⁴⁷². Consistent with these studies, we observed the presence of colonies with various morphologies including fibroblastic-like-MSC colonies and cells with cobblestoned appearance; confirming the heterogeneity of the primary BM mMSC cultures (**data not shown**). The co-existence of contaminating cells in the mMSC cultures may contribute to BM mMSC growth arrest and undesired mMSC differentiation under standard culture conditions. In addition, the inconsistency in mMSC isolation protocols with an impure population of BM mMSC cell lines may provide an explanation on the contradictory reports on the benefits of BM mMSC *in vivo*⁴ (**Table 1.8.1.1** and **Table 1.8.1.2**).

Of note, numerous attempts to establish primary mMSC cell lines from the whole BM fraction ended in failure, consistent with a previous published report⁴⁷⁴. BM mMSC compared to CB mMSC exhibited low growth kinetics and failed to be maintained in *in vitro* tissue culture conditions, with complete loss of cell growth by day 100⁴⁷⁴. Plastic-adherent selected BM mMSC had very low growth potential and hence required longer duration to achieve confluency compared to CB mMSC cultured under normoxia or hypoxia conditions as tested in this thesis. These BM mMSC also underwent senescence and spontaneously differentiated into adipocytes when allowed to propagate long-term in primary mMSC media (20% FBS) under normoxia (**data not shown**). Intracellular lipid vacuoles could be detected by day 21 in

the primary BM mMSC cultures. This observation was similar to another study demonstrating that BM mMSC transdifferentiated into adipocytes when maintained in long-term normal culture conditions and in normoxia⁴⁹². BM mMSC cultured under atmospheric oxygen (21% O₂; normoxia) were shown to induce the expression of p53 and mitochondrial reactive oxygen species (ROS) in mMSC⁴⁹³. These molecules resulted in oxidative stress, increased death and caused growth arrest of BM mMSC⁴⁹³. This may provide a plausible explanation to the failure to maintain BM mMSC multipotency and propagation from whole BM due to long-term atmospheric oxygen exposure that resulted in cell growth arrest. On the contrary, low oxygen tension (2% or 5% O₂) has been shown to support BM mMSC clonogenicity and retained their multipotency⁴⁹³⁻⁴⁹⁵. However, the data presented in this thesis chapter and by previous reports have published that BM mMSC compared to their CB counterparts still have lower clonogenicity and cell yields when established under hypoxia^{481,494}. Hence, for the purpose of this study, mMSC were derived from the CB and were cultured under hypoxia to establish primary CB mMSC cell lines.

Apart from defining oxygen requirements for CB mMSC cultures, optimal conditions to enrich and propagate these cells using the whole CB fraction were also established. CB were depleted of the BM to reduce haematopoietic cells that contaminate mMSC cultures. Collagenase digestion of these bone fragments is an essential step for mMSC progenitors to migrate out of the bone and subsequently attach to tissue culture surfaces in the primary MSC media^{348,475}. The duration of bone digestion in collagenase, as well as the concentration and type of collagenase is critical to release mMSC precursors. If this digestion step is too long, mMSC viability can be impaired³⁴⁸. Additionally, various studies employ different collagenase types (I or II) and digestion protocols^{348,475,476}. Collagenase activity also vary from batch to batch, and hence requires optimization for CB digestion protocols. In this study, the collagenase digestion step of CB for maximal yield of mMSC progenitors was optimized. The

stem cell antigen-1 (Sca-1) has recently emerged as a potential marker to isolate mMSC precursors with higher clonogenicity potential, cell yields and maintains CB mMSC in an undifferentiated state^{476,480,481}. However, haematopoietic stem cells and other stem cell or progenitor cell populations also express Sca-1. Collagenase II (3mg/ml, 1hr) was optimal to enrich for the highest percentage of Sca-1⁺ cells, with no compromise to cell viability. These cells cultured in CFU-F assays for only 5 days in hypoxia (5% O₂) had highest CFU-F numbers, thus confirming that we have enriched for a highly clonogenic CB mMSC precursors. Collagenase I (3mg/ml, 1hr) on the contrary, resulted in a very low yield of Sca-1⁺ cells that correlated with lower CFU-F frequencies and was considered inadequate to release mMSC precursors from the CB.

Mouse MSC were enriched and propagated from CB progenitors using primary MSC media supplemented with 20% FBS and sub-cultured in MSC media with 10% FBS. Previous studies have established that different FBS can affect mMSC clonogenicity, enrichment and expansion⁴⁸⁵⁻⁴⁸⁷. FBS batches vary in quality and composition (eg. growth factors, substrate attachment components, and signaling molecules) which in turn determine MSC attachment to tissue culture flasks, their propagation and maintenance in an undifferentiated state⁴⁸⁵. Although MSC qualified FBS is commercially available, many research laboratories pre-screen these FBS along with normal non-MSC FBS. MSC qualified FBS is generally highly priced compared to normal FBS, vary based on suppliers and are batch tested only on human MSC. In this chapter, the commercially tested human MSC qualified FBS [Invi (MSC)] showed no enrichment of clonogenic mMSC and *ex vivo* propagation with no alterations to their morphology at sub-passaging compared to the non-MSC FBS, Invi (normal). Moreover, no mMSC proliferation arrest was evident in cells cultured up to passage 6 with Invi (normal). These findings validate the non-MSC FBS, Invi (normal) supported mouse MSC maintenance

and expansion in *in vitro* culture conditions as effective as Invi (MSC), and was therefore suitable for CB mMSC propagation.

The T cell immunosuppressive properties of CB mMSC were investigated using a more purified CB mMSC population. CB depleted BM following collagenase II digestion were immunodepleted for lymphocytes (CD45) and cells of erythroid lineages (TER119) that were highly prevalent in the bone marrow by negative selection. These mMSC progenitors from the CB were subsequently preconditioned under hypoxia (5% O₂) at passage 0 to establish the primary mMSC cell lines and were then sub-cultured under normoxia. These CB mMSC progenitors exhibited high growth kinetics as they reached >80% confluency within 5 days. Although the cultures contained the presence of round-adherent trypsin-resistant cells previously reported to be macrophages³⁴⁸, these cells were completely eliminated following 2-3 sub-cultures by reducing the duration of CB mMSC trypsinization³⁴⁸. The resultant CB mMSC obtained from this isolation protocol were therefore highly homogenous (>90% Sca-1⁺, CD29⁺, CD44⁺ and CD90⁺) with absence of contaminating CD11b (monocytes, NK cells, granulocytes, macrophages), endothelial cells (CD31), haematopoietic cells (CD34) and lymphocytes (CD45). CB mMSC were also demonstrated to differentiate into adipocytes and osteoblast when cultured under adipogenic or osteogenic induction conditions, respectively. Taken together, CB mMSC derived from this isolation protocol conformed to the standard mMSC immunophenotypic characteristics and differentiation potential.

Several other groups have explored the option to isolate mMSC from CB however the functional suppressive role of CB mMSC on T cell subsets that closely mimic an allograft rejection response *in vitro* has not been previously reported. In this thesis, T cells isolated from a C57BL/6 mice were allo-stimulated with *ex vivo* generated and matured DC from monocytes of allogeneic donors (BALB/c mice). Autologous CB mMSC (C57BL/6 mice) co-cultured with allogeneic induce T cell proliferation in a one-way MLR potentially inhibited total T cell, CD4⁺

and CD8⁺ T cell proliferation in a dose-dependent manner. A study using combined positive selection of CD90⁺/PDGFR α ⁺ cells to isolate a subpopulation of CB mMSC demonstrated that these cells were capable of inhibiting CD3/CD28 activated T cell proliferation (total, CD4⁺ and CD8⁺) by up to 20% at 1% CB mMSC dose⁴⁸¹. CB mMSC preparation strategy from this chapter compared to the study by Baustian *et. al.*, (2015) was proven to be more superior at suppressing T cells in a context of an alloresponse by up to 76.1% and 56.1% using the same 1% CB mMSC at day 3 and 5 MLR (**Table 6.3.5.1** and **Table 6.3.5.2**).

In **CHAPTERS 2 and 3**, IL-17A preconditioned human BM MSC (MSC-17) functioned as superior suppressors of T cells. In this chapter, CB mMSC pre-treated with IL-17A (CB mMSC-17) were similar to human BM MSC-17, as they did not alter CB mMSC immunophenotype and functional differentiation into adipocytes or osteoblasts. Interestingly however, no enhanced CB mMSC proliferation compared to UT-MSC was obtained following 5 days IL-17A treatment of CB mMSC, contradicting previously published reports that IL-17A can stimulate BM mMSC proliferation^{340,342}. Earlier studies demonstrated that IL-17A functions as a MSC growth factor in both human BM MSC^{2,341,342} and BM mMSC³⁴². IL-17A promoted BM mMSC proliferation through the activation of signaling pathways including AKT, ERK, MEK and p38^{340,342,362}. Discrepancy of our data on the effects of IL-17A on murine MSC could relate to the source of MSC. All previous reports on IL-17A stimulating mMSC growth were conducted using BM mMSC while in this thesis, the CB as a source of MSC was studied.

CB mMSC-17 differ from human MSC-17 as they were ineffective at enhancing suppression of allogeneically stimulated T cell proliferation compared to UT-mMSC. This data is consistent with the recent study showing that treatment of BM mMSC with IL-17 alone is ineffective at increasing the ability of MSC to suppress T cell proliferation *in vitro*³⁶². Inconsistency in the immunosuppressive properties of MSC-17 derived from human MSC and

murine MSC may be due to species differences. It should also be noted that CB mMSC isolated from this thesis protocol expressed only the IL-17A receptor IL-17RC but not IL-17RA. As extensively reviewed in section 5.1, IL-17A homodimers signal through the IL-17RA and IL-17RC dimeric receptor complex to activate downstream IL-17A signaling pathways MAPKs-AP-1, C/EBPs or NF κ B^{383,385}. The findings in this thesis therefore suggest that both IL-17RA and IL-17RC are required for IL-17A to mediate their potent immunoregulatory role on CB mMSC. Han *et. al.*, (2014) showed that BM mMSC expressed IL-17RA³⁶². The expression of IL-17RC, however was not evaluated in this study. Additionally, this study proved that in the murine system, IL-17A acted synergistically with IFN- γ and TNF- α in enhancing BM mMSC immunosuppression *in vivo* in mouse models of hepatitis³⁶². The combination of IL-17A with other proinflammatory cytokines such as IFN- γ and TNF- α in amplifying CB mMSC immunosuppression remains to be elucidated.

Another possibility on the variability of the human and mouse CB MSC-17 data is that CB mMSC were more heterogenic in terms of CD73 expression and lacked CD105. In coherence with preceding publications on the immunophenotype of CB mMSC^{481,484}, only a small fraction of the CB derived mMSC were CD73⁺ and CD105⁺ compared to human BM MSC². The low levels or absence of CD73 and CD105 may be contributing factors for the ineffectiveness of CB mMSC-17 in mediating superior suppression of T cells compared to UT-mMSC. Further studies are therefore required to validate the functional role of CD73 and CD105 in human MSC-17 immunosuppression.

In summary, immunodepletion and hypoxia preconditioning of mouse compact bone (CB) cells represent a novel protocol to isolate highly immunosuppressive MSC that would be beneficial in models of allotransplantation rejection. CB mMSC without IL-17A preconditioning exhibited high purity and potent immunosuppressive effect on CD4⁺ and CD8⁺ T cells, which are the key effector cells mediating mechanisms of allograft rejection. The islet

allograft rejection model was also established, as described in this chapter. STZ induced diabetic mice were transplanted with a bulk of islets from 3 pancreata or 300 islets under the kidney capsule. Mice receiving the smaller islets mass (300 islets/Tx) have poor glycaemic control due to the robust rejection response that may be occurring at an earlier time-point (POD14) compared to mice receiving islets from 3 pancreata (**Figure 6.3.12**). These experiments established that the transplantation of 300 islets in diabetic mice is sufficient to cure mice and result in a robust rejection of the allografts by POD26 and islet graft function declining by POD14. In the future, we aim to investigate the therapeutic efficacy of CB mMSC to prevent rejection and prolong allograft survival and function *in vivo* in these optimized mouse models of islet allotransplantation.

CONCLUSIONS AND FUTURE DIRECTIONS

7.1 Conclusions

Bone marrow-derived mesenchymal stem cells (MSC) have unique immunosuppressive and reparative properties beneficial for allotransplantation cellular therapy. MSC exert direct or indirect immunosuppressive effects on T cells that are key effectors cells mediating mechanisms of allograft rejection. Despite the impressive immunomodulatory properties of MSC *in vitro*, the application of MSC as a monotherapy without conventional drug regimes *in vivo* remains controversial. Therefore, this research aimed to identify novel *ex vivo* strategies to derive MSC with full immunosuppressive potential to prevent allograft rejection and induce transplantation tolerance. Cytokine modified human MSC showed superior immunosuppressive properties compared to unmodified MSC (UT-MSC). These modified MSC may reduce or eliminate the undesired side effects due to the long-term dependence on immunosuppressive drugs. This study described the enhanced immunosuppressive properties of human MSC generated under the influence of the proinflammatory cytokine interleukin-17A (MSC-17) compared to interferon-gamma pre-treated MSC (MSC- γ) and UT-MSC.

In this thesis, it was demonstrated that IFN- γ modified human MSC (MSC- γ) potently suppressed T cell proliferation, activation and effector function as well as promoted the generation of CD4⁺CD25^{high}CD127^{low}FoxP3⁺ Tregs. IFN- γ induced or upregulated the expression of various MSC immunoregulatory factors that may further enhance T cell inhibition by directly or indirectly interacting with T cells. MSC- γ induced or amplified the expression of inhibitory molecules IDO1, PD-L1, IL-6, Cox-1, TLRs and ICAM-1, amongst others. MSC- γ were also enriched for gene sets involved in the regulation of programmed cell death, apoptosis, TGF- β receptor signaling pathway (eg. FMOD, CCL2, MAPK3K1, SMAD6, GDF15 and TGFB2). These molecules detected by either real-time PCR, microarray,

bioinformatics or by protein expression analysis, were previously known to have immunosuppressive roles in MSC. In addition to the generation of a highly immunosuppressive MSC- γ population, these cells have enhanced homing and tissue regenerative properties. Evident by microarray gene expression analysis, MSC- γ were enriched for genes associated with increased chemotaxis. An array of chemokines (eg. CXCL-9, -10, -11, -16, CCL2, CCL8 and CCL13) was upregulated following IFN- γ treatment of MSC, while a set of genes that were induced have biological processes for response to wound healing. Highly immunosuppressive MSC- γ with increased migratory and reparative capacities may aid tissue repair, prolong allograft survival and induce allotransplant tolerance in experimental models.

Nevertheless, there are contradictory *in vivo* observations related to allogeneic or third party (“off-the-shelf”) MSC- γ therapy as discussed in the literature review section of this thesis. Many studies report that “off-the-shelf” MSC are immunogenic due to their inherent expression of major histocompatibility (MHC) molecules. Experiments conducted in **CHAPTERS 3 and 5** confirmed that human MSC- γ induced or upregulated genes and (or) protein expression of MHC class I, MHC class II and T cell co-stimulatory molecule CD40, thereby making them highly immunogenic. Consistent with this observation, DAVID’s bioinformatics analysis revealed that a large number of genes upregulated in MSC- γ were involved in the antigen processing and presentation pathways of MHC class I and MHC class II. Enriched gene sets included aminopeptidases that hydrolyse antigenic peptides for MHC class I peptide binding and antigen presentation (eg. ERAP1 and ERAP2), peptide transporter genes (eg. TAP2) and other genes involved in the antigen processing and presentation pathways (eg. TAPBPL, B2M and CD74). Other biological processes enriched in MSC- γ included immune response activation, humoral response and activation of complement pathways. This microarray data is consistent with the upregulation of MHC molecules in MSC- γ (flow cytometry) and in other studies showing increased MSC- γ immunogenicity *in vivo*.

Hence, the crucial choice is whether the application of autologous or “off-the-shelf” MSC- γ would be more safe and efficacious in the context of allotransplantation. “Off-the-shelf” MSC induce cell- and antibody-mediated alloimmune responses which may decrease MSC longevity and effectiveness *in vivo*. The induction of donor-specific immune responses can also result in either hyperacute, acute or chronic allograft rejection depending on the route, timing (pre- or post-transplant), dose and numbers (single or multiple) of MSC infusion. Similar to models of GvHD and colitis, allogeneic MSC- γ may be effective at preventing acute inflammatory responses in allograft rejection, however their long-term efficacy *in vivo* is questionable. The potential alloimmune responses elicited by “off-the-shelf” MSC- γ suggest that these cells are more immunogenic than UT:MSC and could be detrimental when administered in allotransplant patients. The enhanced alloimmune responses following MSC- γ infusion could relate to the upregulation or induction of MHC molecules and alloproteins upon exposure of MSC to IFN- γ .

When considering the application of UT-MSC or MSC- γ monotherapy in allotransplantation, then autologous MSC would be most effective and safe to prevent rejection and promote long-term allograft survival. There are, however, concerns associated with the declined function of autologous MSC derived from actively ill patients. Besides that, approximately 3 months is required to generate sufficient doses of autologous MSC for patient infusion and this may not be ideal for newly diagnosed patients with acute disease conditions. Under these circumstances, if allogeneic MSC- γ therapy is to be considered, then co-administration with immunosuppressive drugs such as MMF or rapamycin but not CsA could be most effective. Immunosuppressive drugs may control the immune responses directed against allogeneic MSC- γ , prolonging MSC- γ survival and enabling them to exert their beneficiary effects in allotransplantation.

This thesis describes a new method of culturing human bone marrow-derived MSC with the proinflammatory cytokine interleukin-17A (IL-17). MSC pre-treated with IL-17A (MSC-17) represents a novel immunomodulatory strategy and an alternative to IFN- γ treatment of MSC in enhancing MSC immunosuppression on T cells but not their immunogenicity. When co-cultured with phytohemagglutinin (PHA)-activated human T cells, MSC-17 were potent suppressors of T cell proliferation. Furthermore, MSC-17 inhibited surface CD25 expression and suppressed the elaboration of Th1 cytokines, IFN- γ , TNF- α and IL-2 when compared with UT-MSC. MSC-17, but not MSC- γ consistently induced CD4⁺CD25^{high}CD127^{low}FoxP3⁺ regulatory T cells (iTregs) from PHA-activated CD4⁺CD25⁻ T cells. MSC-17-induced iTregs expressed CD39, CD73, CD69, OX40, CTLA-4, and GITR. These suppressive MSC-17 can engender Tregs to potentially suppress T cell activation with minimal immunogenicity and thus represent a superior T cell immunomodulator for clinical application. Human MSC-17 immunomodulation of T cell responses *in vitro* as demonstrated in this thesis is summarised in **Figure 7.1.1**.

Mechanistically, T cell suppression by human MSC-17 correlated with increased IL-6 but not with IDO-1, Cox-1, and TGF- β 1. Based on microarray analysis, an array of chemotactic factors was identified to be upregulated in MSC-17 (ie. CCL2, CCL8 and CXCL6) and was enriched for biological processes of leukocyte migration and chemotaxis. There was also potent induction of MMPs, MMP13 and MMP1 in MSC-17. MSC-17 derived chemokines or MMPs may facilitate T cells into close proximity with MSC-17, enabling potent suppression of proinflammatory T cell responses or in inducing Treg generation. In addition to their immunosuppressive roles, MSC-17 expressed MMPs may facilitate MSC-17 to invade the ECM. MMP expression combined with the strong expression of chemokines, may enable MSC-17 to home specifically to sites of inflammation, thereby allowing them to exert their tissue regeneration of immunosuppression function *in vivo*. MSC-17, unlike MSC- γ , showed

no induction or upregulation of MHC class I, MHC class II, and T cell co-stimulatory molecule CD40, but maintained normal MSC morphology and phenotypic marker expression. “Off-the-shelf” MSC-17 therapy may therefore be less immunogenic *in vivo*.

The final chapter aimed at determining the therapeutic efficacy of MSC-17 in preventing allotransplantation rejection *in vivo* in a preclinical model of islet transplantation. For this purpose, the findings from human MSC-17 were intended to be translated into mouse MSC. Compact bone derived mouse MSC (CB mMSC) were isolated from CB precursor cells by CD45 (lymphocytes) and TER119 (erythroid lineages) immunodepletion, followed by hypoxia preconditioning. These mouse CB mMSC cell lines were subsequently generated under normoxia conditions and were used for mMSC characterization and functional assays. The *in vitro* effects of IL-17A modified CB mMSC (mMSC-17) on MSC immunophenotype, growth kinetics, functional differentiation potential and immunosuppressive properties were extensively studied and compared to UT-mMSC. Mouse MSC-17 exhibited no increase in MSC growth potential, inconsistent with human MSC-17 showing 4-fold increase in MSC proliferation relative to human UT-MSC. Unfortunately, mMSC-17, unlike human MSC-17 showed no enhancement of *in vitro* immunosuppression of allogeneically induced CD4⁺ and CD8⁺ T cell proliferation relative to UT-MSC. The use of mouse MSC in a rodent system is therefore not permissible to study the *in vivo* biological function of IL-17A modified MSC. Nevertheless, CB mMSC, without IL-17A preconditioning mediated potent *in vitro* suppression of T cells even when used at low doses. CB mMSC differ from bone marrow-derived mMSC and may represent a new source to isolate mouse MSC with high purity and potent immunosuppression properties that would be beneficial in a context of allotransplantation rejection.

In conclusion, this thesis showed in detail (*in vitro*) that the IL-17A pre-treated human MSC (MSC-17) are superior modulators of inflammatory responses, which may eventually

lead to new treatment for transplant patients. The potential benefits of human MSC-17 therapy in allotransplantation are summarised in **Figure 7.1.2**. Human MSC-17 potently suppressed inflammatory T cell responses while retaining a normal morphology and immunophenotype compared to UT-MSC. Specifically, these human MSC-17 have translational potential in transplantation protocols because they do not express MHC class II, thereby reducing their immunogenicity, in addition to avidly promote the formation of induced T regulatory cells. The development of a more potent MSC (ie. MSC-17) would foster their application as a monotherapy or combined with low doses of standard drug regimens to prevent rejection in clinical allotransplantation protocols. MSC-17 cell therapy used in conjunction with immunosuppressive drugs may help patients “accept” transplants, thereby reducing detrimental side effects due to long-term systemic immunosuppressive drugs.

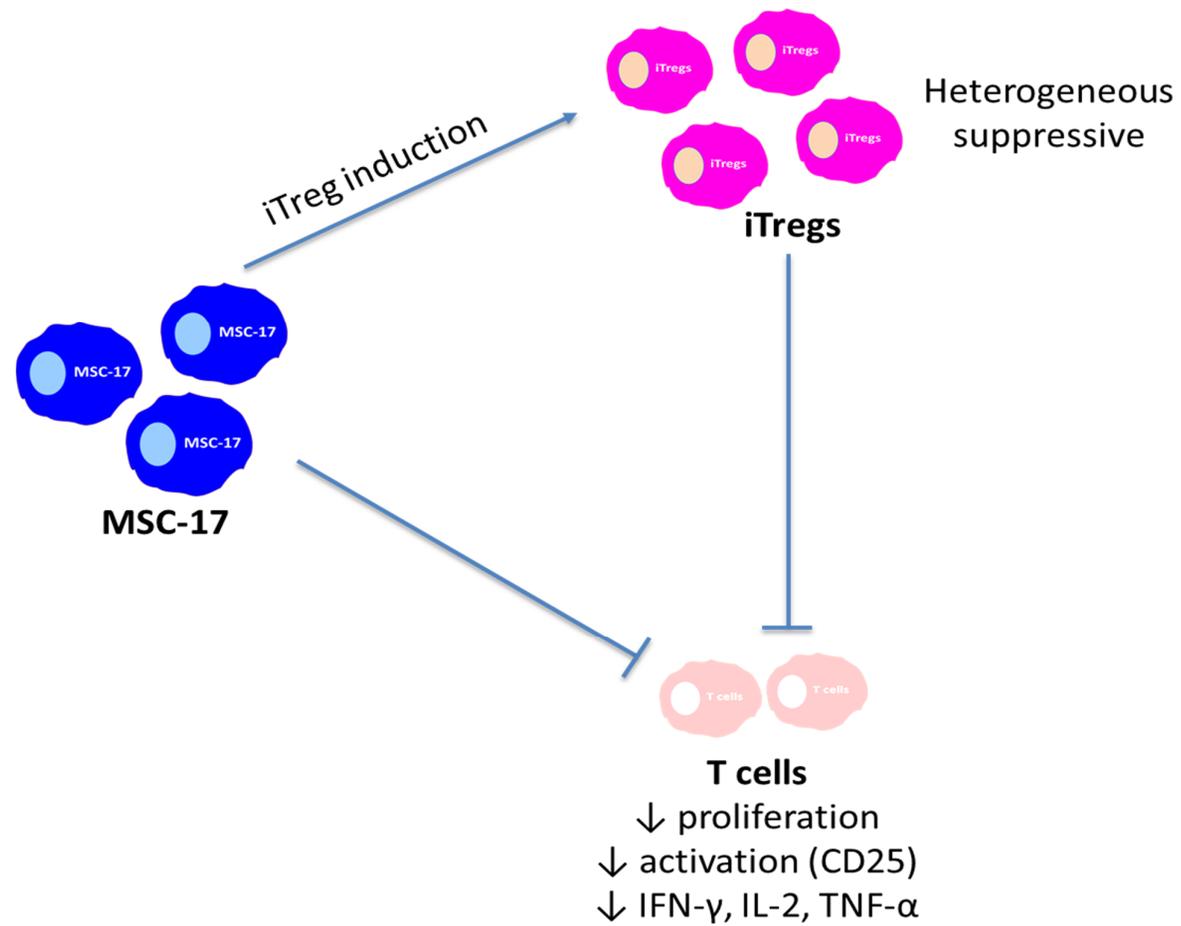


Figure 7.1.1 Human MSC-17 are superior suppressors of T cells.

Summary of human MSC-17 immunomodulation of T cell responses *in vitro*.

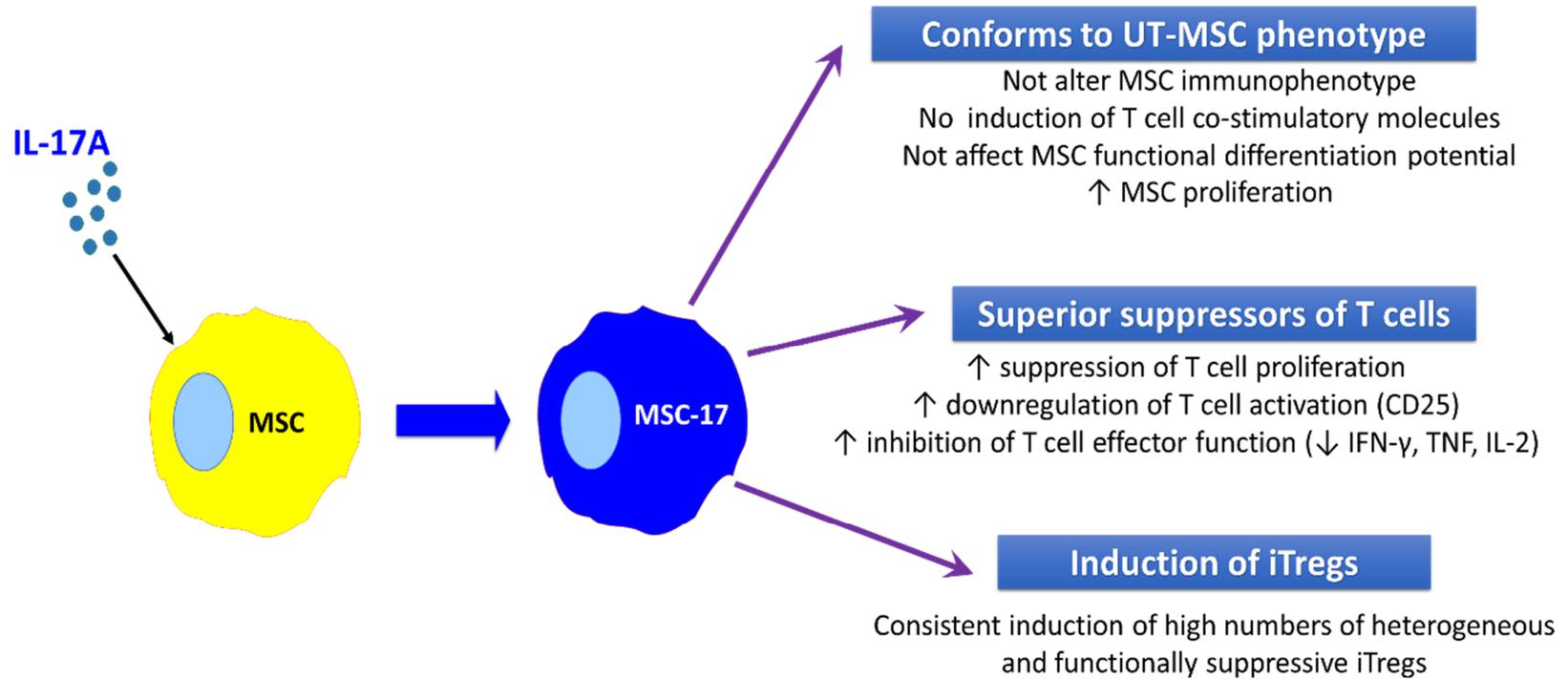


Figure 7.1.2 The potential benefits of human MSC-17 in allotransplantation.

7.2 Future Directions

The work presented in this thesis provides the basis for a number of ongoing studies as outlined in the following section:

7.2.1 *In vitro* mechanisms of human MSC-17 modulation of T cell responses

Aim 1: To determine the functional role of MMP13 in MSC-17 immunoregulation of T cells.

Aim 1a: To validate protein expression of MMP13 in MSC-17 by Western Blot.

Aim 1b: To study IL-17A signaling pathways in MSC-17 that induce MMP13 expression in MSC.

Aim 1c: To determine the functional role of MMP13 in MSC-17 immunosuppression of proinflammatory T cell responses and in the induction of Tregs using MMP13 specific inhibitor CL82198.

Aim 2: To identify other key candidate immunoregulatory molecules in human MSC-17 mediated regulation of T cell responses.

Aim 2a: To validate microarray gene expression data using real-time PCR (gene level) and Western Blot (protein expression).

Aim 2b: To examine the role of MSC-17 expressed chemokines and MMP1 in MSC-17 immunosuppression of proinflammatory T cell responses and in induction of Tregs.

Aim 3: Specific blockade of iTreg generation by MSC (eg. using TGF- β neutralizing antibodies) to determine whether MSC-17 act directly or indirectly via Tregs to promote enhancement of T cell immunosuppression.

7.2.2 *In vivo* fate of “off-the-shelf” MSC-17 therapy

Evaluating and comparing the immunogenicity of allogeneic or third-party (“off-the-shelf”) human MSC-17 to UT:MSC and MSC- γ in humanized mice reconstituted with a human

immune system. The use of a humanized mouse more closely resembles a human biological system and offers a unique opportunity to specifically evaluate the *in vivo* mechanisms of action as well as the efficacy and safety profile of human MSC-17 cellular therapy.

Aim 1: To evaluate the safety and immunogenic status of MSC-17 *in vivo*.

Aim 1a: Evaluating the *in vivo* fate of “off-the-shelf” MSC-17 by real-time imaging techniques.

Aim 1b: To assess the host-immune response to allogeneic MSC-17.

Aim 1c: Understanding mechanisms by which “off-the-shelf” human MSC, particularly MSC- γ are recognised and rejected by the host immune response.

7.2.3 *In vivo* proof-of-concept preclinical study of human MSC-17

Efficacy of human MSC-17 compared to UT-MSC and MSC- γ in improving rejection outcomes in humanized preclinical mouse models of human islet transplantation. These aims may help design future clinical trials for “off-the-shelf” MSC-17 in transplantation protocols.

Aim 2: *In vivo* pilot study to establish the biological plausibility of “off-the-shelf” human MSC-17 in islet allotransplantation.

Aim 2a: To determine the efficacy of MSC-17 to prolong human islet graft survival and function *in vivo*.

Aim 2b: To investigate the ability of human MSC-17 to induce Tregs to promote islet allograft survival and induce allotransplantation tolerance.

Aim 3: *In vivo* tracking of human MSC-17 in humanized mouse models of islet allotransplantation.

Aim 3a: Role of chemokines in regulating MSC-17 homing to sites of inflammation.

Aim 4: To determine mechanisms by which MSC-17 modulate human islet allograft rejection.

Aim 4a: Role of MMP13 in the regulation of MSC-17 immunosuppression of allotransplantation rejection.

Aim 5: To investigate an optimal immunosuppressive dose regime for MSC-17 therapy.

Aim 5a: Synergistic effect of MSC-17 pharmaceutical immunosuppressive drugs (eg. Rapamycin) in prolonging islet allograft survival and function.

7.2.4 *In vivo* efficacy of CB mMSC in mouse models of islet allotransplantation.

Aim 1: To investigate the therapeutic efficacy of CB UT-mMSC to prevent islet allograft rejection *in vivo* in mouse models of islet allotransplantation.

Aim 1a: To determine the efficacy of CB UT-mMSC to prolong islet graft survival and function *in vivo*.

Aim 2b: To investigate the ability of CB UT-mMSC to induce Tregs to promote islet allograft survival and induce allotransplantation tolerance.

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APPENDIX

9.1 Published Papers

9.1.1 Published Paper 1

Sivanathan KN, Gronthos S, Rojas-Canales D, Thierry B, Coates PT. Interferon-gamma modification of mesenchymal stem cells: implications of autologous and allogeneic mesenchymal stem cell therapy in allotransplantation. *Stem Cell Rev.* 2014;10(3):351-375.

Sivanathan, K.N., Gronthos, S., Rojas-Canales, D., Thierry, B. & Coates, P.T. (2014). Interferon-gamma modification of mesenchymal stem cells: implications of autologous and allogeneic mesenchymal stem cell therapy in allotransplantation. *Stem Cell Review and Reports*, 10(3), 351-375.

NOTE:

This publication is included between pages 358 & 359 in the print copy of the thesis held in the University of Adelaide Library.

It is also available online to authorised users at:

<http://dx.doi.org/10.1007/s12015-014-9495-2>

9.1.2 Published Paper 2

Lett B, **Sivanathan KN**, Coates PT. Mesenchymal stem cells for kidney transplantation. *World J Clin Urol.* 2014;3(2):87-95.

Mesenchymal stem cells for kidney transplantation

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Received: April 28, 2014 Revised: June 6, 2014

Accepted: June 27, 2014

Published online: July 24, 2014

Abstract

The long term consequence of immunosuppressive therapy in kidney transplantation has prompted investigation of alternative means to modify the immune response to the allograft. Cell based therapies are potentially attractive as they may provide a long lasting immunomodulatory effect, may repair tissues and reduce the necessity to take immunosuppressive drug therapy. Of the current cell therapies, mesenchymal stem cells have now been trialled in small numbers of human kidney transplantation with apparent safety and potential efficacy. Many issues however need to be resolved before these cells will become mainstays of transplant immunosuppression including *ex vivo* modification to enhance immunomodulatory properties, cell number, route and frequency of administration as well as cellular source of origin.

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Key words: Mesenchymal stem cells; Kidney transplantation; Immunosuppression; Solid organ transplantation; Cellular therapies

Core tip: This review summaries several of the most prominent cellular therapies currently being examined

for use in immunosuppression. From the current evidence the reviewers make the argument that mesenchymal stem cells offer the best chance of a useful and functional cellular therapy for solid organ transplantation.

Lett B, Sivanathan KN, Coates PT. Mesenchymal stem cells for kidney transplantation. *World J Clin Urol* 2014; 3(2): 87-95 Available from: URL: <http://www.wjgnet.com/2219-2816/full/v3/i2/87.htm> DOI: <http://dx.doi.org/10.5410/wjcu.v3.i2.87>

INTRODUCTION

Kidney transplantation remains the optimal treatment for end stage renal disease (ESRD) providing excellent short term outcome with greater quality of life than that provided by dialysis^[1]. Whilst short term graft survival is improving and acute rejection rates are dropping long term graft survival rates remain a major focus for clinical improvement. There are many factors that can impact the prognosis of a kidney transplant, from graft or donor considerations^[2,3], factors involving the immunosuppressant regime^[4,5], and issues concerning the recipient^[6,7].

Tissue typing and stringent exclusion criteria are implemented pre-transplant to reduce the risk of donor related problems^[3]. Issues with the recipient such as non-compliance and co-morbidity are much more difficult to manage and are often beyond a clinician's power to control^[6,7].

When a suitable kidney donor is found, it is then important to make sure that the graft does not reject by suppressing the recipient's immune system. Current immunosuppressive drugs may be classified into five groups based on their mechanism of action: (1) regulators of gene expression; (2) alkylating agents; (3) inhibitors of de novo purine synthesis; (4) inhibitors of de novo pyrimidine synthesis; and (5) inhibitors of kinases and phosphatases^[5]. Targeting each of these mecha-

nisms has its benefits and disadvantages and tailoring a drug schedule has the potential to impact long term graft function and the quality of life of the recipient. However all current drugs are associated with a range of adverse effects including renal toxicity, opportunistic infections, development of malignancy and metabolic complications^[5]. A common trait among all these drug classes is the targeting of T cell function^[5,8-10]. T cells play an important role in rejection via alloantigen recognition and the direction of an effector response that results in graft damage and dysfunction^[11].

Of these issues it is the modification of immunosuppression that is an obvious place to try and improve patient outcomes, as more options will allow for customised treatment programs unique to each patients needs. Towards this end, there has been a recent increase in the development of alternative means of immunosuppression for organ transplantation. Utilizing cell-based therapies for immunosuppression is an alternative approach to traditional pharmacological methods and represents a change in paradigm for transplantation therapies.

CELL THERAPIES FOR ORGAN TRANSPLANTATION

The basic concept of cell therapy is to implant cells with desired properties into a patient in an attempt to treat or cure. Although this idea has been around since the 19th century, it was not until 1968 that it became a viable treatment with the first bone marrow transplant^[12]. Since then, there has been a steady expansion in the type of cells transplanted and the conditions that can be treated. The purpose of this review is to examine the state of several cell types that are being evaluated for preclinical or early clinical trials in solid organ transplantation (SOT), including; T regulatory cells (Tregs), dendritic cells (DCs), and with a particular emphasis on mesenchymal stem cells (MSCs) which have shown the greatest progress and potential as a cellular therapy.

REGULATORY T CELLS

Tregs are naturally occurring T cells which express the cell surface markers CD4⁺CD25⁺ FoxP3⁺ and a variety of differing cell surface markers (CD127, Helios)^[13,14]. Tregs are concerned with the maintenance of immunological self-tolerance by suppressing self-reactive lymphocytes that escape clonal deletion^[14]. Naturally occurring Tregs are formed from naive T cells in the thymus. However these naive T cells can be converted to Tregs *in vitro* using TGF- β induction of FoxP3^[15], providing a second source of Tregs for cell therapy.

Tregs are able to suppress the immune system on many levels, combining inhibitory cytokine secretion (*e.g.*, via TGF- β , IL-10)^[16,17], cytotoxicity and inhibition of NK cells^[18,19], and direct modulation of antigen presenting cells^[20-22]. This multifaceted approach to immunosuppression makes Tregs a promising therapy to facilitate

long term graft survival. Recently there have been advances in the methods for Treg isolation and expansion, with large scale expansion from peripheral blood (PB), umbilical cord blood (UCB), and induced Tregs from naive peripheral blood precursors^[23]. There have also been positive results from experimental animal models^[24]. Of greatest interest are the clinical trials that have used Tregs as a cellular therapy in graft-versus-host disease (GVHD), a major and potentially lethal transplant complication that is particularly prevalent in patients who have undergone a hematopoietic stem cell transplant (HSTC)^[25,26]. With generally positive outcomes from the GVHD trials^[26], it is likely that we will see Tregs initially deployed as an adjunctive therapy in SOT before being used in patients who have a high risk of rejection or who have already experienced adverse effects from standard immunosuppression. This would allow for the efficacy of Tregs to be determined in a way that would be ethical and pose a minimal risk of complications.

In addition to their safety, there are several other important issues that need to be addressed in the pursuit of an effective Treg based therapy. As mentioned above, there have been advances in the isolation and expansion of Tregs. These advances go some way to addressing the large number of cells that would be required for an effective therapy, with some estimates placing the required number at 11×10^8 cells/kg^[27]. Another concern is the source of the Tregs. Currently, the most appropriate source for therapy is unknown, with uncertainty focused on whether alloantigen or antibody mediated expansion is the safest and most effective method^[23]. The stability of Tregs *in vivo* has also been found to be problematic with studies finding that Tregs can lose FoxP3 expression and develop an effector cell phenotype, becoming pathogenic^[28]. Of relevance to the previous point about the source of Tregs is evidence suggesting that induced Tregs lose FoxP3 expression at a much higher rate than natural Tregs^[29,30]. These are just a few of the issues surrounding the use of Tregs for SOT that the ONE study (www.theonestudy.org) hopes to address. Currently the ONE study is examining the use of polyclonally expanded Tregs and alloantigen driven Tregs in kidney transplantation at doses of 1, 3, 6 and 10×10^6 Tregs/kg. As of writing this no results have been published^[23].

DENDRITIC CELLS

Dendritic cells (DCs) are able to function as antigen presenting cells that drive graft rejection (immunogenic DC) or have a role in promoting graft acceptance (tolerogenic DC; ToIDC) depending on their state^[31]. Immunogenic DCs cause T cell activation and proliferation with the use of three signals: (1) they present antigens on MHC molecules; (2) They provide co-stimulatory molecules; and (3) they secrete pro-inflammatory molecules. Only when all three signals are present can DCs activate T cells^[31]. ToIDCs are also able to interact with regulatory

T cells to promote immune tolerance. The role that DCs play in immune tolerance is twofold. Firstly, they play a role in the deletion of self-reactive thymocytes in the thymus^[32]. Secondly, and of relevance to transplantation, they aid in peripheral tolerance. They do this by the presentation of antigens while lacking the co-stimulatory molecules required for T cell activation^[32,33]. This causes T cell unresponsiveness as well as Treg induction^[33].

Two strategies for the use of TolDCs in transplantation are likely to be applied in the setting of allotransplantation. The first involves negative immunization by administering either autologous DCs that have been exposed to alloantigens or donor derived DCs, pre-transplant^[34]. The second method involves the use of recipient derived DCs delivered on the day of transplantation^[35]. Intravenous injection of immature DCs of either donor or recipient origin at the time of transplantation have prolonged allograft survival in SOT models^[36]. There is a large amount of literature on the use of DCs in pre-clinical experimental models^[36,37]. Clinical trials looking at DCs have been carried out in both type-1 diabetes^[38] and rheumatoid arthritis^[39]. This has shown that the use of DCs for immunomodulation is safe and effective.

Many of the issues that face Tregs are also pertinent in the consideration of DCs as a cellular therapy. Cell dose and the best method for the isolation and expansion of the cells is uncertain. The use of either recipient derived DCs or donor DCs is yet to be resolved and adding additional complexity to this issue is the question of negative immunization vs. recipient derived DCs delivered peri-transplant. Again, the ONE study aims to answer these questions and early trials of DCs in SOT are ongoing as of writing this.

MESENCHYMAL STEM CELLS

Mesenchymal stem cells (MSCs) are a multipotent cell lineage that has great potential for use in cellular therapies and is already being widely tested in clinical trials. www.clinicaltrials.gov currently lists 396 studies using MSCs in conditions such as spinal cord injury, diabetes, Alzheimer's disease, and kidney injury.

The International Society for Cellular Therapy (ISCT) has set the minimal criteria for defining MSCs as being plastic adherent, capable of differentiation into osteoblasts, adipocytes, and chondroblasts, and expressing CD105, CD73, and CD90 while lacking expression of CD45, CD34, CD14 or CD11b, CD19, and HLA-DR surface molecules^[40].

MSCs are capable of being isolated from many tissues including bone, fat, and placenta. When cultured they adhere to plastic and have a fibroblast-like appearance, possessing a long, thin body and a small number of protrusions^[40]. MSCs have a role in the formation and homeostasis of connective and structural tissues *via* the production of extracellular matrix, stabilization and regulation of the tissue vascularisation, and the creation of new connective tissue cells^[41,42]. In addition to this, they also play a role in the immune system by inducing

tolerogenic^[42] properties that can be enhanced by *in vitro* treatment^[43]. These roles are able to be exploited to aid in regenerative medicine and in immunosuppression. Combined with the many tissues from which they can be isolated and their ability to remain stable while being expanded *in vitro*^[44] it becomes clear why so much work is now being carried out using MSCs for a large number of clinical applications.

The immunosuppressive abilities of MSCs are mediated by either nitric oxide synthase (iNOS) in mice^[45,46], or indolamine 2,3-dioxygenase (IDO) in humans^[46]. iNOS results in the production of nitric oxide (NO) which is an immunosuppressive agent in high concentrations^[47]. Alternatively, IDO degrades the essential amino acid tryptophan thereby resulting in immunosuppression. The accumulation of the tryptophan metabolite kynurenine is also known to mediate the immunoregulatory effects of MSCs^[48].

The exact mechanisms of how two pathways cause immunosuppression are not fully understood. In addition to these key factors, there are several immunosuppressive molecules secreted by MSCs. These include; PGE-2, IL-10, HO-1, PD-L1, and IL-6^[49].

In reaction to stimulus from interferon-gamma (IFN- γ and proinflammatory cytokines, MSCs also secrete chemokines and adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1)^[45]. This results in a close proximity of immune cells allowing the local immunosuppressive environment to have a more pronounced effect^[49].

A substantial amount of work has been focused on the potential for MSCs to treat GVHD. Ringdén *et al*^[50] treated 8 patients, who had developed steroid-refractory GVHD, with bone marrow derived MSCs. In 6 of these patients acute GVHD ameliorated. The same group later went on to perform a phase II trial consisting of 55 patients with acute GVHD. In this trial, 30 patients completely recovered from GVHD and a further 9 showed improvement. None of the patients developed adverse reactions due to the administration of MSCs^[51]. Another phase one trial administering MSCs for GVHD was carried out by Introna *et al*^[52] This multicentre study looked at 40 patients (15 children and 25 adults) with steroid resistant GVHD and gave them a median of 3 third-party derived MSCs infusions. Here it was found that the MSCs had a 67.5% T cell mediated response rate with a 27.5% complete response, 86 adverse effects were reported however most of these were of an infectious nature (72.1%) and not due to the administration of MSCs^[52]. They concluded that MSCs could safely be administered in addition to conventional immunosuppression (*e.g.*, cyclosporin, steroid). Despite these positive results, there is some concern over a phase III clinical trial that failed to meet its primary clinical end point (NCT00366145)^[53]. In this trial, patients received 8 infusions of 2×10^6 cells/kg over 4 wk and 4 more infusions administered weekly after 28 d. The trial did not meet its primary end point of a significant increase of

complete response of steroid resistant GVHD. Galipeau *et al.*^[54] provides a comprehensive failure analysis of the trial. The main conclusion of this analysis is that there are significant differences between the Martin study and studies from Europe that could account for the failure, in particular the passage number of the cells used^[54]. As such, this study is not damning of MSCs but rather provides more areas that require examination before they can be used more widely.

Unlike the other cell types, there are now completed early clinical trials that have deployed MSCs as a therapy for SOT. The largest comes from Tan *et al.*^[55] In their trial they had 159 kidney transplant patients split into 3 groups, with 2 groups receiving autologous MSCs with either standard dose calcineurin inhibitors (CNIs) or low dose CNIs and the control group receiving standard dose CNIs and anti-IL-2 receptor antibody. The major conclusions from this study were that the MSC groups had a lower incidence of glucocorticoid-resistant rejection, a faster recovery in renal function, and significantly decreased risk of opportunistic infections than the control group^[55]. This study also addresses safety concerns over the use of MSCs as there were no adverse reactions reported in either of the test groups. However this trial was not without its problems. It was noted by the authors that the number of rejection episodes in the control group was higher than what would be expected. This made it appear that the MSC groups performed better than standard immunosuppression when this may not be the case^[55]. Additionally, the major differences in graft function were only noticed in the first 2 wk. It is conceivable that this was due the regenerative abilities of MSCs repairing the reperfusion injury associated with all kidney transplants. And lastly, the major difference in opportunistic infections was noted in the MSC and low dose CNI group. As there was no control low dose CNI group, we cannot be certain that the observed reduction in infection is due to MSCs or simply due to the reduced use of immunosuppressive drugs.

In addition to the work from Tan there have been several case reports looking at the use of MSCs in a small number of SOT patients. Perico *et al.*^[56,57] have performed two pilot studies looking at the use of MSCs in kidney transplantation in 4 patients. In their first study they administered intravenous autologous MSCs 7 d after transplantation and followed the patients for 360 d. From days 7 to 14 post transplant, serum creatinine increased in 1 of their patients, however acute graft rejection was excluded *via* biopsy. They also noted an increase in patient Tregs and a decrease in T cell expansion post-transplant. Long term, both patients showed stable graft and the authors concluded that MSC infusion in kidney transplant recipients is feasible, allows increase of Treg in the peripheral blood, and controls memory CD8⁺ T cell function^[57]. In their second trial, they dosed two living-related kidney transplant recipients with autologous MSCs one day before transplantation. The change in dosing time was an attempt to avoid the acute graft deterioration observed to be caused by intragraft local-

ization of MSCs when dosing 7 d post-transplant. Although both patients had no side effects to the MSC infusion and both had stable graft function at 12 mo, one of their patients did have an acute rejection episode 14 d post-transplant that was resolved with corticosteroid therapy^[57]. The authors attribute the rejection episode to a higher number of HLA mismatches. They concluded that pre-transplant administration of MSCs avoided the cell induced graft dysfunction associated with post-transplant MSC administration and that this method is favourable for future trials. Peng *et al.*^[58] examined the effect of autologous MSCs on renal transplants by giving 6 patients MSCs combined with half doses of tacrolimus and comparing acute rejection, graft function, and graft survival at 12 mo to a control group of 6 patients receiving standard dose tacrolimus. The results of this showed no toxic adverse effects associated with MSC infusion and all patients survived with stable graft function to 12 mo with only 1 acute rejection episode in the control group. The one difference they did notice was elevated B-cell counts in the MSC group at 3 mo compared to the control^[58]. They concluded that MSCs may provide benefits in renal transplantation by reducing the required dose of conventional immunosuppressive drug that is required for long term graft survival. The results of these case reports are consistent with those of the Tan study, with no adverse reactions, stable graft function, reduced rejection, and the ability to lower maintenance immunosuppression (Table 1).

From these early clinical trials, summarised in Table 1, it is evident that MSCs have an acceptable safety profile and have beneficial effects for transplantation. There still remain several very important questions to be answered before MSCs can obtain mainstream clinical use. The issue of whether autologous or allogeneic MSCs are better is significant, with arguments for both being put forward. Tan *et al.*^[55] employed autologous MSCs because of the issues surrounding MSC isolation from deceased donors. Furthermore, the use of autologous MSCs would avoid any potential for rejection of the cells and a subsequent loss of their function. However, there is some evidence that MSCs are immuno-evasive allowing them to escape recognition by the hosts immune system^[59]. If this is the case then allogeneic MSCs are promising as obtaining them will not impact the eventual recipient who may have serious health issues that could be exacerbated by the collection of MSCs or could impact the quality of the MSCs. The immuno-evasive status of MSCs also opens up the potential for third party derived MSCs. This would invalidate concerns about obtaining MSCs in the cases of deceased donors. Nevertheless, issues pertaining to the immunogenicity of allogeneic or third-party derived MSCs has not been substantially addressed *in vivo* and have not been addressed in large animal models. There are preclinical studies demonstrating that allogeneic MSC monotherapy alone failed to prevent allograft rejection^[60-69]. Studies reporting on the benefits of allogeneic MSCs have also shown short term prolongation of graft

Table 1 Summary of clinical trials using mesenchymal stem cells in kidney transplantation

Ref.	Patient number	Cell number	Cell source	Adverse reactions	Graft survival
Tan <i>et al</i> ^[55] , 2012	106	1-2 × 10 ⁶ cells/kg	Autologous, bone marrow	None	100% at 1 yr
Perico <i>et al</i> ^[56] , 2011	2	2 × 10 ⁶ cells/kg	Autologous, bone marrow	Acute graft dysfunction	100% at 360 d
Perico <i>et al</i> ^[57] , 2013	2	2 × 10 ⁶ cells/kg	Autologous, bone marrow	HLA induced rejection	100% at 1 yr
Peng <i>et al</i> ^[58] , 2013	6	5 × 10 ⁶ 1 st dose 2 × 10 ⁶ cells/kg 2 nd dose	Donor derived, bone marrow	None	100% at 1 yr

HLA: Human leukocyte antigen.

survival^[64]. More importantly, in some studies, pre-transplant allogeneic MSC monotherapy accelerated allograft rejection thereby questioning the immunoprivileged status of MSC. There is evidence that allogeneic MSCs can trigger an anti-donor immune response resulting in accelerated allograft rejection^[65-67]. The co-administration of allogeneic MSC with immunosuppressive drugs however showed better outcome of the allograft compared to MSC monotherapy^[63,64,70-72]. Therefore, the synergistic effects of allogeneic MSC with immunosuppressive drugs need to be taken into consideration in MSC therapy. We have previously reviewed in detail the mechanisms associated with allogeneic or third-party derived MSC immunogenicity and the synergistic effects of MSC with immunosuppressive drugs, in Sivanathan *et al*^[43]. Questions around the dose rate, the timing, the route of administration, what happens to the cells and what exactly the MSCs are doing and their mechanism of action still remain unanswered. Given the state of the field it is not possible to accurately speculate on the answers to these questions. Additionally there is the potential for the modification of MSCs that further expands the possible methods of application

MODIFYING MSC FOR ENHANCED IMMUNOSUPPRESSION

The *ex vivo* manipulation of MSCs with proinflammatory cytokines, particularly IFN- γ modification of MSC enhances the immunomodulatory, reparative and homing potential of MSCs^[43]. The enhancement of these MSC properties would be beneficial in a transplant setting and may hasten the translation of MSC therapy into SOT patients.

Of key benefit, the priming of MSCs with IFN- γ is critical to active MSCs immunosuppressive function^[73-75]. IFN- γ primed MSC have an enhanced ability to suppress T cell responses compared to untreated MSC^[76-80]. Increase suppression of T cell responses is mediated by the induction of immunosuppressive factors such as iNOS and IDO^[75,81]. IDO is also well known for its roles in preventing rejection and induction tolerance at the fetal-maternal interface^[82]. In addition, MSC-expressed IDO have been shown to induce tolerogenic DCs and Tregs^[83], which are two other cell based therapies that have gained significant interest in SOT, as we have dis-

cussed above. The upregulation of other MSC immunomodulatory factors, the enhancement of negative T cell signalling, the inhibition of proinflammatory T cell response and the increase in Tregs further support the benefits of administering IFN- γ primed MSC therapy for SOT.

Regardless of the potential therapeutic benefits of IFN- γ primed MSC therapy, it should be noted that IFN- γ upregulate MHC class I and induces MHC class II expression on MSCs^[84-86]. This may render these cells more immunogenic in MHC-mismatched recipients^[43], thereby decreasing their effectiveness at suppressing inflammation as reported in some studies^[87,88]. Only two studies have directly addressed IFN- γ primed MSC immunogenicity *in vivo*^[88,89] and this warrants further investigation. Thus, when considering IFN- γ primed MSC therapy, then administration of autologous MSC may be more beneficial. If allogeneic or third-party IFN- γ primed MSC were to be considered, the co-administration of these cells with immunosuppressive drugs would be necessary as an attempt to control anti-donor immune response towards MSC to enable MSCs to exert their beneficiary effects *in vivo*.

CONCLUSION

In summary, there are numerous cell based therapies that have shown potential for use in the immunomodulation of SOT in pre-clinical, small, and large animal models. Tregs and DCs have shown promise *in vitro* and in animal models as well as displaying safety and efficacy in clinical trials involving GVHD, diabetes, and rheumatoid arthritis. However, only MSCs have completed large clinical trials to date. MSC have shown the most promise having been tested in GVHD and in early clinical trials for kidney transplantation. Based on the GVHD experience and the early transplant work, it appears that MSC have an acceptable safety profile and potential therapeutic effect. However, much needs to be resolved, including the issue of autologous *vs* allogeneic (third party cells), frequency of administration and mechanism of action. The optimal immunosuppressive therapy to be co-administered should also be studied. The results from these early trials are positive but have presented numerous issues that need to be addressed before MSCs gain widespread clinical use.

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P- Reviewers: Niyibizi C, Tamama K, Virador VM, Zocchi E
S- Editor: Ji FF **L- Editor:** A **E- Editor:** Lu YJ



9.1.3 Published Paper 3

Sivanathan KN, Rojas-Canales DM, Hope CM, et al. Interleukin-17A-Induced Human Mesenchymal Stem Cells Are Superior Modulators of Immunological Function. *Stem Cells*. 2015;33(9):2850-2863.

Sivanathan, K.N., Rojas-Canales, D.M., Hope, C.M., Krishnan, R., Carroll, R.P., Gronthos, S., Grey, S.T. & Coates, P.T. (2015). Interleukin-17A-Induced Human Mesenchymal Stem Cells Are Superior Modulators of Immunological Function. *Stem Cells*, 33(9), 2850-2863.

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<http://dx.doi.org/10.1002/stem.2075>