Low-intensity conditioning for the induction of allogeneic tolerance in aged recipients

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Submitted to Monash University in accordance with the requirements for the degree of Doctor of Philosophy

2014

“I almost wish I hadn’t gone down that rabbit-hole - and yet - and yet - it’s rather curious, you know, this sort of life”

~Lewis Carroll (Alice’s Adventures in Wonderland)

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Summary

Organ transplantation is the curative technique for end-stage organ failure. However, the success of this technique is opposed by the immunological rejection of the transplanted tissue. Traditionally strategies to combat allogeneic rejection have been based on life-long immunosuppression, which leads to high levels of morbidity stemming from opportunistic infections and malignancy. This can be overcome via the induction of allogeneic tolerance, where bone marrow transplant (BMT) induced allogeneic mixed chimerism can produce a permanent state of donor-specific tolerance, to both skin and solid organ grafts, across major histocompatibility complex (MHC) barriers.

The clinical translation of such protocols has been hampered by the toxicities associated with recipient preconditioning, involving lethal or sub-lethal doses of irradiation and/or chemotherapy and a concern for the development of graft versus host disease (GvHD). To reduce conditioning-mediated toxicities, non-myeloablative conditioning regimes have been widely explored. However, despite the success of these protocols in small animal models and nonhuman primates translation to clinical trials has been limited.

Age-related thymic involution is often overlooked when trying to induce allogeneic tolerance via BMT. This could pose a significant barrier, considering previous reports demonstrate that to maintain stable allogeneic tolerance in mixed chimeras, there is an absolute requirement for donor-antigen dependent, intrathymic deletion of alloreactive T cells and therefore a functioning thymus. Thymic involution begins at the onset of puberty/sex steroid production and is characterised by a marked disorganisation of the microenvironment, the replacement of lymphoid tissue by adipocytes and a significant decrease in T cell output, leaving the thymus functioning at only 5 % capacity by 10-12 months in the mouse and 40 years in humans. This may be particularly important in a clinical setting, as the majority of transplant recipients are well into adulthood.

This thesis aimed to investigate the ability to induce allogeneic tolerance, via a low-intensity conditioning regime in aged mice. This was achieved by (1) investigating the effects of the chemotherapeutic drug busulfan on the bone marrow (BM), spleen and thymus of both young and aged mice, (2) assessing the level of engraftment achieved when busulfan-treated mice receive both low and high-dose HSCT or allogeneic BMT, (3) investigating the role of sex steroid ablation

(SSA) in boosting donor-cell engraftment and hematopoietic chimerism in both young and aged mice and finally (4) designing a thymus-sparing, low-intensity conditioning protocol that allowed the induction of allogeneic tolerance via mixed chimerism.

Consistent with previous reports, low-dose busulfan was myeloablative, allowing transplanted cells to engraft in the BM, but not immunosuppressive, having only mild effects on the thymus and peripheral lymphoid compartment. Additionally, SSA coupled to chemotherapy increased both thymic cellularity and the number of donor-derived thymocytes following both high and low-dose HSCT. Indicating that SSA could be coupled to non-myeloablative HSCT to boost T cell output.

Importantly, low-dose busulfan could be used in conjunction with T cell depleting antibodies and short-term sirolimus immunosuppression to induce mixed chimerism and allogeneic tolerance following the transplantation of both whole BM (WBM) or purified progenitor cells. Surprisingly, the tolerance generated from BMT was not dependent on the presence of a thymus, but instead the induction of T regulatory cells.

The BM of aged mice was more resistant to chemotherapy than young BM and this lead to a decrease in donor cell engraftment and chimerism following congeneic HSCT. Sensitivity to chemotherapy could be restored by activating BM-progenitor cells with granulocyte colony stimulating factor (G-CSF), however, G-CSF prior to low-dose busulfan and HSCT did not boost engraftment of congeneic progenitor cells. Because the engraftment of progenitor cells was poor and allogeneic T cells can promote donor cell engraftment, the ability for WBM to engraft, promote mixed chimerism and induce allogeneic tolerance in aged mice conditioned with T cell depleting antibodies, sirolimus immunosuppression and low-dose busulfan was investigated. Utilising this protocol robust mixed chimerism and allogeneic tolerance could be achieved in aged mice.

To our knowledge, this is the first study to examine mixed chimerism induced allogenic tolerance in aged mice. In this model age-related thymic involution does not impede the ability to induce donor-specific tolerance. This is an important finding for the clinical application of protocols designed to induce tolerance, considering most transplant recipients are aged individuals, who have experienced significant thymic involution and other signs of immunosenescence.

Declarations

General Declaration

In accordance with Monash University Doctorate Regulation 17 Doctor of Philosophy and Research Master's regulations the following declarations are made:

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes one unpublished publication. The core theme of the thesis is the induction of allogeneic tolerance in aged mice. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the Department of Anatomy and Developmental Biology under the supervision of Professor Richard Boyd.

In the case of Chapter Three, my contribution to the work involved the following: planning, performing and analysing experiments as well as writing and editing the manuscript. The co-authors Jade Hommann, Daniel Layton, Ann Chidgey, Richard Boyd and Tracy Heng assisted with experimental design, technical assistance, interpretation of the results and drafting of the manuscript. Proportional contributions are explained in the declaration.

Thesis chapter	Publication title	Publication status*	Nature and extent of candidate's contribution
Three	Establishment of transplantation tolerance via minimal conditioning in aged recipients	Returned for revision	Performing and analyzing experiments, writing and editing the manuscript.

I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Signed: [redacted]

Date: 24.01.14

Declaration for Thesis Chapter 2

Declaration by candidate

In the case of Chapter 2, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Writing and drafting of manuscript. Experimental design, execution, analysis and interpretation of results.	70

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Jade Homann	Technical assistance	
Tracy Heng	Supervisory role, intellectual input, experimental design interpretation, and drafting of manuscript.	
Ann Chidgey	Supervisory role, intellectual input, experimental design interpretation, and drafting of manuscript.	
Richard Boyd	Supervisory role, intellectual input, experimental design interpretation, and drafting of manuscript.	

Candidate's Signature [redacted] **Date: 15/1/2013**

Declaration by co-authors

The undersigned hereby certify that:

1. (1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
2. (2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
3. (3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
4. (4) there are no other authors of the publication according to these criteria;
5. (5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
6. (6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)	Department of Anatomy and Developmental Biology, Monash University, Clayton Campus.

[Please note that the location(s) must be institutional in nature, and should be indicated here as a department, centre or institute, with specific campus identification where relevant.]

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1	Jade Homann	[redacted]	15/1/2013
2	Tracy Heng		15/1/2013
3	Ann Chidgey		15/1/2013
4	Richard Boyd		15/1/2013

Declaration for Thesis Chapter 3

Declaration by candidate

In the case of Chapter 3, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Writing and drafting of manuscript. Experimental design, execution, analysis and interpretation of results.	70

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

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Ann Chidgey	Supervisory role, intellectual input, experimental design interpretation, and drafting of manuscript.	
Richard Boyd	Supervisory role, intellectual input, experimental design interpretation, and drafting of manuscript.	

Candidate's Signature		**Date: 15/1/2013**

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The undersigned hereby certify that:

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Declaration for Thesis Chapter 4

Declaration by candidate

In the case of Chapter 4, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Writing and drafting of manuscript. Experimental design, execution, analysis and interpretation of results.	70

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

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Richard Boyd	Supervisory role, intellectual input, experimental design interpretation, and drafting of manuscript.	

Candidate's Signature	[redacted]	Date: 15/1/2013

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Publications and Presentations

Publications

I. **Morison, J**., Heng, T., Chidgey, A., Boyd, R. (2013) The immunogenicity of stem cells and thymus-based strategies to minimize immune rejection. In, Dr. P. Fairchild (ed) Immunological Barriers to Regenerative Medicine. Springer Verlag, UK, pp 201-223

II. **Morison. J**., Homann, J., Layton, D., Chidgey, A.P., Boyd, R.L., Heng, T.S.P., Establishment of transplantation tolerance via minimal conditioning in aged recipients. (Manuscript under preparation)

Presentations

I. **2010 -Morison, J**., Heng, T., Chidgey, A., and Boyd, R. Radiation-free conditioning for the induction of transplantation tolerance, Immunology Group of Victoria Annual meeting, Yarra Valley, Australia.

II. **2012 -Morison, J**., Chidgey, A., Boyd, R. & Heng T. Low-intensity conditioning to establish mixed chimerism and transplantation tolerance in aged mice. Transplantation Biology Research Centre, Harvard Medical School, Massachusetts General Hospital, Boston, USA.

III. **2012 -Morison, J**., Chidgey, A., Boyd, R. & Heng T. Low-intensity conditioning to establish mixed chimerism and transplantation tolerance in aged mice. Columbia Centre for Translational Immunology, Columbia University Medical Centre, NYC, USA.

Conference Abstracts

I. **2009 -Morison, J**., Heng, T., Chidgey, A. and Boyd, R. Development of a safe, effective and clinically relevant protocol for the induction of allogeneic tolerance through mixed chimerism, Australasian Society for Immunology Annual Conference, Gold Coast, Australia.

II. **2010 -Morison, J**., Heng, T., Chidgey, A. and Boyd, R. Low-intensity conditioning to establish mixed chimerism and transplantation tolerance. Australasian Society for Immunology Annual Conference, Perth, Australia.

III. **2011** -**Morison, J**., Heng, T., Chidgey, A. and Boyd, R. Low-intensity conditioning to establish mixed chimerism and transplantation tolerance. The California Institute of Regenerative Medicine Grantee Meeting, San Francisco, California, USA.

IV. **2011** -**Morison, J**., Heng, T., Chidgey, A. and Boyd, R. Radiation free conditioning for the establishment of mixed chimerism and allogeneic tolerance. Australasian Society for Immunology Annual Conference, Adelaide, Australia.

V. **2012** -**Morison, J**., Heng, T., Chidgey, A. and Boyd, R. Enablement of allogeneic stem cell therapies via radiation-free conditioning induced mixed chimerism. International Society for Stem Cell Research Annual Meeting, Yokohama, Japan.

VI. **2012** -**Morison, J**., Heng, T., Chidgey, A. and Boyd, R. Low-intensity conditioning to establish mixed chimerism and transplantation tolerance in aged mice. European Congress of Immunology, Glasgow, Scotland.

Acknowledgements

And now I have come to what I think may be one of the most challenging pages of my thesis to put into words, the acknowledgements. **Richard**, you are an inspiring leader and your enthusiasm and passion for all things "choppy offy" was evident from the first time I encountered you in the third year immunology lectures. You have built a lab that is a fantastic place to be a part of and I was really excited (most days) to turn up and do science. Some of my best friends have come out of the Boyd lab and that is because your character attracts some of the best people. I am grateful for the support, encouragement and amazing opportunities you afforded me during my time as a Boydie. **Ann**, thank you for being the mother of the lab, your ability to multi-task like no other has been an inspiration to me. **Tracy**, thank you for your supervision and mentorship throughout my PhD. We made a great team and I will always appreciate your unwavering positiveness towards the project when all I could be was negative. **Chris**, what a great friend you have made. I will always appreciate our coffees at Cinque Lire and our runs on a Thursday night. Most of all I appreciate and will be forever grateful for your help drafting this thesis. **Marky**, thank you for providing so many laughs, you are the most inappropriate of inappropriate –never change. **Jade Homann**, without you, I would have no data and no sanity. Thank you for your patience, for listening, for your highly useful advice, for your crazy emails and for all of the yoga. **Luci**, my other lab-yoga buddy, thank you for your help with my experiments, but more importantly, thank you for keeping me fit with all of those early morning boot camps. **Natalie**, you're the coolest scientist I know and one of the best friends I could ask for. Thank you for so many funny moments (that I really don't want printed here). **DK**, thank you for making the depressing ASIs fun, for eating Japanese food with me and for our continual discussions on all things irrelevant. I really admire your passion for science and the speed at which you can flush bones. **K Wong**, thank you for eating lunch with me and for understanding the importance of a designer handbag. **Adie**, there is not much I can say about our friendship that will do it justice, but I will never forget the time you picked me up following a horrible experiment and an exploded car tire, soaking wet and in tears, on the side of the Monash freeway in the middle of the night, with a post mix diet coke. You and that post mix diet coke saved my life. Thank you for being such a great friend. **Chew Chew**, I knew you before we were even friends (but that was mainly because Adie told me all of your secrets). I don't know another person who I could spend so much time with. Thank you for making lab-life and home-life so much fun. **Marco**, you're fantastic. **Anthony**, thank you for providing massages at my desk when I needed

them. **Lisa**, **Maree**, **Louisa**, **Dan**, **Jade B** and **Sam**, thank you for all of your help and for making the Boyd lab such a fun place.

Emma, **Suz**, and **Trish**, thank you for reminding me that there is a life outside of science and if you give it a chance it can be quite fun. Emma, your friendship has supported me at every level of my education, thank you for standing next to me in line when we were in primary school and for meeting me at Monash when that was the only way we could catch up. **Suz**, thank you for being super-smart and worldly, your advice has meant more to me than you know. **Trish**, you're the loveliest person I have ever met and you make me think about being a better person. Thanks for all the Pho.

McBain and **Jackie**, thank you for looking after me while I was writing my thesis in the UK. McB, you're the brother I never had.

Richard "Kiwi" Watson, thank you for always listening to my rants, for providing sound advice and for your editorial skills. So tolerant. So patient. So deck. I love you.

Prue, **Taylor** and **Alix**, I have never laughed more than I have when I am around you. Thank you for being the best sisters I could hope for and **Prue**, thank you for being a best friend. Having the support of an "older" sister really helped me get through this PhD. **Nanna** and **Grandad McBain**, your love, encouragement and support has meant the world to me. **Nanna Margaret**, your steadfast support of North Melbourne (and Wayne) has taught me passion. Go Roos.

Dad, thank you for supporting me, housing me and providing me with endless opportunities. I will always be grateful and will hopefully be able to make it up to you one day. And finally, **Mum**, you are an inspiration. Thank you for your support. You have shown me what it is to be brave; I hope that you can be as proud of me as I am of you.

Abbreviations

AIRE autoimmune regulator

APC antigen presenting cell

BBB blood brain barrier

BM bone marrow

BMT bone marrow transplant

Bu busulfan

cAMP cyclic adenosine monophosphate

CCL chemokine (C-C motif) ligand

CCR chemokine receptor

CLP common lymphoid progenitors

CMJ cortico-medullary junction

CMP common myeloid progenitors

cTEC cortical thymic epithelial cells

CTLA4 cytotoxic T lymphocyte antigen 4

DC dendritic cell

DN double negative

DP double positive

ETP earliest intrathymic T-lineage progenitor

FoxP3 forkhead box transcription factor 3

G-CSF granulocyte colony stimulating factor

GITR glucocorticoid-induced TNF receptor

HPSC hematopoietic stem progenitor cell

HSC hematopoietic stem cell

HSCT hematopoietic stem cell transplant

ICAM-1 intracellular adhesion molecule 1

IFN-γ interferon gamma

LAG_3 lymphocyte-activating gene 3

IL Interleukin

LP lamina propria

LSK lineage negative, $Sca1^+$, $cKit^+$

MHC major histocompatibility complex

MLN mesenteric lymph node

mTEC medullary thymic epithelial cells

PD1 programmed cell death 1

PI3K phosphoinositide-3-kinase

PSGL-1 P-selectin glycoprotein ligand-1

PTA peripheral tissue antigens

PTH parathyroid hormone

RA retinoic acid

RAG recombinase activating gene

RALDH retinal aldehyde dehydrogenase

RTE recent thymic emigrant

$S1P_1$ sphingosine-1-phosphate receptor-1

SCF stem cell factor

SIP sphingosine-1-phosphate

SP single positive

SSA sex steroid ablation

TBI total body irradiation

TCD T cell depleted

TCR T cell receptor

TEC thymic epithelial cells

TGF soluble transforming growth factor

Th3 T helper 3

TI thymic irradiation

TN triple negative

TNF tumor necrosis factor

Tregs T regulatory cells

CHAPTER ONE

Literature Review

Manuscript Information

Part of this literature review was adapted for the following manuscript: Morison, J., Heng, T., Chidgey, A., Boyd, R. (2013) The immunogenicity of stem cells and thymus-based strategies to minimize immune rejection. In, Dr. P. Fairchild (ed) Immunological Barriers to Regenerative Medicine. Springer Verlag, UK, pp 201-223The full manuscript is contained in the appendices.

Introduction

Organ transplantation is the curative technique for end stage organ failure. However, the success of this technique is hindered by the immunological rejection of transplanted tissue. Traditionally, strategies to combat allogeneic rejection have been based on life-long immunosuppression, which lead to high levels of morbidity stemming from opportunistic infections and malignancy. This can be overcome via the induction of allogeneic tolerance, where bone marrow (BM) transplant (BMT) induced mixed chimerism can produce a permanent state of donor-specific tolerance to transplanted tissue across major histocompatibility complex (MHC) barriers. Initial studies of tolerance induction via mixed chimerism involved lethal total body irradiation (TBI) and host reconstitution with a mixture of both host and donor T cell depleted BM. This induced lifelong hematopoietic chimerism and led to the development of specific tolerance to BM-donor matched skin grafts. A limiting factor in these initial studies was the use of lethal doses of irradiation, which caused significant damage to the host and therefore could not be justified in the absence of malignancy. To overcome the toxic effects of TBI, low-intensity conditioning protocols that reduced or excluded the use of irradiation are now the focus of mixed chimerism based research. Upon the establishment of mixed chimerism, allogeneic tolerance is maintained via the clonal deletion of donor-reactive T cells through the presence of donor-derived dendritic cells (DC) in the thymus. These protocols are highly successful in small animal models, but as yet have had limited success clinically. One major area that has been neglected by previous research is the effect of aging and immunosenescence on the ability to induce immunological tolerance via mixed chimerism. Ironically, the need for most organ transplants arises in an ever-aging population. The hallmark of immunosenescence is thymic atrophy, where functional thymic tissue and T cell output is reduced to less than 5 % following puberty. Considering that mixed chimerism based protocols for the induction of allogeneic tolerance requires a functional thymus, ageing may pose a significant barrier to the induction of tolerance.

Herein, the central and peripheral mechanisms underlying T cell tolerance and how these mechanisms can be manipulated to induce allogeneic tolerance are reviewed. Particular attention is paid to immunosenescence and how thymic atrophy may obstruct the induction of allogeneic tolerance via mixed chimerism and possible strategies that may overcome this hurdle.

The thymus

The thymus, which is the primary site of T cell development (thymopoiesis), is located in the pericardial mediastinum, anterior to the heart in the upper thorax. Despite it's anatomical prominence, it was not until the discoveries of the Australian scientist Jacques Miller in the early 1960's that its function was recognised (1). The organ is a bi-lobed structure, attached by a connective capsule that invaginates to form septa that subdivide the thymus into lobules. Histologically, each lobule can be broadly composed of an outermost subcapsule, a discrete outer cortex, and inner medullary region, separated by a cortico-medullary junction (CMJ) boundary, with each distinct region supporting a specific stage of thymocyte development (2) (Figure 1.1).

Thymic stroma

The thymic stromal microenvironment is comprised of many different cell types, including mesenchyme-derived fibroblasts and endothelium, macrophages and dendritic cells derived from the BM, and most prominently, highly specialised thymic epithelial cells (TEC). Together these cells form a three-dimensional network inside the thymic capsule (2). Unlike most epithelial tissues, which produce two-dimensional structures overlayed on a basement membrane, TECs form a fine reticular network, which shape important niches that are required to maintain thymopoiesis. These microenvironments accommodate for specialised interactions between migrating haematopoietic-derived T cell progenitors and thymic stromal cells (3). The importance of these interactions has been exemplified in a number of natural TEC mutant and knockout mouse strains, where autoimmunity or immunodeficiency results in anomalous TEC development (4). Ultimately, interaction with TEC drives the differentiation of thymocytes and culminates in the export of mature self-tolerant T cells into the periphery to establish and maintain T cell immunity (2).

Development of the thymic epithelium

In early embryogenesis the murine thymic primordium originates from the endodermal layer of the anterior foregut. A population of epithelial cells derived from the third pharyngeal pouch endoderm gives rise to the cortical and medullary regions of the thymus (5). At this stage, a number of the TEC progenitors are bi-potent, with the potential to develop into both cortical (cTEC) and medullary (mTEC) subpopulations (6). Expression of the transcription factor, Forkhead-box N1 (FoxN1), which is restricted to epithelial cells, is initiated at approximately embryonic day (E) 11.5 and is essential for the downstream differentiation into cortical and medullary lineages and colonisation of the anlage by hematopoietic progenitors (7). Following E11.5, FoxN1 is expressed

by all epithelial cells in the rudiment (8) and is maintained throughout thymus development, detectable within the adult TEC subset (9). The function of FoxN1 in the adult steady-state thymus is less understood, but is thought to be involved in the maintenance of the epithelium and homeostasis (10).

The initial stages of TEC formation occurs independently of thymocyte input (11), while the later stages rely on specific interactions between TEC and thymocytes (12). Studies in mice have indicated that signals delivered by thymocytes are crucial for the maturation of the cTEC and mTEC subsets from a common precursor as well as for the support and maintenance of the thymic architecture (3).

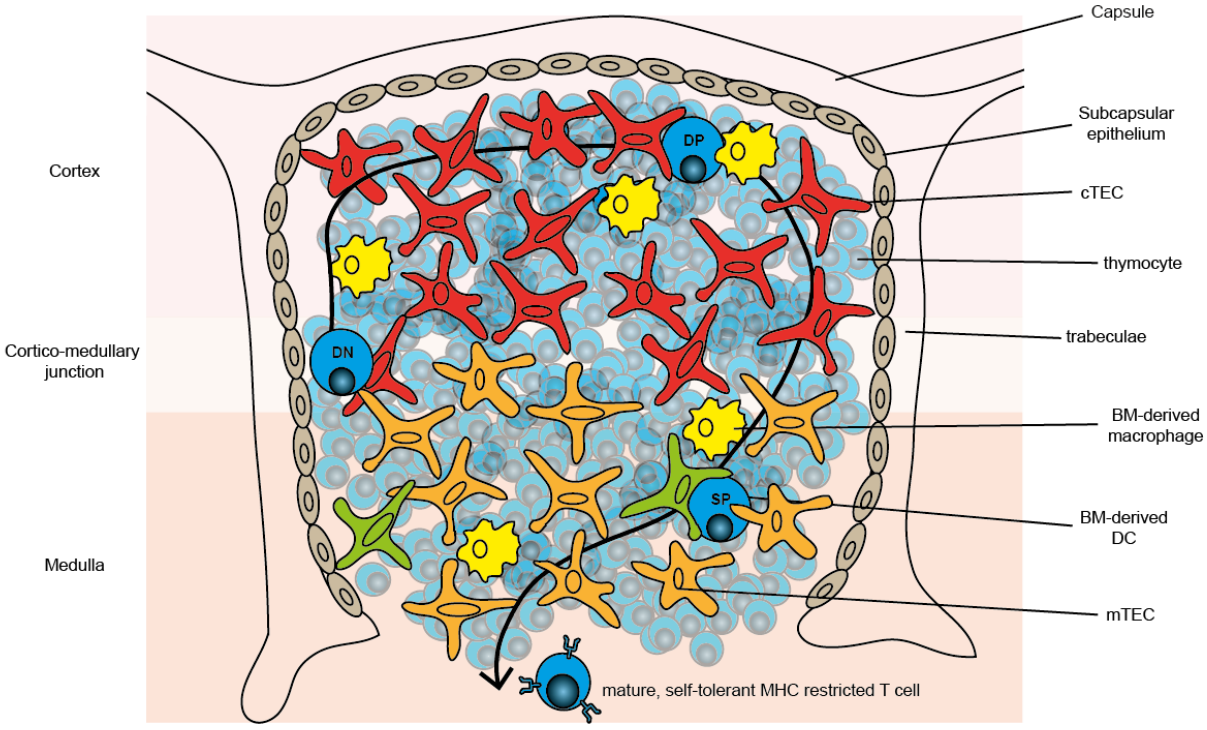
Thymic epithelial progenitor cells

Thymic epithelial progenitor cells (TEPC) have been phenotypically identified as expressing the plet-1 antigen, recognized by the monoclonal antibody MTS24^{+}, as well as being positive for keratin (K) 5^{+} and K8^{+} (13, 14). These cells are located at the CMJ and are responsible for producing both cTEC and mTEC subsets (13, 15, 16). As TECs differentiate, modulation of K5 and K8 antigens ensues with the progressive loss of K5 and retention of K8 in cTECs, whereas mTECs can be marked by the expression of K5 and the loss of K8 (11).

Thymocyte development

The thymus is responsible for providing T cells throughout adult life. However, despite being the principal site of T cell production, the thymus contains no self-renewing T lineage progenitor cells. It instead relies on continual seeding of cells from the circulation, previously released from the bone marrow (BM) in a process known as mobilisation. There are several progenitors that possess the ability to seed the thymus, however the precise identity of the thymus-seeding progenitor cell remains elusive. The classical model of haematopoiesis suggests a strict delineation between myeloid and lymphoid lineages, proposing that only hematopoietic stem cells (HSC) have true mulitpotent potential, giving rise to several downstream lineage-committed cells, such as common myeloid progenitors (CMP -lineage negative, Sca^{+} Fc gamma receptor (FcγR)^{mid} CD34^{mid}) and common lymphoid progenitors (CLP -lineage negative, cKit^{+}, inter leukin-7 receptor (IL7R)$^{+}$). However, more recent studies have shown that within the HSC compartment, there are lineage-biased precursor cells (17, 18). Furthermore, it is now known that T cell progenitors that gain access

Figure 1.1: Thymus structure. The thymus is a three-dimensional network of cells broadly divided into two histologically distinct regions, defined as the cortex and the medulla. T cell precursors enter the thymus at the cortico-medullary junction (CMJ), where they begin differentiation, which is directed by thymic stroma. Uncommitted $CD4^-$ and $CD8^-$ double negative (DN) thymocytes migrate from the CMJ to the cortex. Here DN thymocytes interact with cortical thymic epithelial cells (cTECs) and a undergo proliferation and expansion that results in the expression of the T cell receptor-β chain and both $CD4^+$ and $CD8^+$ co-receptors, acquiring the double positive (DP) phenotype. Self-restricted DP thymocytes migrate back through the cortex and into the medulla differentiating into either $CD4^+$ or $CD8^+$ single positive (SP) thymocytes. Here, SP thymocytes are rendered self-tolerant by medullary thymic epithelial cells (mTECs) and bone marrow-derived dendritic cells (DC). Finally, mature self-MHC restricted, self-tolerant SP T cells egress from the medulla to the periphery.


Capsule
Subcapsular epithelium
cTEC
thymocyte
trabeculae
BM-derived macrophage
BM-derived DC
mTEC
Cortex
Cortico-medullary junction
Medulla
DP
DN
SP
mature, self-tolerant MHC restricted T cell

to the thymus, maintain myeloid differentiation potential (19, 20). Within the stem cell compartment, this is dependent upon the expression of Flt3, PU.1 and GATA-1 (21). It is generally accepted that lymphopoiesis from HSCs involves a transition through a lineage negative $Sca1^+$ $cKit^+$ (LSK) phenotype via a cell classified as a lymphoid-primed multipotent progenitor (LMPP). LMPPs are characterised by the upregulation of lymphoid associated factors such as PU.1 and Ikaros, and the down regulation of GATA-1 and Mpl (22-24). These progenitor cells are released in waves from the BM into the bloodstream and are periodically imported into the thymus via the CMJ (25, 26). Once seeded in the thymus these T cell progenitors, termed earliest intrathymic T-lineage progenitor (ETP), pass through a number of developmentally distinct stages that are mediated by close interactions with the thymic stromal microenvironment (12).

BM egress

Mobilisation of HSPC cells out of the BM is a multistage process, beginning with the uncoupling of adhesion molecules followed by their transmigration across specialised BM sinusoidal endothelium (27). Many specific adhesion interactions are involved in the maintenance/migration of progenitor cells from the BM niche including very late antigen (VLA)-4/vascular cell adhesion molecule 1 (VCAM)-1, VLA-5/Fibronectin, cKit/mSCF, calcium receptor (CaR)/calcium, and most importantly, the chemokine receptor CXCR4, expressed on HPSCs, and it's ligand CXCL12 (SDF-1a), expressed on BM stroma. The CXCR4/CXCL12 interaction plays a crucial role in regulating the localisation of progenitor cells within the BM niche (27, 28). Disruption of these interactions with factors such as granulocyte colony stimulating factor (G-CSF) and parathyroid hormone (PTH) results in rapid mobilisation of progenitor cells into the periphery (29-32). Exploitation of this phenomenon has been successfully implemented in the clinic, enabling mobilisation of HPSC in donors for hematopoietic stem cell transplants (HSCT). This allows sufficient numbers of HSPC to be collected from blood, rather than invasive extraction from the BM. G-CSF has been widely used to promote mobilisation, releasing HSPC through modulating CXCL12 and CXCR4 expression, and creating a proteolyic environment whereby neutrophils release of elastase and cathespin G lead to the cleavage and degradation of CXCL12 as well as other adhesion molecules such as c-kit (33).

Thymic entry

The expression of chemokine receptors 7 and 9 (CCR7 and CCR9), and P-selectin glycoprotein ligand-1 (PSGL-1) by circulating progenitor cells and their respective ligands/receptors on thymic stromal cells is crucial for entry of progenitors into the thymus (34-36). The entry of progenitors

into the thymus is absolutely dependent on CCR9 expression by progenitor cells, which in turn is dependent on Flt3 signalling in the BM (37). Flt3 expression is also associated with lymphoid priming, suggesting an intrinsic link between lymphoid commitment and thymic entry (37). Thymic entry occurs in multiple steps, initiated by a chemokine gradient formed with chemokine (C-C motif) ligand 25 (CCL-25), followed by the interaction between P-selectin, expressed by the thymic endothelium and PSGL-1, expressed by progenitor cells (34, 38). From this stage, progenitor cells can interact with both intracellular adhesion molecule 1 (ICAM-1) and VCAM-1 expressed by endothelial cells, firmly attaching the progenitors to the thymic endothelium (38).

Occupancy within intrathymic microenvironmental niches negatively regulates the importation of progenitors into the thymus, via the opening and closing of so-called thymic microvascular gates (39). This process is thought to occur in coordination with the wave-like periodic release of progenitors from the BM, suggesting that a feedback loop exists between the BM and the thymus (26, 40). This feedback loop is under the control of the early response gene 1 transcription factor and the levels of circulating sphingosine-1-phosphate (SIP), which controls the egress of naive T cells from the thymus, the expression of P-selectin on thymic stroma and the chemokine CCL-25 (41, 42).

Intrathymic T cell development

Once in the thymus, ETPs form an intimate interaction with the supporting stromal microenvironment, passing through several well-defined stages of development, which result in the production of naive, self-tolerant, MHC-restricted T cells. Once in the thymus, Notch signalling quickly leads to the commitment of ETPs to the T cell lineage (43). ETPs fall within a subset of thymocytes that are classified by the triple negative (TN) phenotype, lacking the expression of the CD3, CD4 and CD8 co-receptors. TN cells themselves can be divided into 4 subtypes, based on the expression of CD25 and CD44. ETPs remain in the TN1 stage for around 10 days in the thymus, surrounded by cTECs, which express the highest level of Delta-like-ligand 1. This is required for initiation of Notch signalling and the provision of other signals such as stem cell factor (SCF), Hedgehog, and low levels of IL-7 to the developing thymocytes (44-47). Within the T cell lineage, TN cells remain uncommitted and retain the potential to develop into either γδ or αβ T cell receptor (TCR) expressing T cells (48).

Definitive T cell commitment occurs at the onset of TCR expression. Upregulation of CD25 (IL-2Rα) promotes expression of the pre-TCR signalling complex, which can be directly regulated by Notch signalling (49, 50). αβ-TCR gene rearrangement occurs at the TN3 stage, where the pre-T α chain couples to the TCRβ chain and CD3/ζ, forming the pre-TCR complex. This requires recombination activating genes (RAG) 1 and 2, which allow DNA rearrangements of the TCRβ chain (51, 52). Functionality and signalling ability of the pre-TCR complex is tested at this point, in a process known as β-selection. If deemed non-functional, maturation of thymocytes will be arrested here. Thymocytes that survive β-selection undergo a proliferative burst and progress to the double negative (DN) stage ($CD3^+$ $CD4^-$ $CD8^-$), where rearrangement of the TCRα chain leads to the formation of a mature TCR. This correlates with the upregulation of both CD4 and CD8 co-receptors and entry into the double positive (DP) phenotype classification (51-54). At this stage of development DP thymocytes enter the process known as central tolerance.

Central tolerance

Central tolerance is the process by which developing thymocytes become MHC restricted and self-tolerant. In a healthy individual, the thymus must produce a T cell repertoire that can respond to a seemingly infinite number of potential foreign antigens, whilst remaining non-responsive to self-antigens expressed by various tissues throughout the body. The emigrating T cell pool is therefore delicately balanced between self-antigen driven tolerance and pathogen driven immunity, with a shift towards either end of the scale (lack of T cells and immunodeficiency, or excessive T cell responses and autoimmunity) often resulting in pathophysiological conditions and overt disease. Central tolerance is a coordinated process, exerted at two levels within the thymus. The first, positive selection, enriches for cells that can recognise self MHC (55); the second, negative selection, purges the T cell pool of potentially autoreactive cells (56) (Figure 1.1). Together these two processes strongly bias the naive T cell repertoire against immunodeficiency and autoimmunity.

Positive selection

Positive selection occurs in the cortex, where DP thymocytes represent 80-90% of all cells found in the thymus. During the DP stage, thymocytes rearrange their TCRα-chain and express the mature TCR on their surface (57). DP thymocytes undergo selection based on their ability to recognise self-peptides bound to MHC class I and II, which are expressed on cTECs. Recognition of this complex

transmits a survival signal, allowing continued thymocyte maturation (58). This survival signal is mediated by Ikaros, which suppresses thymocyte proliferation and induces the expression of anti-apoptotic proteins, including Bcl2 and Bag3, upon TCR induced positive selection. This is important to prevent clonal expansion and to maintain a random TCR repertoire during thymocyte differentiation (59). Failure of the TCR to engage the peptide-MHC complex expressed by cTECs leads to death by neglect. The majority of thymocytes are lost here, with 90% succumbing to apoptosis (56). DP thymocytes that have passed positive selection migrate towards the medulla and downregulate either a CD4 or CD8 coreceptor, maturing into MHC-restricted $CD4^+$ or $CD8^+$ single positive (SP) T cells. Recognition of peptide presented by MHC class I molecules selects for $CD8^+$ cytotoxic T cells and recognition of peptide presented by MHC class II molecules selects for $CD4^+$ helper T cells (60). SP T cells, which represent all possible T cells clones, then migrate into the thymic medulla, where they undergo further developmental checkpoints, which purge the T cell repertoire of potentially autoreactive thymocytes.

Negative selection

Negative selection is an apoptotic mechanism that ensures the positively selected thymocytes do not have too strong affinity for their selecting MHC-peptide. This process is guided by two prominent antigen presenting cell (APC) types: mTEC and BM-derived DCs (61). During negative selection, developing thymocytes are exposed to extra-thymic self-peptides they may encounter in the periphery (62).

Medullary TECs are distinct from those found in the cortex, both in terms of both their physical organisation and gene expression, and play a major role in the induction of self-tolerance via their unique ability to promiscuously express and transcribe a diverse range of genes usually constrained to peripheral tissues (63). The expression of these peripheral tissue antigens (PTAs) is under the control of the transcriptional regulator, autoimmune regulator (AIRE), which theoretically allows mTECs to present the entire peptide repertoire of an individual (62). Through this mechanism, diverse tissues can be represented in the mTEC and to date, more than 5000 different self-antigens have been identified (64).

In addition to mTECs, DCs play an important role in the induction of self-tolerance in the thymus. DCs in peripheral tissues have been shown to cross-present antigens to T cells (65-69). Cross presentation is an important mechanism for generating immunity to viruses and tumours, as well as

for the induction of tolerance to self-antigens (65, 70). Thymic DCs also have the capacity to cross-present self-antigens, in this case supporting mTEC antigen presentation for stringent control of central tolerance induction (71). The importance of cross-presentation mediated by intrathymic DCs is highlighted by low frequency of TRA expressing mTECs (63, 72) and the fact that mTECs may be poor APCs (73), suggesting that intrathymic DCs provide a backup mechanism for stringent tolerance induction.

Negative selection results when the engagement between the TCR and peptide-MHC complex occurs at a high affinity, generating signals that lead to either the functional inactivation of thymocytes (anergy) (74), or their apoptotic death (55). Recognition of self-antigens with inappropriately high avidity induces the intrinsic or mitochondrial death pathway, which is regulated by the Bcl-2 family of proteins. Of particular importance are Bak and Bax, which disrupt the mitochondrial membrane, causing the leakage of cyctohrome c into the cytosol, the formation of the apoptosome, and cell death (75, 76).

Developing thymocytes that have successfully undergone negative selection remain in the medulla for a further 14 days (77). During this time, SP thymocytes upregulate the expression of transcription factors that regulate immune responses, and co-stimulatory molecules and cytokine receptors on the cell surface, leading to functional maturation and immunocompetence (78-80). Finally, mature, self-tolerant, MHC-restricted, $CD4^+$ or $CD8^+$ SP T cells migrate through the post-capillary venules at the cortico-medullary junction, emigrating from the thymus at a rate of 1-2% of total thymocytes per day (81).

Peripheral tolerance

Although the ideal outcome of central tolerance is a pool of mature T cells, with a vast TCR repertoire that can successfully bind MHC molecules while remaining unresponsive to autoantigens and even though negative selection is a highly efficient process by virtue of the plasticity inherent in the TCR repertoire, it is never complete. Thus, potentially autoreactive T cells can escape the thymus and enter the periphery. Typically, these cells have a low affinity for self-peptide, as evidence suggests that negative selection is most efficient at deleting thymocytes expressing TCRs with a high avidity for self peptide-MHC complexes presented by mTECs and DCs (82). In the periphery, autoreactive T cells pose a significant risk to the development of autoimmune diseases throughout life. However, while circulating autoreactive T cells can be found in all individuals, the

prevalence of autoimmune disorders affects only a small proportion of the population, suggesting mechanisms of peripheral tolerance operate to silence potentially pathogenic T cells. There are currently four recognised mechanisms of peripheral tolerance: ignorance, anergy, deletion and suppression (83) (Figure 1.2).

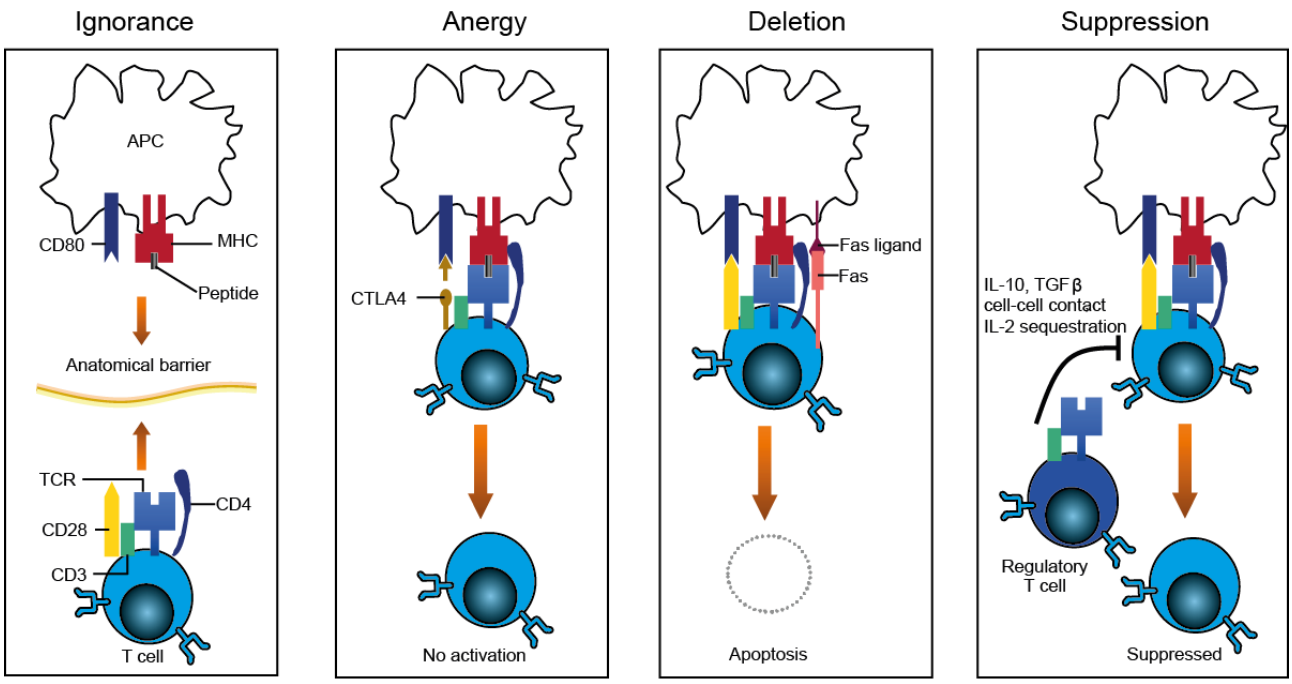
Ignorance

Immunological ignorance refers to the inability of T cells to interact with antigen. Several mechanisms can induce immunologic ignorance, these include: lack of an activation signal, whereby the level of antigen required to induce the activation or deletion of autoreactive T cells may be below the required threshold to induce a response (84); a deficiency in co-stimulation (discussed in detail below), which prevents T cell maturation and proliferation following antigen presentation (85); the absence of help from $CD4^+$ T cells, whereby $CD8^+$ T cells cannot be activated (86); and finally, lack of contact with antigen or physical separation of antigen from T cells (for example blood-brain barrier (BBB) and the eye) (87). Although it has now been shown that sites originally thought to be immune privileged are subject to circulating lymphocytes (88), immune responses that occur in such sites are still decreased and distinct when contrasted against traditional immune responses in other peripheral sites. This can be attributed to a number of factors, which include: limited penetration of the BBB by antibodies, cytokines and lymphocytes from systemic circulation; a lack of lymphatic vessels to drain sites of immune privilege of APCs and antigens to peripheral lymph nodes and thereby initiate an immune response; inability of cells within sites of immune privilege to initiate and sustain immune responses; and low level expression of MHC molecules by immune privileged tissue (89). Thus, T cells will not become pathogenic if they are ignorant of self-antigens.

Anergy

Anergy is defined as a state of long-lasting unresponsiveness of a T cell following incomplete activation (90). T cell activation requires two responses, TCR signalling, induced by recognition of cognate antigen-MHC complexes presented on APCs, and a secondary co-stimulatory signal, provided by receptors on each of the communicating cells at the immunological synapse. In the absence of a co-stimulatory signal, T cells cannot undergo maturation or proliferation and become unresponsive to antigen. The co-stimulatory signal is provided by CD28, which is constitutively expressed on the surface of both $CD4^+$ and $CD8^+$ T cells. CD28 binds to the co-stimulatory receptors, B7-1 (CD80) and B7-2 (CD86), on the surface of APCs and ligation of these molecules

Figure 1.2: Mechanisms of peripheral tolerance. Anatomical barriers can prevent T cell activation, thereby preventing unwanted T cell responses via immunological ignorance. The absence of co-stimulation (ligation of CD28 and CD80) or the induction of inhibitory signals (ligation of CTLA4 and CD80) following TCR signalling leads to T cell anergy, rendering T cells unresponsive to TCR stimulation. High affinity interactions between T cells and APCs can lead to the activation induced cell death pathway. This is mediated by ligation of Fas/FasL and induces T cell apoptosis. Tregs, derived from the thymus or the periphery suppress unwanted T cell responses via the secretion of cytokines (TGF-β, IL-10), cytolysis of target cells, metabolic destruction of target cells, sequestration of available APCs or competitive consumption of IL-2.


Ignorance
APC
CD80
MHC
Peptide
Anatomical barrier
TCR
CD4
CD28
CD3
T cell
Anergy
CTLA4
No activation
Deletion
Fas ligand
Fas
Apoptosis
Suppression
IL-10, TGFβ
cell-cell contact
IL-2 sequestration
Regulatory
T cell
Suppressed

provides the second signal required for T cell activation (85, 91). Following T cell activation, the cytotoxic T lymphocyte antigen 4 (CTLA4) is expressed by mature T cells; CTLA4 binds to B7-2 with a higher affinity than CD28 and delivers an inhibitory signal to T cells, inducing down regulation of CD28 molecules on the surface of activated T cells, thereby suppressing T cell proliferation (91-93).

Deletion

Deletion is another mechanism of peripheral tolerance, functioning to control aberrant T cell responses by inducing the apoptotic cell death pathway. Deletion usually occurs when T cells encounter high antigen concentrations or high affinity interactions with APCs. The process is termed activation induced cell death (AICD) and involves the ligation of Fas (CD95) and Fas ligand (FasL or CD95L) (94, 95). T cells that receive high avidity stimulation express both Fas and FasL on their surface. The ligation of these two molecules leads to the activation of caspase cascades, which inturn induces cells death (96). The importance of this mechanism is highlighted by the severe lymphoproliferative disease experienced by patients who are defective in Fas (97, 98). Additionally, some sites of immune privilege, such as the eye, constitutively express Fas L. Consequently, when Fas^+ T cells enter these sites, they undergo apoptosis without damaging the tissue (99).

Suppression

The best-characterised population of "suppressor cells" are the $FoxP3^+$ $CD25^+$ $CD4^+$ T regulatory cells (Tregs) (100, 101). The first evidence of for a T cell mediated regulatory mechanism occurred in the 1970s, with the discovery of the T suppressor cell (102, 103). However the cellular and molecular basis of this phenomenon was not clearly established and studies in this area were largely abandoned in 1980s. In spite of these failures, a number of groups continued to investigate dominant T cell mediated immunoregulatory networks. In these models, induction of lymphopenia and/or neonatal thymectomy led not only to impaired immune responses but also, paradoxically, to autoimmunity (104). Importantly, transfer of normal lymphocytes could prevent disease (105). The development of monoclonal antibodies allowed the further characterisation of the cell populations that could mediate suppression, with evidence in T cell transfer experiments that both pathology and protection from disease were mediated by T cells (106, 107). These suppressor T cells, now termed Tregs have become a major focus of modern cellular immunological investigations.

Several types of Tregs have been discovered in both mice and humans (108). The best-described population of Tregs are the naturally occurring nTregs (109, 110), arising in the thymus from $CD4^+$ T cells expressing the α-chain of the IL-2 receptor (CD25). In addition to nTregs, other types of Tregs can be induced in the periphery; these cells are known as induced or adaptive Tregs (iTregs). These regulatory cells are induced outside the thymus by a number of diverse mechanisms during the course of an immune response (111). There are at least two additional populations of induced regulatory cells: Th3 cells and Tr1 cells. Th3 cells were first discovered due to their role in oral tolerance, through the secretion of soluble transforming growth factor (TGF)-β (112) and have subsequently been shown to inhibit the outcome of some autoimmune disorders (113-115). Tr1 cells are similar to Th3 cells, but secrete large amounts of IL-10 (116, 117) and were characterised on the basis of their role in preventing autoimmune colitis (118). Other T cell populations with demonstrable immunosuppressive function such as natural killer (NK) T cells (119, 120) and $CD8\alpha\alpha^+$ intraepithelial lymphocytes (IELs) (121-123) have also been implicated in different models of autoimmune diseases, however this review will focus on nTregs and iTregs.

Naturally occurring $FoxP3^+$ $CD4^+$ $CD25^+$ Tregs

Naturally occurring Tregs were first identified as T cells expressing high levels of CD25 that were capable of suppressing autoimmune reactions (100). Subsequent identification of additional surface markers, such as the glucocorticoid-induced tumor necrosis factor (TNF) receptor (GITR), CTLA4 and most notably, the forkhead box transcription factor FoxP3, a T-lineage specific transcription factor found in mice, allowed further characterisation of nTregs (124-127). nTregs develop in the thymus (128), however how Tregs gain functional maturity is a somewhat contentious issue. Early studies that examined double-transgenic mice expressing a monoclonal TCR and cognate neoautoantigen suggested that within the thymus, there was a skewing of mature $CD4^+$ SP thymocytes towards a $CD25^+$ $FoxP3^+$ regulatory phenotype (129-131) and selection of these nTregs occurred following high-affinity T cell interactions between TCR and MHC/self-peptide complexes during thymic development (130-132).

nTreg development occurs as a two-step process within the thymus, characterised by differential dependence TCR signals (133). In the first stage of development, thymocytes undergo a TCR and co-receptor dependent selection that gives rise to a $CD4^+$ $CD25^+$ GITRhi $FoxP3^-$ precursor cell (134, 135). This $FoxP3^-$ precursor population is enriched for the nTreg specific TCR and undergoes the second, TCR-independent, step of nTreg selection. This step is mediated by common-γ chain

specific cytokines (predominantly IL-2 and, to a lesser degree, IL-7 and IL-15) and results in mature FoxP3 expressing nTregs (133, 134).

CD28 co-stimulation is also important to the development of nTregs, synergising with the TCR and promoting cell survival (136). However, although the role of CD28-mediated signalling the homeostasis and survival of peripheral Tregs is well characterised (137-139), the precise mechanism by which the CD28 co-stimulatory signals regulate Treg development within the thymus remains poorly characterised. The interaction between B7, expressed on APCs within the thymus, and CD28 on thymocytes is thought to strengthen the contact between the APC and the developing thymocyte, thereby promoting thymocyte survival via the production of IL-2 and the upregulation of the anti-apoptotic protein Bcl_{XL} (140-142). The importance of co-stimulation in the selection of nTregs is highlighted by the loss of nTregs in CD28 and B7 knockout mice (136-138, 143).

Beyond TCR signalling and CD28 co-stimulation, a number of experimental conditions drive the expression of FoxP3 and by consequence induce Treg production. These include constitutive NF-κB signalling, loss of maintenance DNA methyltransferase activity, deficiency in mammalian target of rapamycin (mTOR) or sphingosine-1-phosphate receptor-1 ($S1P_1$), and a reduction in phosphoinositide-3-kinase (PI3K) signalling (144-149). Together supporting the notion that not only TCR signalling, but also the appropriate coordination of other cell intrinsic and extrinsic factors induce Treg differentiation.

The loss of FoxP3 expression, demonstrated in Scurfy mice (which have a naturally occurring *FoxP3* deletion), also results in a loss of nTregs and leads to a severe T cell dependent autoimmunity and inflammatory disease (150, 151). This phenomenon can also be observed in humans, with a mutation in the *FoxP*3 gene resulting in a spontaneous inflammatory disease called immune dysregulation polyendocrinopthy enteropathy X linked syndrome (IPEX)(152-154). FoxP3 expression plays an indispensible role in nTreg development, indicated by the inability of *FoxP3*$^-$ BM cells to give rise to nTregs in disease free, lethally irradiated recipient mice (128). Supporting these observations, the ectopic expression of *FoxP3* in mice can phenotypically and functionally convert non-regulatory T cells into Tregs (155), with the level of FoxP3 protein directly proportional to the suppressive activity of the Treg (156). Furthermore, sustained FoxP3 expression in mature Treg cells is required for the maintenance of the Treg phenotype and suppressor function, with conditional deletion of *FoxP3* corresponding to a loss of suppressive function, Treg phenotype

and the gain of T effector properties, including the production of immune-response promoting cytokines IL-2, IL-4, IL-7 and interferon-γ (IFN-γ) (157). In mice FoxP3 is a good phenotypic marker that allows the identification of Treg cells (158, 159); in humans however, FoxP3 does not allow unambiguous discrimination between conventional T cells and Tregs as its expression is induced upon TCR stimulation (much the same way as CD25) in human $CD4^+$ T cells (160-162).

Peripherally induced $FoxP3^+$ $CD4^+$ $CD25^+$ Tregs

In addition to the development of nTregs in the thymus, T cells with a regulatory function can develop from the adult T cell pool in the periphery, in response to antigenic challenges and the local microenvironment. Such induced regulatory T cells include the $FoxP3^+$ (iTregs) and $FoxP3^-$ (Th3, Tr1, iTr35 and $CD8^+$ $CD28^-$) populations of cells (163).

The development of iTregs is thought to occur following the recruitment of naive $CD4^+$ T helper cells following the detection of infection and/or tissue injury. Here, naive $CD4^+$ T cells are induced to commit to a particular T cell lineage, based on the mode of antigen presentation, antigenic concentration, co-stimulation and cytokine milieu at the immunological synapse (164). Each lineage is then characterised by the expression of it's own cytokine profile: Th1-IFN-γ, Th2-IL-4, Th17-IL-17 and Treg-IL-10/TGF-β and chemokine receptors: Th1-$CXCR_5$/$CXCR_3$, Th2-$CRTH_2$/CCR_4 and Th17-CCR_6 (165-168). Each of the individual $CD4^+$ T cell lineage committed cells are specialised to perform specific biological roles, including immunity against intracellular pathogens (Th1), humoral immunity (Th2), clearance of extracellular infections (Th17) and regulation of immune system activation (Tregs) (169, 170), with the local microenvironment functioning as a deciding factor in the differentiation of the appropriate $CD4^+$ T cell lineage.

Conditions favouring the differentiation of Tregs in the periphery include sub-optimal activation and antigen presentation by DCs, sub-immunogenic doses of agonist peptide, mucosal administration of peptide, and/or T cell activation in the presence of the correct cytokine milieu, notably TGF-β and IL-2 (171-176). Importantly, iTregs can be generated in TCR-transgenic mouse models that are completely devoid of a thymus, indicating that the production of iTreg cells from $CD4^+$ T cells can occur independently of nTregs (177).

DCs within the gut have been shown to play a crucial role in the induction of iTregs and the maintenance of oral tolerance. Although the exact mechanism is not yet fully understood, it has

been hypothesised that dietary antigens are carried from the gut mucosa to the draining mesenteric lymph nodes (MLN) by DCs in a chemokine receptor (C-C motif) receptor-7 (CCR7) dependent process (351). Here the antigens are presented to naïve T cells in a manner that leads to the expression of FoxP3 and their conversion to iTregs (352). These tolerising DCs originate in the gut lamina propria (LP), express the αE intigrin CD103 and have a high capacity to metabolise Vitamin A to retinoic acid (RA) via the retinal aldehyde dehydrogenase (RALDH) enzyme (353, 354). Once settled in the MLN, the $CD103^+$ DCs cooperate with MLN stromal cells to shape a micro-environmental niche that supports Treg induction (355). $CD103^+$ DCs can induce the expression of FoxP3 in naïve T cells, both *in vivo* and *in vitro* via the production of TGF-β and RA (353). Upon induction *in vivo*, $FoxP3^+$ iTregs home back to the LP, undergo expansion and induce oral tolerance via the production of IL-10 (352). Loss of APC and iTreg homing between the LP and MLN abrogates oral tolerance and can lead to the development of inflammation (356).

In a similar process iTregs can be induced by alveolar macrophages in the lung. These macrophages, like $CD103^+$ DCs, express RALDH and induce FoxP3 expression in naïve T cells via the production of both TGF-β and RA functioning to prevent asthmatic lung inflammation and airway hypersensitivity (357).

TCR specificity also plays a crucial role in the generation of iTregs, first demonstrated by the transfer of $FoxP3^-$ $CD4^+$ T cells into lymphopenic mice. The resultant $FoxP3^+$ cells had a distinct TCR repertoire from the $FoxP3^-$ cells, suggesting that the generation of $FoxP3^+$ cells was not an automatic process arising from of $CD4^+$ $FoxP3^-$ cells that could give rise to both $FoxP3^+$ and $FoxP3^-$ clones (178). Strengthening these observations, more recent data has identified colonic iTreg cells which feature distinct TCRs from those displayed by Tregs in other tissues, recognising antigens expressed by commensal microbiota (179). Together this data implies that some adult T cells with certain TCR specificities are more suited to differentiate into Tregs than other T cells and this phenomenon may be due to a higher than average avidity for self-MHC/peptide complexes (163). However, unlike the differentiation of nTregs within the thymus, which occurs in response to increased affinity interactions with self-peptide-MHC complexes, differentiation of peripheral iTregs occurs in response to non-self antigens, such as allergens, food, commensal microbiota and non-self transplanted tissue (129, 130, 179-182).

Mechanism of action

Tregs suppress immune responses via a number of mechanisms. These include, cell-cell contact-dependent inhibition (183-185), secretion of inhibitory cytokines (186-190), cytolysis of target cells (191, 192), metabolic disruption (193), modulation of APC function (194, 195), and competition for environmental IL-2 (196). The mechanism by which Treg cells "choose" to suppress immune responses is context dependent and thought to be dictated by the degree and mode of inflammation. Importantly, Tregs are not limited to a single mechanism. For example, Tregs can simultaneously deliver suppressive factors such as cyclic adenosine monophosphate (cAMP) via gap junctions (197), modulate APC function via membrane-bound suppressive TGF-β (198), and induce suppressive signalling via CTLA4 (199) and lymphocyte-activating gene 3 (LAG_3) (200).

Cell surface molecules have been proposed as a means by which Tregs suppress conventional T cells. CD25, a subunit of the IL-2 receptor (IL-2R), is upregulated on effector T cells and constitutively expressed on Treg cells. This high level of IL-2R expression on Treg cells may starve effector T cells of IL-2, thereby inhibiting proliferation and expansion of activated T cells (201). In a similar mechanism, the cell surface marker CTLA4 is highly expressed in Treg cells. CTLA4 binds to co-stimulatory molecules and delivers an inhibitory signal, via B7 (CD80 and CD86), to both conventional T cells (202, 203) and antigen presenting cells (204). The importance of CTLA4 to Treg-mediated suppression is highlighted by the loss of suppressive function when CTLA4 expression is selectively inhibited in Tregs (205) or blocked by anti-CTLA4 monoclonal antibodies (206). In addition to cell surface molecule expression, a number of secreted cytokines have been implicated in Treg mediated suppression. These include IL-10, IL-35, granzyme B, IL-9 and TGF-β (207).

Cytolytic activity has also been suggested as a possible mechanism by which Tregs suppress aberrant immune responses. Activated FoxP3-expressing T cells can secrete granzyme A when stimulated with CD3 and CD46 monoclonal antibodies. This results in porforin-dependent killing of activated $CD4^+$ and $CD8^+$ effector T cells and B cells. However, it is not known if antigen mediated activation can stimulate the same responses *in vivo* (208).

To this point, no distinct differences have been demonstrated between nTreg and iTreg function, suggesting that the mechanism of suppression is dictated by the context of the immune response, rather than the mode by which the Treg cells have been induced. Indeed, both nTreg and iTreg cells

are equally as effective at preventing DC-mediated antigen presentation to autoreactive T cells (194). Despite this, it is generally accepted that nTregs function to prevent the development of autoimmune diseases and that iTregs limit inflammation to neo-antigens and commensal microflora (163).

Other T cells with a suppressive regulatory function

In addition to Tregs, a number of other T cells with inducible suppressive properties have been described. These include members of both the $CD4^+$ (Th3, Tr1, iTr35), $CD8^+$ ($CD8^+CD28^-$) and $CD4^-CD8^-$ double negative Treg lineages. Of these T cells, the most controversial is the T helper 3 (Th3) subset. Th3 cells were first described as a Th2-like regulatory subset, which secrets TGF-β, derived from orally tolerised animals, induced by mucosal stimulation with antigen (112). It was then demonstrated that Th3 cells could be induced via cognate stimulation of $CD4^+$ T cells by APCs together with B7 co-stimulation in the presence of TGF-β and IL-4 and, unlike $FoxP3^+$ Tregs, further growth and division of Th3 cells is dependent on IL-4, not TGF-β and IL-2 (114, 209, 210). Th3 cell suppression is mediated by the secretion of TGF-b following ligation of the CTLA4 antigen (211).

Type 1 regulatory T cells (Tr1) are $FoxP3^-$ T cells that are induced in the periphery in a TCR-dependent and antigen-specific manner. The Tr1 cell phenotype and effector function are induced following repeated stimulation with antigen or following antigen presentation by immature DCs (212, 213) or IL-10, with or without IFN-α (214). Thus, in humans, Tr1 cells are produced following antigen presentation by IL-10 producing, tolerogenic DCs (215). Additionally, IL-10 producing Tr1 cells can be induced following engagement of the complement receptor CD46 (216). Following the induction of the Tr1 phenotype, the $CD4^+$ T cells become anergic, respond poorly to antigen and produce little IL-2 and IL-4, exerting their suppressive function via the production of TGF-β and IL-10. Distinct from other suppressive T cell phenotypes, Th1 cells also secrete IFN-γ and IL-5 (118). Although Tr1 cells do not constitutively express *FoxP3*, they do share a number of suppressive mechanisms with traditional Tregs. These include engagement of CTLA4 and programmed cell death 1 (PD1) (217, 218), metabolic disruption via CD39 and CD73 (219), and cytolysis of APCs through granzyme B and perforin (220).

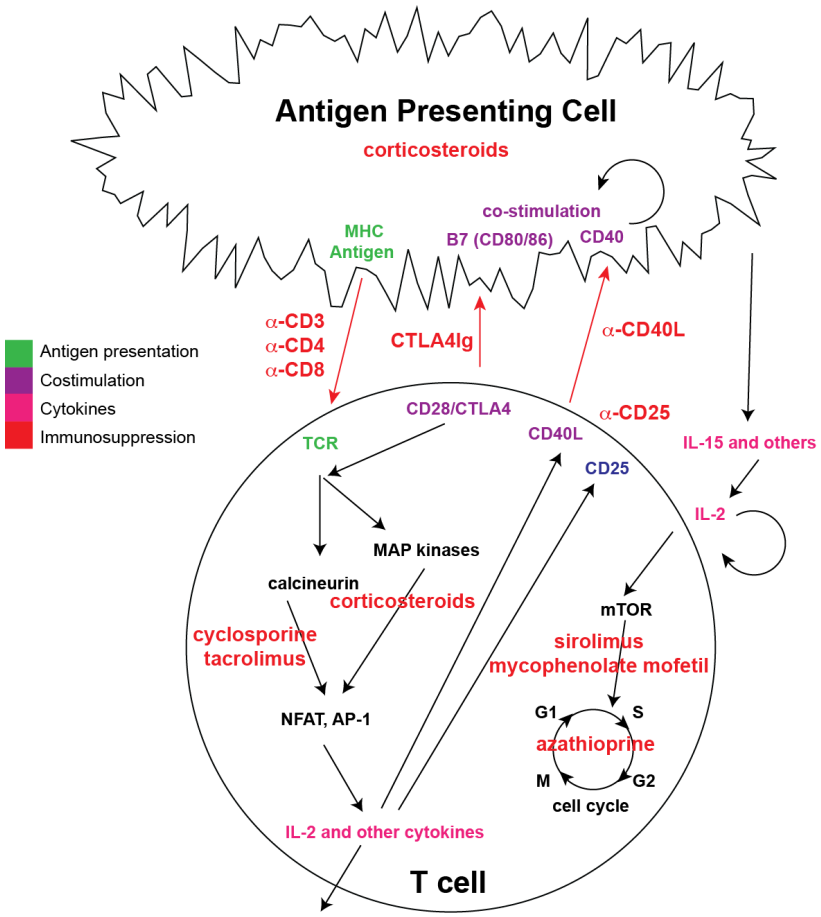
IL-35 is a cytokine member of the IL-12 family, originally described as a suppressive cytokine produced by $FoxP3^+$ Tregs. Secretion of IL-35 can induce the development of $CD4^+$ $FoxP3^-$ T cells

that secrete IL-35, but not TGF-β or IL-10. These suppressive T cells are hyporesponsive to re-stimulation, can be induced in both mice and humans, and are known as iTr35 cells (190). Although the role of iTr35 cells in immune physiology is currently unknown, ectopic expression of IL-35 on pancreatic β-cells can protect against the development of experimental autoimmune diabetes (221).

T cells with regulatory functions are not restricted to the $CD4^+$ lineage, but also include some $CD8^+$ and $CD4^-$ $CD8^-$ TCR^+ cell populations. $CD8^+$ regulatory T cells are induced following stimulation of naive $CD8^+$ T cells by CD40L-activated plasmacytoid DCs in an IL-10 dependent manner (222). These cells produce high levels of the suppressive cytokine IL-10 and low levels of IFN-g, but not IL-4, IL-5 or TGF-β and can suppress $CD8^+$ T cell alloresponses via the production of IL-10 (222). Additionally, repeated stimulation of $CD8^+$ T cells with antigen can induce $CD8^+$ $CD28^-$ T cells that have a similar molecular profile (FoxP3, GITR, CTLA4 and CD25) to $CD4^+$ $FoxP3^+$ Tregs (223, 224). The loss of CD28 by $CD8^+$ T cells is a well-documented phenomenon that occurs naturally with ageing and corresponds to a decrease in the ability of $CD8^+$ T cells to carry our effector function (225, 226). However, the exact mechanism by which $CD8^+$ suppressive T cells modulate immune responses is unknown, but is thought to involve inhibition of co-stimulation between T cells and DCs (227, 228).

T cells that are $\alpha\beta TCR^+$ $CD4^-$, $CD8^-$ $NK1.1^-$ (DN Tregs) can also act as regulatory T cells (229). These, $FoxP3^-$ DN Tregs were first characterised in TCR transgenic mice and have now also been identified in mice and humans. DN Tregs are antigen-specific regulatory cells that can exert control over autoimmune responses (230), graft versus host disease (GVHD) (229), and both allogenic and xenogenic rejection responses (231-234). The origin of DN Tregs is controversial, with a number of studies suggesting that peripheral DN Tregs are thymus-derived (235, 236), while other studies hypothesise that peripheral DN Tregs represent a primitive αβ T cell subset that bypasses the thymus completely (237, 238) or alternatively, escaped further differentiation into $CD4^+$ or $CD8^+$ T cells, traversing alternative developmental pathways (235, 239). DN Tregs employ multiple mechanisms to control immune responses. These include the acquisition of antigen-specific surface molecules (trogocytosis) from APCs, followed by binding and killing $CD8^+$ T cells with the same antigen specificity (240); the secretion of IFN-γ (231) and IL-10 (241); inhibition of Signal 2 via interactions with DCs that lead to a decrease in the expression of the co-stimulatory molecules CD80 and CD86 via CTLA4 engagement (242, 243); apoptosis of APCs via the induction of the

Figure 1.3: Schematic of mechanisms of action by immunosuppressive drugs. T cell proliferation results from activation after presentation of donor antigen by APCS in conjunction with MHC and co-stimulation. This mechanism results in the activation of calcineurin, which leads to the production of IL-2. Autocrine stimulation by IL-2 results in cell proliferation, mediated by a pathway that involves the target of rapamycin (mTOR) and cyclin/cyclin dependent kinase. Immunosuppressive agents (shown in red) exert their effects on a number of different targets to prevent T cell proliferation. G1 (first growth phase), S (synthesis of DNA), G2 (second growth phase) and M (cell division) represent the phases of cell cycle.


Antigen Presenting Cell
corticosteroids
co-stimulation
MHC
Antigen
B7 (CD80/86)
CD40
α-CD3
α-CD4
α-CD8
CTLA4Ig
α-CD40L
Antigen presentation
Costimulation
Cytokines
Immunosuppression
CD28/CTLA4
α-CD25
CD40L
TCR
IL-15 and others
CD25
IL-2
MAP kinases
calcineurin
corticosteroids
mTOR
cyclosporine
tacrolimus
sirolimus
mycophenolate mofetil
NFAT, AP-1
G1
S
azathioprine
M
G2
cell cycle
IL-2 and other cytokines
T cell

Fas pathway (242); and finally, perforin and granzyme-mediated killing of B cells and NK cells (232, 244).

Manipulation of tolerance for the induction of transplantation tolerance

The induction of tolerance, for the purpose of organ transplantation has been a long-time goal of both basic and clinical immunology research. Currently, organ transplantation is feasible due to the implementation of life-long immunosuppressive regimes (Figure 1.3). However, continual immunosuppression is associated with a number of clinical morbidities, these include diabetes mellitus, nephrotoxicity, neurotoxicity, osteonecrosis, leukopenia, hypertension, hyperlipidemia, cardiovascular events, opportunistic bacterial and viral infections, organ fibrosis, and graft loss (245-249). Strikingly, 50% of all renal transplant recipients will lose their graft to one of the aforementioned complications (250). The induction of immunological tolerance is a means by which long-term immunosuppressive regimes can be overcome, eliminating negative side effects, whilst still preventing both acute and chronic rejection. This can be achieved through the induction of deletional tolerance via the establishment of mixed hematopoietic chimerism (251). Considering that T cell output from the thymus relies on continual seeding by BM precursors (25), it is possible to supply donor-derived hematopoietic precursors to induce a state of mixed chimerism; the co-existence of donor and host haematopoietic cells in the same tissue. If the haematopoietic cells are matched to donor tissue, the thymus provides all of the necessary attributes to "teach" the body to accept the donor graft, essentially re-programming the immune system for the life of the recipient (Figure 1.4)

Mixed chimerism

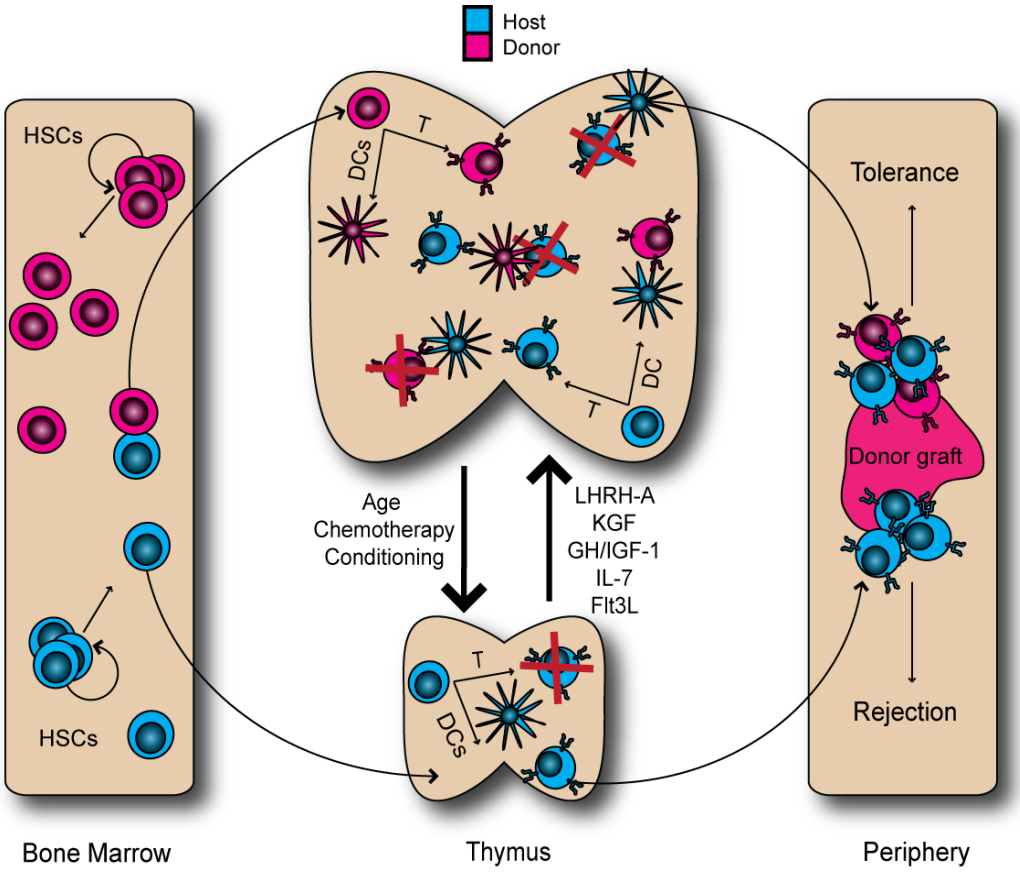
Mixed chimerism has been used successfully in many rodent models for the specific induction of allogeneic (tissue derived from genetically disparate MHC molecules) tolerance. Owen first demonstrated this process over 60 years ago, showing that a mixture of two genetically distinct erythrocytes can be found long after the birth in fraternal bovine twins, which had shared a common maternal circulation (252). Shortly after, Medawar and colleagues demonstrated that skin grafts between these bovine chimeric twins were accepted indefinitely, indicating that each had acquired tolerance to the others tissue (253). Following on from this discovery, Billingham and colleagues showed that this form of tolerance could be actively induced between MHC-disparate mice, provided the skin graft recipient had been exposed to donor antigen in the neonatal stages of development (254).

In the context of a bone marrow graft, the establishment of mixed chimerism has been relatively simple to achieve in adult mouse models, with the proviso that successful engraftment of HSCs can be achieved and the existence of an active thymus. Recipients are given a bone marrow transplant (BMT) or hematopoietic stem cell transplant (HSCT) from an allogeneic donor that is MHC-matched to the tissue (such as skin) to be transplanted. These cells engraft in the BM and differentiate to lymphoid and myeloid progenitors. T cells and DC precursors from both host and donor migrate to the thymus whereby the process of negative selection purges the emerging T cell pool of host-reactive, as well as donor-reactive, T cells (251, 255). When the appropriate immunosuppressive regimes are applied to eliminate pre-existing mature alloreactive T cells in the periphery, tolerance to fully MHC-mismatched skin grafts can be consistently achieved (256) (Figure 1.4). This phenomenon has also been achieved in the clinical setting, where patients who have received BMT to treat haematological malignancies subsequently became tolerant to skin (257) and kidney (258) transplants originating from the same donor, with the additional contribution of newly produced thymus derived-donor specific Tregs (259).

Host-derived donor-specific Tregs are also involved in the induction and maintenance of allogeneic tolerance via their ability to promote donor cell engraftment (260) and prevent both allogeneic graft rejection (261) and GvHD (262, 263), indicating that they play an important role in the long-term survival of the graft by providing a natural, more specific means of immunosuppression (264). Furthermore, donor-derived Tregs have been implicated in promoting donor cell engraftment and chimerism following BMT. Tregs are induced from donor-derived $CD4^+$ T cells by plasmacytoid DC precursors known as facilitating cells, which are carried over from the BMT, inducing donor specific tolerance in the absence of GVHD (180, 265-267).

To allow donor BM cells or HSCs to engraft within the recipient, existing mature alloreactive T cells must be eliminated and "space" must be created within the recipient BM. This process is referred to as conditioning. To induce a permanent state of immunological tolerance, cells that have engrafted in the BM must have lifelong multi-lineage repopulating ability in order to provide the thymus with a constant source of donor antigens. The simplest and most reliable method for creating "space" in the BM is lethal total body irradiation (TBI) prior to transplantation of T cell depleted BM. The dose of TBI is both myeloablative, allowing space for donor BM to engraft, and immunosuppressive, eliminating T cells that respond to donor antigens and initiate host versus graft disease (HvGD). This approach successfully induces tolerance to fully MHC-mismatched allogeneic and xenogeneic donor antigens (251). Although highly successful in both small and large

Figure 1.4: Tolerance to an allogenic graft can be induced by generating hematopoietic chimerism. Host cells are shown in blue and donor cells are shown in pink. Following appropriate BM conditioning, transplanted donor HSCs will engraft in the host bone marrow (BM), producing T cell and DC precursors, which migrate to the thymus. In the thymus precursors differentiate into T cells, Tregs and DCs. Reactive cells are deleted upon encounter with their cognate self-antigen presented by thymic epithelial cells (TEC) as well as host and donor-derived DCs, a process known as negative selection. T cell output is significantly hindered in the atrophied thymus, which occurs through ageing, chemotherapy and BM conditioning. Thymic atrophy can be reversed through the ablation of sex steroids and/or the provision of growth factors. Educated T cells along with both host and donor-derived Tregs migrate to the periphery, where tolorogenic host and donor-derived Tregs induce anergy in any reactive T cells that have escaped negative selection, promoting tolerance and graft acceptance.


Host
Donor
HSCs
HSCs
DCs
T
DC
T
Age
Chemotherapy
Conditioning
LHRH-A
KGF
GH/IGF-1
IL-7
Flt3L
T
DCs
Tolerance
Donor graft
Rejection
Bone Marrow
Thymus
Periphery

animal models, the clinical translation of such protocols has been hampered by toxicities associated with recipient preconditioning, which involves lethal/sub-lethal doses of irradiation and/or chemotherapy and a concern for the development of graft versus host disease (GvHD).

Low-intensity conditioning

While initial studies of tolerance induction provided a proof-of-principal that mixed chimerism could induce donor-specific tolerance, the TBI used to condition the BM to receive HSCT induced toxic side effects in the transplant recipients (251). Although the use of lethal doses irradiation and chemotherapy are acceptable for the treatment of malignant disease, their use cannot be justified in patients requiring only an organ transplant. Therefore, considerable effort has been devoted to the development of non-lethal low-intensity conditioning regimes (268, 269).

In initial studies, Cobbold and colleagues showed that non-lethal TBI (at least 6 Gy) in combination with T cell depletion *in vivo* was sufficient to allow MHC-mismatched allogeneic BM to engraft in recipients and induce donor-specific tolerance (270). Sharabi and colleagues further improved this protocol by showing that T cell-depleting antibodies removed peripheral T cells, but not thymic T cells and the addition of 7 Gy thymic irradiation (TI) allowed the dose of TBI to be reduced to 3 Gy (268). Early studies also showed that mixed chimerism based tolerance requires continual education of T cells by the thymus and that established tolerance could be abrogated when donor-derived cells were depleted with donor-MHC specific depleting antibodies (271). In these studies, tolerance was established in euthymic mice and then broken by depleting cells that expressed donor-MHC, therefore removing the source of donor antigen required by the thymus to produce tolerant T cells. A loss of tolerance corresponded with the emergence recent thymic emigrants (RTE) in the periphery that expressed donor-reactive TCRs. Thymectomising mice prior to the depletion of donor cells preserved tolerance, because no new donor-reactive RTEs could be produced (271). These protocols have had success in both nonhuman primate models and clinical trials (272-276).

Other studies into low-intensity conditioning regimes attempted to replace thymic irradiation and T cell depletion via the implementation of co-stimulation blockade following cytoreductive conditioning. This strategy, instead of depleting T cells, rendered them anergic and therefore non-responsive when encountering donor-derived antigens (277). Clinically, these regimes were attractive because T cell recovery from the thymus following conditioning may be slow, and further exacerbated by ageing (discussed in detail below). Co-stimulation blockade was achieved via

CD40L monoclonal antibodies and CTLA4-immunoglobulin (278-283). Additionally, Co-stimulation blockade in the absence of cytoreductive conditioning could achieve BM engraftment, chimerism and tolerance, when supraphysiological doses (an approximate 12-fold increase) of BM were administered (284, 285). The feasibility of BMT and co-stimulation blockade was further increased when a short course of a conventional immunosuppressive drug, such as busulfan, was added to the protocol (279). However, despite the success of these protocols in small animal models, translation to nonhuman primates and clinical trials have been hampered by the feasibility of high-dose BMT and thromboembolic complications associated with CD40L blockade (286). The current clinical practice for reduced intensity conditioning regimes includes chemotheraputic drugs, such as fludarabine, busulfan or cyclophosphamide and/or a low-dose of total body irradiation (TBI) and some protocols include the use of T cell depletion with anti-thymocyte globulin (ATG) or anti-CD52 (Campath) (287).

Immunosenescence

A problem that is frequently overlooked with the above approach is that the thymus, the principal organ responsible for generating a pool of T cells tolerant to both donor and host tissue, undergoes severe atrophy with age. Thymic function is most active during the foetal stages of development, with a decline in function evident from as early as the first year of human life. Thymic degeneration is progressive and most apparent at puberty, with approximately 95 % of thymus function lost by 50 years of age (288).

Thymic atrophy

This age related thymic involution is characterised by gradual changes in the thymic microenvironment, including a loss of distinction between cortical and medullary regions, extensive vacuolisation of epithelial cells and the replacement of thymopoietic tissue with perivascular spaces and adipose deposits (288). Detailed analysis of thymic stromal subsets has revealed an increase in the proportion of non-TECs such as fibroblasts and a decrease in TEC number and proportion, in particular the mTEC subset (289). In addition to thymic stromal changes, there is a decline in early T cell progenitors, which also display a reduced capacity for differentiation (290).

Together these processes result in a decreased production and export of naive T cells from the thymus, leaving homeostatic proliferation in the periphery to compensate for this loss. As T cell maintenance in the elderly relies on the expansion of mature T cell subsets rather than the

production of naive RTEs, the diversity of the T cell pool undergoes a bias towards antigens that have already been encountered by the immune system. Within this constricted TCR repertoire, the likelihood of matching the appropriate TCR to novel antigenic epitopes decreases, ultimately limiting the immune system's ability to recognise and respond to unfamiliar challenges and predisposing the individual to the development of autoimmunity (291).

The mechanisms behind thymic atrophy are unclear and several factors have been implicated. In humans, a reduction of thymic mass starts at one year of age and becomes most evident at puberty (292, 293, 294, 287, 295). In mice, a diminished capacity to promote proliferation has been noted as early as 2 weeks after birth and a reduction in thymic size is visible approximately 6 weeks after birth (292, 296). These findings have lead to the hypothesis that thymic involution begins at puberty, where increased circulation of sex steroid hormones marks the beginning of a significant steady degradation of thymic tissue (288, 297). Additionally, the thymus undergoes a transient involution in response to stress of pregnancy, induced by an increase in glucocorticoids or sex steroids respectively (298, 299). Thymic recovery after stress and post partum is rapid, unlike age-related involution, which is chronic (300, 301).

Physical castration performed on mice demonstrates a profound thymic hypertrophy that is reversible upon testosterone injection, confirming the observational link between the onset of puberty and thymic atrophy (297, 302, 303). Further studies have revealed that the thymus is influenced by positive and negative feedback loops operating between the hypothalamus, pituitary gland, gonads, thymus and bone marrow (304) (Figure 1.5).

Sex steroid production begins in the hypothalamus, which releases lutenising hormone-releasing hormone (LHRH, also known as gonadotrophin-releasing hormone GnRH). LHRH is detected by LHRH-receptors in the anterior pituitary gland, leading to the subsequent production of luteinising hormone (LH) and follicle stimulating hormone (FSH). LH and FSH then stimulate the gonads to produce testosterone and estrogen (305). The sex steroids then feedback to the hypothalamus and directly regulate the production of LHRH, LH and FSH (Figure 1.5). Sex steroids and LHRH have an opposing effect on the immune system; sex steroids dampen thymic activity, thymopoeisis and B cell production, while LHRH imposes a directly stimulatory effect on lymphocytes (306, 307). As such, castration has a positive effect on the immune system by both removing sex steroids from the feedback loop and potentiating the effect of LHRH on lymphocyte function.

Tregs and ageing

Despite decreases in thymic output with age, the proportion and number of $CD4^+$ $FoxP3^+$ Treg cells increases with age in both mice and humans (308, 309). This is primarily due to the increased resistance of aged Tregs to apoptosis (310). Additionally, the level of both *in vitro* and *in vivo* suppression achieved by these aged Tregs is equal to, if not better, than their young counterparts (308, 311-313). Increases in Treg populations with ageing leads to an increase in Treg mediated suppression, increasing the susceptibility of the elderly to infectious diseases and cancer (308, 314).

Impacts of ageing on the establishment of chimerism

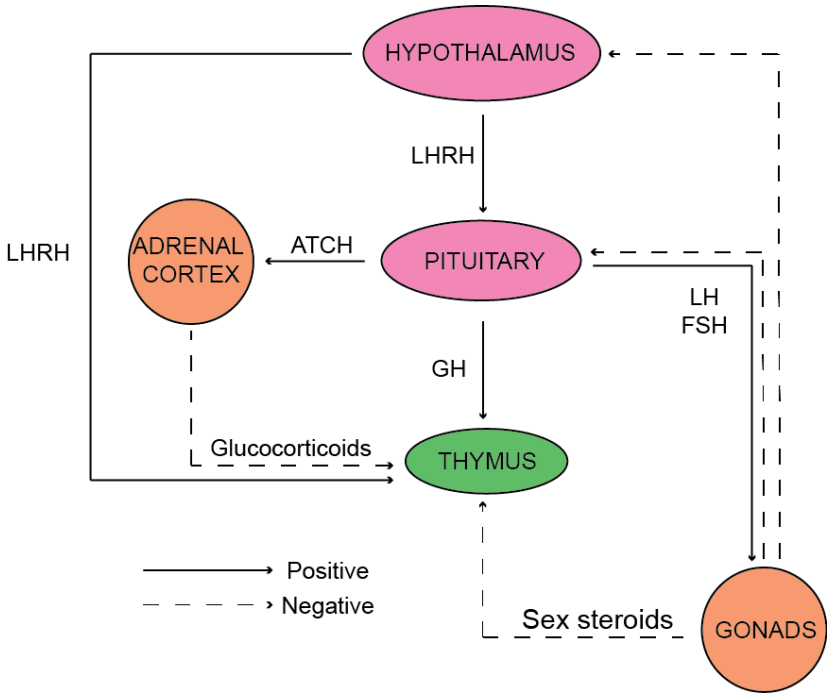
One of the fundamental requirements for the induction of tolerance via mixed chimerism is a functional thymus (271, 315). Age-related thymic atrophy may therefore present a significant challenge for the development of chimerism-based approaches for the induction of allogeneic tolerance.

Contributing further difficulties to the induction of transplantation tolerance via mixed chimerism in the aged immune system is the poor prognosis of patients undergoing BMT. Allogeneic BMT is relatively safe and more effective in younger patients (316, 317). However, with increasing age (patients >55-60 years of age), allogeneic BMT is associated with a poorer prognosis and an increase in complications including poor stem cell engraftment, the development of GVHD, increased incidence of infections and prolonged immune deficiency (316, 318-320). Differences in the aged host environment including the atrophic thymus and poorer humoral and cell-mediated immunity may underlie the limitations of immune recovery in adults (321, 322). It is therefore logical to couple strategies that manipulate thymic selection mechanisms to induce allogeneic tolerance to strategies that can restore thymic function. Several pre-clinical and clinical models to target thymic regeneration have been proposed. These include hormone blocking therapies (described in detail below); administration of growth factors such as keratinocyte growth factor (KGF) (323-326), growth hormone (327-329), IL-7 (330-333) and FMS-like tyrosine kinase 3 ligand (Flt3L) (334, 335); the *de novo* generation of thymic tissue via a thymic epithelial progenitor cell (13, 336); or more recently, by the use of embryonic stem cells (337).

Thymic regeneration via sex steroid ablation

Evidence for residual thymic function, albeit very limited, in aged individuals gives credence to the possibility of inducing thymus regeneration *in vivo* via the removal of inhibitory factors. Of the

Figure 1.5: The neuroimmunoendocrine axis. The thymus is negatively influenced by sex steroids and glucocorticoids and positively influenced by LHRH and GH. LH and FSH may also be immunostimulatory. LHRH release from the hypothalamus is pulsative, due to negative feedback from the gonads.


HYPOTHALAMUS
LHRH
LHRH
ADRENAL CORTEX
ATCH
PITUITARY
LH
FSH
GH
Glucocorticoids
THYMUS
Positive
Negative
Sex steroids
GONADS

former, sex steroids have been strongly linked to thymic atrophy, since they have considerable effects on both lymphoid development and immune function (288). Sex steroids exert direct effects on thymic stromal cells, which express sex steroid receptors on their surface (338). Consequently, the removal of sex steroids through castration (chemical or surgical) is associated with marked rejuvenation of the thymic microenvironment in aged mice following chemically induced thymic damage (339). This is evident in both thymic architecture (289) and thymic cellularity (339, 340). Specifically, regeneration is demonstrated by the restoration of both cortical and medullary regions, TEC to fibroblast ratios, as well as TEC and thymocyte numbers. Importantly, sex steroid ablation (SSA) increases thymic export of functional naive T cells, resulting in enhanced viral clearance and cytolytic activity upon infection (341), and a decrease in the number of inducible tumours observed in aged SSA mice (342).

Additionally, these improvements correlate with an increase in BM lymphopoeisis. In particular, an increase in the number of IL-7 responsive progenitor cells, an increase in B cell export, as well as enhanced B cell function (338), therefore contributing to an overall improvement in immune competence (343, 344). The clinical relevance of SSA is further demonstrated by the accelerated recovery from chemotherapy and irradiation induced damage (340, 341, 345, 346). Of particular relevance to donor-derived tolerance induction, the removal of sex steroids has also shown to improve engraftment in the BM, and peripheral reconstitution following allogeneic BMT (347).

SSA can be achieved in a reversible manner with the use of a LHRH agonist (LHRH-A). These analogues cause a flare in SSA production, resulting in a sensitisation and down regulation of sex steroid receptors in the hypothalamus and a shutdown of sex steroid production (304). This process is reversed upon cessation of the treatment. Clinical application of SSA in the context of immune regeneration has been demonstrated by improved thymic and immune recovery of LHRH-A-treated patients who received autologous or allogeneic HSCT for the treatment of haematological malignancies (341). Most importantly, in the allogeneic setting, SSA increased the rate of BM engraftment with no evidence of GVHD (347). Collectively, these studies suggest that transient SSA could be used as a prophylactic therapy to enhance post-transplant immune reconstitution in allogeneic HSCT/BMT, as it is known that the successful implementation of mixed chimerism induced tolerance requires a functional thymus (271, 315, 348).

Although these studies were focused on enhancing HSCT recovery in fully allogeneic chimeras following myeloablative conditioning, there is great potential for SSA to be used in the induction of

mixed allogeneic chimeras following non-myeloablative conditioning for transplantation tolerance. Following an alternative strategy, Zhao and colleagues have shown that the ability to induce allogeneic tolerance with CD45RB monoclonal antibody treatment is lost in aged mice (349). Although this protocol does not induce allogeneic tolerance via BMT, significantly, CD45RB monoclonal antibody mediated allogeneic tolerance induction is reliant on a functional thymus to produce antigen-specific Tregs (350). Formal proof of this concept was demonstrated in experiments where thymic regeneration was instituted by surgical or chemical castration, which subsequently restored the ability of recipient aged mice to be tolerized with CD45RB monoclonal antibody (349). If SSA can increase thymic seeding by donor progenitor cells and thymic output of donor-tolerant naive T cells, this strategy could complement BMT protocols for the generation of hematopoietic chimerism for tolerance and may play a pivotal role in the development of more clinically applicable regimes for the induction of mixed chimerism and transplantation tolerance in aged individuals (Figure 1.4).

Conclusion

The concept of re-educating the immune system and establishing transplant tolerance through mixed chimerism is highly appealing, as it allows permanent survival of a transplant without the need for chronic immunosuppression. Current methods used to prevent organ rejection focus on long-term immunosuppression, are associated with adverse side effects and, in some instances, can ultimately lead to graft rejection (250). The induction of donor-specific tolerance via mixed haematopoietic chimerism removes the need for chronic immunosuppression and has demonstrated preclinical success and clinical promise for replacing immunosuppressive drugs. However, strategies for inducing mixed chimerism and allogeneic tolerance have rarely been tested in aged recipients, who comprise the largest proportion of transplant recipients. This is an important issue as thymic atrophy and immunosenescence may pose a major barrier to tolerance induction. Furthermore, recipient preconditioning required for bone marrow transplantation is associated with severe toxicities that have precluded the clinical translation of such protocols in non-malignant conditions. With these restrictions in mind, this thesis aimed to develop a low-intensity conditioning regime, to induce mixed chimerism and allogeneic tolerance, and examine the ability to apply such protocols to the aged immune system.

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CHAPTER TWO

Reduced-intensity conditioning coupled to sex steroid ablation positively impacts lymphocyte output following autologous hematopoietic stem cell transplantation

Abstract

This study aimed to assess if sex steroid ablation (SSA) coupled to busulfan-mediated conditioning could enhance the bone marrow (BM) to receive hematopoietic stem cell (HSC) transplants. Sex steroid ablation (SSA) has been shown to enhance immune reconstitution following autologous and allogeneic HSC transplantation (HSCT) following myeloablative BM conditioning regimes. Here we hypothesised that SSA would boost donor hematopoietic cell engraftment allowing the dose of busulfan to be decreased. Busulfan was found to deplete progenitor cells in the BM as well as the thymus, in the absence of any diminishing effects on lymphocytes and monocytes in the periphery. Importantly, SSA did not abrogate the depletion of BM precursors following chemotherapy, but did protect thymocytes from chemotherapy-mediated damage. A linear correlation between donor cell chimerism and transplant cell dose was observed in SSA and sham-treated groups indicating that SSA did not enhance the recipient BM microenvironment or confer a selective growth advantage on incoming donor cells. However SSA did induce a significant increase in thymic cellularity, which correlated with an expansion in the number of donor-derived T cells, indicating that SSA could be coupled to low-dose busulfan to boost T cell output following congenic HSCT.

Key words: busulfan, HSCT, sex steroids, non-myeloablative

Introduction

Haematopoietic stem cell transplantation (HSCT) has become a standard therapy for many inherited and acquired disorders of the immune system. Both autologous and allogeneic HSCT have been utilised in the treatment of haematological malignancies and autoimmune diseases. Furthermore allogeneic HSCT has been widely investigated as means to induce tolerance in immunosuppression-free solid organ transplants. However recipient pre-conditioning for HSCT is a complex procedure, involving lethal or sub-lethal doses of irradiation and/or chemotherapy, leading to a number of treatment-associated morbidities. These include opportunistic bacterial, viral and fungal infections, resulting from sever immuno-depletion, gastro-intestinal toxicity, such as mucositis and in the case of allogeneic HSCT, the development graft versus host disease (GvHD), which can be lethal. These resultant, conditioning-associated states have hindered further development of HSCT for the treatment of autoimmune disorders, immunodeficiency and the induction of allogeneic tolerance; as such side effects cannot be justified in the absence of malignant disease (1-3). For these reasons, low-intensity conditioning regimes have been the subject of intense research.

Despite clinically applicable conditioning regimes varying in intensity, they all result in some form of post-transplant immunosuppression. A delay in immune reconstitution can not-only lead to opportunistic infections, but may also result in reactivation of latent infections, relapse of original disease or development of new, secondary malignancies (4, 5). Therefore a reduction in treatment-associated morbidities can be achieved through swift reconstitution of the recipient's immune system; demonstrating strategies should focus on improving conditioning-related immune-deficiency to enhance patient outcomes (6).

An attractive agent to address this problem is busulfan, a bifunctional alkylating agent typically used in high-dose-chemotherapy conditioning regimes (7), as lymphoid reconstitution following high-dose busulfan is prompt and not associated with lasting lymphocyte defects (8). The alkyl groups on busulfan (1,4-di (methanesulfonoxy) butane) binds-to and cross-links DNA, resulting in significant myelosuppression (9) but not immunosuppression (10). Preclinical and clinical studies that substitute total body irradiation (TBI) for busulfan in combination with cyclophosphamide-mediated immunosuppression show efficient engraftment and host reconstitution following both autologous and allogeneic bone marrow transplants (BMT) (10). Additionally post-transplant reconstitution by donor lymphocytes, in mice given high-dose busulfan and autologous HSCT without immunosuppression, is equivalent to that observed following TBI (11). However, doses of

busulfan lower than 20 mg/kg show slower and incomplete reconstitution (11, 12). Studies conducted in our laboratory have shown that sex steroid ablation (SSA) enhances recovery of the hematopoietic system and increases donor cell output following both autologous and allogeneic HSCT in both mice and humans (13-15). Considering this, it was hypothesised that SSA prior to conditioning with low-dose busulfan would enhance engraftment of donor cells in the BM, and boost donor cell output in the thymus and the periphery allowing lower-doses of busulfan to be used in HSCT.

Methods

Animals

Male C57BL/6 (H-2^b, Ly5.2) and B6.SJL-ptprc (H-2^b, Ly5.1) were purchased at 6-8 weeks of age form Monash Animal Services (Melbourne, Australia), the Walter and Elisa Hall Institute (Melbourne, Australia) or from the Animal Resources Centre (Perth, Australia). Animals were housed under specific pathogen-free conditions (SPF) at the Monash University Animal Research Laboratories. All animal experiments were performed in accordance with the Australia Code of Practice for the Care and Use of Animals for Scientific Purposes (2004, 7th edition), after approval by the Monash University School of Biomedical Sciences Animal Ethics Committee.

Conditioning and hematopoietic stem cell transplantation

For chemotherapy experiments, mice received a single intra-peritoneal (IP) injection of busulfan, at a dose of 10 mg/kg or 20 mg/kg (Bulsulfex, PDL BioPharma, Fremont, CA). For hematopoietic stem cell transplantion (HSCT) experiments, mice received a single IP dose of 10 mg/kg busulfan 4 days prior to transplantation with either 5 x 10^4 (high-dose) or 1 x 10^4 (low-dose) Lineage negative $Sca1^+$ $cKit^+$ (LSK) progenitor cells, administered via lateral tail vein injection. LSK progenitor cells were purified from single cell suspensions of BM mononuclear cells flushed from the femurs and tibiae of C57Bl/6 (congenic) mice and sorted on a BD/Cytopeia Influx II flow cytometer (BD Bioscience, Franklin Lakes, N.J).

Sex steroid ablation

Male mice were anaesthetized with Isofluorane (2-3% in Oxygen) (Delvet, NSW, Australia) and a small scrotal incision was made to reveal the testes. The testes were tied-off and removed along with the surrounding fatty tissue. The wound was closed using sutures. Sham-sex steroid ablated (SSA) mice followed the same surgical procedure, except for the tying-off and removal of the testes. SSA was performed 1 day prior to chemotherapy.

Cell preparation

Single cell suspensions of thymocytes or splenocytes were prepared via manual mincing between frosted glass slides. BM mononuclear cells were flushed from the femurs and tibiae with cold FACS buffer (phosphate buffered saline (PBS)/1% v/v fetal calf serum (FCS)/ 0.1% w/v sodium-

azide) using a 23G needle. Cells were pelleted at 350_{gmax} for 5min at 4˚C. Red blood cells (RBC) were removed by resuspending cell pellets in lysis buffer (0.9 % w/v ammonium chloride/ 10 % v/v 0.1M Tris-hydrochloride/pH 7.5 ±0.2) at room temperature for 1 minute. Cells were washed in FACs buffer and 3 x 10^6 cells were incubated for 30 minutes at 4^oC with primary antibodies, and then washed twice with FACs buffer.

Cell counts were determined by gating on viable cells based on size using a Z2 Coulter Counter (Bekman Coulter).

Flow cytometric analysis

LSK progenitor populations in the BM were assessed via staining with a PE-conjugated lineage cocktail consisting of CD3 (145-2C11), CD4 (RM4-5), CD8 (53-6.7), CD19 (1D3), NK1.1 (PK136), CD11b (M1/70), Gr-1 (RB6-8C5), TER-119 (TER-119), and CD11c (HL3) in addition to APC-conjugated CD117 (cKit) (2B8) and PECy7-conjugated Sca1 (D7). T cell populations were assessed via PerCP Cy5.5-conjugated CD8 (53-6.7), PeCy7-conjugated CD4 (RM4-5, eBioscience), and APC-conjugated TCRβ (H57-597). Peripheral lymphoid and myeloid cells in the spleen were assessed via PerCP Cy5.5-conjugated CD45R (B220) (RA3-6B2) and APC-conjugated CD11b (M1/70). For all staining an FcR block (2.4G2, laboratory produced) was used. Unless otherwise stated all antibodies were obtained from BD Biosciences. All flow cytometry experiments were performed on a BD FACSCanto II (BD Bioscience, Franklin Lakes, N.J).

Statistical analysis

Statistical analysis was performed using an unpaired, two-tailed t test. A p value ≤ 0.05 was considered to be significant.

Results

Low-dose Busulfan depletes BM progenitor cells and thymocytes, without depleting peripheral lymphocyte and monocyte populations.

Conditioning prior to HSCT is required to generate space in the BM for transplanted cells to engraft. To assess the hematopoietic recovery in response to low or high dose chemotherapy, mice received a single dose of either 10 or 20 mg/kg busulfan (12). To determine at which point the BM cell nadir occurred, cell populations within the BM were analysed via flow cytometry 1, 4 and 7 days following chemotherapy. Both 10 mg/kg and 20 mg/kg busulfan depleted BM progenitor cells, defined as Lineage negative, $Sca1^+$ $cKit^+$ (LSK) (Figure 2.1A and C) without depleting whole BM (WBM) cell numbers (Figure 2.1B). 80-90% of LSK cells had been depleted from the BM 4-days following chemotherapy (Figure 2.1F). LSK cells did not immediately return to homeostatic levels, with progenitor cell proportions and numbers still significantly depleted up-to 7-days following chemotherapy (Figure 2.1A and C). Both 10 mg/kg and 20 mg/kg busulfan depleted $B220^+$ B cells and $CD11b^+$ macrophages in the BM 1-day following chemotherapy, with homeostasis returning within 4-days (Figure 2.1D and E). Interestingly, in mice treated with 10 mg/kg busulfan, B cells were significantly increased compared to both the untreated and sham-SSA control mice 7 days post chemotherapy.

To assess changes in the thymus following low-dose busulfan mediated chemotherapy, thymii from mice treated with 10 or 20 mg/kg busulfan were harvested 1, 4 and 7 days following chemotherapy and analysed via flow cytometry. Unlike in the BM (Figure 2.1), a significant differential effect was observed between the 10 and 20 mg/kg dose. The lower dose of 10 mg/kg had a mild depleting effect 4-days following chemotherapy (Figure 2.2A and B). This was attributed to a decrease in the $CD4^+$ $CD8^+$ (DP) thymocyte subset, with homeostasis returning 7-days following chemotherapy (Figure 2.2B and C). A dose of 10 mg/kg busulfan did not deplete $CD4^-$ $CD8^-$ (DN), $CD4^+$ or $CD8^+$ thymocytes subsets (Figures 2.2D-F). The higher dose of 20 mg/kg busulfan significantly depleted thymocytes immediately following chemotherapy. This was observed across all thymocytes subsets; with thymocytes remaining significantly depleted 7-days following chemotherapy (Figure 2.2A-F).

To analyse the effects of busulfan on peripheral immune cells, the spleens from mice treated with 10 or 20 mg/kg busulfan were harvested 1, 4 and 7 days following chemotherapy. At the lower dose of 10 mg/kg, no significant changes were observed in the total number of splenocytes (Figure 2.3A)

Figure 2.1: Low-dose busulfan depletes progenitor cells in the BM, without depleting WBM cell numbers. (A) Flow cytometric plots showing the proportion of Lineage negative $Sca1^+$ $cKit^+$ (LSK) progenitor cells in the BM following administration of busulfan at 10 mg/kg and 20 mg/kg. As a control BM from an untreated (UT) mouse is shown. Figures above boxes represent the proportion of LSKs. **(B)** Quantification of total BM cellularity and absolute number of **(C)** LSK, **(D)** $B220^+$ and **(E)** $CD11b^+$ cells following administration of busulfan at 10 mg/kg and 20 mg/kg. **(F)** The proportion of LSK progenitor cells depleted from the BM, calculated as a ratio of total LSK cells in treated mice compared to untreated mice, and expressed as a percentage. White bars indicate untreated mice, grey bars indicate 10 mg/kg busulfan and black bars indicate 20 mg/kg busulfan. Data is representative of three individual experiments, expressed as mean ± SE and analysed using an unpaired, two-tailed T-test. * ($p< 0.05$), ** ($p< 0.01$) represent significant differences compared to untreated mice. ^ ($p< 0.05$), ^^ ($p< 0.01$), ^^^ ($p< 0.001$) represent significant differences when comparing sham-SSA and SSA groups.

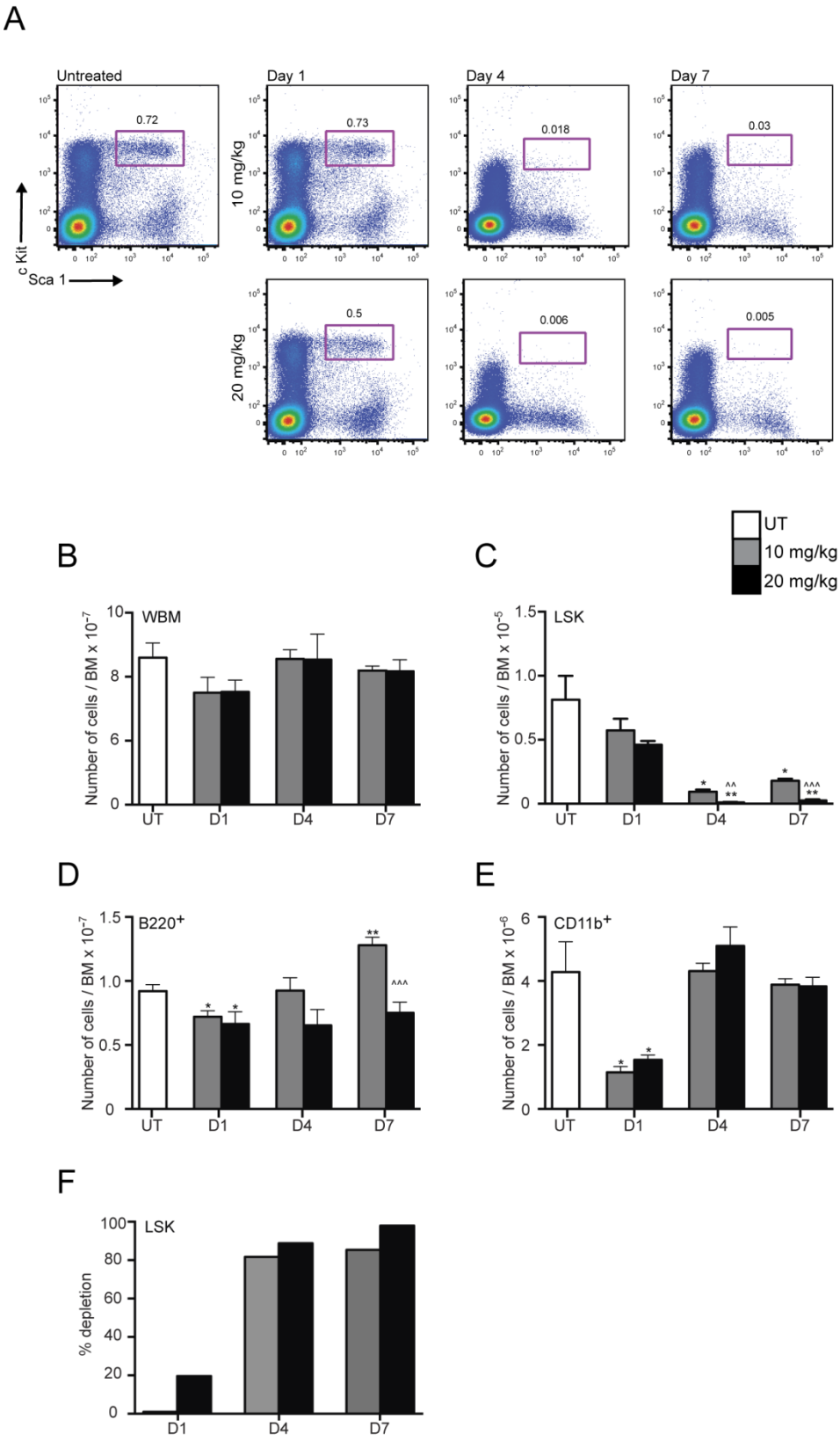
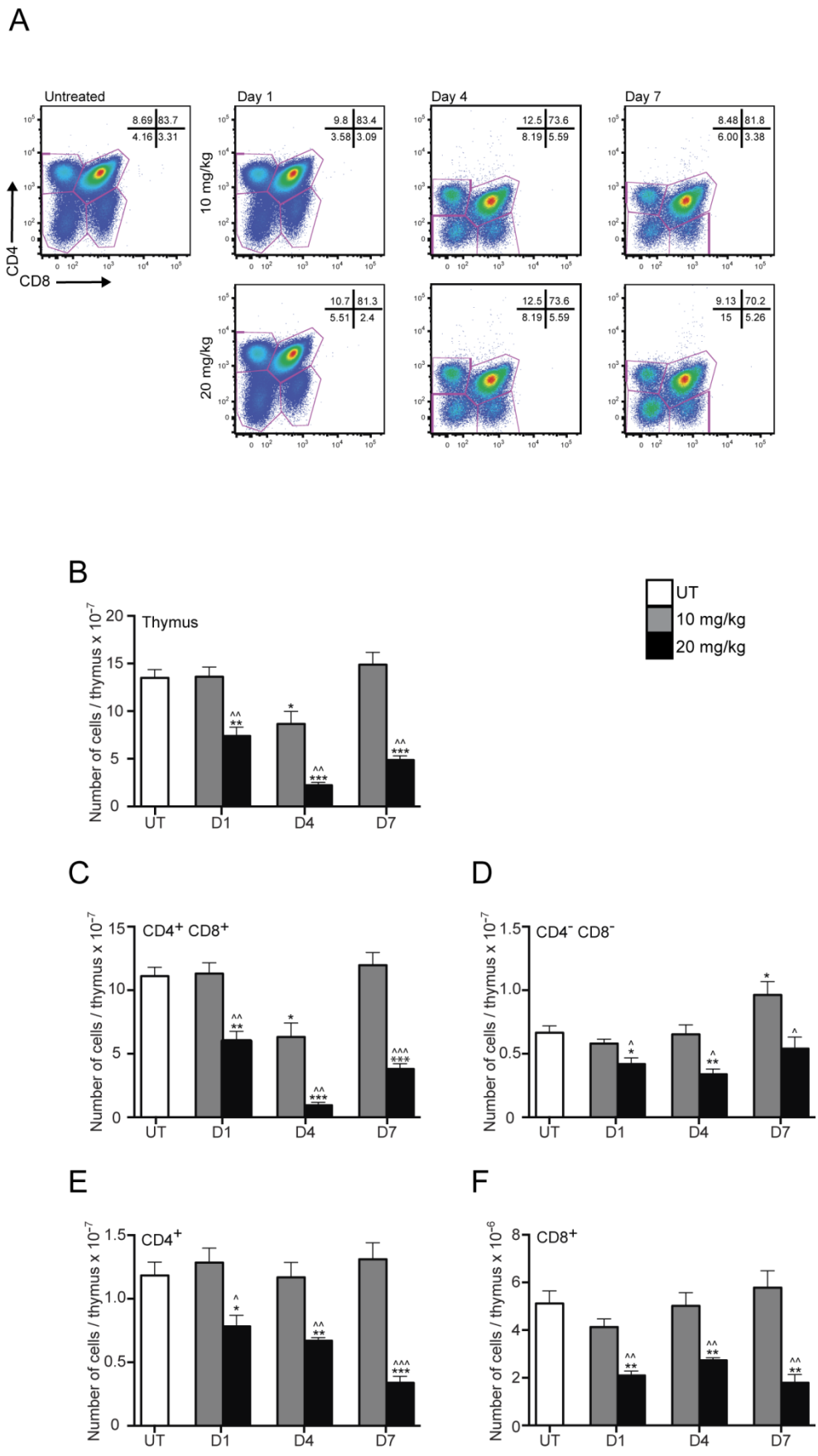

A
Untreated
Day 1
Day 4
Day 7
0.72
0.73
0.018
0.03
10 mg/kg
0.5
0.006
0.005
20 mg/kg
c Kit
Sca 1
B
WBM
Number of cells / BM x 10⁻⁷
UT
D1
D4
D7
C
LSK
Number of cells / BM x 10⁻⁵
UT
10 mg/kg
20 mg/kg
D
B220⁺
Number of cells / BM x 10⁻⁷
E
CD11b⁺
Number of cells / BM x 10⁻⁶
F
LSK
% depletion

Figure 2.2: Low-dose busulfan depletes thymocytes. (A) Flow cytometric plots showing the proportion of thymocyte subsets, expressing CD4 and CD8 co-receptors following administration of busulfan at 10 mg/kg and 20 mg/kg. As a control BM from an untreated (UT) mouse is shown. Figures in quadrants represent the proportions of $CD4^+CD8^+$ double positive, $CD4^-CD8^-$ double negative, $CD4^+$ and $CD8^+$ single positive thymocyte subsets. **(B)** Quantification of the total thymic cellularity and **(C)** absolute number of $CD4^+CD8^+$ double positive, **(D)** $CD4^-CD8^-$ double negative, **(E)** $CD4^+$ single positive and **(F)** $CD8^+$ single positive thymocytes. White bars indicate untreated mice, grey bars indicate 10 mg/kg busulfan and black bars indicate 20 mg/kg busulfan. Data is representative of three individual experiments, expressed as mean ± SE and analysed using an unpaired, two-tailed T-test. * ($p< 0.05$), ** ($p< 0.01$) represent significant differences compared to untreated mice. ^ ($p< 0.05$), ^^ ($p< 0.01$), ^^^ ($p< 0.001$) represent significant differences when comparing sham-SSA and SSA groups.


A
Untreated
Day 1
Day 4
Day 7
8.69 83.7 4.16 3.31
9.8 83.4 3.58 3.09
12.5 73.6 8.19 5.59
8.48 81.8 6.00 3.38
10 mg/kg
10.7 81.3 5.51 2.4
12.5 73.6 8.19 5.59
9.13 70.2 15 5.26
20 mg/kg
CD4
CD8
B
UT
10 mg/kg
20 mg/kg
Thymus
Number of cells / thymus x 10-7
UT D1 D4 D7
C
CD4+ CD8+
D
CD4- CD8-
E
CD4+
F
CD8+
Number of cells / thymus x 10-6

including mature T-cell receptor-expressing $CD4^+$ (Figure 2.3B) and $CD8^+$ T cells (Figure 2.3C), $B220^+$ B cells (Figure 2.3D) and $CD11b^+$ macrophages (Figure 2.3E). At 20 mg/kg, busulfan significantly decreased all these mature cell subsets (Figures 2.3B-E), however cell numbers returned to normal levels by 7 days following chemotherapy.

Collectively, this data indicates that conditioning with either 10 or 20 mg/kg busulfan leads to the depletion of host BM LSK cells. However, in the thymus and periphery, 20 mg/kg is significantly more toxic, depleting both thymocytes and peripheral immune cells. A dose of 10 mg/kg was chosen for further experiments, as it caused minimal short-term effects on the thymus and spleen.

SSA protects the thymus from Busulfan-mediated damage, without abrogating the depletion of progenitor cells in the BM.

The thymus is responsible for controlling and maintaining the T cell compartment following cytoreductive conditioning. To ensure swift T cell reconstitution following HSCT, it is important to have an intact thymus. We have previously shown that SSA enhances immune reconstitution following cytotoxic antineoplastic conditioning with cyclophosphamide (16). To determine if the thymus could be protected from busulfan-mediated damage, mice were sham-SSA or SSA via physical castration 1-day prior to 10 mg/kg busulfan and thymocyte populations assessed 4 days following chemotherapy. Day 4 was assessed, as this is the time at which the LSK progenitor cell nadir occurs (Chapter 3). Similar to previous experiments it was found that the thymus of sham-SSA mice that received 10 mg/kg busulfan were significantly depleted compared to their untreated counterpart (Figure 2.4A and B). SSA prior to chemotherapy protected the thymus from busulfan-mediated damage, significantly increasing thymocyte numbers compared to sham-SSA mice, to a number equivalent to that of untreated mice (Figure 2.4B). This was observed in both the proportion (Figure 2.4A) and absolute number of DP (Figure 2.4C), DN (Figure 2.4D), $CD4^+$ (Figure 2.4E) and $CD8^+$ (Figure 2.4F) thymocyte subsets.

Because space is required to allow transplanted progenitors to engraft in the BM, it is important that SSA prior to busulfan-chemotherapy did not negate the depletion of the LSK subset. In support of previous experiments, LSKs were significantly depleted in sham-SSA mice that received 10 mg/kg busulfan 4-days following chemotherapy, compared to untreated mice (Figure 2.5A and C). Similarly SSA did not abrogate the effects of low-dose busulfan on LSK cells, with the proportions

and number of these progenitors being significantly lower in comparison to untreated mice (Figure 5C). This equated to a loss of 80 % of LSK cells in both sham-SSA and SSA mice (Figure 2.5D). Interestingly both SSA and sham-SSA mice had significantly increased BM derived $B220^+$ B cells (Figure 2.5E) and $CD11b^+$ macrophages (Figure 2.5F) compared to untreated mice. There was also a significant decrease in the WBM cell number observed in both the sham-SSA and SSA busulfan treated mice (Figure 2.5B) this can be attributed to the stress of the surgery as this was not observed in experiments that assessed busulfan treatment alone, even at the higher dose of 20 mg/kg (Figure 2.1B). SSA had no effect on T-cells, B-cells and monocytes in the spleen when mice were analysed 4-days following treatment with 10 mg/kg busulfan (Figure 2.5G-K).

SSA increases the number of donor-derived thymocytes following congenic HSCT.

Previous studies have reported that mice conditioned with 10 mg/kg busulfan prior to transplantation with a mixture of congenic BM and spleen derived stem cells results in a slow and incomplete level of donor cell engraftment that cannot be compared to results achieved with total body irradiation (TBI) and HSCT (11). Because SSA has been shown to enhance immune reconstitution following autologous and allogeneic HSCT (13, 14, 17), we hypothesised that SSA prior to conditioning with 10 mg/kg busulfan would increase donor cell engraftment and host reconstitution when mice are transplanted with congenic LSK progenitor cells.

Conditioning with 10 mg/kg busulfan allowed donor cells to engraft in the BM following transplantation of 5 x 10^4 congenic LSK cells (Figure 2.6A), with approximately 50 % of LSK progenitor cells of donor-origin (Fig 2.6Av) when mice were analysed 28 days following HSCT. Interestingly, SSA induced an expansion of total $B220^+$ cells (Figure 2.6Civ), which was not reflected in the donor $B220^+$ subset (Figure 2.6Cv-vi). No changes were observed in regards to $CD11b^+$ monocytes in response to SSA (2.6Bvii-ix).

Donor-derived LSK progenitor cells had the capacity for both myeloid and lymphoid differentiation, however SSA treatment appeared to have disparate effects in primary and secondary lymphoid organs. For example, there was no change in the total cell number in WBM in SSA mice compared to sham-treated controls (Fig 2.6Ai). This was in contrast to the spleen, where a significant increase in total cell number (Fig 2.6Ci), corresponding to an expansion of T-cells, B-cells and monocytes (Fig 2.6Civ), was demonstrated. Given this observation it was intriguing to note that SSA did not affect the proportion or number of donor-derived hematopoietic cells in the

Figure 2.3: High dose, but not low dose busulfan depletes myeloid and lymphoid cells in the spleen. (A) Total splenic cellularity following administration of busulfan at 10 mg/kg and 20 mg/kg. As a control spleen cells from an untreated (UT) mouse are shown. **(B)** The absolute numbers of TCR^+CD4^+ T cells, **(C)** TCR^+CD8^+ T cells, **(D)** $B220^+$ B cells and **(E)** $CD11b^+$ Macrophages. White bars indicate untreated mice, grey bars indicate 10 mg/kg busulfan and black bars indicate 20 mg/kg busulfan. Data is representative of three individual experiments, expressed as mean ± SE and analysed using an unpaired, two-tailed T-test. * ($p< 0.05$) represents significant differences compared to untreated mice. ^ ($p< 0.05$) represents significant differences when comparing 10 mg/kg and 20 mg/kg busulfan treated groups.

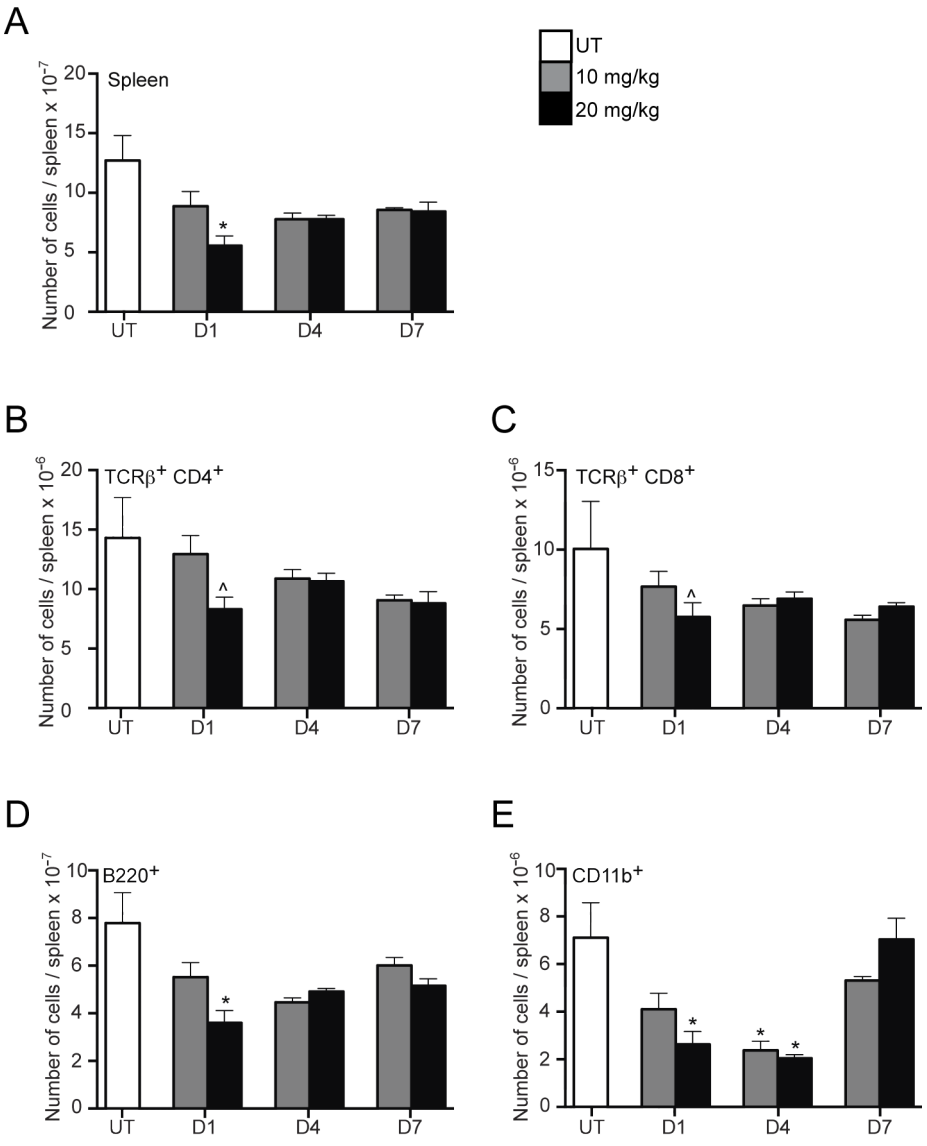
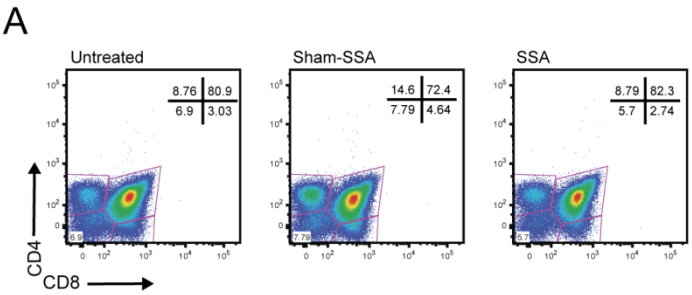
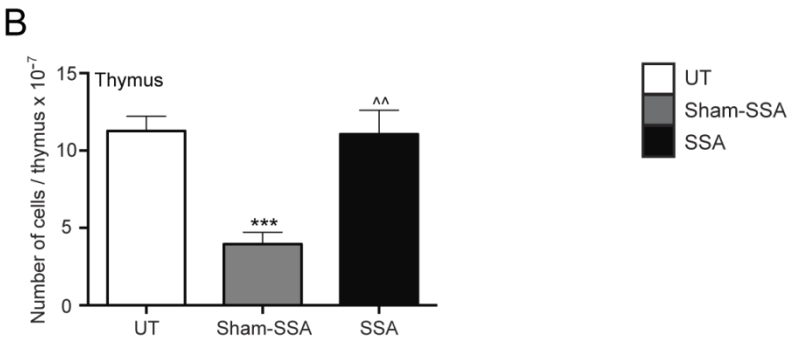
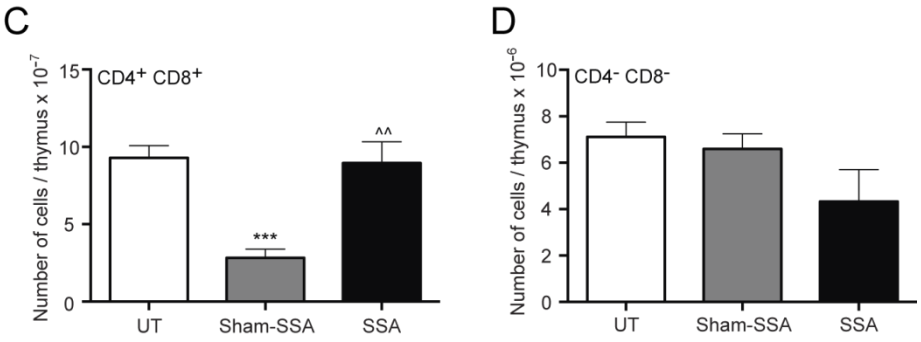

A
UT
10 mg/kg
20 mg/kg
Spleen
Number of cells / spleen x 10^-7
0
5
10
15
20
UT
D1
D4
D7
*
B
TCRβ+ CD4+
Number of cells / spleen x 10^-6
0
5
10
15
20
UT
D1
D4
D7
^
C
TCRβ+ CD8+
Number of cells / spleen x 10^-6
0
5
10
15
UT
D1
D4
D7
^
D
B220+
Number of cells / spleen x 10^-7
0
2
4
6
8
10
UT
D1
D4
D7
*
E
CD11b+
Number of cells / spleen x 10^-6
0
2
4
6
8
10
UT
D1
D4
D7
*
*
*

Figure 2.4: SSA protects thymocytes from low-dose busulfan-mediated damage. (A) Flow cytometric dot plots showing the proportion of thymocyte subsets expressing CD4 and CD8 co-receptors following SSA and administration of busulfan at 10 mg/kg. As a control BM from untreated (UT) mouse is shown. Figures in quadrants represent the proportions of $CD4^+CD8^+$ double positive, $CD4^-CD8^-$ double negative, $CD4^+$ and $CD8^+$ single positive thymocyte subsets. **(B)** Total thymic cellularity and **(C)** absolute number of $CD4^+CD8^+$ double positive, **(D)** $CD4^-CD8^-$ double negative, **(E)** $CD4^+$ single positive and **(F)** $CD8^+$ single positive thymocytes. White bars indicate untreated mice, grey bars indicate sham-SSA and black bars indicate SSA. Data is representative of three individual experiments, expressed as mean ± SE and analysed using an unpaired, two-tailed T-test. * ($p< 0.05$), ** ($p< 0.01$), *** ($p< 0.001$) represent significant differences compared to untreated mice. ^ ($p< 0.05$), ^^ ($p< 0.01$) represent significant differences when comparing sham-SSA and SSA groups.


A
Untreated
Sham-SSA
SSA
8.76
80.9
6.9
3.03
14.6
72.4
7.79
4.64
8.79
82.3
5.7
2.74
CD4
CD8


B
Thymus
Number of cells / thymus x 10⁻⁷
UT
Sham-SSA
SSA
***
^^


C
CD4⁺ CD8⁺
Number of cells / thymus x 10⁻⁷
***
^^
D
CD4⁻ CD8⁻
Number of cells / thymus x 10⁻⁶

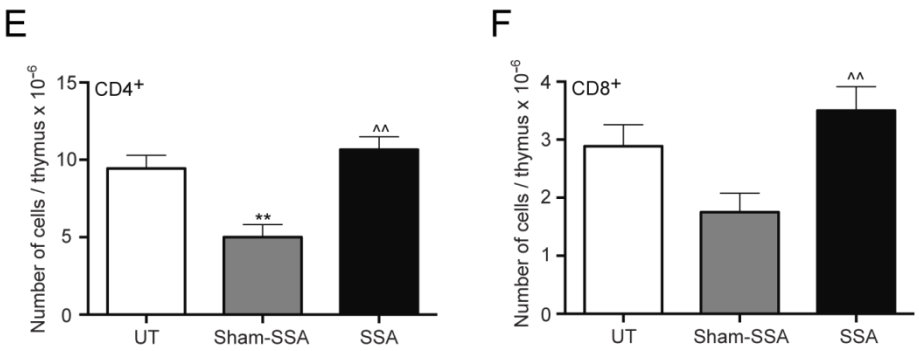

E
CD4⁺
Number of cells / thymus x 10⁻⁶
**
^^
F
CD8⁺
Number of cells / thymus x 10⁻⁶

Figure 2.5: SSA does not protect LSK progenitor cells from low-dose busulfan-mediated damage. (A) Flow cytometric dot plots showing the proportion of Lineage negative $Sca1^+$ $cKit^+$ (LSK) progenitor cells in the BM following sex steroid ablation (SSA) and administration of busulfan at 10 mg/kg. As a control BM from an untreated (UT) mouse is shown. Figures above boxes represent the proportion of LSKs. **(B)** Quantification of total BM cellularity, **(C)** absolute number of LSK progenitor cells and **(D)** proportion of LSK progenitor cells depleted from WBM. **(E)** Absolute number of $B220^+$ B cells and **(F)** $CD11b^+$ macrophages in the BM. **(G)** Total splenocyte number. **(H)** Absolute number of TCR^+CD4^+ T cells, (I) TCR^+CD8^+ T cells, (J) $B220^+$ B cells and **(K)** $CD11b^+$ Macrophages in the spleen. White bars indicate untreated mice, grey bars indicate sham-SSA and black bars indicate SSA. Data is representative of two individual experiments, expressed as mean ± SE and analysed using an unpaired two-tailed T-test. * ($p< 0.05$), ** ($p< 0.01$), **** ($p< 0.0001$) represent significant differences compared to untreated mice. ^ ($p< 0.05$), ^^ ($p< 0.01$) represent significant differences when comparing sham-SSA and SSA groups.

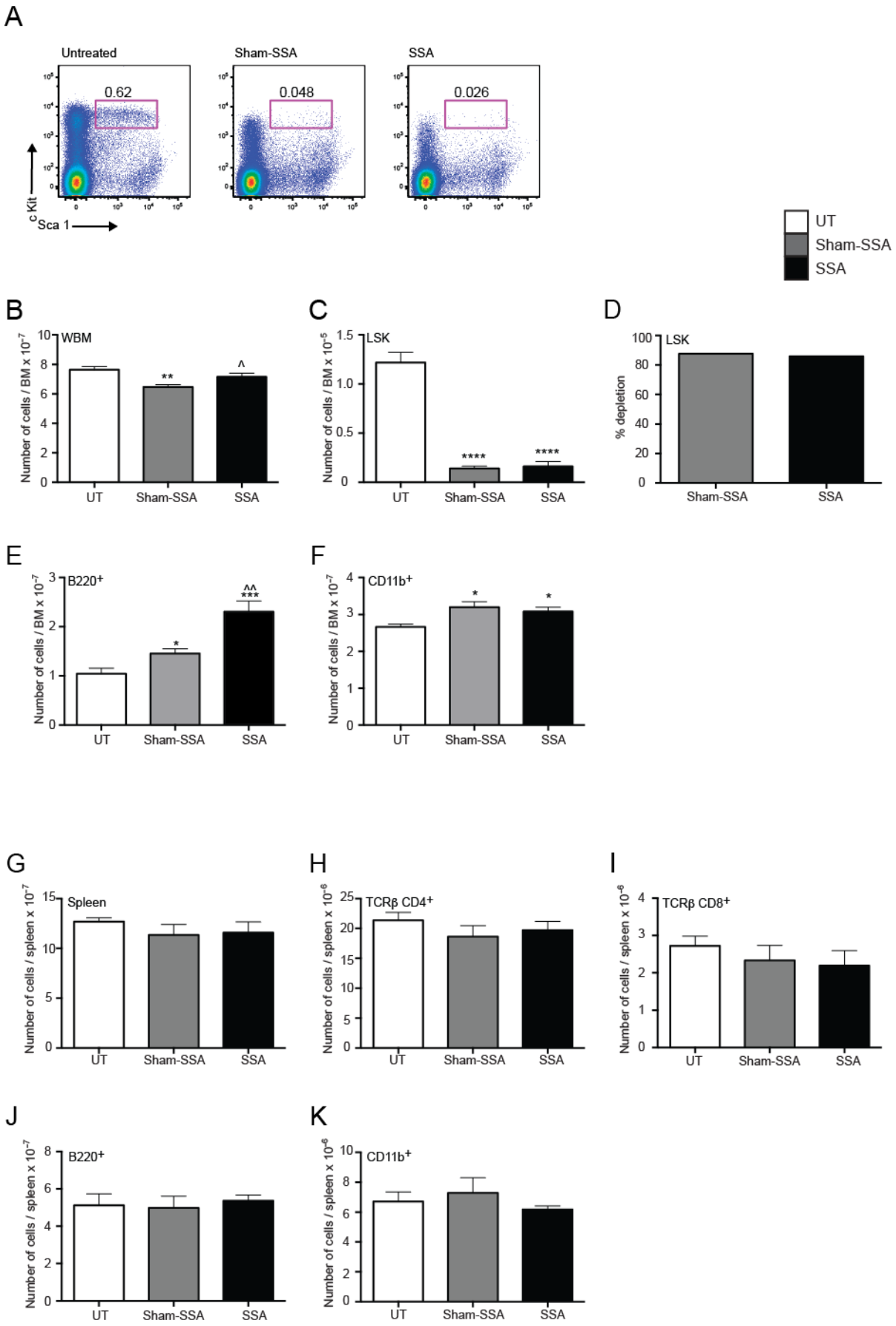

A
Untreated
Sham-SSA
SSA
0.62
0.048
0.026
c Kit
Sca 1
UT
Sham-SSA
SSA
B
WBM
Number of cells / BM x 10⁻⁷
**
^
C
LSK
Number of cells / BM x 10⁻⁵
****
D
LSK
% depletion
E
B220+
Number of cells / BM x 10⁻⁷
^^
***
*
F
CD11b+
Number of cells / BM x 10⁻⁷
*
G
Spleen
Number of cells / spleen x 10⁻⁷
H
TCRβ CD4+
Number of cells / spleen x 10⁻⁶
I
TCRβ CD8+
Number of cells / spleen x 10⁻⁶
J
B220+
Number of cells / spleen x 10⁻⁷
K
CD11b+
Number of cells / spleen x 10⁻⁶

Figure 2.6: SSA increases number of both total and donor-derived thymocytes and peripheral lymphoid cells following high-dose congenic HSCT. Mice were transplanted with 5 x 10^4 congenic LSK cells. Five days prior to HSCT mice were sham-SSA or SSA via physical castration and four days prior to HSCT received 10 mg/kg busulfan. Mice were analysed via flow cytometry 28 days following HSCT **(A)** (i) Total BM cell number, (ii) proportion of donor cells in WBM and (iii) absolute number of donor cells in WBM. (iv) Total Lineage negative $Sca1^+$ $cKit^+$ (LSK) progenitor cell number, (v) proportion of donor cells in LSK progenitor cell subset and (vi) absolute number of donor-derived LSKs. (vii) Total number of $B220^+$ and $CD11b^+$ cells in WBM, (viii) proportion of donor-derived $B220^+$ and $CD11b^+$ cells in WBM and (ix) absolute number of donor-derived $B220^+$ and $CD11b^+$ cells in WBM. **(B)** (i) Total number of thymocytes, (ii) proportion of donor cells in the thymus and (iii) absolute number of donor-derived thymocytes. (iv) Total number of $CD4^+$ and $CD8^+$ single positive, $CD4^+CD8^+$ double positive and $CD4^-CD8^-$ double negative thymocytes, (v) proportion of donor cells across $CD4^+$ and $CD8^+$ single positive, $CD4^+CD8^+$ double positive and $CD4^-CD8^-$ double negative thymocytes subsets and (vi) absolute number of donor-derived $CD4^+$ and $CD8^+$ single positive, $CD4^+CD8^+$ double positive and $CD4^-CD8^-$ double negative thymocytes. **(C)** (i) Total number of splenocytes, (ii) proportion of donor cells in the spleen and (iii) absolute number of donor-derived splenocytes. (iv) Total number of TCR^+CD4^+ and TCR^+CD8^+ T cells, $B220^+$ B cells and $CD11b^+$ macrophages, (v) proportion of donor cells across TCR^+CD4^+ and TCR^+CD8^+ T cells, $B220^+$ B cells and $CD11b^+$ macrophages splenocyte subsets and (vi) absolute number of donor-derived TCR^+CD4^+ and TCR^+CD8^+ T cells, $B220^+$ B cells and $CD11b^+$ macrophage, splenocyte subsets. White bars indicate untreated mice, grey bars indicate sham-SSA and black bars indicate SSA. Data is representative of two individual experiments, expressed as mean ± SE and analysed using an unpaired two-tailed T-test. * ($p< 0.05$), ** ($p< 0.01$) represent significant differences compared to untreated mice. ^ ($p< 0.05$), ^^ ($p< 0.01$), ^^^ ($p< 0.001$), ^^^^ ($p< 0.0001$) represent significant differences when comparing sham-SSA and SSA groups.

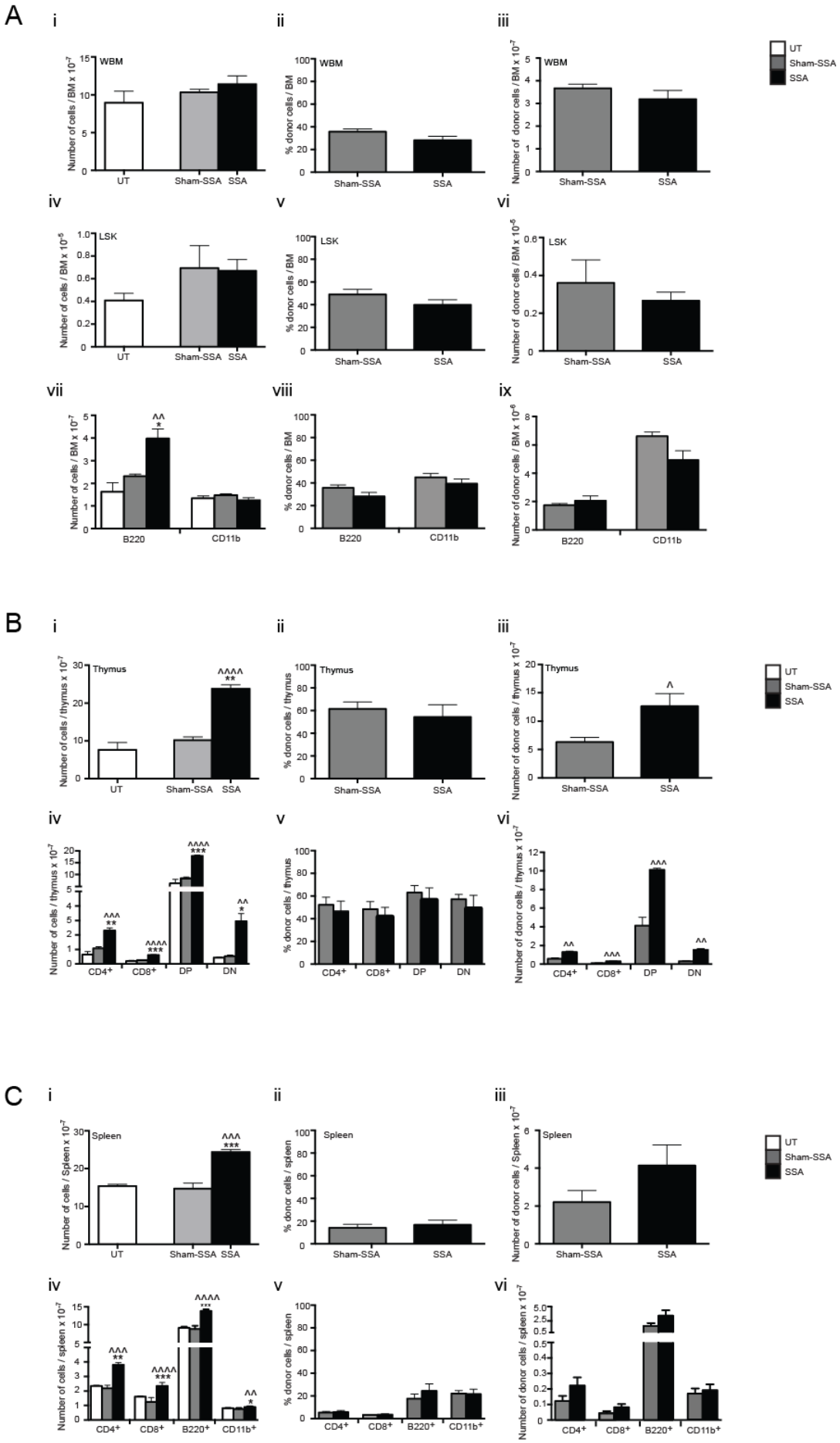
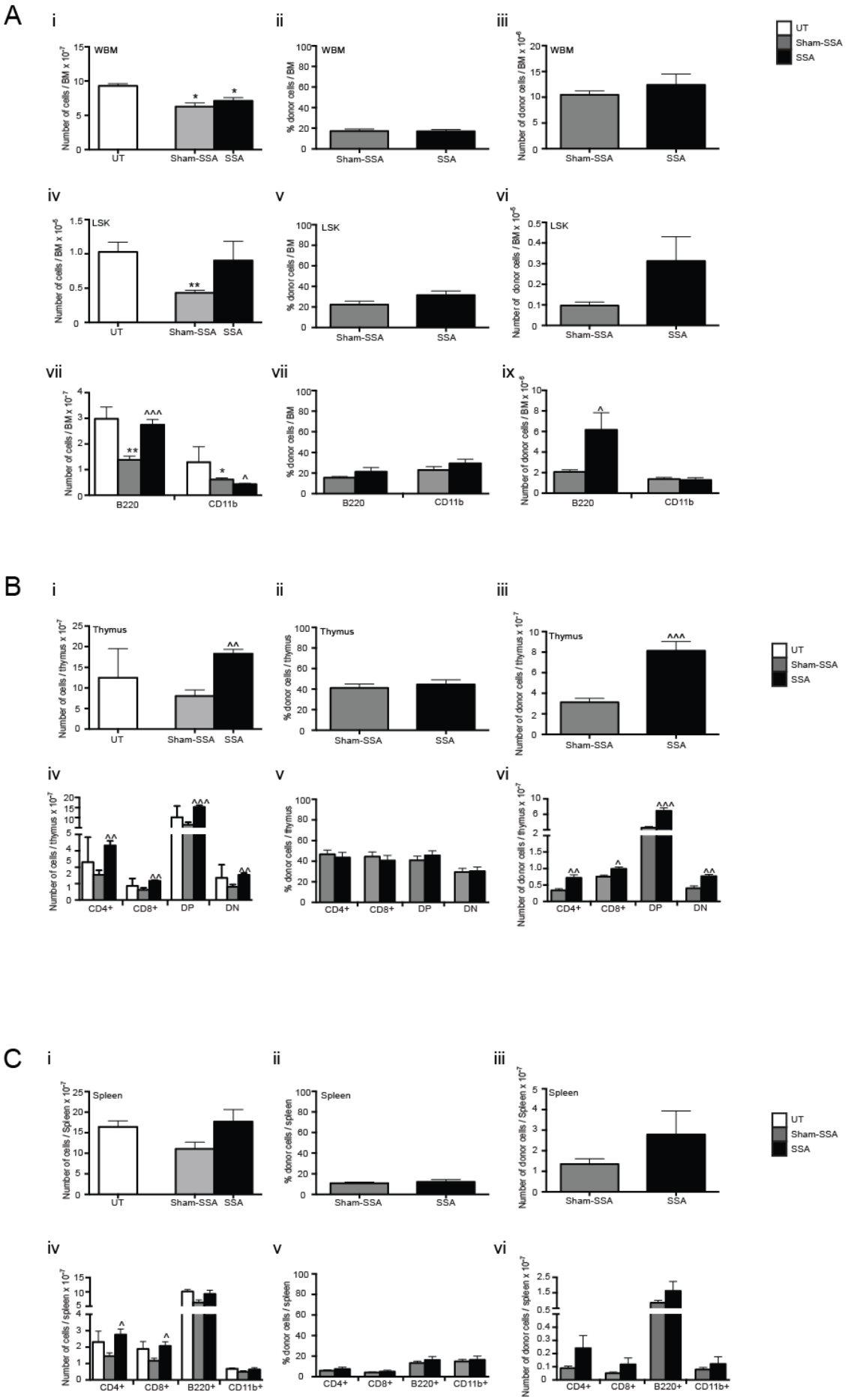

A
i
ii
iii
iv
v
vi
vii
viii
ix
WBM
LSK
Number of cells / BM x 10^-7
% donor cells / BM
Number of donor cells / BM x 10^-7
Number of cells / BM x 10^-5
Number of donor cells / BM x 10^-6
UT
Sham-SSA
SSA
B220
CD11b
B
Thymus
Number of cells / thymus x 10^-7
% donor cells / thymus
Number of donor cells / thymus x 10^-7
CD4+
CD8+
DP
DN
C
Spleen
Number of cells / Spleen x 10^-7
% donor cells / spleen
Number of donor cells / Spleen x 10^-7
Number of cells / spleen x 10^-7
Number of donor cells / spleen x 10^-7
B220+
CD11b+

Figure 2.7: SSA increases number of total and donor-derived thymocytes but not peripheral lymphoid cells following low-dose congenic HSCT. Mice were transplanted with 1 x 10^4 congenic LSK cells. Five days prior to HSCT mice were sham-SSA or SSA via physical castration and four days prior to HSCT received 10 mg/kg busulfan. Mice were analysed via flow cytometry 28 days following HSCT **(A)** (i) Total BM cell number, (ii) proportion of donor cells in WBM and (iii) absolute number of donor cells in WBM. (iv) Total Lineage negative $Sca1^+$ $cKit^+$ (LSK) progenitor cell number, (v) proportion of donor cells in LSK progenitor cell subset and (vi) absolute number of donor-derived LSKs. (vii) Total number of $B220^+$ and $CD11b^+$ cells in WBM, (viii) proportion of donor-derived $B220^+$ and $CD11b^+$ cells in WBM and (ix) absolute number of donor-derived $B220^+$ and $CD11b^+$ cells in WBM. **(B)** (i) Total number of thymocytes, (ii) proportion of donor cells in the thymus and (iii) absolute number of donor-derived thymocytes. (iv) Total number of $CD4^+$ and $CD8^+$ single positive, $CD4^+CD8^+$ double positive and $CD4^-CD8^-$ double negative thymocytes, (v) proportion of donor cells across $CD4^+$ and $CD8^+$ single positive, $CD4^+CD8^+$ double positive and $CD4^-CD8^-$ double negative thymocytes subsets and (vi) absolute number of donor-derived $CD4^+$ and $CD8^+$ single positive, $CD4^+CD8^+$double positive and $CD4^-CD8^-$ double negative thymocytes. **(C)** (i) Total number of splenocytes, (ii) proportion of donor cells in the spleen and (iii) absolute number of donor-derived splenocytes. (iv) Total number of TCR^+CD4^+ and TCR^+CD8^+ T cells, $B220^+$ B cells and $CD11b^+$ macrophages, (v) proportion donor cells across TCR^+CD4^+ and TCR^+CD8^+ T cells, $B220^+$ B cells and $CD11b^+$ macrophages splenocyte subsets and (vi) absolute number of donor-derived TCR^+CD4^+ and TCR^+CD8^+ T cells, $B220^+$ B cells and $CD11b^+$ macrophage, splenocyte subsets (C vi). White bars indicate untreated mice, grey bars indicate sham-SSA and black bars indicate SSA. Data is representative of two individual experiments, expressed as mean ± SE and analysed using an unpaired two-tailed T-test. * ($p< 0.05$), ** ($p< 0.01$) represent significant differences compared to untreated mice. ^ ($p< 0.05$), ^^ ($p< 0.01$), ^^^ ($p< 0.001$) represent significant differences when comparing sham-SSA and SSA groups.


A
i
WBM
Number of cells / BM x 10^-7
UT
Sham-SSA
SSA
ii
WBM
% donor cells / BM
Sham-SSA
SSA
iii
WBM
Number of donor cells / BM x 10^-6
Sham-SSA
SSA
UT
Sham-SSA
SSA
iv
LSK
Number of cells / BM x 10^-5
v
LSK
% donor cells / BM
vi
LSK
Number of donor cells / BM x 10^-6
vii
Number of cells / BM x 10^-7
B220
CD11b
vii
% donor cells / BM
ix
Number of donor cells / BM x 10^-6
B220
CD11b
B
i
Thymus
Number of cells / thymus x 10^-7
ii
Thymus
% donor cells / thymus
iii
Thymus
Number of donor cells / thymus x 10^-7
iv
Number of cells / thymus x 10^-7
CD4+
CD8+
DP
DN
v
% donor cells / thymus
vi
Number of donor cells / thymus x 10^-7
C
i
Spleen
Number of cells / Spleen x 10^-7
ii
Spleen
% donor cells / spleen
iii
Spleen
Number of donor cells / Spleen x 10^-7
iv
Number of cells / spleen x 10^-7
CD4+
CD8+
B220+
CD11b+
v
% donor cells / spleen
vi
Number of donor cells / spleen x 10^-7

BM (Figure 2.6Aii-iii) or spleen (Figure 2.6Cii-iii). In contrast, SSA was able to significantly increase both thymic cellularity and the number of donor-derived thymocytes compared to sham-SSA controls (Figure 2.6Bi-iii). These changes all correlated with increases in the number of $CD4^-$ $CD8^-$ double negative, $CD4^+CD8^+$ double positive and, $CD4^+$ and $CD8^+$ single positive thymocyte subpopulations, without their proportions being skewed (Fig 2.6Biv-vi).

Following these investigations we next determined the hematopoietic response in mice transplanted with a relatively lower numbers of progenitors. Mice were transplanted with 1 x 10^4 congenic LSK progenitor cells 4-days following 10 mg/kg busulfan. Mice were also sham-SSA or SSA at 1-day prior to chemotherapy and analysed 28 days following HSCT. Whilst the responses were not as robust in animals that received the higher transplant cell dose, it was found that 1 x 10^4 LSK cells could engraft in the BM yielding multi-lineage hematopoietic cells of donor origin (Figure 2.7A-C). Predictably donor cell engraftment was reduced from 40 % in mice transplanted with 5 x 10^4 LSKs (Figure 2.6A) to 20 % in mice transplanted with 1 x 10^4 LSK cells (Figure 2.7A). SSA did not increase the proportion or number of total donor cells observed in the BM or spleen, but it did significantly increase the total number of $B220^+$ B cells, the number of donor-derived $B220^+$ B cells and the total number of $CD4^+$ and $CD8^+$ T cells observed in the spleen (Figure 2.7A and C). Similar to what was previously observed, SSA increased both thymic cellularity and number of donor-derived thymocytes across all four $CD4^+$ $CD8^+$ double positive, $CD4^-$ $CD8^-$ double negative, $CD4^+$ and $CD8^+$ single positive subsets, following transplantation with 1 x 10^4 LSK cells (Figure 2.7B).

These data indicate that both high and low dose HSCT can induce detectable levels of chimerism when mice are conditioned with 10 mg/kg of busulfan and that SSA prior to chemotherapy and HSCT significantly improves thymus cellularity and donor T cell output.

Discussion

The DNA alkylating agent busulfan is an integral component in many HSCT conditioning regimes (18). However busulfan-dosing is associated with variable efficacy and toxicities (19). In this study we aimed to investigate the ability of low-doses of the drug busulfan to condition the BM of mice to receive HSCT. We also assessed the ability for SSA prior to busulfan-mediated conditioning to enhance the engraftment of donor cells in the BM, and boost donor cell output in the thymus and the periphery, in the hope that this would allow lower-doses of busulfan to be used in conditioning regimes for HSCT and provide a platform for the development of low-dose busulfan mediated conditioning for mixed chimerism induced transplantation tolerance.

Busulfan is an ideal drug to condition the BM for autologous HSCT as it is myelosuppressive, allowing space for donor-cells to engraft and proliferate, but is not immunosuppressive and therefore does not induce post-transplant immunosuppression (8). This decreases the risk of developing treatment-associated morbidities, such as opportunistic infections and malignancy. Clinically busulfan has been used to condition patients with adenosine deaminase (ADA)–deficient severe combined immunodeficiency (SCID) preceding the transplantation of gene-corrected autologous HSCT, with the success of treatment directly related to the engraftment and expansion of genetically corrected HSCs (1). Supporting this observation, studies in mice have shown that the level of donor-reconstitution following HSCT is directly proportional to the dose of busulfan, with a dose of 50 mg/kg producing equivalent engraftment to a sub-lethal TBI, when mice are transplanted with a combination of 10-15 x 10^6 WBM and 35-50 x 10^6 splenocytes (11). In another study, where busulfan and HSCT were used to treat Type 1 Gaucher disease, a lysosomal storage disorder, it was found that 25 mg/kg of busulfan was the threshold dose able to induce detectable levels of chimerism (12). When allogeneic whole BM (WBM) is transplanted a dose of 20 mg/kg busulfan is typically employed, with increasing chimerism corresponding to dosage increases, as mice are relatively more resistant to busulfan than humans (11, 20, 21). Collectively these studies highlight a clear need to improve the level of engraftment achieved with lower doses of busulfan. Considering doses higher than 20 mg/kg busulfan are associated with impaired weight gain and the development of mucocistis (8), in this study we aimed to assess the ability for SSA to improve donor cell engraftment and enhance donor cell reconstitution following autologous HSCT, when mice were conditioned with low-dose busulfan. We have previously shown that the removal of sex steroids improves donor cell engraftment in the BM and peripheral reconstitution following autologous or allogeneic HSCT (14, 17) and hypothesised that SSA prior to conditioning and HSCT

would allow the chemotheraputic dose of busulfan to be decreased, lessening the risk of conditioning associated morbidities, whilst still promoting donor-cell engraftment in the BM and output in the periphery.

Compelling evidence suggests that sex steroids play a major role in inducing thymic atrophy and the diminution in immune cell function (22). Whilst SSA was achieved via physical castration in this study, chemical SSA using the clinically-available hormone, lutenising hormone releasing hormone (LHRH) agonist (LHRH-A), has been shown to have a similar effects as surgical gonadectomy leading to a profound regenerative effect on the immune system. Operating via the hypothalamus-pituatary-adrenal/gonadal axis, LHRH-As control the production and release of the pituitary hormones, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which subsequently control the production of sex steroids (22). Chemical castration is advantageous, because unlike physical castration, it is reversible, displaying few, if any long-term side effects (13). Furthermore, LHRH-As are commonly used to treat a range of disorders, including precocious puberty (23), endometriosis (24), prostate (25) and breast cancer (26) and chemotherapy-induced sterility (27), providing readily available information on pharmacokinetics, dosing toxicity and efficacy. Importantly, human studies in our laboratory have shown that LHRH-A administration prior to both autologous and allogeneic HSCT significantly increases the number of naive T cells produced and their function in the periphery (15).

In initial experiments, the changes in lymphoid and myeloid populations in mice injected with 10 mg/kg or 20 mg/kg busulfan were determined. At a concentration of 10 mg/kg, busulfan selectively depleted the LSKs within the BM, with 80 % of this progenitor cell subset depleted 4-days following chemotherapy. The LSK phenotype classifies a subset of progenitors enriched for HSCs, with 1 in 10 LSKs containing true HSC capacity (28), indicating that via a depletion of LSKs, low-dose busulfan had cleared space for transplanted progenitor cells to engraft. Similar to the 10 mg/kg dose, 20 mg/kg busulfan significantly depleted progenitor cells in the BM, without depleting WBM cell numbers, confirming previous reports that state at a dose lower than 20 mg/kg busulfan, is not completely myeloablative; selectively depleting hematopoietic precursors (8, 29, 30).

Other studies that have examined conditioning-associated morbidities following busulfan-chemotherapy have neglected to investigate in detail its effect on the thymus. The thymus is important, as it is responsible for thymopoiesis and therefore required to generate T cells, which are a critical component of the adaptive immune system. This aspect is of particular importance as

protracted immune deficiency following conditioning and HSCT is associated with significant transplant-related morbidities and is unquestionably pertinent in SCID patients, where such individuals experience profound T cell deficiency (31). Although busulfan does not overtly deplete mature peripheral lymphocytes (8), as confirmed by our own studies in this report, the effects of busulfan on immature thymocyte populations has not been investigated. A dose of 20 mg/kg busulfan is seemingly toxic to the thymus, significantly depleting each of the $CD4^+$, $CD8^+$ DP and DN thymocyte subsets, with cell numbers remaining significantly low for more than 7 days following chemotherapy. The higher dose of 20 mg/kg is also significantly more toxic to the thymus compared to the 10 mg/kg dose, which only had a mild effect on the DP thymocyte subset, as numbers and proportions returned to homeostasis within 7 days. SSA has previously been shown to protect the thymus from chemotherapy-mediated damage, restoring the thymic architecture and enhancing thymocyte proliferation (16). It was hypothesised that SSA, prior to conditioning with 10 mg/kg busulfan would protect the thymus from chemotherapy-mediated damage. As demonstrated herein SSA was able to protect all thymocyte subpopulations from the depleting effects of 10 mg/kg busulfan.

SSA also has been shown to have positive effects in the BM following chemotherapy in mice, increasing BM cellularity and progenitor cell numbers (16). Considering busulfan was investigated as a conditioning agent to "create space" in the BM for HSCT engraftment, it was important that SSA did not abrogate the depletion of LSK progenitor cells in the BM following chemotherapy. We found that SSA had no significant effect on the LSK compartment of busulfan-treated mice; with progenitor cell numbers still significantly decreased compared to the untreated control mice. A 90 % depletion of the LSK compartment was observed, which was equivalent to that observed in sham-SSA mice. These results are consistent with previous studies from our laboratory, that show it is the common lymphoid progenitor cell subset (Lineage negative, $IL7Ra^+$ $cKit^+$) within the BM that is increased by SSA, not the LSK population, in 6-8 week old mice (16). A slight increase in the B cell compartment following SSA was also observed, this is again consistent with previous observations, that show SSA induces B cell proliferation and expansion in the BM (16), whether this is due to direct stimulation or homeostatic expansion through creation of space is unknown.

Not only does the chemotherapeutic dose of busulfan administered (11, 32) and the level of host reconstitution following HSCT (33, 34) influence the level of engraftment achieved, but also the number of progenitor cells transplanted. To investigate the role of SSA in boosting donor-cell engraftment following conditioning with busulfan, two different cell doses were selected, 5×10^4

LSKs (equivalent to 2 x 10^6 cells/kg), representing high-dose HSCT and 1 x 10^4 LSKs (equivalent to 4 x 10^5 cells/kg), representing low-dose HSCT (34, 35). Previous studies have shown that busulfan administered in doses lower than 20 mg/kg result in slow and incomplete donor cell engraftment following high-dose BMT (11, 12). Because SSA prior to lethal TBI and autologous BMT, significantly enhances BM reconstitution and increases the number of donor-derived HSCs observed in the BM (17), it was hypothesised that this would be true for busulfan-conditioned mice. However, SSA did not increase the number of donor-derived LSKs when mice received either high or low dose HSCT following conditioning with busulfan. This can be explained by considering that at low-doses, such as 10 mg/kg, busulfan in not myeloablative, selectively depleting only the progenitor compartment (36). Previous studies from our laboratory that have shown an increase in BM cellularity following SSA have used harsh myeloablative treatments such as TBI and cyclophosphamide, significantly depleting WBM cell numbers and allowing "space" in the niche for an increase in BM cellularity, which inturn was reflected by an increase in the number of donor cells (14, 16, 37). Although an improvement in donor cell engraftment in the BM of SSA mice prior to HSCT was not demonstrated, transplantation of 5 x 10^4 LSKs and 1 x 10^4 LSK progenitor cells achieved approximately 40% and 20% BM chimerism respectively. These findings are highly encouraging, as chimerism levels at approximately 10% have successfully treated lysosomal storage diseases (12, 38, 39) and primary immunodeficiency disorders (33, 40). Additionally, microchimerism levels as low as 1 % has been used to induce robust allogeneic tolerance to donor specific organs, tissues and cells (41).

Although SSA did not have any significant effects on the BM, it did enhance thymic reconstitution following both high and low-dose HSCT. These changes encompassed a significant increase in thymic cellularity, as well as an expansion in the total number of $CD4^+$, $CD8^+$, DP and DN thymocyte subsets and peripheral T cells. SSA also significantly increased the number of donor-derived thymocytes. This is an important finding in the context of transplantation of gene-corrected HSCs to treat immunodeficiency, such as SCID; as it may enhance the number of gene-corrected T cells selected in the thymus and exported to the periphery, as it would confer a selective growth advantage to the transplanted gene corrected cells. Although SSA would not be necessary for the treatment of X-linked SCID, as transplanted wild type lymphocytes have a significant growth advantage, but may improve transplant outcomes for other forms of SCID such as, ADA-deficient or Jak3-3 deficient SCID, where no selective advantage is conferred on genetically modified cells (33, 42).

Busulfan is already a standard-of-practice chemotherapeutic drug in many clinical conditioning regimes (7, 43), however, previous studies that have examined conditioning associated morbidities following busulfan chemotherapy (7-9, 19, 44-50) have neglected to investigate in any detail the effect of chemotherapy on the thymus, an important point to consider when looking to use the protocol to scenarios for HSCT that require a thymus, such as those that utilise mixed chimerism to induce allogeneic tolerance. Herein we have demonstrated that lowering the dose of busulfan can still achieve clinically feasible levels of chimerism following both low and high dose autologous HSCT, whilst significantly lowering the level collateral damage caused to the thymus. These findings serve as an important platform on which to build new, clinically feasible BM conditioning regimes.

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CHAPTER THREE

Establishment of transplantation tolerance via minimal conditioning in aged recipients

Manuscript information

The following manuscript is being prepared to submit to the American Journal of Transplantation. The candidate, Jessica Morison, was responsible for planning, performing and analysing experiments as well as writing and editing the manuscript. The co-authors Jade Hommann, Daniel Layton, Ann Chidgey, Richard Boyd and Tracy Heng assisted with experimental design, technical assistance, interpretation of the results and drafting of the manuscript. Proportional contributions are explained in the declaration.

Establishment of transplantation tolerance via minimal conditioning in aged recipients

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Running title: Transplantation tolerance in aged mice

Key words: mixed chimerism, transplantation, tolerance induction, non-myeloablative conditioning

Abbreviations: BMT, bone marrow transplantation; WBM, whole bone marrow; LSK, Lineage$^-$Sca-1$^+$c-kit$^+$; SIR, sirolimus; Bu, busulfan; MST, mean survival time; TCD, T cell-depleted; Tx, thymectomised; ShTx, sham-thymectomised; Treg, regulatory T cell

Abstract

Mixed haematopoietic chimerism is a powerful means of generating donor-specific tolerance, allowing long-term graft acceptance without lifelong dependence on immunosuppressive drugs. To avoid the need for whole body irradiation and associated side effects, we utilised a radiation-free minimal conditioning regime to induce long-term tolerance across major histocompatibility barriers. We found that low-dose busulfan, in combination with host T cell depletion and short-term sirolimus-based immunosuppression, facilitated efficient donor engraftment. Tolerance was achieved when mice were transplanted with whole or T cell-depleted bone marrow, or purified progenitor cells. Tolerance induction was associated with an expansion in regulatory T cells and was not abrogated in the absence of a thymus, suggesting a dominant peripheral mode of tolerance. Importantly, we were able to generate durable chimerism and tolerance to donor skin grafts in both young and aged mice, despite age-related thymic atrophy and immune senescence. Clinically, this is especially relevant as the majority of transplant recipients are older patients whose immune recovery might be dangerously slow and would benefit from radiation-free minimal conditioning regimes that allow efficient donor engraftment without fully ablating the recipient immune system.

Introduction

Bone marrow transplantation (BMT)-induced mixed chimerism can produce a permanent state of donor-specific tolerance, thereby removing the need for chronic immunosuppression in organ transplantation (1). As recipient preconditioning toxicities have precluded the clinical translation of such BMT-based protocols in non-malignant conditions, non-myeloablative conditioning regimes, including costimulation blockade (2-7) and administration of supraphysiological doses of BM (8, 9) have been widely explored, but clinical translation has been hampered by thromboembolic complications associated with CD40L blockade (10). Other studies have investigated low-dose total body irradiation (TBI) or chemotherapy and peripheral T cell depletion, but focal thymic irradiation (11, 12) is required to deplete donor-reactive thymocytes (13). Although these protocols have been effective in nonhuman primate models and clinical trials (14-18), T cell selection may be compromised by focal thymic irradiation.

The maintenance of stable allogeneic tolerance in mixed chimeras requires intrathymic deletion of alloreactive T cells (19). However, this may be compounded by age-related thymic involution, which is characterised by a disorganised microenvironment, adipocytic replacement of lymphoid tissue and decreased thymic output, such that the thymus is functioning at only 5% capacity by 10-12 months in the mouse (20) and 40 years in humans (21). As the majority of transplant recipients are well into adulthood, treatments that preserve or even enhance thymic function are clinically attractive. The aim of this study was to design a low-intensity, thymus-sparing, conditioning regime for the establishment of mixed chimerism and allogeneic tolerance in both young and aged mice.

Materials and Methods

Animals

Male C57BL/6 (H-2^b, Ly5.2), B6.SJL-ptprc (H-2^b, Ly5.1 congenic), B10.BR (H-2k) and BALB/c (H-2^d) were purchased at 6-8 weeks of age or 11-12 months of age from Monash Animal Research Platform, the Walter and Eliza Hall Institute and the Animal Resources Centre. Animals were housed under specific pathogen-free conditions at the Monash University Animal Research Laboratories. All animal experimentation and procedures were approved by the Monash University Animal Ethics Committee (SOBSA/MIS/2007/55, SOBSA/MIS/2010/98).

Conditioning and bone marrow transplantation

For TBI experiments, 6-8 weeks old B6.SJL-ptprc mice received 3Gy TBI, before same-day transplantation with $4x10^7$ B10.BR whole bone marrow (WBM) cells. For busulfan experiments, 6-8 weeks old or 11-12 months old mice received 10 mg/kg (Busulfex, PDL BioPharma, Fremont, CA) on day -4, and intraperitoneal injections of 0.1mg anti-CD4 (GK1.5) and 0.1mg anti-CD8 (2.43) (Bio X Cell, West Lebanon, NH) on day -3 and -1. On day 0, mice were transplanted with $4x10^7$ B10.BR WBM cells, $3.88x10^7$ Thy1-depleted BM cells or $5x10^4$ Lineage$^-$Sca-1$^+$c-kit$^+$ (LSK) cells from C57Bl/6 (congenic) or B10.BR (allogeneic) donor mice, followed by daily intraperitoneal injections of 3mg/kg/day sirolimus (Rapamycin, LC Laboratories, Woburn, MA) for 28 days.

Thymectomy and skin grafting

Thymectomy was performed 11 days prior to BMT, as previously described (32). Skin grafting was performed 12 weeks (donor graft) and 24 weeks (third-party graft) after BMT. Mice were anaesthetised with 2-3% isoflurane (Delvet, NSW, Australia) in oxygen and full-thickness tail skin (1x1cm) from 6-8 weeks old donor B10.BR (allogeneic) and BALB/c (third-party) mice were grafted onto the lateral flank. Bandages were removed 7 days after surgery and grafts were monitored daily. Rejection was recorded when more than 75% of the graft's epithelial tissue had broken down.

Flow cytometric analysis

Multi-lineage chimerism was assessed via flow cytometric analysis. Cells were labelled with CD45.2 (104), CD3 (145-2C11), CD49b (DX5, Biolegend), CD45.1 (A20), IA/IE (MS/114,

Miltenyi), CD45R (RA3-6B2), CD11b (M1/70) and CD11c (HL3). BM progenitor populations were assessed with a lineage cocktail consisting of CD3 (145-2C11), CD4 (RM4-5), CD8 (53-6.7), CD19 (1D3), NK1.1 (PK136), CD11b (M1/70), Gr-1 (RB6-8C5), TER-119 (TER-119) and CD11c (HL3), in addition to CD117 (2B8) and Sca-1 (D7). T cell populations were assessed with CD4 (RM4-5, eBioscience), CD8 (53-6.7), TCRβ (H57-597) and CD25 (PC61, Biolegend) and intracellular staining with Foxp3 (FJK-16a, eBioscience). Peripheral lymphoid and myeloid cells were assessed with CD11c (HL3), Gr-1 (RB6-8C5), CD45R (RA3-6B2), CD11b (M1/70), I-A/I-E (M5/114.15.2, Biolegend). For all staining, an FcR block (2.4G2, laboratory produced) was used. Unless otherwise stated, all antibodies were obtained from BD Biosciences. All data were acquired on a BD FACSCanto II and analysed using Flowjo software.

Mixed lymphocyte reaction

$4x10^5$ responder splenocytes from chimeric mice were co-cultured with $4x10^5$ 20Gy-irradiated stimulator splenocytes from untreated B10.BR (allogeneic), BALB/c (third party) or Ly5.1 (syngeneic) mice at 37^oC, $5\%CO_2$ for 96hr. Cells were pulsed with 1μCi of ^3H-thymidine (Amersham Biosciences) for a further 18hr and ^3H-thymidine incorporation was measured as previously described (32).

Statistical analysis

Statistical analysis was performed using an unpaired, two-tailed t test or a two-way ANOVA. A p value ≤ 0.05 was considered to be significant.

Results

Low-dose TBI, sirolimus and BMT induces allogeneic tolerance

Initial studies aimed to determine if low-dose irradiation with short-term immunosuppression was sufficient to allow stable mixed chimerism and allogeneic tolerance. B6.SJL-Ly5.1 (H-2^b) mice were conditioned with 3Gy TBI, the minimal dose required for long-term engraftment of syngeneic haematopoietic stem cells (22), prior to same-day transplantation with $4x10^7$ allogeneic B10.BR (H-2^k) whole bone marrow (WBM) cells and sirolimus (SIR) monotherapy for 28 days (3Gy/SIR group). These mice initially developed robust mixed chimerism (Figure 3.1A), and 3 out of 4 mice accepted allogeneic donor skin grafts (Figure 3.1B), but total blood chimerism steadily degraded over time.

The addition of monoclonal antibodies to deplete host $CD4^+$ T cells (3Gy/αCD4/SIR group), or both $CD4^+$ and $CD8^+$ T cells (3Gy/αCD4/αCD8/SIR group), prior to BMT significantly increased initial blood chimerism, but chimerism again declined over time (Figure 3.1A). These mice were tolerant and accepted B10.BR allogeneic skin grafts (Figure 3.1B). Treatment with T cell-depleting antibodies further reduced the proportion of circulating $CD3^+$ T cells (Figure 3.1C), while increasing the initial proportion of donor-derived T cells observed in peripheral blood (Figure 3.1D). However, no significant differences were observed 12 weeks after BMT and the proportion of donor-derived T cells in all 3 groups gradually declined over time (Figure 3.1D). Donor-derived $B220^+$ B cell (Figure 3.1E) and $CD11b^+$ macrophage (Figure 3.1F) proportions were initially very high across all treatment regimes, but steadily decreased. Despite a gradual decline in blood chimerism, donor cell engraftment was still detected in the BM in all 3 groups 56 weeks after BMT (Figure 3.1G), with approximately 20% of $Lineage^-Sca\text{-}1^+c\text{-}kit^+$ (LSK) progenitor cells derived from donor (Figure 1H). Therefore, mixed chimerism and allogeneic tolerance can be achieved with low-dose TBI in conjunction with short-term immunosuppression without the need for host T cell depletion.

Low-dose busulfan, sirolimus, T cell depletion and BMT induces allogeneic tolerance

As TBI disrupts the cellular composition and organisation of thymic stroma and impairs thymopoiesis (23), we next investigated whether irradiation could be replaced with a less damaging alternative conditioning treatment. Busulfan (Bu) is an attractive agent for low- intensity conditioning and suitable alternative to TBI as it depletes refractory non-cycling primitive stem

cells to achieve lasting and high levels of donor haematopoietic engraftment (24). We found that 10 mg/kg busulfan depleted LSK progenitors in the BM, with LSK numbers remaining low 8 weeks after treatment (Figure 3.2A). WBM counts were low at 1 and 4 weeks after treatment, returning to untreated levels 1 week following each nadir (Figure 3.2A). Busulfan had a mild depleting effect on thymus cellularity, mainly due to a loss of $CD4^+CD8^+$ double-positive thymocytes, which returned to untreated levels 3 weeks after treatment (Figure 3.2B). A decrease in mature splenic lymphoid and myeloid cells was also observed at 1 week after treatment but not thereafter (data not shown). Hence, conditioning with 10 mg/kg busulfan leads to depletion of host BM LSK cells but has minimal short-term effects on the thymus and spleen.

We next assessed whether busulfan could replace TBI to allow mixed chimerism and allogeneic tolerance. B6.SJL-Ly5.1 mice were conditioned with 10 mg/kg busulfan 4 days prior to transplantation with $4x10^7$ allogeneic B10.BR WBM cells. As busulfan is myelosuppressive but not immunosuppressive (25), mice received a short course of sirolimus for 28 days following BMT (Bu/SIR). Mice treated with Bu/SIR developed a low level of peripheral blood chimerism, which declined following sirolimus withdrawal and donor cells could not be detected at 16 weeks after BMT (Figure 3.2C). These mice did not develop tolerance and rejected allogeneic donor skin (mean survival time (MST) 8.75 days) (Figure 3.2D). However, depletion of both $CD4^+$ and $CD8^+$ host T cells prior to BMT (Bu/αCD4/αCD8/SIR) resulted in robust and stable chimerism (Figure 3.2C). These mice were tolerant and accepted B10.BR allogeneic skin grafts for over 250 days (Figure 3.2D). Importantly, these mice were immunocompetent, maintaining the ability to reject third-party BALB/c ($H\text{-}2^d$) allogeneic skin grafts (MST 5 days) (Figure 3.2D). Sirolimus was absolutely required for the development of stable chimerism and allogeneic tolerance, as busulfan-conditioned mice that received T cell-depleting antibodies, but not sirolimus (Bu/αCD4/αCD8), exhibited transient chimerism that could not be detected 8 weeks after BMT (Figure 3.2C), and rejected donor skin grafts (MST 21 days) and third-party BALB/c skin graft (MST 7 days) (Figure 3.2D).

Host T cell depletion is required to purge pre-existing donor-reactive cells. Unlike 3Gy/SIR (Figure 1C), Bu/SIR did not deplete circulating T cells at early time points (Figure 3.2E).

Figure 3.1: Low-dose irradiation establishes allogeneic tolerance. 6-8 week old B6.SJL-Ly5.1 (H-2^b) mice received PBS (black square), anti-CD4 (blue triangle), or anti-CD4 and anti-CD8 (red circle) monoclonal antibodies on day -3 and -1. On day 0 mice received 3Gy TBI, before same-day transplantation with $4x10^7$ B10.BR (H-2^k) allogeneic WBM cells. Sirolimus was administered at 3mg/kg/day for 28 days following BMT. 28 weeks later, chimeric mice received an allogeneic skin graft from B10.BR (H-2^k) tail skin. **(A)** The proportion of donor cells in peripheral blood over time. **(B)** Donor skin graft survival. **(C)** The proportion of $CD3^+$ T cells in peripheral blood. **(D)** The proportion of donor $CD3^+$ T cells, **(E)** $B220^+$ B cells and **(F)** $CD11b^+$ macrophages in peripheral blood over time. **(G)** The proportion of donor cells and **(H)** donor LSK progenitor cells in the BM at 56 weeks after BMT. Data are representative of one individual experiment, expressed as mean±SE and analysed using two-way ANOVA, with n=4 mice/group.

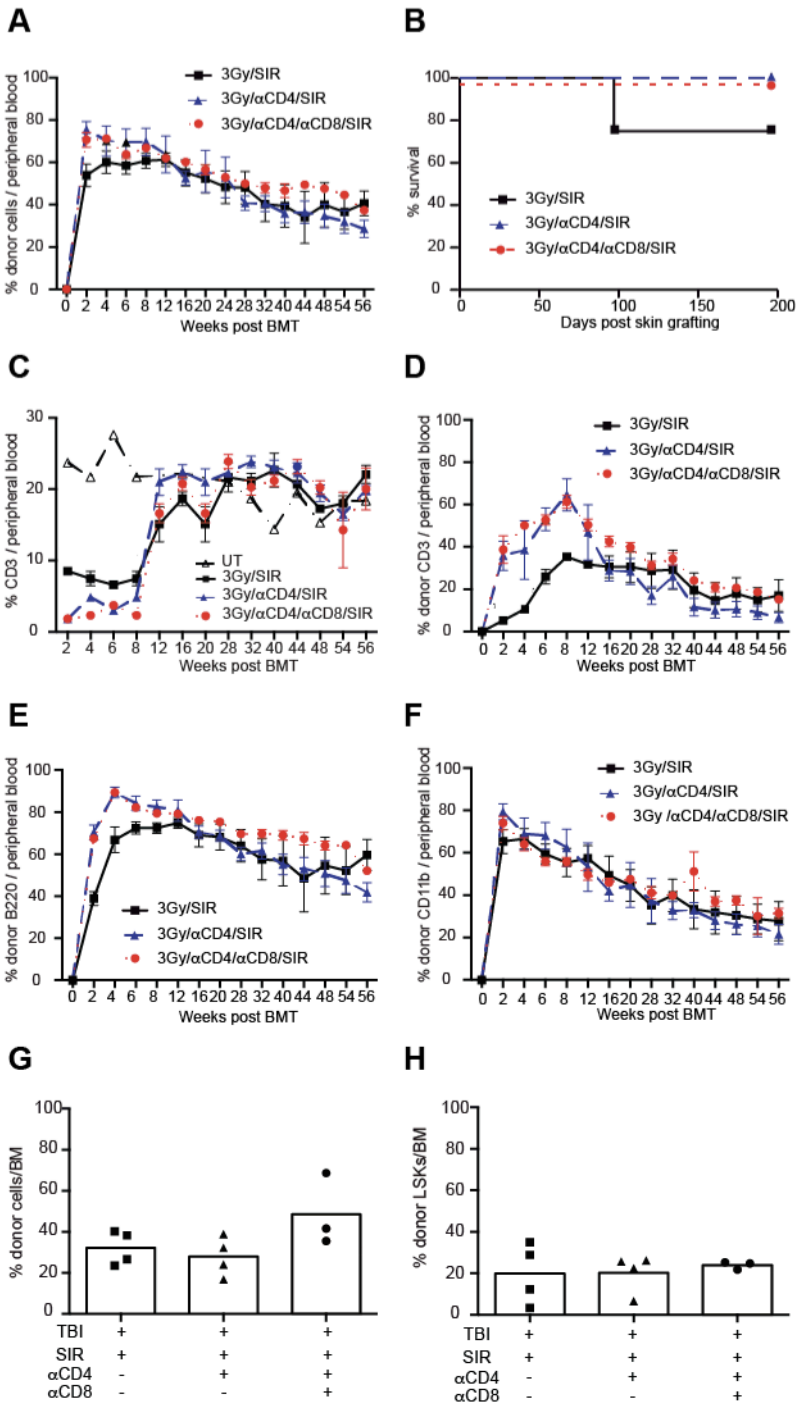
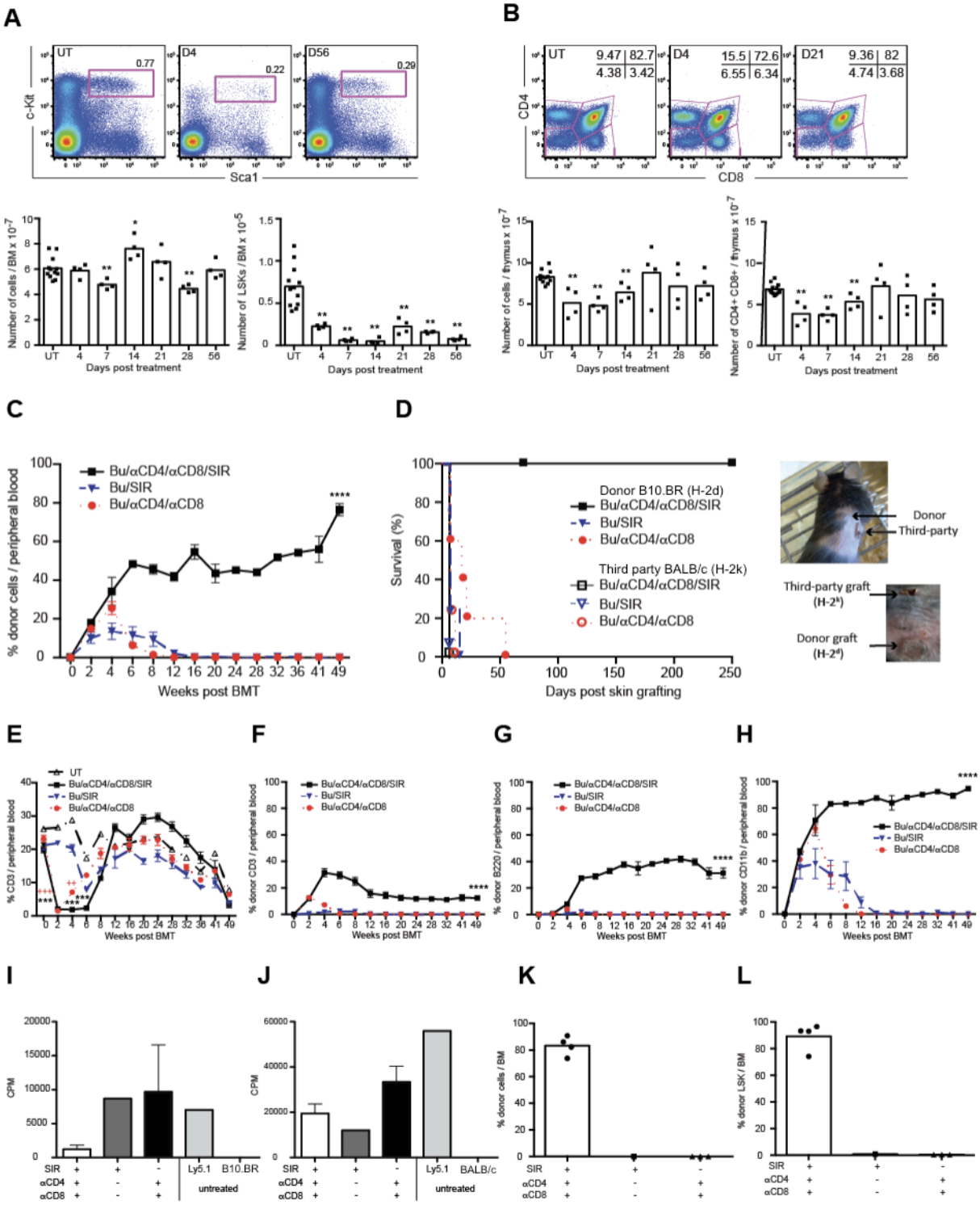

A
% donor cells / peripheral blood
3Gy/SIR
3Gy/αCD4/SIR
3Gy/αCD4/αCD8/SIR
Weeks post BMT
B
% survival
3Gy/SIR
3Gy/αCD4/SIR
3Gy/αCD4/αCD8/SIR
Days post skin grafting
C
% CD3 / peripheral blood
UT
3Gy/SIR
3Gy/αCD4/SIR
3Gy/αCD4/αCD8/SIR
Weeks post BMT
D
% donor CD3 / peripheral blood
3Gy/SIR
3Gy/αCD4/SIR
3Gy/αCD4/αCD8/SIR
Weeks post BMT
E
% donor B220 / peripheral blood
3Gy/SIR
3Gy/αCD4/SIR
3Gy/αCD4/αCD8/SIR
Weeks post BMT
F
% donor CD11b / peripheral blood
3Gy/SIR
3Gy/αCD4/SIR
3Gy /αCD4/αCD8/SIR
Weeks post BMT
G
% donor cells/BM
TBI
SIR
αCD4
αCD8
H
% donor LSKs/BM
TBI
SIR
αCD4
αCD8

Figure 3.2: Low-dose busulfan, in combination with host T cell depletion and short-term immunosuppression, induces mixed chimerism and long-term allogeneic tolerance. (A) Representative profile of LSK cells in the BM, total BM cellularity and number of LSK cells after treatment with 10 mg/kg busulfan. **(B)** Representative profile of thymocyte subsets, total thymic cellularity and number of $CD4^+CD8^+$ double-positive thymocyte cells after treatment with 10 mg/kg busulfan. Data are expressed as mean±SE, representative of 4 independent experiments and analysed using two-way ANOVA or an unpaired two-tailed t test, with n=4 mice/group. *($p \leq 0.05$) and **($p \leq 0.01$) represent significant differences compared to untreated (UT) mice. **(C)** 6-8 week old B6.SJL-Ly5.1 ($H\text{-}2^b$) mice received 10 mg/kg busulfan on day -4, anti-CD4 and anti-CD8 monoclonal antibodies on day -3 and -1, $4x10^7$ B10.BR ($H\text{-}2^k$) allogeneic WBM cells on day 0, followed by daily sirolimus treatment for 28 days (black square). Some mice received PBS instead of T cell-depleting antibodies (blue triangle), or CMC vehicle control instead of sirolimus (red circle). 12 and 28 weeks after BMT, mice received an allogeneic skin graft from donor B10.BR ($H\text{-}2^k$) and third-party BALB/c ($H\text{-}2^d$) strain respectively. The proportion of donor cells in peripheral blood was measured over time. **(D)** Donor and third-party skin graft survival. **(E)** The proportion of $CD3^+$ T cells in peripheral blood. **(F)** The proportion of donor $CD3^+$ T cells, **(G)** $B220^+$ B cells and **(H)** $CD11b^+$ macrophages in peripheral blood. **(I)** *In vitro* allo-responsiveness was tested in a one-way MLR with stimulator splenocytes from B10.BR (donor) or **(J)** third-party unrelated BALB/c mice. **(K)** The proportion of total donor cells and **(L)** donor LSK progenitor cells in the BM at 49 weeks after BMT. Data are representative of two individual experiments. ****($p \leq 0.0001$) represents significant differences compared to other treatment groups.


A
UT
D4
D56
0.77
0.22
0.29
c-Kit
Sca1
B
UT
D4
D21
9.47 82.7
4.38 3.42
15.5 72.6
6.55 6.34
9.36 82
4.74 3.68
CD4
CD8
Number of cells / BM x 10^-7
Number of LSKs / BM x 10^-5
Number of cells / thymus x 10^-7
Number of CD4+ CD8+ / thymus x 10^-7
Days post treatment
C
Bu/αCD4/αCD8/SIR
Bu/SIR
Bu/αCD4/αCD8
% donor cells / peripheral blood
Weeks post BMT
D
Donor B10.BR (H-2d)
Third party BALB/c (H-2k)
Survival (%)
Days post skin grafting
Donor
Third-party
Third-party graft (H-2k)
Donor graft (H-2d)
E
% CD3 / peripheral blood
F
% donor CD3 / peripheral blood
G
% donor B220 / peripheral blood
H
% donor CD11b / peripheral blood
I
CPM
Ly5.1
B10.BR
untreated
J
BALB/c
K
% donor cells / BM
L
% donor LSK / BM
SIR
αCD4
αCD8

Treatment with T cell-depleting monoclonal antibodies significantly reduced the proportion of T cells in peripheral blood of Bu/αCD4/αCD8/SIR-treated mice at early time points, compared to Bu/SIR-treated mice (Figure 3.2E). This corresponded to stable chimerism across the T cell (Figure 3.2F), B cell (Figure 3.2G) and macrophage (Figure 3.2H) compartments over time. MLR data supported skin graft results, with Bu/αCD4/αCD8/SIR-treated mice showing a reduced responsiveness when challenged with B10.BR stimulator cells (Figure 3.2I). Mice treated with Bu/SIR or Bu/αCD4/αCD8 were not tolerant to haematopoietic antigens and responded to restimulation with B10.BR splenocytes (Figure 3.2I). All 3 groups retained the ability to proliferate in response to third-party BALB/c stimulator cells (Figure 3.2J).

BM engraftment of donor cells is absolutely required for long-term tolerance induction. We found the proportion of donor cells that engrafted in the BM was higher in Bu/αCD4/αCD8/SIR-treated mice (80%) (Figure 3.2K), compared with mice that received 3Gy/SIR (50%) (Figure 3.1G). Within the LSK compartment, 90% of progenitor cells were donor-derived in Bu/αCD4/αCD8/SIR-treated mice (Figure 3.2L), compared to 20-25% in 3Gy-irradiated mice (Figure 3.1H). In summary, treatment with 10 mg/kg busulfan in combination with host T cell depletion and short-term sirolimus leads to robust, stable chimerism and allogeneic tolerance whilst maintaining immunocompetence.

Allogeneic tolerance via mixed chimerism is achievable in aged mice

Aging is associated with thymic atrophy and a diminished ability to generate functional T cells and antigen-specific responses following BMT (26). Indeed, tolerance cannot be achieved in mice 1 year and older via conditioning with CD45RB monoclonal antibodies without thymic regeneration (27), or without TBI (28). Our challenge, ultimately, was to induce allogeneic tolerance in aged mice using a radiation-free conditioning regime that caused minimal damage to the involuted thymus and did not require additional thymic enhancement.

We therefore treated 1-year old B6.SJL-Ly5.1 mice with 10 mg/kg busulfan and T cell-depleting antibodies before transplanting $4x10^7$ allogeneic B10.BR WBM cells, followed by short-course sirolimus. The proportion of donor cells detected in peripheral blood over time was lower in aged mice (20-30%) (Figure 3.3A), compared to young mice (40-50%) (Figure 3.2A). The proportion of circulating $CD3^+$ T cells was also lower in aged mice (Figure 3.3B), as were donor-derived T cells (Figure 3.3C), B cells (Figure 3.3D) and macrophages (Figure 3.3E). Nevertheless, allogeneic

tolerance was still achievable in Bu/αCD4/αCD8/SIR-treated aged mice, with no signs of donor B10.BR skin graft rejection for over 150 days (Figure 3.3F). Importantly, aged mice remained immunocompetent, rapidly rejecting third-party BALB/c skin (MST 2.6 days), with MLR data supporting skin graft results (Figure 3.3G and H). Thus, treatment with 10 mg/kg busulfan in combination with host T cell depletion and short-term sirolimus leads to stable chimerism and allogeneic tolerance in aged mice whilst maintaining immunocompetence.

Purified BM progenitor cells can induce allogeneic tolerance

Donor BM contains mature T cells that can promote haematopoietic engraftment and reconstitute T cell immunity, but transplantation with allogeneic T cell-replete BM is often associated with graft-versus-host disease in the clinic (29). Although we have not observed disease in our experiments, it is clinically more desirable to use T cell-depleted (TCD) BM or purified progenitor cells as the source of donor antigen. To determine the lowest possible progenitor cell dose required to engraft in the recipient BM in our model, B6.SJL-Ly5.1 mice received 10 mg/kg busulfan 4 days prior to transplantation with purified LSK cells from C57Bl/6 mice. Transplantation with $5x10^4$ LSK cells produced 20% chimerism within 6 weeks, a significantly higher level than all other LSK doses which produced less than 5% chimerism, while chimerism was not detected in mice transplanted with $1x10^3$ LSK cells (Figure 3.4A).

Having determined $5x10^4$ LSK cells as the minimum donor progenitor cell dose that would engraft in the recipient BM following conditioning with 10 mg/kg busulfan (Figure 3.4A), we transplanted busulfan-conditioned B6.SJL-Ly5.1 mice with either WBM cells, TCD BM cells or purified LSK cells from allogeneic B10.BR mice. Mice that received TCD BM showed the same pattern of haematopoietic chimerism and allogeneic tolerance as mice that received WBM cells, demonstrating that TCD BM can be used as the source of donor antigen (Figure 3.4B-F). Mice that received purified LSK cells displayed haematopoietic chimerism at levels slightly lower than mice that received WBM (Figure 3.4B). Similarly, the proportion of donor-derived $CD3^+$ T cells (Figure 3.4C), $B220^+$ B cells (Figure 3.4D) and $CD11b^+$ macrophages (Figure 3.4E) were slightly lower in

Figure 3.3: Allogeneic tolerance is achievable in aged mice. 12-month-old B6.SJL-Ly5.1 (H-2^b) mice received 10 mg/kg busulfan on day -4, anti-CD4 and anti-CD8 monoclonal antibodies on day -3 and -1, $4x10^7$ B10.BR (H-2^k) allogeneic WBM cells on day 0, followed by daily sirolimus treatment for 28 days (black square). 12 and 28 weeks after BMT, mice received an allogeneic skin graft from B10.BR (H-2^k) and BALB/c (H-2^d) respectively. **(A)** The proportion of donor cells in peripheral blood over time. **(B)** The proportion of $CD3^+$ T cells in peripheral blood. **(C)** The proportion of donor $CD3^+$ T cells, **(D)** $B220^+$ B cells and **(E)** $CD11b^+$ macrophages in peripheral blood. **(F)** Donor and third-party skin graft survival. **(G)** *In vitro* allo-responsiveness of splenocytes isolated from conditioned mice (B6.SJL-Ly5.1) was tested in a one-way MLR against stimulator splenocytes isolated from either donor B10.BR mice or **(H)** third-party unrelated BALB/c mice. Data are representative of one individual experiment, expressed as mean±SE and analysed using two-way ANOVA or an unpaired two-tailed t test, with n=5-7 mice/group.

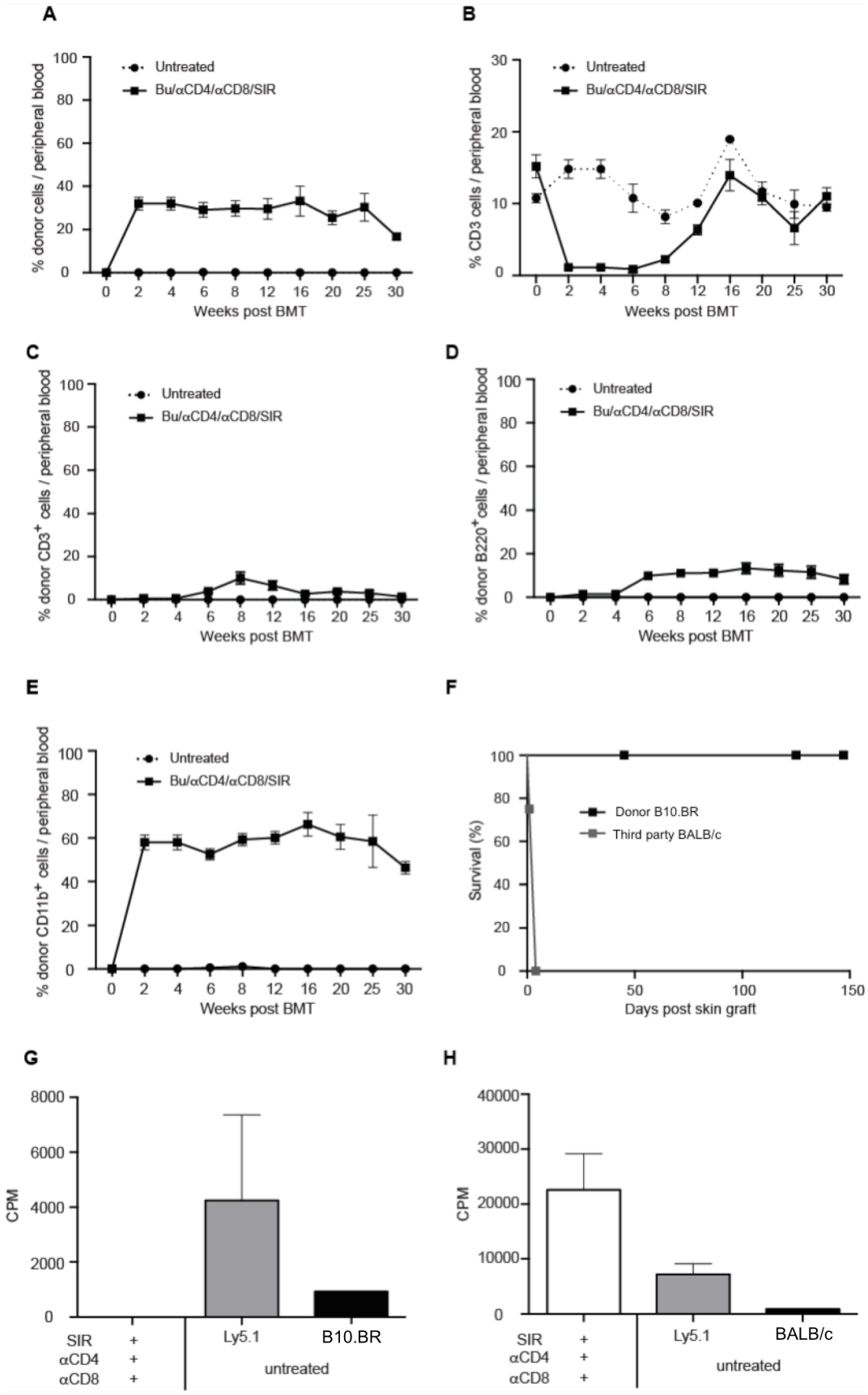
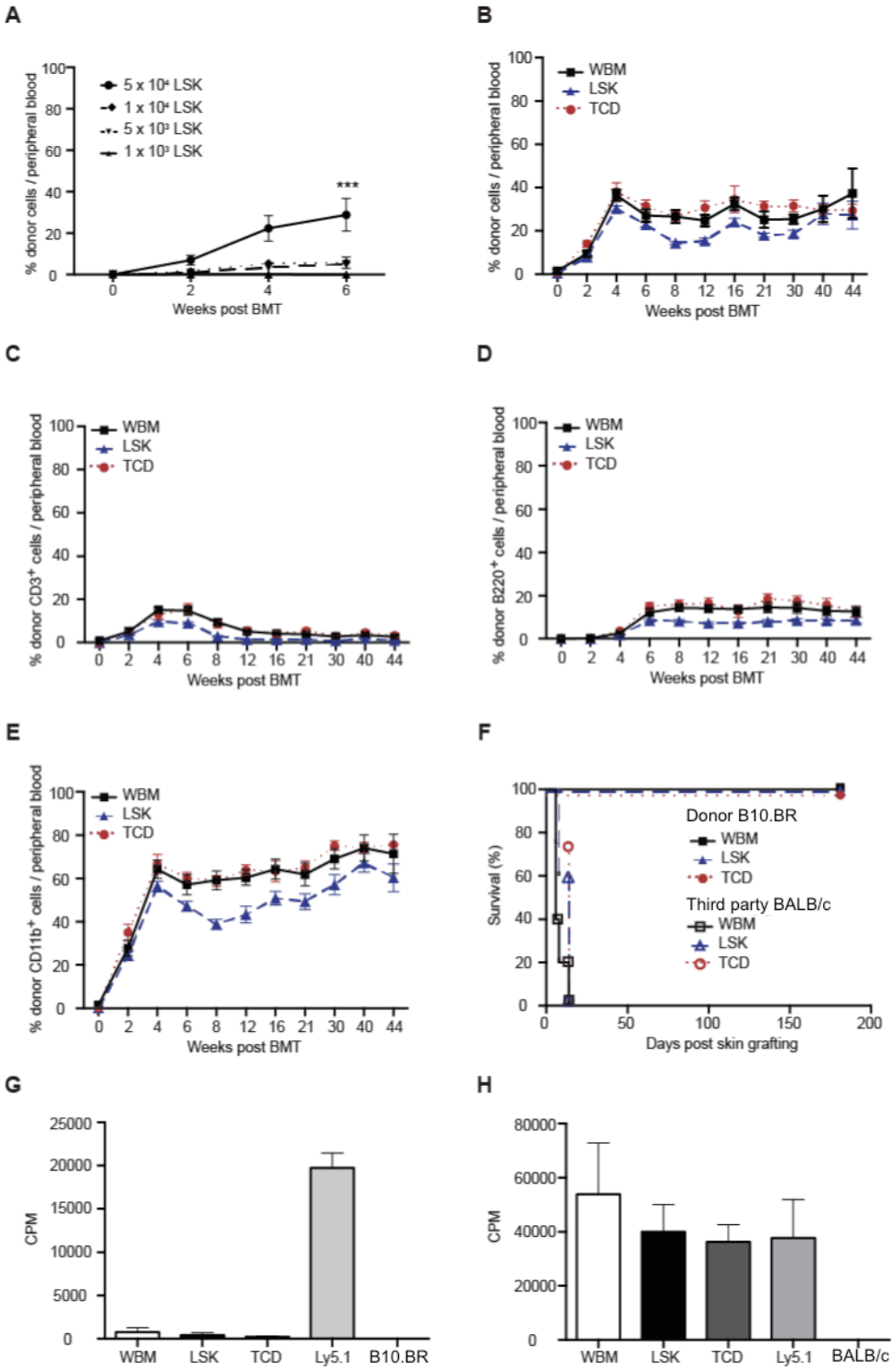

A
% donor cells / peripheral blood
Untreated
Bu/αCD4/αCD8/SIR
Weeks post BMT
B
% CD3 cells / peripheral blood
Untreated
Bu/αCD4/αCD8/SIR
Weeks post BMT
C
% donor CD3+ cells / peripheral blood
Untreated
Bu/αCD4/αCD8/SIR
Weeks post BMT
D
% donor B220+ cells / peripheral blood
Untreated
Bu/αCD4/αCD8/SIR
Weeks post BMT
E
% donor CD11b+ cells / peripheral blood
Untreated
Bu/αCD4/αCD8/SIR
Weeks post BMT
F
Survival (%)
Donor B10.BR
Third party BALB/c
Days post skin graft
G
CPM
SIR +
αCD4 +
αCD8 +
Ly5.1
B10.BR
untreated
H
CPM
SIR +
αCD4 +
αCD8 +
Ly5.1
BALB/c
untreated

Figure 3.4: Mixed chimerism and graft tolerance are achievable with purified BM progenitors, as well as T cell-depleted BM cells. **(A)** 6-8 weeks old B6.SJL-Ly5.1 mice were treated with 10 mg/kg busulfan on day -4, then transplanted with various numbers of congenic LSK cells from C57Bl/6 mice on day 0. The proportion of donor cells detected in peripheral blood following transplantation with $5x10^4$, $1x10^4$, $5x10^3$ or $1x10^3$ LSK cells was measured over time. **(B)** 6-8 week old B6.SJL-Ly5.1 (H-2^b) mice received 10 mg/kg busulfan on day -4, anti-CD4 and anti-CD8 monoclonal antibodies on day -3 and -1, $4x10^7$ B10.BR (H-2^k) allogeneic WBM cells (black square), LSK cells (blue triangle) or T cell-depleted BM (TCD) (red circle) on day 0, followed by daily sirolimus treatment for 28 days. 12 and 28 weeks after BMT, mice received an allogeneic skin graft from B10.BR (H-2^k) and BALB/c (H-2^d) respectively. The proportion of donor cells in peripheral blood was measured over time. **(C)** The proportion of donor $CD3^+$ T cells, **(D)** $B220^+$ B cells and **(E)** $CD11b^+$ macrophages in peripheral blood. **(F)** Donor and third-party skin graft survival. **(G)** *In vitro* allo-responsiveness was tested in a one-way MLR with stimulator splenocytes from donor B10.BR or **(H)** third-party unrelated BALB/c mice. Data are representative of one individual experiment, expressed as mean±SE and analysed using analysed using two-way ANOVA or an unpaired two-tailed t test with n=5 mice/group. ***($p \leq 0.001$) represents significant differences compared to other groups.


A
% donor cells / peripheral blood
5 x 10⁴ LSK
1 x 10⁴ LSK
5 x 10³ LSK
1 x 10³ LSK
***
Weeks post BMT
B
% donor cells / peripheral blood
WBM
LSK
TCD
Weeks post BMT
C
% donor CD3⁺ cells / peripheral blood
WBM
LSK
TCD
Weeks post BMT
D
% donor B220⁺ cells / peripheral blood
WBM
LSK
TCD
Weeks post BMT
E
% donor CD11b⁺ cells / peripheral blood
WBM
LSK
TCD
Weeks post BMT
F
Survival (%)
Donor B10.BR
WBM
LSK
TCD
Third party BALB/c
WBM
LSK
TCD
Days post skin grafting
G
CPM
WBM
LSK
TCD
Ly5.1
B10.BR
H
CPM
WBM
LSK
TCD
Ly5.1
BALB/c

LSK-transplanted mice compared to WBM- or TCD BM-transplanted mice. Nevertheless, LSK-transplanted mice were tolerant to allogeneic B10.BR skin grafts (Figure 3.4F) and were unresponsive to *in vitro* restimulation with B10.BR splenocytes (Figure 3.4G). These mice were immunocompetent, as demonstrated by their rapid rejection of third-party BALB/c skin (MST 10.6 days) (Figure 3.4F), and proliferated when challenged with third-party BALB/c stimulator cells (Figure 3.4H). Thus, stable chimerism and long-term allogeneic tolerance can be achieved by transplantation with TCD BM or purified haematopoietic cells in this minimal conditioning model.

Depletion of both CD4 and CD8 T cell subsets is required to establish tolerance in this model

We next investigated whether depletion of either $CD4^+$ or $CD8^+$ T cell subsets alone was sufficient to establish mixed chimerism and allogeneic tolerance. Depletion of $CD4^+$ T cells in combination with low-dose busulfan and short-course sirolimus (Bu/αCD4/SIR) established stable blood chimerism, but chimerism was lower than mice depleted of both $CD4^+$ and $CD8^+$ T cells and was insufficient to induce durable allogeneic tolerance, with all mice eventually rejecting donor B10.BR grafts (MST 65 days) (Figure 3.5B). However, Bu/αCD4/SIR-treated mice demonstrated allogeneic tolerance to donor haematopoietic antigens and showed reduced responsiveness when challenged with B10.BR splenocytes in an MLR (Figure 3.5C). Mice were immunocompetent and rejected third-party BALB/c allogeneic skin (MST 5 days) (Figure 3.5B) and responded to *in vitro* restimulation with BALB/c splenocytes (Figure 3.5D).

Depletion of $CD8^+$ T cells in combination with low-dose busulfan and sirolimus failed to establish chimerism, which dropped as soon as sirolimus was withdrawn 4 weeks after BMT (Figure 3.5E). This corresponded to a rapid rejection of B10.BR skin grafts (MST 13.6 days) (Figure 3.5F). Similar to Bu/αCD4/SIR, tolerance to B10.BR haematopoietic antigens was induced in Bu/αCD8/SIR-treated mice (Figure 3.5G). Mice were immunocompetent, rejecting third-party BALB/c skin graft (MST 8.6 days) (Figure 3.5F) and responded to restimulation with BALB/c splenocytes in a MLR (Figure 3.5H). Thus, the presence of alloreactive host $CD4^+$ T cells prevents the establishment of mixed haematopoietic chimerism and allogeneic skin graft tolerance, while alloreactive host $CD8^+$ T cells mediates the eventual rejection of allogeneic skin graft even in the presence of mixed chimerism.

Thymus-independent mode of tolerance induction

To determine the role of central tolerance in the establishment of mixed chimerism and allogeneic tolerance in this model, mice were either thymectomised (Tx) or sham-thymectomised (ShTx) prior to treatment with 10 mg/kg busulfan, host T cell depletion and short-term sirolimus. Tx mice developed blood chimerism levels equivalent to mice with an intact thymus (Figure 3.6A). Both Tx and ShTx mice were tolerant to allogeneic B10.BR skin grafts and maintained grafts for over 200 days (Figure 3.6B). Mice remained immunocompetent, with both groups rejecting third-party BALB/c skin grafts (MST 12 and 7 days respectively) (Figure 3.6B). The proportion of peripheral blood $CD3^+$ T cells in Tx mice was significantly lower than ShTx mice (Figure 3.5C). Within the T cell compartment, the proportion of donor cells in Tx mice was significantly higher than ShTx mice at 4 and 21 weeks after BMT, but no differences could be detected at later time points (Figure 3.6D). Both Tx and ShTx groups showed a decreased responsiveness to restimulation with B10.BR splenocytes (Figure 3.6E). However, despite rejecting third-party skin graft (Figure 3.6B), Tx mice displayed a decreased ability to respond to third-party BALB/c haematopoietic antigens (Figure 3.6F), probably as a result of an overall decrease in T cell numbers (Figure 3.6C).

As tolerance induction appears to be operating via thymic-independent mechanisms, we next investigated whether peripheral tolerogenic mechanisms were involved in this model. An increase in overall number, but not proportion, of T cells expressing PD-1 and CTLA-4 was observed in tolerant mice (data not shown). More strikingly, there was a proportional increase of Foxp3-expressing regulatory T (Treg) cells within the $CD4^+$ T cell compartment of mice that received donor WBM (Figure 3.7A), corresponding to a significant expansion in absolute number of Treg cells in the spleen, compared to mice that did not receive donor antigen (Figure 3.7B). These observations were consistent across treatment groups, with mice transplanted with purified allogeneic LSK cells or TCD BM exhibiting an increase in Treg cells. The ability to expand Treg cells was not dependent on the presence of a thymus, as a significant increase in both the proportion (Figure 3.7C) and absolute number of Treg cells was observed in the spleen of Tx mice (Figure 3.7D). This Treg expansion was not observed

Figure 3.5: Depletion of both host $CD4^+$ and $CD8^+$ T cells is required to establish tolerance in this model. (A) 6-8 week old B6.SJL-Ly5.1 (H-2^b) mice received 10 mg/kg busulfan on day -4, anti-CD4 (black triangle) or both anti-CD4 and anti-CD8 monoclonal antibodies (black square) on day -3 and -1, $4x10^7$ B10.BR (H-2^k) allogeneic WBM cells on day 0, followed by daily sirolimus treatment for 28 days. 12 and 28 weeks after BMT, mice received an allogeneic skin graft from B10.BR (H-2^k) and BALB/c (H-2^d) respectively. The proportion of donor cells in peripheral blood was measured over time. **(B)** Donor and third-party skin graft survival. **(C)** *In vitro* allo-responsiveness was tested in a one-way MLR with stimulator splenocytes from donor B10.BR or **(D)** third-party unrelated BALB/c mice. **(E)** To investigate the role of host $CD8^+$ T cells in this model, another group of mice received anti-CD8 monoclonal antibodies (black triangle) with no other variations in the treatment regime. The proportion of donor cells in peripheral blood detected over time was compared to mice that received both anti-CD4 and anti-CD8 monoclonal antibodies (black square). **(F)** Donor and third-party skin graft survival. **(G)** *In vitro* allo-responsiveness was tested in a one-way MLR with stimulator splenocytes from donor B10.BR or **(H)** third-party unrelated BALB/c mice. Data are representative of one individual experiment, expressed as mean±SE and analysed using two-way ANOVA or an unpaired two-tailed t test with n=5 mice/group. ***($p \leq 0.001$) represents significant differences compared to other treatment groups.

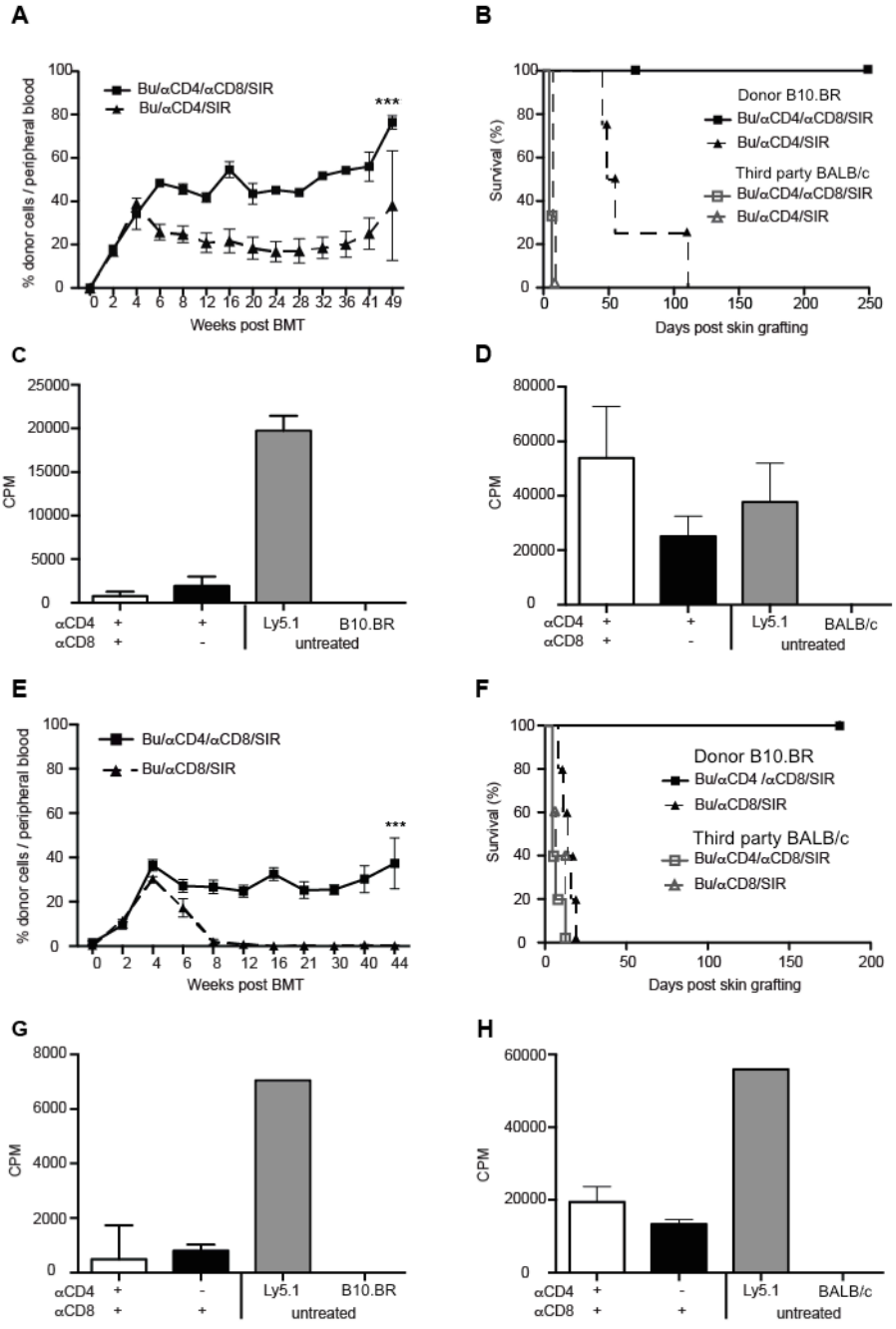

A
% donor cells / peripheral blood
Bu/αCD4/αCD8/SIR
Bu/αCD4/SIR
***
Weeks post BMT
B
Survival (%)
Donor B10.BR
Bu/αCD4/αCD8/SIR
Bu/αCD4/SIR
Third party BALB/c
Bu/αCD4/αCD8/SIR
Bu/αCD4/SIR
Days post skin grafting
C
CPM
αCD4
αCD8
Ly5.1
B10.BR
untreated
D
CPM
αCD4
αCD8
Ly5.1
BALB/c
untreated
E
% donor cells / peripheral blood
Bu/αCD4/αCD8/SIR
Bu/αCD8/SIR
***
Weeks post BMT
F
Survival (%)
Donor B10.BR
Bu/αCD4 /αCD8/SIR
Bu/αCD8/SIR
Third party BALB/c
Bu/αCD4/αCD8/SIR
Bu/αCD8/SIR
Days post skin grafting
G
CPM
αCD4
αCD8
Ly5.1
B10.BR
untreated
H
CPM
αCD4
αCD8
Ly5.1
BALB/c
untreated

Figure 3.6: Thymectomy does not abolish BMT-induced mixed chimerism and graft tolerance. (A) 6-8 week old B6.SJL-Ly5.1 (H-2^b) mice were thymectomised (Tx, black triangle) or sham-thymectomised (ShTx, black square) on day -11 and received 10 mg/kg busulfan on day -4, anti-CD4 and anti-CD8 monoclonal antibodies on day -3 and -1, $4x10^7$ B10.BR (H-2^k) allogeneic WBM cells on day 0, followed by daily sirolimus treatment for 28 days. 12 and 28 weeks after BMT, mice received an allogeneic skin graft from B10.BR (H-2^k) and BALB/c (H-2^d) respectively. The proportion of donor cells in peripheral blood was compared between Tx and ShTx mice over time. **(B)** Donor and third-party skin graft survival. **(C)** The total proportion of $CD3^+$ T cells and **(D)** the proportion of total $CD3^+$ cells that are of donor origin in peripheral blood. **(E)** *In vitro* allo-responsiveness was tested in a one-way MLR with stimulator splenocytes from donor B10.BR or **(F)** third-party unrelated BALB/c mice. Data are representative of one individual experiment, expressed as mean±SE and analysed using two-way ANOVA or an unpaired two-tailed t test with n=5 mice/group. ***($p \leq 0.001$) and ****($p \leq 0.0001$) represent significant differences compared to euthymic ShTx mice.

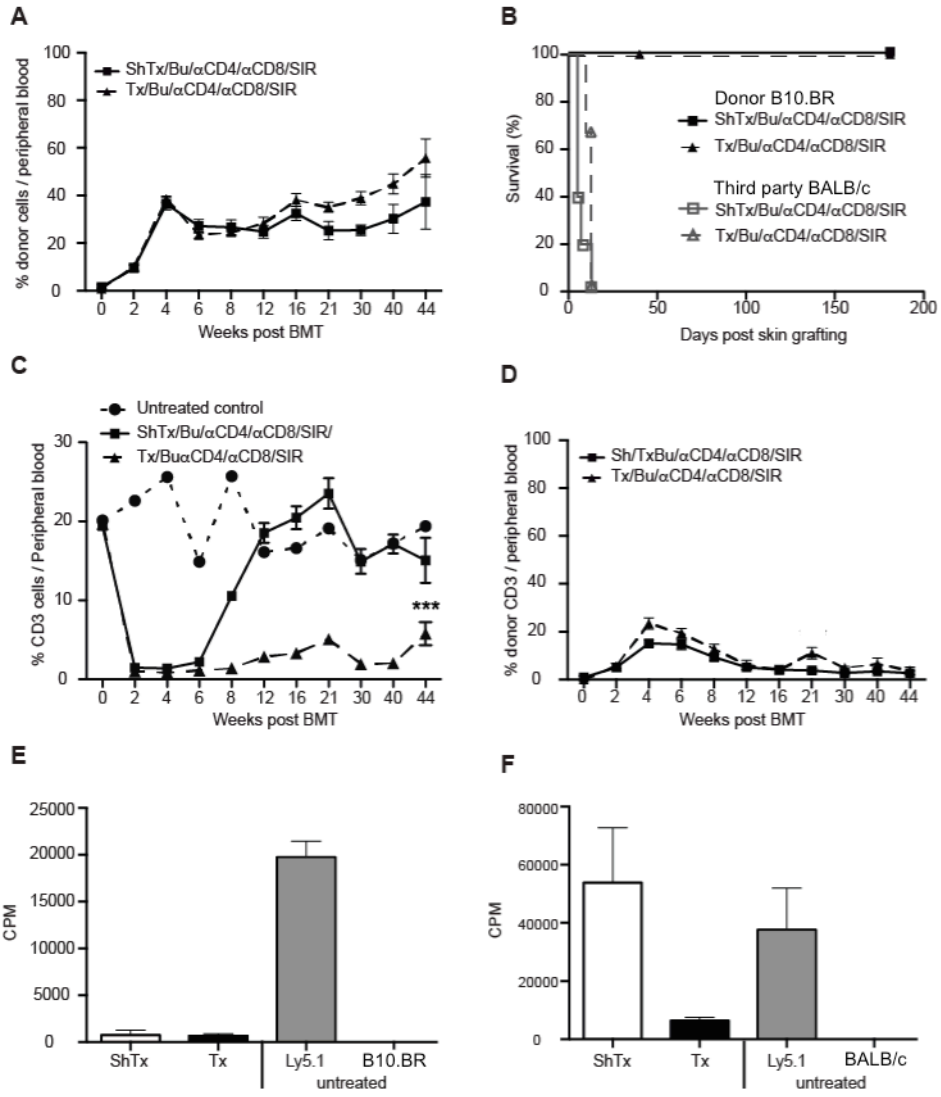
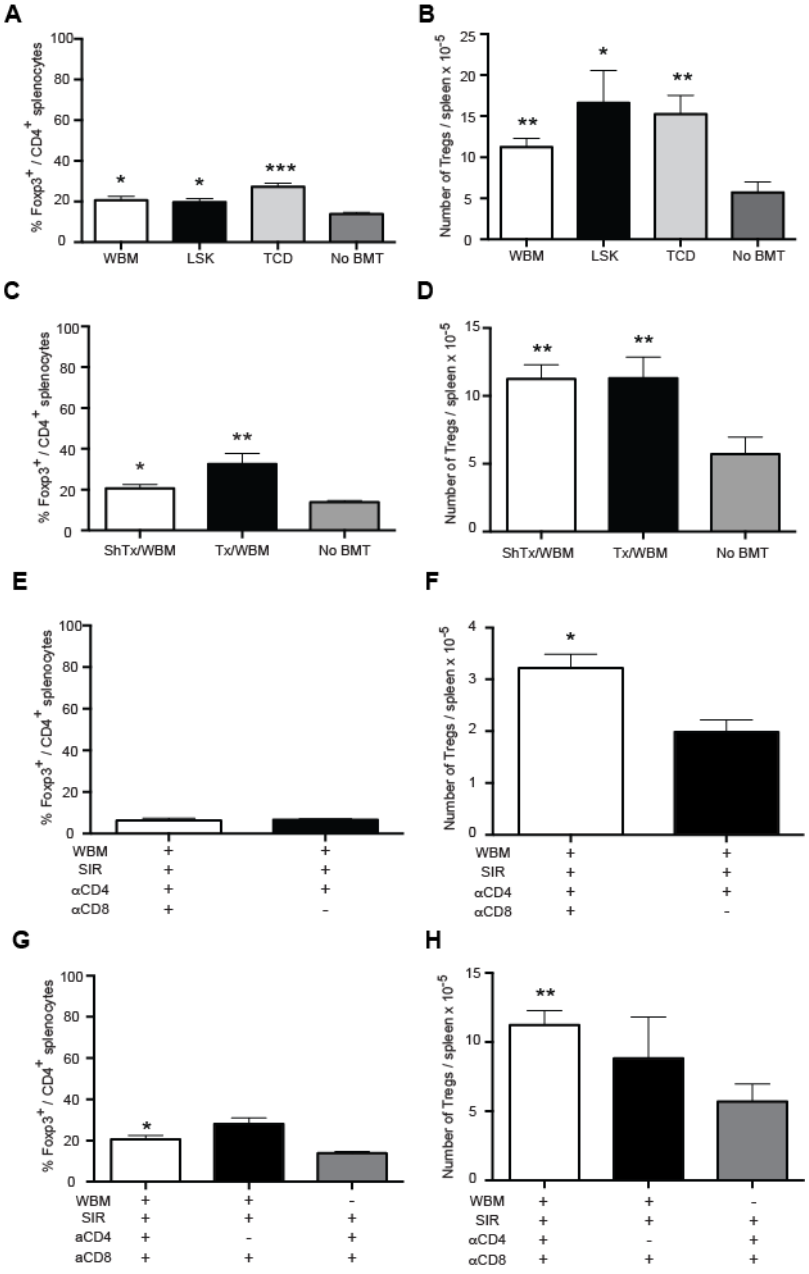

A
% donor cells / peripheral blood
ShTx/Bu/αCD4/αCD8/SIR
Tx/Bu/αCD4/αCD8/SIR
Weeks post BMT
B
Survival (%)
Donor B10.BR
ShTx/Bu/αCD4/αCD8/SIR
Tx/Bu/αCD4/αCD8/SIR
Third party BALB/c
ShTx/Bu/αCD4/αCD8/SIR
Tx/Bu/αCD4/αCD8/SIR
Days post skin grafting
C
Untreated control
ShTx/Bu/αCD4/αCD8/SIR/
Tx/BuαCD4/αCD8/SIR
% CD3 cells / Peripheral blood
***
Weeks post BMT
D
Sh/TxBu/αCD4/αCD8/SIR
Tx/Bu/αCD4/αCD8/SIR
% donor CD3 / peripheral blood
Weeks post BMT
E
CPM
ShTx
Tx
Ly5.1
B10.BR
untreated
F
CPM
ShTx
Tx
Ly5.1
BALB/c
untreated

Figure 3.7: Treg cells are increased in chimeric and tolerant mice. (A) The proportion and **(B)** number of $CD4^+$ $Foxp3^+$ Treg cells in mice transplanted with either donor WBM, LSK cells or T cell depleted (TCD) BM, compared to mice that did not receive donor BMT. **(C)** The proportion and **(D)** the number of Tregs in sham-thymectomised (ShTx/WBM) or thymectomised (Tx/WBM) mice that were transplanted with donor WBM cells compared to mice that did not receive donor BMT. **(E)** The proportion and **(F)** number of Treg cells in mice transplanted with WBM and treated with either anti-CD8 alone, or both anti-CD4 and anti-CD8 monoclonal antibodies. **(G)** The proportion and **(H)** number of Treg cells in mice transplanted with WBM and treated with either anti-CD4 alone, or both anti-CD4 and anti-CD8 monoclonal antibodies. Data are representative of one individual experiment, expressed as mean±SE and analysed using an unpaired two-tailed t test with n=5 mice/group. *($p \leq 0.05$), **($p \leq 0.01$) and ***($p \leq 0.001$) represent significant differences compared to mice that did not received donor BMT.


A
% Foxp3+ / CD4+ splenocytes
WBM
LSK
TCD
No BMT
B
Number of Tregs / spleen x 10-5
C
ShTx/WBM
Tx/WBM
D
E
WBM
SIR
αCD4
αCD8
F
G
aCD4
H

in mice harbouring alloreactive $CD8^+$ (Figure 3.7E and F) or $CD4^+$ T cells (Figure 3.7G and H) that did not demonstrate durable chimerism. Importantly, all treatment groups that showed an increase in Treg cells accepted allogeneic skin grafts. Hence, the induction of allogeneic tolerance via mixed chimerism in this minimal conditioning model is mediated by thymus-independent peripheral mechanisms, presumably by an expansion of Foxp3-expressing Treg cells.

Discussion

Ageing has profound effects on the immune system, including reduced diversity in the T cell receptor repertoire (30), diminished effector T and B cell function (31) and poor responses to viral challenge (32). Decreased immune responsiveness in aged individuals would seem advantageous to inducing allogeneic tolerance; however, the ability to generate functional T cells and induce antigen-specific responses following BMT declines with age (26). The importance of intrathymic deletion of donor-reactive T cell clones in the establishment of allogeneic tolerance has been clearly demonstrated (19, 33), yet protocols have rarely been tested in the aged setting where thymic function is severely compromised. Moreover, conditioning-induced damage to the thymus is compounded by age-related thymic atrophy and contributes to variability observed in the clinical setting. With this in mind, we sought to establish mixed chimerism and long-term allogeneic tolerance via a low-intensity, thymus-sparing protocol that would be applicable in the aged setting.

Mixed chimerism and allogeneic tolerance can be generated in mice receiving T cell-depleting antibodies and 3Gy TBI with 7Gy thymic irradiation (11). In this study, we first investigated whether thymic irradiation could be substituted with a short course of sirolimus, which inhibits T cell proliferation by blocking T effector responses to IL-2 (34) and inducing T cell anergy (35), while maintaining Treg cell function (36). We found that conditioning with 3Gy TBI and sirolimus could induce allogeneic tolerance in mice transplanted with $4x10^7$ WBM cells, a clinically feasible cell dose (5). However, peripheral blood chimerism degraded over time and was not stabilised when T cells were depleted prior to BMT. As the persistence of donor chimerism is required to maintain tolerance (12), we investigated busulfan as an alternative conditioning agent in an attempt to stabilise chimerism. Busulfan is an attractive agent for low-intensity conditioning because, unlike other commonly used chemotherapeutic agents, it depletes non-cycling primitive stem cells, which is essential to achieve lasting and high levels of donor hematopoietic engraftment (24).

Busulfan is typically administered at a dose of 20 mg/kg or higher, with higher levels of chimerism corresponding to dosage increases (6, 37, 38), as mice are relatively more resistant to busulfan than humans. We found that 10 mg/kg busulfan was sufficient to deplete LSK progenitors without severely affecting WBM cell numbers. Host LSK cell counts remained low 8 weeks following BMT (Figure 3.2K), while 80-90% of LSK cells in the BM were donor-derived (Figure 3.2I and J), compared to 20-25% in 3Gy-irradiated mice (Figure 3.1H). The higher levels of donor LSK engraftment could have contributed to the persistence of lymphoid and myeloid chimerism observed in busulfan-treated mice (24), compared to irradiated mice. Important to our aim of

developing a thymus-sparing conditioning protocol, 10 mg/kg busulfan had minimal and short-lasting effects on the thymus.

In a previous study, C57Bl/6 mice treated with a similar protocol using 20 mg/kg busulfan, T cell-depleting antibodies and transplanted with full-mismatched BALB/c WBM, followed by 14-day sirolimus immunosuppression, showed stable chimerism but nevertheless rejected donor skin grafts (38). We found that decreasing the busulfan dose to 10 mg/kg and increasing the sirolimus course to 28 days could efficiently establish long-term allogeneic tolerance to donor skin graft and haematopoietic antigens in 100% of mice. Importantly, aged mice treated with this regimen demonstrated stable chimerism and tolerance while maintaining immunocompetence.

Intrathymic clonal deletion is the principal mechanism by which allogeneic tolerance is induced in mixed BM chimeras (19). Models of transplantation tolerance that do not depend on BMT-induced mixed chimerism have also demonstrated a requirement for an active thymus (27, 39, 40). We found that thymectomised mice, surprisingly, were able to accept donor skin grafts and reject third-party unrelated skin grafts. Hence, tolerance was still generated in the absence of intrathymic deletion. Our data thus indicate peripheral mechanisms play a dominant role in tolerance induction in this model. In all mice that received sirolimus and either donor WBM or LSK cells, we found an increase in both the proportion and absolute number of Treg cells. This was not observed in mice that only received sirolimus. As sirolimus increases Treg survival and function in mice (36, 41) and humans (42, 43), our data suggest that Treg cells are induced in the presence of alloantigen and preferentially survive under the influence of sirolimus (36). Additionally, alloreactive T cell depletion is essential in this mode of tolerance, as neither depletion of host $CD4^+$ or $CD8^+$ T cells by itself was sufficient to induce tolerance.

A previous study utilising CD45RB monoclonal antibodies showed that tolerance could not be achieved in aged mice due to thymic atrophy (27), because this mode of tolerance induction requires an active thymus to produce new Treg cells (39). In the present study, 100% of aged mice became tolerant to allogeneic skin grafts, suggesting that tolerance induction in our model is not hindered by thymic atrophy and/or compensated by peripheral mechanisms. Our thymectomy data suggest that Treg cells are being produced in response to donor antigens in peripheral tissues, rather than when antigen sampled in the periphery is recirculated to the thymus (44). Facilitating cells in WBM have been shown to induce antigen-specific Treg cells that enhance progenitor cell engraftment (45), but this is unlikely to be the sole mechanism, as Treg cells and allogeneic

tolerance were also generated when mice were transplanted with purified LSK progenitor cells lacking the facilitating cell population. These results do not indicate that peripheral tolerance is mutually exclusive from central tolerance and it is likely that both central deletion and peripheral suppression influence tolerance induction in euthymic mice.

Recently it was reported that, despite increased memory T cell frequencies, tolerance can be established in aged mice by costimulation blockade or host T cell depletion, in conjunction with low-dose irradiation (28). We now demonstrate that radiation-free, low-intensity conditioning with busulfan facilitates the generation of allogeneic tolerance via BMT-induced mixed chimerism in aged mice. In this model, peripheral regulation operates in the absence of a thymus and age-related thymic involution does not impede the ability to induce donor-specific tolerance. Our findings have important implications for the clinical application of tolerance induction protocols, especially in aged patients whose immune recovery might be dangerously slow due to thymic atrophy.

Acknowledgements

The authors would like to thank Ms. Luciana Thompson and Ms. Jade Barbuto for excellent technical assistance. This work was supported by an Early Translational Award from the Victoria-California Stem Cell Alliance between the Californian Institute for Regenerative Medicine and the State Government of Victoria, Australia (TR1-01245). J.M. is the recipient of an Australian Postgraduate Award from the National Health and Medical Research Council and a Postgraduate Supplementary Scholarship from the Australian Stem Cell Centre. T.H. is the recipient of an Australian Postdoctoral Research (Industry) Fellowship from the Australian Research Council (LP110201169).

Disclosure

The authors of this manuscript have no conflicts of interest to disclose as described by the American Journal of Transplantation.

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CHAPTER FOUR

The impact of reduced intensity conditioning on the bone marrow of aged mice and strategies to boost hematopoietic chimerism in aged mice following autologous and allogeneic bone marrow transplantation

Abstract

Age-related immunosenescence, preceded by compromised thymus and bone marrow, is often overlooked when trying to induce allogeneic tolerance via BMT. Here we assessed the ability of low-dose busulfan to condition the BM to facilitate BMT engraftment in aged mice, and further if age-related thymic involution is a significant barrier to the induction of allogeneic tolerance via mixed chimerism. The BM of aged mice was more resistant to busulfan chemotherapy than young BM, leading to decreased donor cell engraftment and chimerism following congeneic HSCT. To investigate why chimerism was decreased in aged mice, we analysed the ability of low-dose busulfan to condition aged-BM compared to young-BM. We found that aged BM was relatively resistant to busulfan-mediated chemotherapy and that sensitivity to chemotherapy could be restored by pre-treatment with G-CSF, freeing space within the niche. However, G-CSF prior to low-dose busulfan did not boost engraftment of congeneic LSKs. Because the engraftment of purified donor-LSKs was poor, we investigated the ability of allogeneic WBM, which contains T cells that have been proposed to promote HSC engraftment, to promote mixed chimerism and induce allogeneic tolerance in aged mice. Mice were conditioned with T cell depleting antibodies, sirolimus immunosuppression and low-dose busulfan. Under these conditions we found that engraftment can be achieved in aged mice and that in this model age-related thymic involution does not impede the ability to induce allogeneic tolerance.

Key words: age, immunosenescence, BMT, HSCT, congenic, allogeneic, tolerance

Introduction

The most logical approach to inducing donor tolerance to any transplant would be to harness the thymus-based mechanism of self-tolerance and apply this to the allograft setting. The basic premise is that the donor HSCs will engraft in the host bone marrow (BM) and haematopoietic progenitors will migrate to the thymus to become both T cells and dendritic cells, capable of inducing host tolerance to donor MHC. While this principle is well accepted, the influence of age on the capacity to induce tolerance via BM transplantation (BMT) -induced mixed chimerism has been poorly characterised. BMT is remarkably effective and safe in young patients (1), however increasing age is associated with poorer outcomes and a higher occurrence of complications, such as graft versus host disease (GVHD) and poor engraftment following allogeneic BMT (2-6). The development of low-intensity conditioning regimes have improved outcomes in older patients who receive allogeneic BMT to treat haematological malignancies (7), however, the most successful pre-clinical and clinical studies that examine low-intensity conditioning protocols for mixed chimerism induced tolerance have been performed on young-middle aged individuals (8-12).

The applicability of mixed chimerism induced transplantation tolerance in older patients may be further complicated by age-induced thymic involution. This is characterised by a progressive reduction in thymic size, a marked disorganisation of the microenvironment, the replacement of functional lymphoid tissue with adipocytes and a significant decrease in T cell output (13), leaving the thymus functioning at only 5 % capacity by 10-12 months in the mouse (14) and 40 years in humans (15). It has previously been shown that to maintain stable allogeneic tolerance in mixed chimeras, there is an absolute requirement for donor-antigen dependent, intrathymic deletion of alloreactive T cells, which therefore requires an adequately functioning thymus (16); age may thus present a significant barrier to the induction of tolerance via BMT. Compounding age-related thymic involution is the use of cytoreductive treatments, required to promote BMT engraftment, but which cause further thymic damage - some even specifically target the thymus such as focal irradiation (17, 18).

Immunosenescence, which broadly defines the reduced responsiveness of the immune system to novel antigens with age, can be attributed to functional decreases in a number of cell types including, hematopoietic stem cells (HSCs) (19), effector T cells and Tregs (20), B cells (21, 22), macrophages and neutrophils (23, 24). These aged-related changes to the immune system result in increased susceptibilities to autoimmunity (25, 26) and malignancy (27, 28) as well as increases in

infection rates and poorer responses to vaccination (29-31). Immunosenescence also presents further challenges to the induction of tolerance in the aged; while a decreased responsiveness to immunogenic stimuli would seem favourable, inducing tolerance is an active process requiring functional responses in both the thymus (16) and the periphery (32). Supporting this Zhao *et al*. show that allogeneic tolerance, induced via CD45RB monoclonal antibodies fails in mice 1-year old and above (33). Although the study by Zhao and colleagues did not use BMT-induced mixed chimerism to generate transplantation tolerance, it does support the hypothesis that ageing hampers the ability to induce tolerance.

Reversing thymic atrophy and hence immunosenescence would thus be expected to enhance tolerance induction in aged mice; this can be achieved via both physical and chemical sex steroid ablation (SSA) (34, 35). SSA increases thymic cellularity, which leads to increased T cell emigration and improved peripheral T cell function (36, 37). Supporting this, Zhao *et al*, showed that the inability to induce tolerance in aged mice with CD45RB monotherapy is restored when mice undergo SSA prior to treatment (33). SSA can also protect the thymus during chemotherapy; this is true for both busulfan-mediated chemotherapy (see Chapter 2) and other chemotherapeutic drugs (38). Additionally SSA enhances recovery of the hematopoietic system following both autologous and allogeneic HSCT (39-41), suggesting that SSA coupled to protocols that induce allogeneic tolerance in aged mice may improve outcomes.

We have recently characterised the effects of low-dose busulfan on young mice, focusing on reducing thymic damage whilst still permitting "space" in the BM for donor cells to engraft (see Chapter 2). In young mice, low-dose busulfan depleted Lineage negative, $Sca1^{+}$ $cKit^{+}$ (LSK) progenitor cells, a population enriched for HSCs (42), but did not cause overt damage to the thymus or peripheral lymphoid cells, allowing congenic LSK progenitor cells to engraft and promote robust levels of peripheral chimerism. Additionally, SSA prior to HSCT boosts thymic cellularity and donor-cell out put (Chapter 2). We have also shown that in combination with T cell depleting antibodies and short-term sirolimus immunosuppression, low-dose busulfan promotes the engraftment of whole BM (WBM) or LSK progenitor cells, inducing multi-lineage mixed chimerism and allogeneic tolerance in both young and aged mice (Chapter 3). In this present study, we examined the ability of low-dose busulfan to condition the BM of aged mice to allow the engraftment of both congenic and allogeneic HSCs (as either LSKs or WBM) and establish mixed chimerism as the foundation for tolerance induction.

Methods

Animals

Male C57BL/6 (H-2^b, Ly5.2), B6.SJL-ptprc (H-2^b, Ly5.1 congenic), B10.BR (H-2k) and BALB/c (H-2^d) were purchased at 6-8 weeks of age or 11 to 12 months of age form Monash Animal Services (Melbourne, Australia), the Walter and Elisa Hall Institute (Melbourne, Australia) or the Animal Resources Centre (Perth Australia). Animals were housed under specific pathogen-free conditions (SPF) at the Monash University Animal Research Laboratories. All animal experiments were performed in accordance with the Australia Code of Practice for the Care and Use of Animals for Scientific Purposes (2004, 7th edition), after approval by the Monash University School of Biomedical Sciences Animal Ethics Committee.

Conditioning and hematopoietic stem cell transplantation

For chemotherapy experiments, mice received a single intra-peritoneal (IP) injection of busulfan at a dose of 10 mg/kg or 20 mg/kg (Bulsulfex, PDL BioPharma, Fremont, CA). For hematopoietic stem cell transplantion (HSCT) experiments, mice received a single IP dose of 10 mg/kg busulfan 4 days prior to transplantation with either 5 x 10^4 (high-dose) or 1 x 10^4 (low-dose) Lineage negative $Sac1^+$ $cKit^+$ (LSK) progenitor cells, administered via lateral tail vein injection. LSK progenitor cells were purified from single cell suspensions of BM from femurs and tibiae of C57Bl/6 (congenic) mice and sorted on a BD/Cytopeia Influx II flow cytometer (BD Bioscience, Franklin Lakes, N.J). For allogeneic transplant experiments, 12-month old mice received 10 mg/kg Busulfan (Bulsulfex, PDL BioPharma, Fremont, CA), 4 days prior to transplantation with 4 x 10^7 B10.BR WBM cells. Mice received two IP injections of depleting antibodies to CD4 (GK1.5, 0.1 mg/dose) and CD8 (2.43, 0.1 mg/dose) (Bio X Cell, West Lebanon, NH) on days -3 and -1 relative to BMT. Sirolimus (3mg/kg/dose) (Rapamycin, LC Laboratories, Woburn, MA) was administered daily, via IP injection, for 28 days following BMT. Single cell suspensions of BM were flushed from femurs and tibiae of C57Bl/6 (congenic) or B10.BR (allogeneic) donor mice and administered via lateral tail vein injection.

Sex steroid ablation

Male mice were anaesthetized with Isofluorane (2-3% in Oxygen) (Delvet, NSW, Australia) and a small scrotal incision was made to reveal the testes. Testes were tied-off and removed along with the surrounding fatty tissue. The wound was closed using sutures. Sham-sex steroid ablated (SSA)

mice followed the same surgical procedure, except for the tying-off and removal of the testes. SSA was performed 1 day prior to chemotherapy.

Pharmacological inhibition of adipocyte formation

Mice received daily IP injections of BADGE (Fluka), at 30 mg/kg in 10% DMSO (Sigma), 5 days prior to and 14 days following HSCT. Control animals receive the equivalent volume of 10 % DMSO.

Bone marrow mobilisation

Mice received subcutaneous (SC) injections of G-CSF (Filgrastrim, Amgen, Thousand Oaks, CA, USA) at 250 μg/kg in saline 100 μl/10 g body weight twice daily, 6-8 hours apart, for 4 consecutive days prior to chemotherapy with 10 mg/kg busulfan. Control animals received the equivalent volume of saline.

Skin grafting

Skin grafting was performed on Isofluorane (2-3% in Oxygen) (Delvet, NSW, Australia) anesthetized mice by grafting full thickness tail skin (1 x 1 cm), from donor B10.BR (allogeneic) and BALB/c (third-party) (6-8 week old) mice, onto the lateral flanks of B6.SJL-ptprc mice. Bandages were removed 7 days after surgery and grafts were monitored daily. Rejection was recorded when more than 75% of the graft's epithelial tissue had broken down.

Cell collection and staining

Peripheral blood was collected via retro-orbital bleeds. Red blood cells (RBC) were removed by suspension in lysis buffer (0.9 % w/v ammonium chloride/ 10 % v/v 0.1M Tris-hydrochloride/pH 7.5 ±0.2) at room temperature for 1 minute. Single cell suspensions of BM cells, thymocytes or splenocytes were prepared via manual mincing between frosted glass slides. Cells were washed in FACS buffer (phosphate buffered saline (PBS)/1% v/v fetal calf serum (FCS)/ 0.1% w/v sodium-azide) and 3 x 10^6 cells were incubated for 30 minutes at 4^oC with primary antibodies, and then washed twice with FACs buffer.

Single cell suspensions of thymocytes or splenocytes were prepared via manual mincing between frosted glass slides. BM mononuclear cells were flushed from the femurs and tibiae with cold

FACS buffer (phosphate buffered saline (PBS)/1% v/v fetal calf serum (FCS)/ 0.1% w/v sodium-azide) using a 23G needle. Cells were pelleted at 350_{gmax} for 5min at 4˚C. Red blood cells (RBC) were removed by resuspending cell pellets in lysis buffer (0.9 % w/v ammonium chloride/ 10 % v/v 0.1M Tris-hydrochloride/pH 7.5 ±0.2) at room temperature for 1 minute. Cells were washed in FACs buffer and 3 x 10^6 cells were incubated for 30 minutes at 4^{o}C with primary antibodies, and then washed twice with FACs buffer. Cell counts were determined by gating on viable cells based on size using a Z2 Coulter Counter (Bekman Coulter).

Flow cytometric analysis

Multi-lineage chimerism was assessed via flow cytometric analysis. Cells were labelled with CD45.2 (104), CD3 (145-2C11), CD49b (DX5, Biolegend), CD45.1 (A20), IA/IE (MS/114, Miltenyi), CD45R (RA3-6B2), CD11b (M1/70) and CD11c (HL3). BM progenitor populations were assessed with a lineage cocktail consisting of CD3 (145-2C11), CD4 (RM4-5), CD8 (53-6.7), CD19 (1D3), NK1.1 (PK136), CD11b (M1/70), Gr-1 (RB6-8C5), TER-119 (TER-119) and CD11c (HL3), in addition to CD117 (2B8) and Sca-1 (D7). T cell populations were assessed with CD4 (RM4-5, eBioscience), CD8 (53-6.7), TCRβ (H57-597) and CD25 (PC61, Biolegend) and intracellular staining with Foxp3 (FJK-16a, eBioscience). Peripheral lymphoid and myeloid cells were assessed with CD11c (HL3), Gr-1 (RB6-8C5), CD45R (RA3-6B2), CD11b (M1/70), I-A/I-E (M5/114.15.2, Biolegend). For all staining, an FcR block (2.4G2, laboratory produced) was used. Unless otherwise stated, all antibodies were obtained from BD Biosciences. All data were acquired on a BD FACSCanto II and analysed using Flowjo software.

Statistical analysis

Statistical analysis was performed using an unpaired, two-tailed t test or a two-way ANOVA. A p value ≤ 0.05 was considered to be significant.

Results

Aged mice have decreased chimerism compared to young mice following congenic HSCT and chimerism is not improved with SSA.

We have previously shown that conditioning of young recipient (young) mice with low-dose busulfan prior to congenic HSCT allowed donor cells to engraft and produce multi-lineage chimerism. SSA prior to busulfan-mediated conditioning, increased total thymocyte number, of both donor and host origin, and their release into the periphery, following both high-dose (5 x 10^4) and low-dose (1x10^4) HSCT (Chapter 2). This chimerism was compared with aged recipient (aged) mice (with significant immunosenescence and thymic atrophy) that were surgically castrated (SSA) or sham-castrated (sham-SSA) prior to conditioning with 10 mg/kg busulfan and transplanted with a high-dose (5 x 10^4) of congenic LSK cells. 56 days following HSCT mice were analysed via flow cytometry and total BM cellularity was decreased in aged mice compared to the young (Figure 4.1A) and SSA did not increase BM cell numbers in either age group. In the BM, the proportion of donor cells observed in aged mice following high-dose congenic HSCT was decreased 5-fold compared to young, leading to a 3-fold decrease in the absolute number of donor-cells in aged mice compared to young mice (Figure 4.1A). This clearly indicated that the aged BM was greatly compromised in its ability to engraft transplants. SSA had no effect on the total number, or proportion and absolute number of donor-derived LSKs, B cells or macrophages in the BM of aged mice transplanted with 5 x 10^4 congenic LSK cells (Figure 4.1).

The proportion of donor cells observed in the thymus of aged mice transplanted with high dose (5 x 10^4) LSKs was not significantly reduced compared to young, however, the absolute number of donor thymocytes decreased by 3 fold in aged mice (Figure 4.1B). Supporting results from Chapter 2, we found that SSA boosted thymic cellularity in young mice following high-dose HSCT and 10 mg/kg busulfan. This was also true for aged mice, with SSA significantly increasing total thymic cellularity compared to sham-SSA mice (Figure 4.1Bi). SSA also significantly increased the number of thymocytes in aged mice, but not to young-untreated levels (Figure 4.1B). This was also observed in the $CD8^+$ and DP thymocyte subsets (Figure 4.2B). Similar to the BM, both the proportion and number of donor-derived thymocytes was decreased in aged mice compared to young mice (Figure 4.1B). Despite the lack of proportional changes observed in the thymus, SSA did significantly increase the number of donor-derived thymocytes observed in aged mice (Figure 4.1B), which was also evident in the $CD8^+$ and DP thymocyte subsets (Figure 4.2B). This was in contrast to the aged spleen; whereby SSA significantly increased splenic cellularity compared to

sham-SSA in young but not aged mice (Figure 4.1C). Supporting this, SSA did not significantly increase total number of $CD4^+$ or $CD8^+$ T cells, B cells or macrophages in the spleen of aged mice (Figure 4.2C). Again, similar to both the BM and thymus, the proportion and number of donor-derived splenocytes was decreased in the aged compared to young and SSA did not significantly improve chimerism in either age groups (Figure 4.1C). This was also observed in both the proportion and number of $CD4^+$ and $CD8^+$ T cells, B cells and macrophages within the spleen (Figure 4.2C).

To assess if SSA could improve low-dose HSCT, aged mice were transplanted with 1 x 10^4 congenic LSK progenitor cells following 10 mg/kg busulfan and analysed 28 and 56 days following chemotherapy. In this low-dose regime, SSA significantly increased total cellularity in the BM compared to untreated control mice; this was observed at both 28 and 56 days following HSCT. Additionally at day 56, SSA also significantly increased total cellularity compared to sham-SSA mice. SSA had no effect on the proportion or number of donor cells in the BM at either time point (Figure 4.3A). This is consistent with previous experiments conducted in young mice, which show SSA has no significant effect on donor proportion or number 28 days following HSCT (Chapter 2).

Similar to BM, SSA significantly increased total thymocyte number; this was observed at both 28 and 56 days following low-dose HSCT. SSA also significantly increased both the proportion and number of donor cells at day 28, however this effect was lost by 56 days following HSCT (Figure 4.3B).

SSA significantly increased total splenocytes, compared to both untreated and sham-SSA controls at day 28 and compared to sham-SSA mice at day 56 following SSA. Splenic cellularity of sham-SSA mice was significantly depleted compared to untreated control mice. SSA had no significant effect on the proportion or number of donor-derived splenocytes (Figure 4.3C). Together these data indicate that SSA prior to busulfan-chemotherapy and congenic HSCT does not boost donor cell engraftment or output in the BM of aged mice.

Aged BM is more resistant to busulfan-chemotherapy than young BM.

Conditioning prior to HSCT is required to generate space in the BM for transplanted cells to engraft. To assess if the low level of engraftment observed in aged mice is due a lack of depletion in

Figure 4.1: The proportion and number of donor cells observed in the BM, thymus and spleen of aged recipient mice is decreased compared to young recipient mice following congenic high-dose HSCT and is not improved with SSA. (A) (i) Quantification of total BM cellularity in young recipient mice, (ii) quantification of total BM cellularity in aged recipient mice (iii) the proportion of donor cells in WBM of young recipient mice, (iv) the proportion of donor cells in WBM of aged recipient mice, (v) the absolute number of donor cells in WBM of young recipient mice (vi) the absolute number of donor cells in WBM of aged recipient mice. **(B)** (i) Quantification of total thymus cellularity in young recipient mice, (ii) quantification of total thymus cellularity in aged recipient mice (iii) the proportion of donor cells in the thymus of young recipient mice, (iv) the proportion of donor cells in the thymus of aged recipient mice, (v) the absolute number of donor cells in thymus of young recipient mice (vi) the absolute number of donor cells in the thymus of aged recipient mice. **(C)** (i) Quantification of total spleen cellularity in young mice, (ii) quantification of total spleen cellularity in aged recipient mice (iii) the proportion of donor cells in the spleen of young recipient mice, (iv) the proportion of donor cells in the spleen of aged recipient mice, (v) the absolute number of donor cells in spleen of young recipient mice (vi) the absolute number of donor cells in the spleen of aged recipient mice. Mice were sex steroid ablated (SSA) 1 day prior to chemotherapy with 10 mg/kg busulfan. 4 days following chemotherapy mice were transplanted with 5 x 10^4 congenic LSK cells from 8 week old mice and chimerism was analysed 56 days following HSCT. Untreated control mice (white bars), Sham-SSA (grey bars) and SSA (black bars). Data is representative of two individual experiments, expressed as mean ± SE and analysed using an unpaired, two-tailed T-test. * ($p< 0.05$), ** ($p< 0.01$) represent significant differences compared to untreated mice. ^ ($p< 0.05$), ^^ ($p< 0.01$) represent significant differences when comparing sham-SSA and SSA groups.

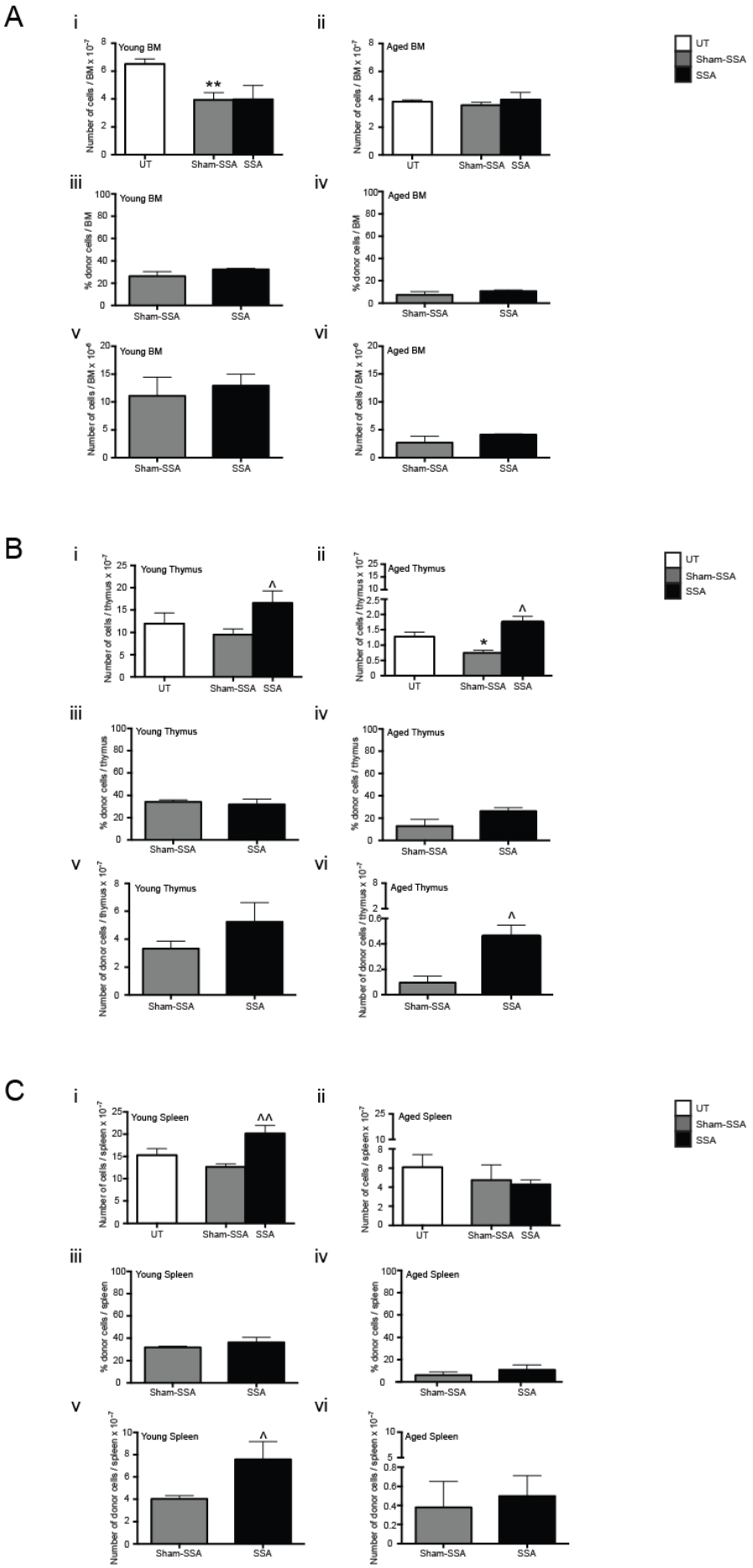

A
i
Young BM
Number of cells / BM x 10^-7
**
UT
Sham-SSA
SSA
ii
Aged BM
Number of cells / BM x 10^-7
UT
Sham-SSA
SSA
UT
Sham-SSA
SSA
iii
Young BM
% donor cells / BM
Sham-SSA
SSA
iv
Aged BM
% donor cells / BM
Sham-SSA
SSA
v
Young BM
Number of cells / BM x 10^-6
Sham-SSA
SSA
vi
Aged BM
Number of cells / BM x 10^-6
Sham-SSA
SSA
B
i
Young Thymus
Number of cells / thymus x 10^-7
^
UT
Sham-SSA
SSA
ii
Aged Thymus
Number of cells / thymus x 10^-7
*
^
UT
Sham-SSA
SSA
UT
Sham-SSA
SSA
iii
Young Thymus
% donor cells / thymus
Sham-SSA
SSA
iv
Aged Thymus
% donor cells / thymus
Sham-SSA
SSA
v
Young Thymus
Number of donor cells / thymus x 10^-7
Sham-SSA
SSA
vi
Aged Thymus
Number of donor cells / thymus x 10^-7
^
Sham-SSA
SSA
C
i
Young Spleen
Number of cells / spleen x 10^-7
^^
UT
Sham-SSA
SSA
ii
Aged Spleen
Number of cells / spleen x 10^-7
UT
Sham-SSA
SSA
UT
Sham-SSA
SSA
iii
Young Spleen
% donor cells / spleen
Sham-SSA
SSA
iv
Aged Spleen
% donor cells / spleen
Sham-SSA
SSA
v
Young Spleen
Number of donor cells / spleen x 10^-7
^
Sham-SSA
SSA
vi
Aged Spleen
Number of donor cells / spleen x 10^-7
Sham-SSA
SSA

Figure 4.2: SSA increases total and donor thymocyte cell numbers following congenic high-dose HSCT but has no significant effect on the BM or spleen. (A) (i) Total Lineage negative $Sca1^+$ $cKit^+$ (LSK) progenitor cell number in aged mice, (ii) proportion of donor cells in LSK progenitor cell subset of aged mice and (iii) absolute number of donor-derived LSKs in aged mice. **(B)** (i) Total number of $CD4^+$ and $CD8^+$ single positive, $CD4^+CD8^+$ double positive and $CD4^-CD8^-$ double negative thymocytes in aged mice, (ii) proportion of donor cells across $CD4^+$ and $CD8^+$ single positive, $CD4^+CD8^+$ double positive and $CD4^-CD8^-$ double negative thymocytes subsets in aged mice and (iii) absolute number of donor-derived $CD4^+$ and $CD8^+$ single positive, $CD4^+CD8^+$ double positive and $CD4^-CD8^-$ double negative thymocytes in aged mice. **(C)** (i) Total number of TCR^+CD4^+ and TCR^+CD8^+ T cells, $B220^+$ B cells and $CD11b^+$ macrophages, (ii) proportion of donor cells across TCR^+CD4^+ and TCR^+CD8^+ T cells, $B220^+$ B cells and $CD11b^+$ macrophages splenocyte subsets and (iii) absolute number of donor-derived TCR^+CD4^+ and TCR^+CD8^+ T cells, $B220^+$ B cells and $CD11b^+$ macrophage splenocyte subsets. Mice were sex steroid ablated (SSA) 1 day prior to chemotherapy with 10 mg/kg busulfan. 4 days following chemotherapy mice were transplanted with 5×10^4 congenic LSK cells and chimerism was analysed 56 days post HSCT. Untreated control mice (white bars), Sham-SSA (grey bars) and SSA (black bars). Data is representative of two individual experiments, expressed as mean ± SE and analysed using an unpaired, two-tailed T-test. * ($p < 0.05$) represents significant differences compared to untreated mice. ^ ($p < 0.05$) represents significant differences when comparing sham-SSA and SSA groups.

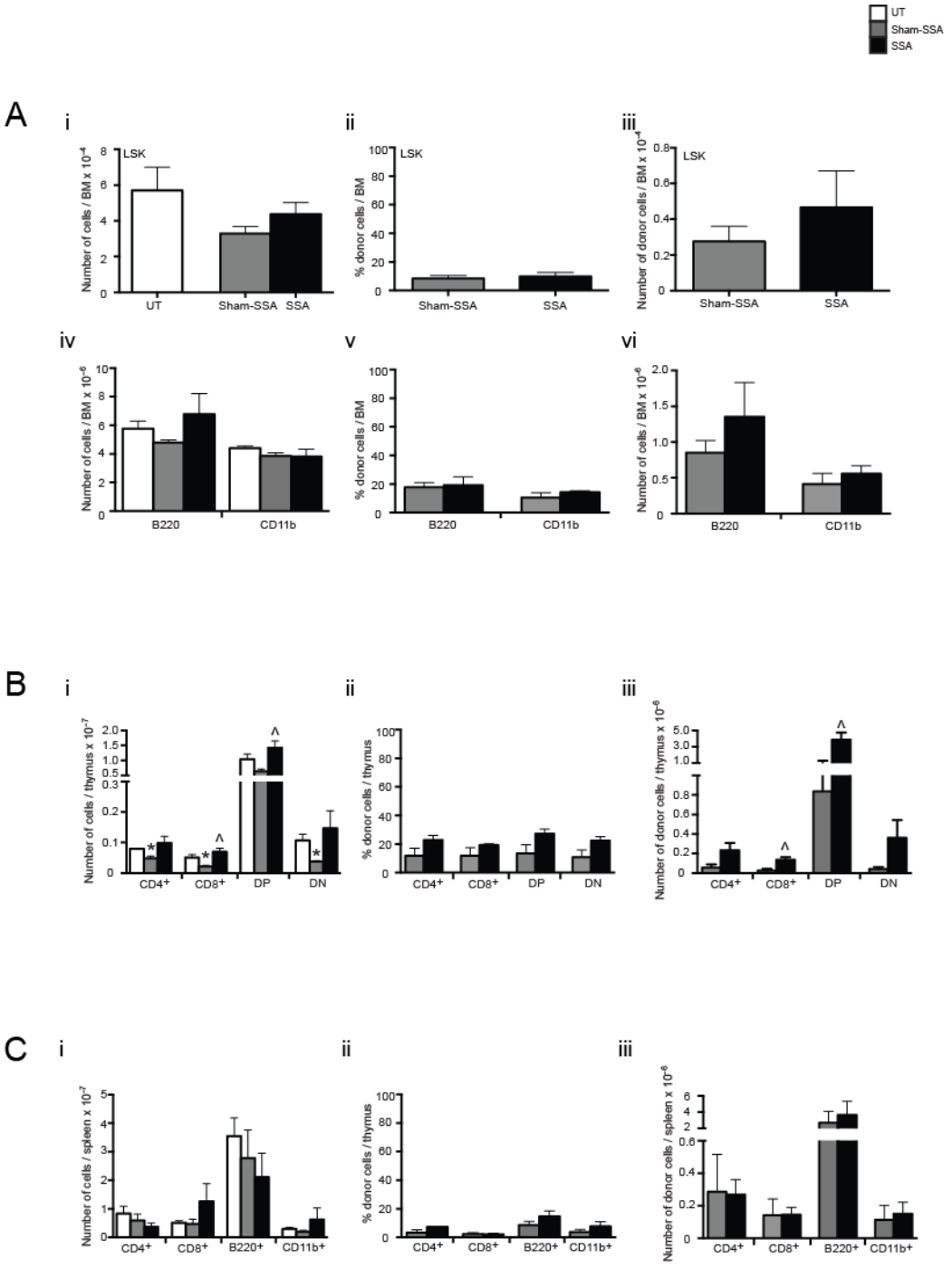

UT
Sham-SSA
SSA
A
i
LSK
Number of cells / BM x 10⁻⁴
UT
Sham-SSA
SSA
ii
LSK
% donor cells / BM
Sham-SSA
SSA
iii
LSK
Number of donor cells / BM x 10⁻⁴
Sham-SSA
SSA
iv
Number of cells / BM x 10⁻⁶
B220
CD11b
v
% donor cells / BM
B220
CD11b
vi
Number of cells / BM x 10⁻⁶
B220
CD11b
B
i
Number of cells / thymus x 10⁻⁷
CD4+
CD8+
DP
DN
ii
% donor cells / thymus
CD4+
CD8+
DP
DN
iii
Number of donor cells / thymus x 10⁻⁶
CD4+
CD8+
DP
DN
C
i
Number of cells / spleen x 10⁻⁷
CD4+
CD8+
B220+
CD11b+
ii
% donor cells / thymus
CD4+
CD8+
B220+
CD11b+
iii
Number of donor cells / spleen x 10⁻⁶
CD4+
CD8+
B220+
CD11b+

Figure 4.3: SSA increases the total number of cells in WBM, thymus and spleen, but does not increase the proportion or number of donor cells following low-dose congenic HSCT in aged mice. (A) (i) Quantification of total BM cellularity in aged mice, (ii) the proportion of donor cells in WBM of aged mice, (iii) the absolute number of donor cells in WBM of young mice. **(B)** (i) Quantification of total thymus cellularity in aged mice, (ii) the proportion of donor cells in the thymus of aged mice, (iii) the absolute number of donor cells in the thymus of aged mice. **(C)** (i) Quantification of total spleen cellularity in aged mice, (ii) the proportion of donor cells in the spleen of aged mice, (iii) the absolute number of donor cells in the spleen of aged mice. Mice were sex steroid ablated (SSA) 1 day prior to chemotherapy with 10 mg/kg busulfan. 4 days following chemotherapy mice were transplanted with 1 x 10^4 congenic LSK cells and chimerism was analysed at 28 and 56 days following HSCT. Untreated control mice (white bars), Sham-SSA (grey bars) and SSA (black bars). Data is representative of two individual experiments, expressed as mean ± SE and analysed using an unpaired, two-tailed T-test. * ($p< 0.05$), ** ($p< 0.01$) represent significant differences compared to untreated mice. ^ ($p< 0.05$), ^^ ($p< 0.01$), ^^^ ($p< 0.001$) represent significant differences when comparing sham-SSA and SSA groups.

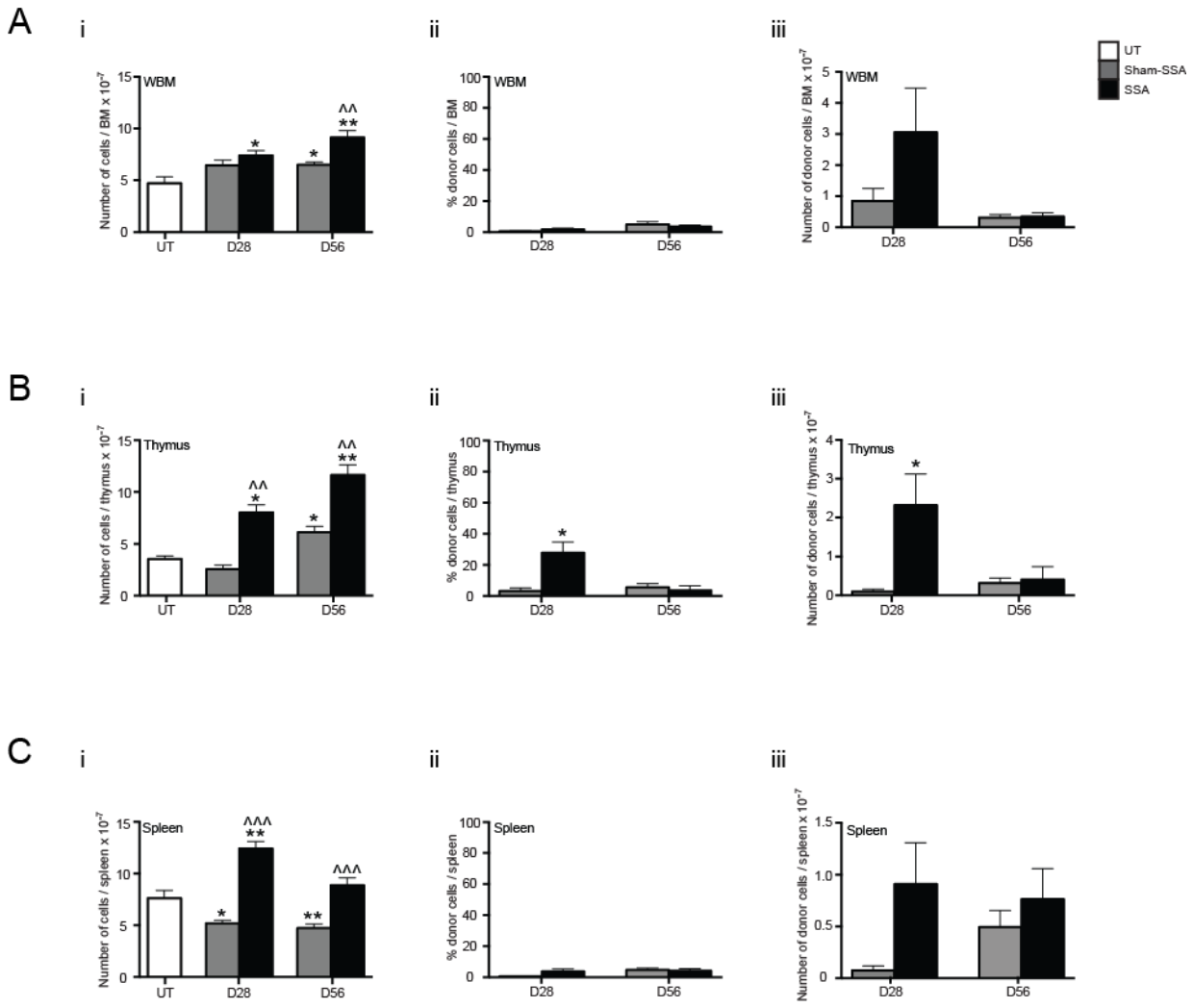
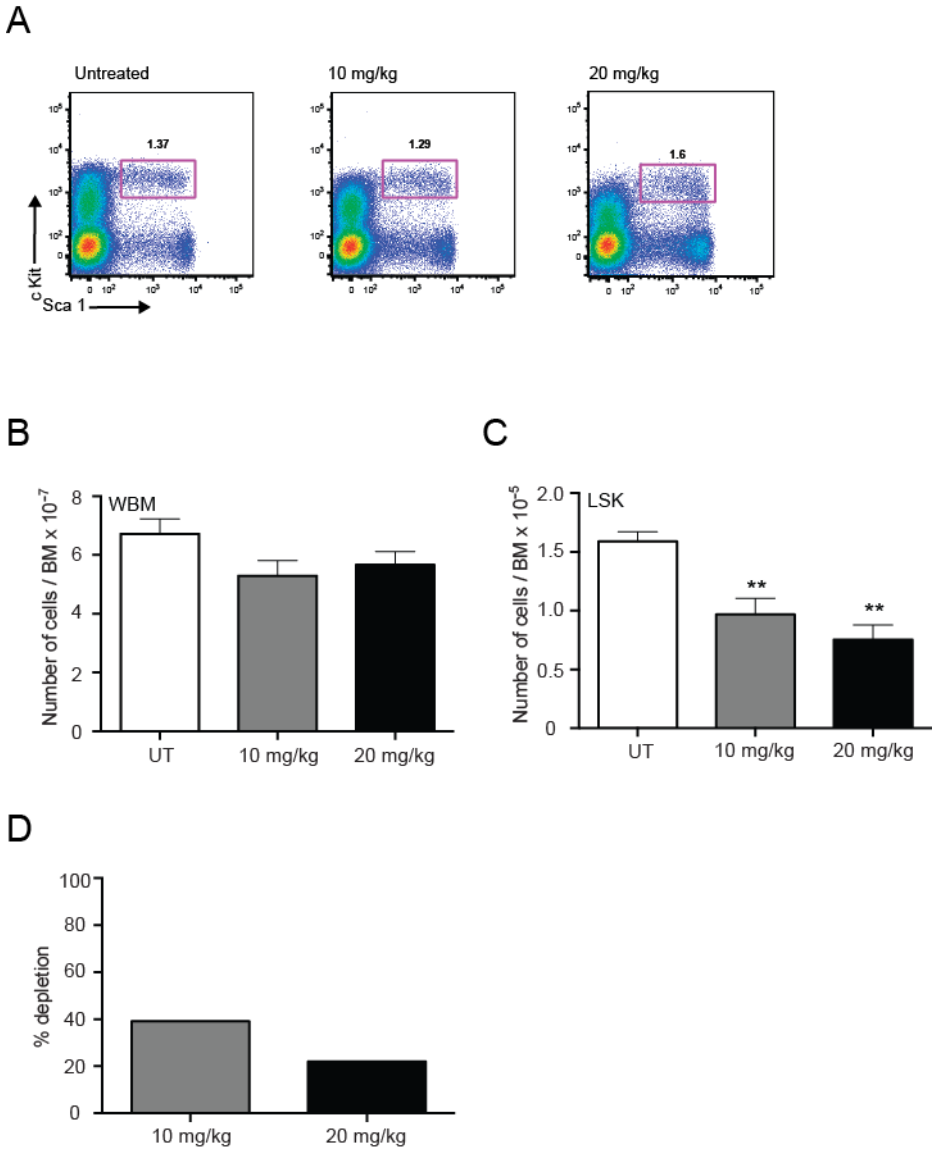

A
i
ii
iii
WBM
Number of cells / BM x 10⁻⁷
% donor cells / BM
Number of donor cells / BM x 10⁻⁷
UT
Sham-SSA
SSA
UT
D28
D56
B
Thymus
Number of cells / thymus x 10⁻⁷
% donor cells / thymus
Number of donor cells / thymus x 10⁻⁷
C
Spleen
Number of cells / spleen x 10⁻⁷
% donor cells / spleen
Number of donor cells / spleen x 10⁻⁷

Figure 4.4: Progenitor cells in the BM of aged mice are resistant to busulfan-mediated chemotherapy. (A) Flow cytometric plots showing the proportion of Lineage negative $Sca1^+$ $cKit^+$ (LSK) progenitor cells in the BM following administration of busulfan at 10 mg/kg and 20 mg/kg. As a control BM from an untreated (UT) mouse is shown. Figures above boxes represent the proportion LSKs. **(B)** Quantification of total BM cellularity and absolute number of **(C)** LSK cells following administration of busulfan at 10 mg/kg and 20 mg/kg. **(F)** The proportion of LSK progenitor cells depleted from the BM, calculated as a ratio of total LSK cells in treated mice compared to untreated mice, and expressed as a percentage. White bars indicate untreated mice, grey bars indicate 10 mg/kg busulfan and black bars indicate 20 mg/kg busulfan. Data is representative of two individual experiments expressed as mean ± SE and analysed using an unpaired, two-tailed T-test. ** ($p< 0.01$) represents significant differences compared to untreated mice.


A
Untreated
10 mg/kg
20 mg/kg
1.37
1.29
1.6
c Kit
Sca 1
B
WBM
Number of cells / BM x 10⁻⁷
UT
10 mg/kg
20 mg/kg
C
LSK
Number of cells / BM x 10⁻⁵
**
**
UT
10 mg/kg
20 mg/kg
D
% depletion
10 mg/kg
20 mg/kg

the BM, mice received a single dose of either 10 or 20 mg/kg busulfan. Cell populations within the BM were analysed via flow cytometry 4 days following chemotherapy, as we have previously shown that the LSK progenitor cell nadir occurs at this time point (Chapter 3). Neither 10 mg/kg busulfan. nor 20 mg/kg depleted BM total cell numbers in aged mice (Figure 4.4B). Importantly, however, both doses significantly depleted the total number of BM progenitor cells, defined as Lineage negative, $Sca1^+$ $cKit^+$ (LSK) cells, compared to untreated control mice (Figure 4.4C). No proportional changes were observed for either chemotherapy dose within the LSK subsets (Figure 4.4A). Doubling the dose of busulfan to 20 mg/kg did not deplete more LSKs than the 10 mg/kg dose (Figure 4.4C). The 10 mg/kg dose depleted 40 % of LSK progenitor cells and 20 mg/kg depleted 30 % of LSKs within the aged BM (Figure 4.4D), compared to 80 and 90% in young mice (Chapter 2). This clearly indicates that the aged-BM is relatively resistant to LSK reduction by busulfan-chemotherapy, which would be a logical explanation for the compromised engraftment in HSCT.

Pharmacological inhibition of adipogenesis prior to busulfan-chemotherapy does not increase donor cell engraftment following high-dose HSCT.

Adiopgenesis within the BM increases with age (43) and adipocyte infiltration of the BM follows both irradiation and chemotherapy (44, 45). Adipocytes can also reduce chemotherapy-induced apoptosis (46, 47). To investigate if adipocytes were protecting LSK progenitor cells from busulfan-mediated chemotherapy, adipogenesis was blocked pharmacologically with the peroxisome proliferator-activated receptor-γ (PPAR-γ) inhibitor bisphenol A diglycidyl ether (BADGE), which inhibits adipocyte formation (48), prior to 10 mg/kg busulfan and high-dose HSCT (5×10^4 LSKs). BADGE was continued for 14 days following HSCT and mice were analysed via flow cytometry at day 14. BADGE treatment in combination with busulfan-chemotherapy significantly increased both the proportion and number of donor-derived cells within the BM compared to mice that only received BADGE, but had no significant effect compared to mice that only receive 10 mg/kg busulfan (Figure 4.5A).

With respect to LSK, BADGE in combination with busulfan, significantly increased both the proportion and number of donor-derived LSK progenitor cells, compared to mice that only received BADGE, but did not improve chimerism compared to mice that received chemotherapy alone. Both the proportion and number of donor-derived progenitor cells are significantly low (<10%) in both the busulfan alone and BADGE-busulfan mice (Figure 4.5A). Like WBM, BADGE had no

significant effect on the number of cells observed in the thymus (Figure 4.5B). Again BADGE in combination with busulfan significantly improved the proportion and number of donor-derived cells in the thymus, compared to BADGE alone mice, but not compared to busulfan alone mice (Figure 4.5B). This indicates that inhibition of adipogenesis with BADGE prior to busulfan-chemotherapy did not improve chimerism observed in the BM or thymus of aged mice.

Pre-treatment with G-CSF restores sensitivity of the BM to busulfan-chemotherapy, but does not improve donor cell engraftment following congenic HSCT or allogeneic BMT.

In young mice busulfan depletes early and late cycling progenitor cells (49, 50). With age, the number of LSK progenitor cells within the BM increases (51), however these cells are less functional, more quiescent and more resistant to apoptosis (52). They are thus more resistant to busulfan than the young, as shown in Figure 4.4. We reasoned that cytokine activation of LSKs may reverse this resistance. Accordingly, we investigated if granulocyte-colony stimulating factor (G-CSF) activation of the BM can promote sensitivity of aged-LSK progenitor cells to low-dose busulfan. Mice were treated with G-CSF at 250 μg/kg twice daily for 4 consecutive days prior to conditioning with 10 mg/kg busulfan and were analysed 4 days following chemotherapy, via flow cytometry. Busulfan alone significantly depleted whole BM cells, compared to untreated control mice, but not G-CSF alone or G-CSF and busulfan treated mice (Figure 4.6B), although there was very little difference between these three groups. Neither busulfan nor G-CSF alone depleted the proportion or number of LSK progenitor cells in the BM. Importantly, however, busulfan in combination with G-CSF significantly depleted both the proportion and absolute number of LSK progenitor cells, compared to untreated control mice, busulfan alone and G-CSF alone treatment groups (Figure 4.6A and B). Busulfan in combination with G-CSF depleted 80 % of LSK progenitor cells in the BM of aged mice (Figure 4.6C), restoring sensitivity equivalent to that of the young (Chapter 2).

In previous studies, G-CSF has been shown to enhance progenitor cell engraftment following low-dose irradiation (53). We therefore examined if the combination of G-CSF and low dose busulfan could result in higher levels of chimerism in adult mice undergoing HSCT. Aged mice received G-CSF at 250 μg/kg twice daily for 4 consecutive days prior to 10 mg/kg busulfan and high-dose HSCT (5 x 10^4 congenic LSKs). Mice were analysed via flow cytometry 28 days following HSCT.

Figure 4.5: Pharmacological inhibition of adipogenesis prior to chemotherapy does not increase donor cell engraftment in aged mice. (A) (i) Quantification of total BM cellularity, (ii) proportion of donor cells in WBM, (iii) absolute number of donor cells in WBM, (iv) total Lineage negative $Sca1^+$ $cKit^+$ (LSK) progenitor cell number, (v) proportion of donor cells in LSK progenitor cell subset and (vi) absolute number of donor-derived LSKs in the BM of aged mice. (**B)** (i) Total number of thymocytes, (ii) proportion of donor cells in the thymus and (iii) absolute number of donor-derived thymocytes in aged mice. Mice received daily IP injections of bisphenol A diglycidyl ether (BADGE) (30 mg/kg) or a vehicle control 5 days prior to and 14 days following HSCT with 5 x 10^4 congenic LSK progenitor cells busulfan (10 mg/kg) was administered 4 days prior to HSCT. Mice were analysed 14 days post HSCT. White bars indicate untreated mice, light grey bars indicate busulfan alone, dark grey bars indicate BADGE alone, black bars indicate both busulfan and BADGE. Data is representative of one individual experiment, expressed as mean ± SE and analysed using an unpaired, two-tailed T-test. * ($p< 0.05$), ** ($p< 0.01$) represent significant differences compared busulfan -chemotherapy alone. ^ ($p< 0.05$), ^^ ($p< 0.01$), ^^^ ($p< 0.001$), ^^^^ ($p< 0.0001$) represent significant differences compared to mice that only receive BADGE.

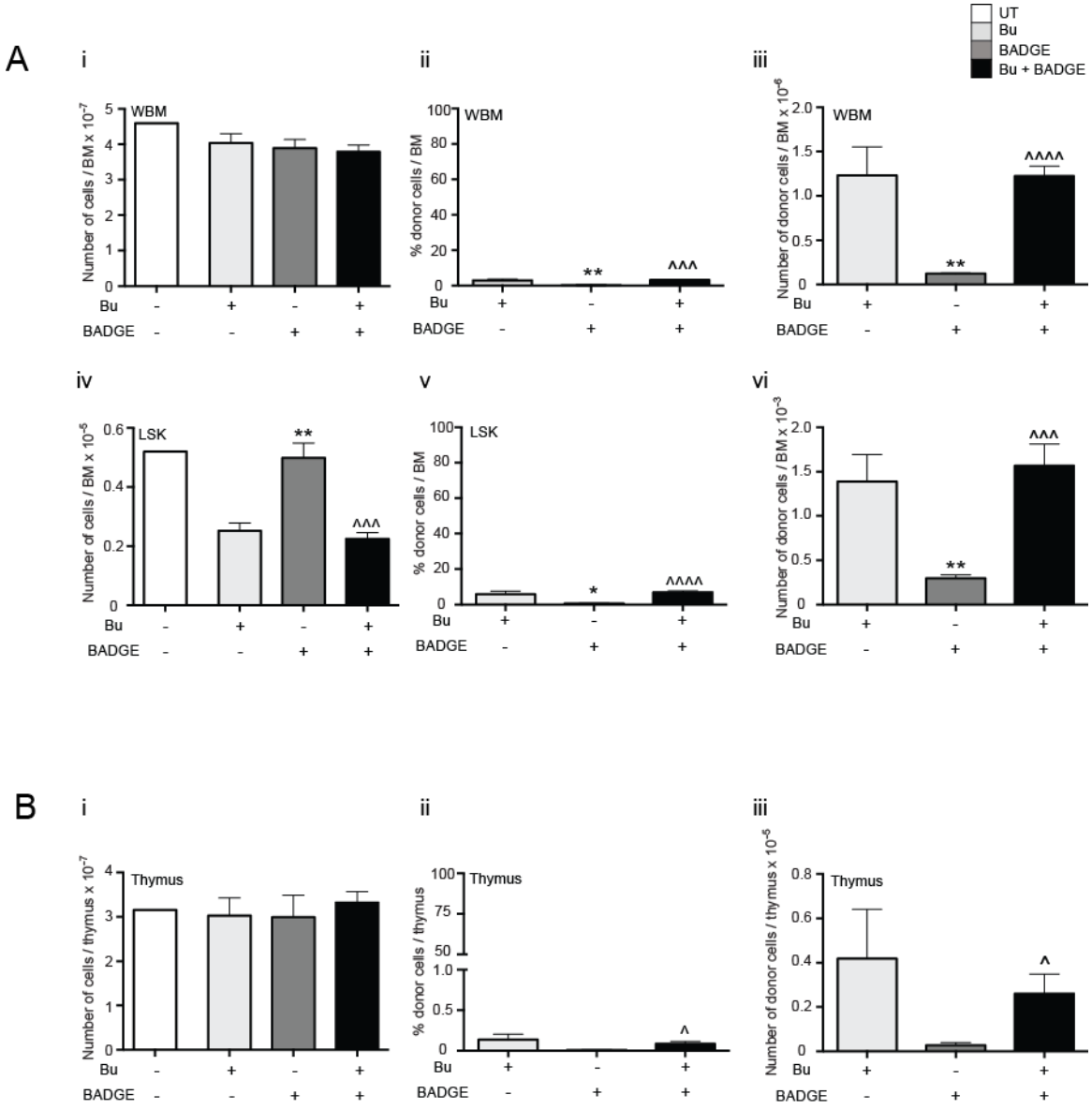

A
i
ii
iii
UT
Bu
BADGE
Bu + BADGE
WBM
Number of cells / BM x 10^-7
% donor cells / BM
Number of donor cells / BM x 10^-6
Bu
BADGE
iv
v
vi
LSK
Number of cells / BM x 10^-5
% donor cells / BM
Number of donor cells / BM x 10^-3
B
Thymus
Number of cells / thymus x 10^-7
% donor cells / thymus
Number of donor cells / thymus x 10^-5

Figure 4.6: Mobilising the BM in aged mice with G-CSF restores sensitivity to busulfan-mediated chemotherapy. (A) Flow cytometric plots showing the proportion of Lineage negative $Sca1^+$ $cKit^+$ (LSK) progenitor cells in the BM following administration of busulfan, G-CSF or busulfan in combination with G-CSF. As a control BM from an untreated (UT) mouse is shown. Figures above boxes represent the proportion of LSKs. **(B)** (i) Quantification of total BM cellularity, (ii) and absolute number of LSK cells in aged mice. **(C)** The proportion of LSK progenitor cells depleted from the BM, calculated as a ratio of total LSK cells in treated aged mice compared to untreated aged mice, and expressed as a percentage. Mice received G-CSF (250 μg/kg) or a vehicle control twice daily for 4 days prior to chemotherapy with 10 mg/kg busulfan. Mice were analysed 4 days following chemotherapy. White bars indicate untreated mice, light grey bars indicate busulfan alone, dark grey bars indicate G-CSF alone and black bars indicate both busulfan and G-CSF. Data is representative of two individual experiments, expressed as mean ± SE and analysed using an unpaired, two-tailed T-test. * ($p< 0.05$), ** ($p< 0.01$) represents significant differences compared to untreated mice. ^^ ($p< 0.01$), represent significant differences compared busulfan-chemotherapy alone. #### ($p< 0.001$) represent significant differences compared to mice that only receive G-CSF.

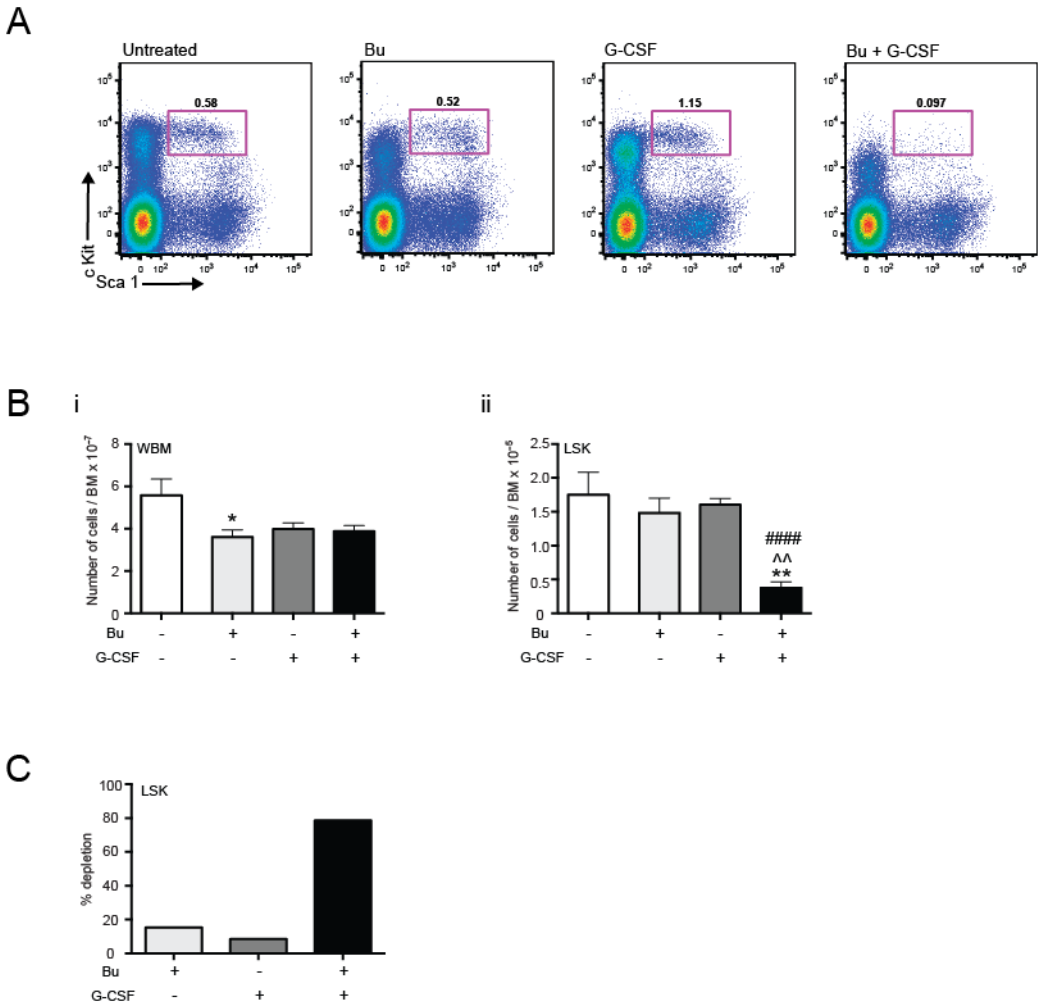
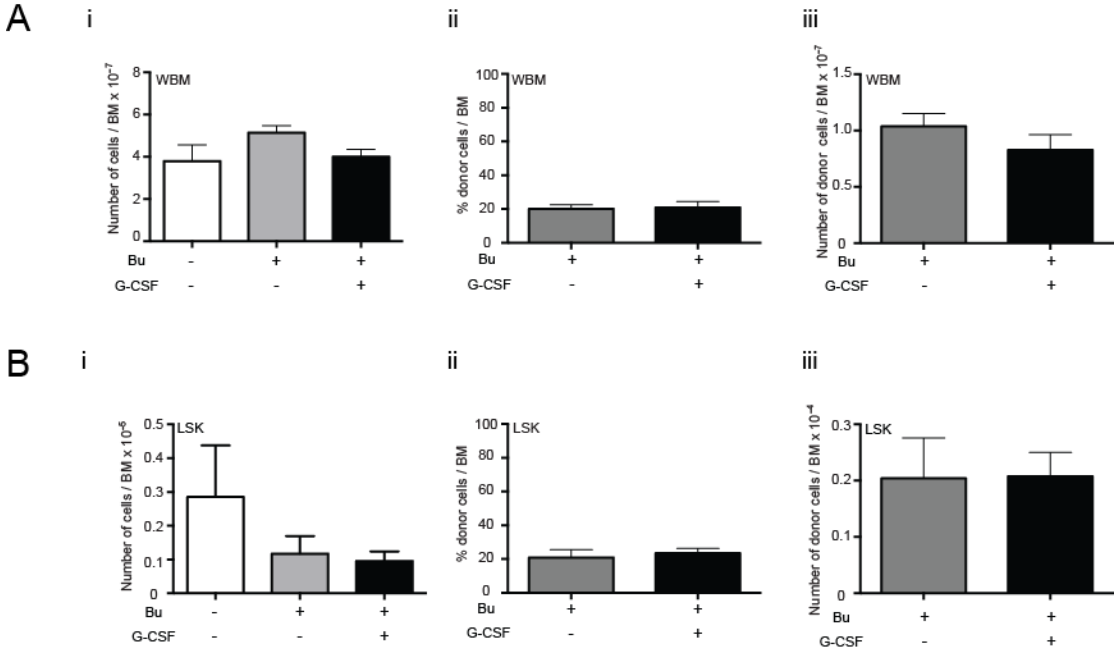

A
Untreated
Bu
G-CSF
Bu + G-CSF
0.58
0.52
1.15
0.097
c Kit
Sca 1
B
i
WBM
Number of cells / BM x 10⁻⁷
*
Bu
G-CSF
ii
LSK
Number of cells / BM x 10⁻⁵
####
^^
**
C
LSK
% depletion

Figure 4.7: Mobilising the BM with G-CSF prior to busulfan-mediated chemotherapy and congenic high dose HSCT does not increase donor cell engraftment in aged mice. (A) (i) Quantification of total BM cellularity, (ii) proportion of donor cells in WBM, (iii) absolute number of donor cells in WBM of aged mice. **(B)** (i) total Lineage negative $Sca1^+$ $cKit^+$ (LSK) progenitor cell number, (ii) proportion of donor cells in LSK progenitor cell subset and (iii) absolute number of donor-derived LSKs in the BM of aged mice. Mice received G-CSF (250 μg/kg) or a vehicle control twice daily for 4 days prior to chemotherapy with 10 mg/kg busulfan. 4 days following chemotherapy mice were transplanted with 5 x 10^4 congenic LSK progenitor cells and mice were analysed 28 days following HSCT. White bars indicate untreated mice, dark grey bars indicate busulfan alone and black bars indicate both busulfan and G-CSF. Data is representative of two individual experiments, expressed as mean ± SE and analysed using an unpaired, two-tailed T-test.


A
i
ii
iii
WBM
Number of cells / BM x 10⁻⁷
% donor cells / BM
Number of donor cells / BM x 10⁻⁷
Bu
G-CSF
B
LSK
Number of cells / BM x 10⁻⁶
Number of donor cells / BM x 10⁻⁴

No significant changes were observed in the total cellularity of WBM when mice were treated with busulfan alone or in combination with G-CSF (Figure 4.7A). Similarly, G-CSF pre-treatment had no significant effect on the proportion or number of total donor cells observed in the BM of aged mice following HSCT (Figure 4.7A). Furthermore, despite the reduction in LSK number with the G-CSF- busulfan combination (Figure 4.6B), there were no significant differences in the total number of LSK cells between untreated control mice, busulfan treated mice or mice that received both busulfan and G-CSF (Figure 4.7B). Busulfan alone or with G-CSF pre-treatment also failed to increase the proportion or number of donor-derived LSK cells observed in the BM of aged mice (Figure 4.7B). This indicates that while G-CSF pre-treatment promotes busulfan-induced loss of host LSK, it does not improve donor cell engraftment when mice are conditioned with low-dose busulfan.

Robust engraftment, chimerism and allogeneic tolerance are achieved in aged mice when WBM is transplanted. G-CSF is not required as an adjuvant to boost chimerism.

We have previously reported that low-dose busulfan can be used in conjunction with T cell depleting antibodies and short-term sirolimus immunosuppression to induce mixed chimerism and allogeneic tolerance following transplantation of both WBM and LSK progenitor cells in both young and aged mice (Morison *et al.* manuscript in preparation and Chapter 3). To assess if G-CSF could boost donor cell engraftment in an aged allogeneic setting, 1-year-old mice receive G-CSF at 250 µg/kg twice daily for 4 consecutive days, prior to conditioning with 10 mg/kg busulfan and transplantation of 4 x 10^7 WBM cells 4 days following chemotherapy. Mice were monitored via flow cytometry for 27 weeks following BMT. No significant differences were observed in the total BM cellularity between treatment groups (Figure 4.8A). Unlike mice transplanted with high-dose (5 x 10^4) congenic LSK progenitor cells (Figure 4.7), transplantation of 4 x 10^7 allogeneic WBM resulted in robust engraftment, with 60 % of cells within the BM of both treatment groups derived from donor (Figure 4.8A). Pre-treatment with G-CSF did not significantly increase the proportion or number of donor-derived cells observed in the BM (Figure 4.8A). Donor cell engraftment, following transplantation with WBM resulted in multi-lineage chimerism in peripheral blood, which could be mainly attributed to the myeloid compartment (Figure 4.9). Chimeric mice were tolerant to donor skin transplants, with 7/7 mice conditioned with 10 mg/kg busulfan alone, 4 days prior to transplantation with 4 x 10^7 WBM cells accepting allogeneic skin grafts (Figure 4.9E). 7/8 mice treated with G-CSF prior to the standard conditioning regime (10 mg/kg busulfan 4 days prior

to transplantation with 4 x 10^7 WBM) accepted allogeneic skin transplants for more than 100 days following skin grafting (Figure 4.9E). The chimeric mice were immunocompetent, with 100% of mice in each treatment group rejecting third-party unrelated skin in less than 10 days following grafting (Figure 4.9E). This data indicated that when WBM is transplanted, robust donor cell engraftment is achieved in the BM of aged mice and this leads to the development of stable mixed chimerism and allogeneic tolerance.

Figure 4.8: Mobilising the BM with G-CSF prior to busulfan-mediated chemotherapy and allogeneic BMT does not increase donor cell engraftment in aged mice. (A) (i) Quantification of total BM cellularity, (ii) proportion of donor cells in WBM, (iii) absolute number of donor cells in WBM of aged mice. **(B)** (i) Total Lineage negative $Sca1^+$ $cKit^+$ (LSK) progenitor cell number, (ii) proportion of donor cells in LSK progenitor cell subset and (iii) absolute number of donor-derived LSKs in the BM of aged mice. Mice received G-CSF (250 μg/kg) or a vehicle control twice daily for 4 days prior to chemotherapy with 10 mg/kg busulfan. 4 days following chemotherapy mice were transplanted with 4 x 10^7 B10.BR allogeneic ($H\text{-}2^k$) WBM cells. Anti-CD4 and anti-CD8 monoclonal antibodies were administered on days -3 and -1 relative to BMT. Mice were immunosuppressed by daily sirolimus treatment for 28 days following BMT and analysed 27 weeks post transplant. White bars indicate untreated mice, dark grey bars indicate Busulfan alone and black bars indicate both Busulfan and G-CSF. Data is representative of two individual experiments, expressed as mean ± SE and analysed using an unpaired, two-tailed T-test.

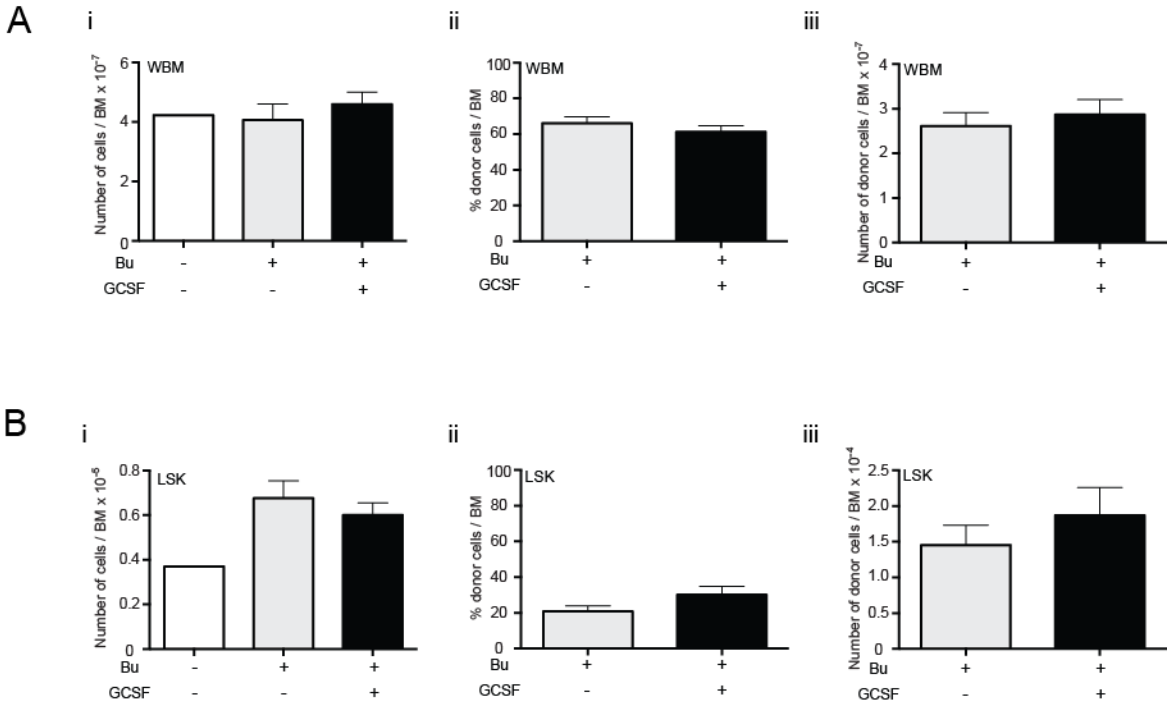
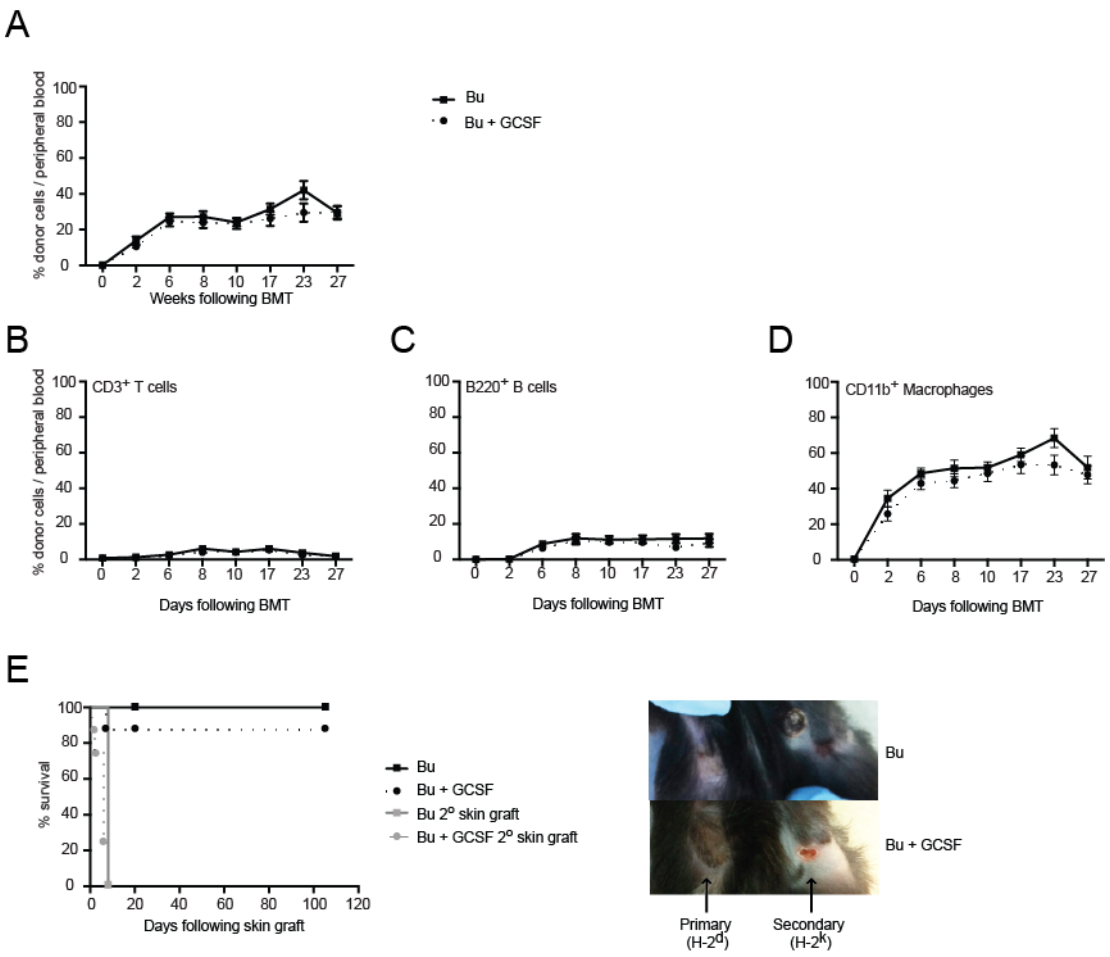

A
i
ii
iii
WBM
Number of cells / BM x 10⁻⁷
% donor cells / BM
Number of donor cells / BM x 10⁻⁷
Bu
GCSF
B
LSK
Number of cells / BM x 10⁻⁵
% donor cells / BM
Number of donor cells / BM x 10⁻⁴

Figure 4.9: Allogeneic tolerance is achievable in aged mice when WBM is transplanted and does not require adjuvant therapy. (A) The proportion of donor cells in peripheral blood over time. **(B)** The proportion of $CD3^+$ T cells, **(C)** $B220^+$ B cells and **(D)** $CD11b^+$ macrophages in peripheral blood over time. Busulfan and G-CSF treated mice are represented with a dashed line and circles and busulfan and vehicle control mice are represented with a solid line and squares. **(E)** Donor (black) and third-party (grey) skin graft survival. Mice received G-CSF (250 μg/kg) or a vehicle control twice daily for 4 days prior to chemotherapy with 10 mg/kg busulfan. 4 days following chemotherapy mice were transplanted with 4 x 10^7 B10.BR allogeneic ($H\text{-}2^k$) WBM cells. Anti-CD4 and anti-CD8 monoclonal antibodies were administered on days -3 and -1 relative to BMT. Mice were immunosuppressed by daily sirolimus treatment for 28 days following BMT and analysed 27 weeks post transplant. 12 and 28 weeks after BMT, mice received an allogeneic skin graft from B10.BR and BALB/c ($H\text{-}2^d$) respectively. Data is representative of two individual experiments, expressed as the mean ± SE and analysed using two-way ANOVA with n = 7-8 mice/group.


A
% donor cells / peripheral blood
Weeks following BMT
Bu
Bu + GCSF
B
CD3+ T cells
Days following BMT
C
B220+ B cells
Days following BMT
D
CD11b+ Macrophages
Days following BMT
E
% survival
Days following skin graft
Bu
Bu + GCSF
Bu 2° skin graft
Bu + GCSF 2° skin graft
Primary (H-2d)
Secondary (H-2K)

Discussion

HSCT in the aged population is significantly more challenging than it is in the young, which presents a major clinical challenge given that most regenerative medicine transplants are going to be required for this age demographic. While immunosenescence does not overtly influence healthy individuals, ageing is however associated with increased opportunistic infections, recovery from immune depletion states, such as irradiation and chemotherapy (required to destroy cancer cells but also generate space in the BM for donor-HSC engraftment), is severely compromised with age (7). High morbidity and mortality rates are associated with HSCT in the aged and can be attributed to the delay in immune reconstitution following myeloablative conditioning. This immunodeficiency can result in opportunistic or reactivation of latent infections, inflammation and an increase in malignancy relapse. The development of low-intensity conditioning regimes has been credited with overcoming to some extent, the age barrier for HSCT in cancer patients; the reasons for this include fewer conditioning associated toxicities and better patient monitoring for infectious disease (7). Because of this, low-intensity conditioning regimes for HSCT have been reasonably well characterised in the treatment of malignancies, but in the aged population, the use of these regimes to induce allogeneic tolerance has been neglected.

We have recently developed a low-intensity radiation-free protocol for the induction of allogenic tolerance via mixed chimerism (Morison *et. al.* manuscript in preparation and Chapter 3). In this protocol we showed that low-dose busulfan could be used in conjunction with T cell depleting antibodies and short-term sirolimus immunosuppression to induce tolerance when young or aged mice are transplanted with 4 x 10^7 WBM cells. Additionally, transplantation of 5 x 10^4 allogeneic LSK progenitor cells can be used to induce tolerance with this conditioning regime in young mice. In this present study we aimed to characterise the ability of low-dose busulfan to promote engraftment of purified LSK progenitor cells in aged mice. Previous reports in young mice have shown that conditioning with doses of busulfan lower than 20 mg/kg resulted in slow and incomplete reconstitution following congenic HSCT (54, 55). Given that the level of donor cell engraftment following myeloablative conditioning in the aged is two-three times lower than that of the young (2, 4), we hypothesised that engraftment of congenic LSKs would be greatly reduced in aged mice compared to young following low-dose busulfan. Furthermore since SSA increases engraftment following myeloablative conditioning (39, 41), we proposed that SSA would boost engraftment and chimerism in aged mice.

Supporting our hypothesis we have shown that engraftment and chimerism were decreased in aged mice compared to young when 5 x 10^4 congenic LSKs are transplanted. In the BM of aged mice the proportion of donor cells observed was decreased 5 fold. Although the proportion of donor cells observed in the thymus was not significantly decreased compared to young mice, the number of donor cells in the thymus decreased by 3 fold. This can be attributed to age induced thymic atrophy, where the total number of thymocytes in aged mice decreased 10 fold. A decreased output of donor cells from the thymus resulted in a low level of donor cells being detected in the periphery.

SSA increases cellularity and progenitor cell number in the BM of young mice following myeloablative chemotherapy (38). Here we show that SSA prior to low-dose busulfan chemotherapy and HSCT also increased BM cellularity when mice were transplanted with 1 x 10^4 congenic LSK cells at both 28 and 56 days following HSCT. This was not observed for the higher dose of 5 x 10^4 LSK cells, but supported previous observations from young mice (Chapter 3). We attribute this to chemotherapy dosage; at 10 mg/kg busulfan is not myeloablative, selectively depleting only the progenitor compartment (Chapter 2). Previous studies, that have shown an increase in BM cellularity following SSA, have used harsh myeloablative treatments such as TBI and high dose cyclophosphamide, which significantly deplete WBM cell numbers, presumably by creating "space" in the niche to increase donor engraftment and subsequent cellularity (38, 39, 56). However, SSA herein did not increase long-term engraftment or donor cell output in aged mice.

Interestingly, at the day 28 time point following low-dose HSCT, SSA mice had a significantly higher proportion of donor cells in the thymus, compared to sham-SSA mice. This increase had disappeared by 56 days following HSCT and suggests that the limiting factor for SSA potentiated donor cell output is LSK engraftment, not thymic output. In young mice 10 mg/kg busulfan depletes 80 % of LSK progenitor cells in the BM, allowing engraftment of donor LSK progenitor cells (Chapter 2). Here we show in aged mice, the level of depletion achieved with 10 mg/kg busulfan alone is reduced to 40 % and the number of LSK progenitor cells in the BM of aged mice is increased, supporting previous observations that show the LSK compartment can increase numerically up to 5 fold in aged mice, not withstanding them being functionally compromised (19, 51, 57, 58).

The decreased sensitivity of aged BM LSK progenitor cells to busulfan could have been attributed to a dose limiting effect, given there is an increase in the number of progenitor cells. However doubling the dose of busulfan to 20 mg/kg did not increase sensitivity. Alternatively, resistance in

aged mice may be an increased ability for LSK progenitor cells to efflux busulfan. Within the LSK subset is a population of cells characterised by their ability to exclude the DNA-binging dye Hoechist 33342, these cells, termed side population (SP) cells, undergo a 30 fold increase with age (51). SP cells exclude Hoechist 33342 through the cell surface transporter ATP-binging cassette (ABC)/G2, which can also efflux chemotherapeutic compounds, promoting cell survival (59). An increase in cells that can exclude busulfan could thus result in a population of LSK cells that are more resistant to depletion.

Ageing is also associated with an increased number of adipocytes in the BM (43). Again this could be contributing to the increased busulfan resistance that occurs in aged mice, as adipocytes have been shown to protect cells from chemotherapy mediated apoptosis (46, 60, 61). Because busulfan is highly lipophilic (62), adipocytes could be sequestering the drug, decreasing its availability. Potentiating this effect, chemotherapeutic agents, including busulfan, induce adipocyte infiltration of the BM (45). Naveiras *et al*. have shown that pharmacological inhibition of adipocyte formation enhances donor cell engraftment, following lethal irradiation and congenic HSCT. Adipogenesis can be blocked using the pharmacological agent BADGE prior to and following HSCT. However, BADGE in combination with busulfan and HSCT did not increase the proportion or number of donor cells observed in aged mice. This may be because BADGE prevents the formation of new adipocytes, but does not deplete pre-formed adipocytes within the marrow, which could still protect progenitor cells.

Alternatively, adipocytes may protect LSK progenitor cells from depletion by decreasing the number of cells in cycle (44). In this regard SP cells, which increase with age, are quiescent (52). Busulfan depletes both early and late cycling progenitor cells in the BM (49, 50), indicating that an increase in the number of quiescent progenitor cells, would decrease sensitivity to chemotherapy. As a corollary, since G-CSF activates LSK progenitors in the BM (63), we have shown that mobilisation of the BM with G-CSF does indeed restore their sensitivity to low-dose busulfan, depleting 80 % of LSK progenitors, a level of depletion equivalent to young mice (Chapter 2). Although G-CSF does not increase the proportion of cells cycling in the BM during mobilisation (63), our data suggests that some level of cycling occurs, because the cells are being depleted. Despite having restored sensitivity to busulfan, G-CSF mobilisation prior to chemotherapy and HSCT does not increase engraftment or donor cell output in aged mice. This was surprising, considering G-CSF prior to low-dose irradiation potentiates engraftment when mice are

transplanted with $Sca1^+$ BM cells (53). Together with our data, this indicates that in the aged setting, cell extrinsic factors, such as the BM niche are also affecting engraftment.

Previous studies in our laboratory have shown SSA shifts the molecular profiling of the aged BM niche towards a young phenotype (Chidgey *et al*. manuscript in preparation) Functionally, SSA prior to lethal irradiation and HSCT increases the availability niche space, promoting the entry of progenitors to the microenvironment and increasing engraftment (Khong *et al*. manuscript in preparation). Although our data herein suggest that the BM niche is limiting the ability to generate robust donor chimerism following low-dose busulfan in aged mice, SSA did not improve engraftment. Taken together our data suggest a combination of factors, that could include, adipocytic content of aged BM, an increase in the ability for progenitor cells to efflux busulfan, a decrease in cell cycling or a decrease in receptivity of the BM niche contribute to the decrease in donor cell engraftment observed in aged mice. Perhaps combining SSA, to improve the niche and G-CSF, to increase depletion, prior to low-dose busulfan would increase the level of engraftment and chimerism following HSCT in aged mice.

Despite the low level of engraftment observed following congenic HSCT, we show that allogeneic tolerance can be achieved in aged mice transplanted with allogeneic WBM. In these mice, 60 % of the BM compartment was of donor origin. The most likely explanation for this is the presence of allogeneic T cells within the transplant, which may be promoting a higher level of engraftment in these mice (64-67); these donor T cells may also protect transplanted marrow from rejection by Natural Killer cells, enhancing donor-derived myelopoiesis, via the release of growth promoting cytokines and cell-cell contact between host and donor derived T cells (68).

In our studies, donor cell engraftment in aged mice lead to multi-lineage mixed chimerism and allogeneic tolerance. Supporting congenic HSCT results, G-CSF did not increase engraftment or peripheral chimerism observed following transplantation of WBM in aged mice. Although we have shown that allogeneic tolerance can be achieved in aged mice when WBM is transplanted, clinically, the transfer of allogeneic T cells leads to the development of GVHD. This is an untenable clinical risk and is of particular concern in the aged setting, where the risk of developing GVHD is increased even further (2). Transplanting purified progenitor cells prevents the occurrence of GVHD, making it safer for patients.

In our hands, it appears that it is the BM and not the thymus that is the major limitation to successful HSCT in the aged following low-intensity conditioning regimes. However even a low level of engraftment by donor progenitor cells may facilitate the induction of allogeneic tolerance, exemplified by liver and kidney transplants, whereby small number of donor HSCs can migrate out of the transplanted organ, engraft and induce allogeneic tolerance (69-73). However the success of such experiments has often been attributed to the young age of recipients (70, 72). We are currently investigating the ability for purified LSK progenitor cells to induce allogeneic tolerance in aged mice with our protocol utilising low-dose busulfan.

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CHAPTER FIVE

General discussion

Organ transplantation is the only current curative procedure for end stage organ disease. The first successful organ transplant was performed in Boston in 1954, where a kidney was transplanted between identical twins. Although several attempts at organ transplantation had been made prior to this report, Murray and colleagues are credited with the first proof-of principle transplantation with long-term success, attributed to immunological acceptance or tolerance induction of haploidentical tissue (1).

An understanding of immunological rejection came from experimentation with skin grafting in the 1930s. In early experiments, skin allografts from related and unrelated donors were used to treat burns patients with insufficient intact skin for autografts. Although allografts did not survive, they did protect burn wounds long enough to allow re-epithelialisation and infection control. Interestingly, allograft survival time could not be predicted, but it was observed that related skin survived longer than unrelated skin (2). A better understanding of tolerance versus rejection came from experiments in skin grafting between monozygotic and dizygotic twins. The first observation was that skin grafts, transplanted between monozygotic twins survived permanently, providing some hope for tissue and organ replacement therapies. Following these seminal observations by Brown (3), Gibson and Medawar provided further insights, demonstrating that subsequent transplantation of a second allograft from the same donor was rejected more rapidly that the first (4). This was a key finding in the understanding of immunological rejection, indicating tissue loss was not a fixed process, rather an acquired response, with the potential for manipulation. Subsequently, Medawar, along with Billingham and Brent confirmed this hypothesis, demonstrating that immunological tolerance could be acquired in mice who were exposed to donor cells in the neonatal stages of life (5). This work was a culmination of many observations beginning in Freemartin cattle, where dizygotic twins, are chimeric, share a placental circulation (6) and can indefinitely accept skin grafts from the respective twin (7). Medawar and colleagues recapitulated these experiments by injecting donor cells into neonatal mice of a genetically disparate strain. When these mice were mature, their blood systems were chimeric and they could accept skin grafts from mice that matched the donor cell strain but not from other stains of mice, demonstrating that specific immunological tolerance could be induced (8).

Despite these important observations, it was the development of immunosuppressive drugs that led to the widespread feasibility of organ transplantation. Modern immunosuppressive regimes have decreased the incidence of acute renal transplant rejection by approximately 10% in the first few months following transplantation (9). However, even with the continual development of new

immunosuppressive drugs, anti-rejection therapies are associated with a array of side effects, including diabetes mellitus, nephrotoxicity, neurotoxicity, osteonecrosis, leukopenia, hypertension, hyperlipidemia, cardiovascular events, opportunistic bacterial and viral infections, organ fibrosis and graft loss (10-14). Currently 50% of all renal transplant recipients will lose their graft to one of the aforementioned complications (14, 15).

The induction of immunological tolerance is a means by which long-term immunosuppressive regimes can be overcome, eliminating negative side effects whilst still preventing both acute and chronic rejection and it is because of this that studies have continued to build on Billingham and Medawar's pioneering investigations. Initial studies of tolerance induction through mixed chimerism in the adult mouse involved the lethal irradiation of recipient mice and reconstitution with a mixture of both host and donor T cell depleted bone marrow (BM) (16). The hematopoietic and lymphocyte compartments of these mice remained chimeric for the duration of animal's life and led to the development of specific tolerance to donor-matched skin grafts.

Originally, myeloablative conditioning prior to BM transplantation (BMT) and hematopoietic stem cell (HSC) transplantation (HSCT) was required to kill leukemic cells and the BMT/HSCT was used as a rescue therapy for the hematopoietic destruction caused by the lethal doses of irradiation. However, the myeloablative conditioning itself causes significant collateral damage, including complications within the gastrointestinal tract, loss of hematopoietic and leukocyte compartments, central nervous system dysfunction, pulmonary complications, multi-organ co-morbidities, the development of malignancies and in the worst case scenarios, it led to the death of the patient (17, 18). Because of these complications, lethal doses of irradiation are considered too toxic to justify in the absence of a malignancy. Therefore, to make mixed chimerism based approaches for the induction of allogeneic tolerance more clinically feasible, research focus has shifted to the development of non-myeloablative conditioning regimes.

Non-myeloablative conditioning regimes prior to BMT/HSCT have been widely explored. In this context the most successful regimes in murine models have employed co-stimulation blockade by CD40L monoclonal antibody therapy and CTLA4-immnoglobulin (19-24). Co-stimulation blockade in the absence of cytoreductive conditioning can also achieve BM engraftment when supraphysiological doses of BM are administered (25, 26). Despite the success of these conditioning regimes in small animal models, translation to nonhuman primates and clinical trials have been hampered by the feasibility of high-dose BMT and thromboembolic complications

associated with CD40L blockade (27). Other studies have reduced the collateral damage associated with high dose whole body irradiation by combining low dose whole body irradiation and/or chemotherapy with focal thymic irradiation and peripheral T cell depletion (28, 29). These protocols have had success in both nonhuman primates and in clinical trials (30-34). The current clinical practice for reduced intensity conditioning regimes include chemotheraputic drugs, such as fludarabine, busulfan or cyclophosphamide, and/or a low-dose of total body irradiation (TBI) and some protocols include the use of T cell depletion with anti-thymocyte globulin (ATG) or anti-CD52 (Campath) (35).

A large number of studies have been conducted into translating the ability to generate tolerance via mixed chimerism in the clinical setting. The research to date, conducted in rodents, large animals, pre-clinical and clinical models has been focused on four main aspects: 1) understanding the mechanisms by which mixed chimerism induces tolerance; 2) myelosuppressive conditioning to allow the engraftment of transplanted hematopoietic cells (lethal irradiation, sub-lethal irradiation or chemotherapy); 3) immunosuppressive treatment, to protect the transplanted tissue from rejection and to prevent the development of graft versus host disease (GVHD) (irradiation, antibody mediated T cell depletion or drug based non-specific immunosuppression); and 4) the content of the HSCT (WBM, T cell depleted BM, peripheral blood stem cells, purified $CD34^+$ stem cells, purified HSCs or combinations of either). Whilst research in tolerance induction schemes implementing mixed chimerism have yielded some significant findings, reviewed by Kawai and Sachs (36), a major limitation of these studies is that they have not collectively taken into account or investigated the effects of immunosenescence and by extension, the ability to induce tolerance in an aged immune system. This is an important point, considering aged recipients (>50 years) constitute the bulk of people on transplant waiting lists and patients receiving organ transplants (37-39). Additionally, and strengthening this observation, the elderly are the fastest growing segment of the population with end-stage organ disease, and the largest population demographic requiring an organ transplant (40).

Immunosenescence has a profound effect on the immune system, which undergoes a complex and continuous remodelling with increasing age. This can be characterised by both qualitative and quantitative changes in specific subpopulations of immune cells, rather than a global deterioration of the system as a whole. For the induction of tolerance, the most pertinent age-related immunological dysregulation occurs in the thymus, which undergoes a progressive decline in structure and function (41). The direct consequence of which is a reduction in export of naive T

cells, a homeostatic compensatory increase in peripheral memory T cells and the generation of allospecific cytotoxic T cells (42). These age-specific changes in the T cell repertoire have a major impact on the recognition of and response to allogeneic tissue and consequently graft rejection. Additionally, Khan and colleagues have demonstrated that to maintain stable allogeneic tolerance in mixed chimeras, a functioning thymus is absolutely required (43).

The central theme of this thesis was based on the premise that the induction of tolerance is reduced in an un-manipulated aged immune system. This notion has been established based on the following evidence: 1) the maintenance of stable allogeneic tolerance in mixed chimeras requires intrathymic deletion of alloreactive T cells, which is severely compromised by age-induced thymic atrophy (43, 44); 2) the senescent immune system is less able to respond to novel antigens and the induction of tolerance is an active process, requiring antigen recognition and regulatory cell activation, division and functionality (45-47); and finally, 3) conditioning-induced damage to the thymus may be compounded by thymic atrophy and contributes to the variability observed in the clinical setting. Collectively, this evidence indicates that for a clearer understanding of the therapeutic potential of BMT-mediated tolerance induction, experiments incorporating immune senescence together with optimising preconditioning regimens need to be thoroughly examined. The central hypothesis of this thesis was that the ageing thymus would impose a significant barrier to the induction of transplantation tolerance via mixed chimerism. To this end, the studies presented herein aimed to investigate the ability of low-doses of the chemotherapeutic drug busulfan to condition the bone marrow of young (Chapter 2 and 3) and aged (Chapters 3 and 4) mice to receive either selected or a whole BM transplant. Furthermore, this thesis investigated whether age reduced the ability of autologous and allogeneic cells to engraft in the BM of aged mice compared to young mice (Chapter 4) and if the level of engraftment achieved in aged mice permitted the establishment of allogeneic tolerance (Chapters 3 and 4).

Mixed chimerism and allogeneic tolerance can be achieved in mice receiving 3Gy total body irradiation (TBI) with 7Gy thymic irradiation (28). In Chapter 3 of this thesis, it was investigated if thymic irradiation could be substituted with a short course of sirolimus, which inhibits T cell proliferation by blocking T effector responses to IL-2 (48) and induces T cell anergy (49) when mice receive 3Gy TBI. Sirolimus with 3 Gy TBI could indeed induce allogeneic tolerance when mice were transplanted with $4x10^7$ whole BM (WBM) cells, a high, but clinically feasible cell dose (23). However, peripheral blood chimerism was degraded over time and not stabilised when T cells were depleted prior to BMT. Considering persistent chimerism is required to maintain tolerance

(29), in Chapters 2, 3 and 4 we investigated the chemotherapeutic agent busulfan as an alternative conditioning agent to irradiation, in an attempt to achieve sufficient levels of hematopoietic chimerism that could lead to positive outcomes. Busulfan was the agent of choice for these studies because, unlike other chemotherapeutic agents, it depletes both cycling and non-cycling primitive stem cells, which is essential to achieving long-lasting and high levels of hematopoietic engraftment (22).

Although busulfan is already a standard-of-practice chemotherapeutic drug in many clinical conditioning regimes (35, 50), previous studies that have examined conditioning associated morbidities following busulfan chemotherapy (50-60) have neglected to investigate in any detail the effect of chemotherapy on the thymus, an important point to consider when looking to apply the protocol to the aged setting. Busulfan is typically administered at a dose of 20 mg/kg or higher, with higher levels of chimerism corresponding to dosage increases (20, 61, 62), as mice are relatively more resistant to busulfan than humans. In Chapters 2, 3 and 4 it was found that in young mice, 10 mg/kg of busulfan was sufficient to deplete BM progenitors (LSK cells), without severely affecting cells numbers in WBM or in the peripheral lymphoid pool. One important observation was that there were minimal and short-lasting effects on thymocyte numbers, indicating that systemic administration of busulfan in young mice at 10 mg/kg was thymus-sparing. In Chapter 4, the effect of low-dose busulfan on the BM and thymus in young and aged mice was investigated. Here, it was shown that aged mice were more resistant to low-intensity conditioning with this chemotherapeutic agent. This increased resistance was not due to a dose-limiting effect of the drug, owing to an increase in the number of BM progenitors in aged mice (63) (Chapter 4) or sequestration of the drug by adipocyte infiltrate in the BM, which occurs with increasing age and following chemotherapy (64, 65), suggesting that cell extrinsic factors, such as the BM niche are also affecting engraftment in the aged.

Significantly, sensitivity equivalent to young mice could be restored by treating the mice with granulocyte-colony stimulating factor (G-CSF), which activates quiescent BM progenitor cells (66), prior to busulfan chemotherapy. Supporting the hypothesis, in Chapter 4 it was also shown that busulfan-mediated chemotherapy is more toxic to the already fragile thymus of aged mice. Considering that the removal of sex steroids can significantly enhance thymic reconstitution and protect the thymus from chemotherapy-mediated damage (67), it was hypothesised that sex steroid ablation (SSA) prior to conditioning would protect the thymus from busulfan-mediated damage. This was indeed the case, with SSA protecting both the young (Chapter 2) and aged (Chapter 4)

thymus. Importantly, when mice were conditioned with 10 mg/kg busulfan, both autologous HSCs and allogeneic WBM cells could engraft and induce robust and stable hematopoietic chimerism. In a previous study, young C57Bl/6 mice were conditioned using a protocol similar to the one developed in Chapter 3. This protocol utilised 20 mg/kg busulfan, T cell depleting antibodies, and the mice were transplanted with fully major histocompatibility complex (MHC)-mismatched BALB/c BM, followed by 14-day sirolimus immunosuppression. Although these mice developed stable chimerism, allogeneic tolerance could not be established and skin grafts were rejected (62). In contrast, our protocol used a more clinically applicable donor-recipient strain combination (C57BL/6 and B10.BR) whereby major, but not minor, MHC molecules were mismatched. Following this preconditioning regimen, it was demonstrated that decreasing the busulfan dose to 10 mg/kg and increasing the sirolimus course to 28 days led to the establishment of long-term allogeneic tolerance in 100% of treated mice (Chapters 3 and 4). Surprisingly, aged mice treated with this regime developed stable cellular chimerism that led to tolerance induction, whilst maintaining immune competence, which was assessed by the rejection of third-party (BALB/c) skin grafts.

Intrathymic clonal deletion is the principal mechanism by which allogeneic tolerance is established in mixed BM chimeras (34). Additionally, other tolerance inducing protocols that do not depend on BMT-induced mixed chimerism have also demonstrated a requirement for an active thymus (45-47). Considering this, it was surprising to observe that thymectomised mice were able to accept donor skin grafts and reject third-party unrelated skin (Chapter 3). This suggests that tolerance was generated in the absence of intrathymic deletion, indicative that peripheral tolerance mechanisms play a dominant role in tolerance induction in this model. Tolerance is likely to be established due to the increase in T regulatory cells (Treg) (Chapter 3), which are induced by alloantigen and preferentially expanded by the presence of sirolimus (68).

A previous study, utilising CD45RB monoclonal antibodies showed that tolerance could not be achieved in aged mice (one year old and above) due to thymic atrophy (45), as this mode of tolerance induction requires an active thymus to produce new Tregs (46). In Chapter 3 of this thesis we showed that 100% of aged mice become tolerant to allogeneic skin grafts, suggesting that tolerance induction in our model was not hindered by thymic atrophy and/or compromised by senescent cells in the periphery. The thymectomy data suggests that Tregs are being produced in response to donor antigens in peripheral tissues, rather than antigen being sampled in the periphery and recirculated to the thymus (69). The current literature on changes in Treg function with aging is

controversial. Some studies indicate that both increases and decreases in cell number occur in aged animals, however, most studies are congruent that aged Tregs are dysfunctional and unable to suppress aged effector T cells (40, 70-74). Data presented herein suggests that this is not the case and that Tregs can be induced and suppress alloresponsive effector T cells in this model. Although these studies indicate that ageing does not pose a barrier to tolerance induction when conditioning strategies are targeted to both clonal deletion and peripheral regulation, a limiting factor of this research is that the protocol was not tested in mice older than one year of age. This may be an important point, considering a small number of recent thymic emigrants, demonstrating thymic function have been found in mice up to two years of age.

Induction of tolerance for some experiments described in this thesis was achieved using donor WBM. This cellular mixture contained, among other hematopoietic cell types, mature T cells that can promote hematopoietic engraftment and reconstitute T cell immunity; however, transplantation with allogeneic T cell-replete BM is often associated with GVHD in the clinic (75). Although no GVHD was observed, it is clinically more desirable to use T cell depleted (TCD) BM or purified progenitor cells as a source of donor antigen. In Chapter 3 it was shown that transplantation of TCD-BM and purified progenitor cells can engraft in the BM and induce robust peripheral blood chimerism, at a level comparable to whole BM when young mice were transplanted. In contrast, when aged mice were transplanted with autologous purified progenitor cells, the level of engraftment achieved was 5 fold less than the engraftment observed in the young (Chapter 4). This was not surprising, considering previous studies, that employ myeloablative conditioning, show two-to-three times less donor cell engraftment in aged mice compared to young counterparts (76, 77). In this model, neither SSA nor G-CSF prior to conditioning and autologus HSCT or allogeneic BMT improved the level of chimerism observed in aged mice (Chapter 4). However, this is not a limiting factor for tolerance induction, as chimeric mice were tolerant to donor skin grafts in 100 % of transplanted mice. High levels of donor cell engraftment and subsequent chimerism do not appear to be necessary for BMT-induced tolerance in young or aged mice, as chimerism as low as 1% has been sufficient to induce tolerance in young animals (16).

In this thesis, it was shown that a low-intensity conditioning regime utilising busulfan, in combination with host T cell depletion and short-term immunosuppression, facilitated the engraftment of donor bone marrow cells. Tolerance to MHC-disparate donor antigens was demonstrated using skin grafting; the most stringent transplantation model available to immunology researchers. Importantly, this model was applied in aged mice whereby stable chimerism was

achieved, leading to long-term tolerance to allogeneic skin grafts whilst maintaining functional immunity. Chimerism and tolerance could also be achieved in thymectomised mice, suggesting that peripheral regulation, rather than intrathymic deletion was the dominant mechanism of tolerance induction operating in this model. This led to the conclusion that age-induced immunesenescence is not a barrier to the successful induction of allogeneic tolerance via mixed chimerism in this model.

The attainment of immunological tolerance in the absence of lifelong immunosuppression has long been a major goal of organ transplantation. Ironically, the need for most transplants arises in an ever aging population, yet these are the very patients in whom donor tolerance is the most difficult to achieve. In recent years, only two clinical studies (78, 79) using genetically disparate transplants have demonstrated stable graft survival without maintenance of immunosuppression in patients under 46 years of age (median age 39 years). Therefore, tolerance induction protocols need to be rigorously tested in the aged setting for clinical translation to be successful. In this respect, tolerance induction has rarely been achieved in aged recipients, an impasse considered to be due to thymic atrophy and immunosenescence. Currently, to the best of our knowledge, only two research studies have attempted to induce tolerance in aged animals, in the first study Zhao *et al*. 2011 *Sci Trans Med*, tolerance could not be induced in aged mice, unless mice were castrated to enable thymic regeneration. In a second study, Hock *et al.* 2013 *Transplantation*, tolerance was established using whole body irradiation. However, the toxic side effects associated with the use of irradiation are not clinically justifiable for organ transplant recipients without malignant disease.

This thesis shows, for the first time, the ability to induce long-term allogeneic tolerance in the aged setting using radiation-free, non-myeloablative conditioning without any additional form of endocrine modulation to enhance thymic function, suggesting that strategies targeting peripheral regulation, rather than thymic function may be more effective in achieving and maintaining transplantation tolerance in aged recipients. The implications of the findings presented herein are significant and may serve as a platform for the successful application of tolerogenic protocols in the clinic, not only for solid organ transplantation but also for applications employing HSCT to treat metabolic or autoimmune diseases. With certainty, any allogeneic stem cell based therapy requires a tolerance strategy. Considering that it has been increasingly demonstrated in pre-clinical models that stem cell-derived tissues may have the ability to cure any number of debilitating diseases (80), there is a need, now more than ever, for clinically suitable conditioning regimes for the induction of allogeneic tolerance.

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Appendix 1

Chapter 11
The Immunogenicity of Stem Cells and Thymus-Based Strategies to Minimise Immune Rejection

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Abstract Stem cell research is advancing at a rapid pace, offering the possibility of personalised, "made to order" reparative stem cell treatments. A major challenge, however, is the immunological rejection of the transplanted tissue or 'allograft' that is not derived from self. Current clinical practice for overcoming graft rejection is to administer immunosuppressive drugs. Unfortunately these are associated with a number of side effects, including severe and often prolonged immune deficiency, which can lead to complications associated with opportunistic infections. Rather than prolonged global suppression of the immune system, strategies that focus on inducing graft-specific tolerance will provide a more robust and sustained approach to enabling successful translation of stem cell therapies to the clinic.

11.1 Introduction

It has been increasingly demonstrated in preclinical models that stem cells have the potential to cure a number of debilitating diseases [1]. However, the successful application of stem cell-derived therapies relies on the ability of the host immune system to accept the graft [2]. Unless the grafted tissue is derived from self, it will ultimately be rejected by the host immune system. Traditionally, strategies to overcome non-self or allogeneic graft rejection have been based on lifelong immunosuppression, which leads to high levels of morbidity stemming from opportunistic infections and malignancy.

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P. J. Fairchild (ed.), *The Immunological Barriers to Regenerative Medicine*, Stem Cell Biology and Regenerative Medicine, DOI: 10.1007/978-1-4614-5480-9_11,

A logical solution to overcoming these problems is adaptation of the body's own mechanisms for inducing 'central tolerance' to self-antigens, allowing long-term acceptance of the graft, while maintaining immuno-competence. This can be achieved using donor hematopoietic stem cell (HSC) transplantation (HSCT) which, after bone marrow (BM) engraftment together with host HSCs and subsequent seeding of appropriate progenitor cells to the thymus, results in a mixed chimera of both self and donor hematopoietic and lymphoid cells [3]. Following this thymus-based "central tolerance" induction, the new T cell repertoire will be tolerant to both host and donor cell antigens. As successful as this has been in young animals, a major problem arises around puberty, when the thymus undergoes a natural, prolonged and ultimately profound decline in function with age. Although the direct mechanisms behind thymic atrophy have yet to be determined, a number of approaches have demonstrated that atrophy is at least partially reversible, with substantial restoration of thymic function [4]. Hence, strategies to generate central tolerance to stem cell grafts should be complemented with thymic regeneration.

11.2 Stem Cells

The idea that one cell holds the possibility to treat any number of diseases is fast becoming a reality with the advent of stem cell biology and research. A number of different stem cell types exist, including embryonic stem (ES) cells, adult (also including placental) stem cells and induced pluripotent stem (iPS) cells. Of these, ES cells are commonly considered the most pluripotent but iPS cells may well be equivalent; both thus hold great clinical potential, not only for treatment but also understanding disease processes. ES cells are derived from the inner cell mass of blastocyst-stage embryos and were, therefore, associated with a number of ethical and safety-related issues, although these have now been adequately addressed in most countries. ES cells cannot only self-renew indefinitely, but can differentiate into each of the three embryonic germ layers, endoderm, mesoderm and ectoderm. By the addition of specific growth factors, ES cells can be induced to differentiate into a wide variety of somatic tissues for potential therapeutic application [5, 6], including neurons and glia, cardiac muscle cells, blood progenitor cells, hepatocytes, retinal precursor cells, lung epithelial cells and β-cells of pancreatic islets [1]. They are characterised by the ability to spontaneously form differentiated structures known as embryoid bodies (EB) upon transfer onto non-adherent plates, or in vivo by their ability to form teratomas following injection into severe combined immunodeficient (SCID) mice. These teratoma structures are essentially tumours that contain a mixture of cell types from the three germ layers. In addition to immune rejection, it is this property, which precludes the ease of usage of ES cell-derived products clinically, as any contaminants may seed teratoma formation. This illustrates the critical requirement to remove any undifferentiated cells from

the ES or iPS cell-derived therapeutic cell population to be transplanted, to avoid the risk of tumour formation.

Unlike ES cells, adult stem cells are found throughout the body post-embryonic development. These stem cells are responsible for the homeostatic maintenance, repair and regeneration of the tissue or organ in which they reside. Some of the most characterised adult stem cells include HSCs of the BM, epithelial stem cells of the skin and satellite cells of skeletal muscle. However, despite their ability to self-renew and differentiate, they are restricted in their potential to give rise to other tissue types [7]. Some adult stem cells have been reported to possess ES cell-like characteristics and maintain the ability to give rise to all three germ layers in vitro. These include amnion epithelial cells, isolated from placental membranes [8]. Mesenchymal stromal cells (MSC) also exhibit multiple functions and differentiation potential. Initially isolated from the BM [9], adherent fibroblastic MSCs can expand in culture and give rise to bone, fat and cartilage in vitro. MSCs have since been isolated from various tissues including umbilical cord, placenta and adipose tissue [10]. MSCs also have characteristic anti-inflammatory/immunosuppressive properties, which are important for repressing inflammatory conditions and preventing immune rejection (at least in the short term) [11]. Despite being less "plastic" than ES cells, one important aspect of the use of adult stem cells for tissue regeneration is that they do not form teratomas upon transplantation, eliminating the safety issues associated with their embryonic counterparts [9]. There are now several clinical trials involving MSCs for a variety of clinical conditions including cardiac, osteopathic, hematopoietic and autoimmune diseases [12–15].

iPS cells are ES-like cells that were originally generated from terminally differentiated somatic cells, through the addition of pluripotency-associated genes. This revolutionary technology has now progressed dramatically, with the use of a wide variety of target cells (including adult stem cells) and more defined factors (including small molecules [16] and proteins [17]); without the original oncogenic transcription factors such as c-Myc [18]. iPS cells exhibit similar morphology, growth patterns and gene expression profiles to ES cells. Upon injection into SCID mice, iPS cells are also able to form teratomas [19]. These iPS cells hold the promise of overcoming immune rejection—a skin cells, for example, could be de-differentiated into an iPS cells, and then re-differentiated into a therapeutic cell population for transplantation into the patient. However, whilst, iPS cells may hold the same therapeutic potential as ES cells without being associated with ethical controversies, there remains safety issues associated with the profound genetic re-organisation and, in some systems, the use of viral vectors to deliver the relevant genes [19]. More recently, the potential for spontaneous dedifferentiation of the therapeutic cell population has become evident [19]. The former is now being overcome using proteins [16], mini-circle DNA [17] together with small molecules as delivery systems, rather than incorporation of genes permanently into the genome [20].

11.2.1 Stem Cell Transplantation

The therapeutic potential of stem cell transplantation is best exemplified by the use of bone marrow transplants (BMT) for over 40 years.[1] HSC are the most important cell type in BMT, because they not only replenish the hematopoietic compartment but they do so permanently. Both autologous and allogeneic HSCT are now well established and constitute a curative technique for many conditions including primary immunodeficiency disorders, autoimmune conditions, BM failure syndromes, non-malignant hematological disorders, as well as hematological malignancies [21]. Whereas autologous HSCT will not involve any rejection, a major problem with allogeneic HSCT is not so much the rejection of the foreign graft by the recipient's immune system (because they are immune suppressed from the conditioning), but the induction of graft-versus-host disease by T cells within the graft. This remains a major clinical problem, particularly since it needs to be delicately balanced with the beneficial effects of the graft versus leukaemia (GvL) effect mediated by the donor T cells [22].

11.2.2 The Immunogenicity of Stem Cells

Initially, there was a widely held belief that many types of stem cells evaded immune rejection because they themselves produced anti-inflammatory molecules or because they did not express sufficiently high levels of immune stimulating molecules. Indeed some studies have shown that undifferentiated ES cells can be transplanted across a minor histocompatibility (mH) barrier, seemingly without eliciting an immune response [23]. However, in most cases the transfer of cells, tissues or organs from one individual to another results in immunological rejection through host immune recognition of donor antigens.

There are three classes of "transplantation" antigens, namely major histocompatibility complex antigens (MHC), mH antigens (H) [24] and the blood group (ABO) antigens [25]. Of these, MHC mismatch is the major cause of allograft rejection. MHC proteins are classified as MHC class I, which is expressed on almost all nucleated cells, and MHC class II, which is expressed only on cells in the body that can present antigen. In humans, MHC proteins are known as human leukocyte associated antigens (HLA). There are three genes for each of the HLA classes, namely HLA-A, HLA-B and HLA-C for class I and HLA-DP, HLA-DQ and HLA-DR for class II. The HLA genes are the most polymorphic genes in the human genome, with some possessing several hundred alleles. Additionally, at least six different HLA alleles are expressed at any one time, in a co-dominant fashion [26].

[1] http://emedicine.medscape.com/article/1014514-overview

The probability of selecting a complete MHC match for any allogeneic transplanted tissue, including ES cells and their derivatives, is thus almost impossible. It has been suggested that creating an ES cell bank comprising 150 donors encompassing all ABO blood groups, or 100 universal blood group O donors, would include enough HLA haplotypes for matching the general population [27]. However, the success of this approach would be limited, since a single mismatch at any one locus can generate an immune response and ultimately induce rejection [23]. Moreover, ES cells and some stem cells derived from adult tissues express low levels of HLA class I, which is up-regulated upon differentiation into more mature cell lineages [28] or following exposure to pro-inflammatory cytokines [29]. Accordingly, there have been recent reports that ES cells are indeed immunogenic, eliciting readily detectable immune responses [30, 31].

T cells recognise alloantigen by two distinct pathways, direct recognition, whereby T cells recognise intact allogeneic MHC molecules, together with peptide on the surface of donor-derived dendritic cells (DCs) present in the graft. Indirect recognition involves the presentation of alloantigen by host derived antigen presenting cells (APC), that has been phagocytosed and processed for presentation by host-MHC [32]. Unlike tissue allografts, ES cell transplants do not contain DCs, professional APCs which express MHC class II. DCs are widely distributed throughout the body and, because of their role in priming the immune response via MHC class II antigen presentation to $CD4^+$ T cells, they play an important role in allograft recognition by the immune system. An absence of DCs from the ES cell graft eliminates direct antigen recognition [32]. Nevertheless, several studies have now indicated that ES cell-derived grafts undergo a progressive infiltration of inflammatory cells, which include neutrophils, macrophages, granulocytes, B cells and T cells [30, 31]. Furthermore, host-derived DCs and other APCs accumulate in the ES cell graft over time, leading to indirect graft recognition [30].

There is also evidence suggesting ES cell-grafts induce a humoral immune response. In support of this contention, high levels of allo-antibodies are found in transplanted mice, accompanied by high levels of T helper cell (Th)-2 cytokines, including interferon-γ and IL-4 [31]. Together this suggests that rejection of ES cell-grafts is mediated by both strong cellular and humoral immune responses.

A type of adult stem cell that appears to show some "immune privilege" is the MSC. Following transplantation, MSCs can home to sites of damage and produce cytokines and growth factors which suppress inflammation and induce tissue repair [33]. Early reports have shown that MSCs do not elicit overt allogeneic responses in vitro [34] suggesting that they will evoke little immunity when transplanted [11]. Hence MSCs have now been subjected to many clinical trials, highlighting their ability to restrain graft-versus-host disease, promote hematopoietic engraftment [10, 11] and repair bone fractures [14, 35]. In some cases, MSC induction of tissue repair may be by direct differentiation into the damaged cell type [36] or by indirect mechanisms, such as cytokine/growth factor production to reduce inflammation to enable tissue repair to progress [37]. Regardless, there is now accumulating evidence suggesting that allogeneic MSCs, like ES cells, are being recognised by host T cells, resulting in both cellular and humoral immune

responses [38–40]. This will need to be resolved, particularly if multiple dosing of MSCs from the same source is required for treating more chronic conditions.

11.2.3 Overcoming Immunological Barriers

In terms of avoiding transplantation rejection, iPS cells derived from the patient and Somatic Cell Nuclear Transfer (a technique where the nucleus of a donor somatic cell is removed and placed in an enucleated oocyte, creating a more primitive phenotype and plastic cell) [41] are promising alternatives. These techniques can be utilised to revert differentiated cells to a more pluripotent or even partially "stem cell" state, and then differentiate them into disease specific therapeutic cells, thus providing patients with a source of autologous tissue for transplantation. For example, disease-corrected iPS cells have been developed from patients with both hematological and neurodegenerative disorders [42]. However, many questions remain with regard to their clinical utility. As yet, it is not known if iPS cells will revert to their original diseased cell type, if they will form tumours and how they function in comparison to their natural counterparts.

The successful application of non-self stem cell-derived therapies relies on the ability of the host immune system to accept the graft. As previously mentioned, conventional strategies to overcome graft rejection are based on long-term, often lifelong, immunosuppressive regimes. While these can successfully aid graft acceptance in the short-term, they also lead to generalised immunodeficiency, precipitating opportunistic infections and even malignancy. A logical solution to overcoming these problems is to adapt the body's own highly successful mechanisms for inducing permanent tolerance to self-antigens to create a donor-specific tolerance, thereby facilitating long-term acceptance of the graft, while maintaining immune competence and minimising the use of immunosuppressive drugs. The organ responsible for "teaching" the body to distinguish self from non-self is the thymus.

11.3 The Thymus

The adult thymus is a 3-dimensional stromal network; composed of discrete cortical and medullary epithelial regions, mesenchyme-derived fibroblasts, BM-derived DCs and macrophages, as well as endothelial cells. Thymocytes interact with and migrate through these stromal cells as they differentiate into mature self-tolerant T cells before migrating to the periphery, to establish and maintain the T cell arm of immunity [43]. This thymic microenvironment provides a niche where specialised interactions between hematopoietic T cell progenitors and thymic stromal cells can occur, each contributing to the development and maintenance of the other, in a sophisticated course of events [44].

11.3.1 Thymus Development

In early embryogenesis the thymus originates from the endodermal layer of the anterior foregut. A homogenous population of epithelial cells, derived from the third pharyngeal pouch endoderm gives rise to the cortical and medullary regions of the thymus [45]. At this stage at least some of the thymic epithelial cells (TEC) are bi-potent, with the potential to differentiate into both cortical TECs (cTECs) and medullary TECs (mTECs) [46]. Expression of the Forkhead-box transcription factor N1 (FoxN1), restricted to epithelial cells, is initiated at approximately embryonic day (E) 11.5, a process essential for the downstream differentiation into cortical and medullary lineages and colonisation of the anlage by hematopoietic progenitors [47]. Following E11.5, FoxN1 is expressed by all epithelial cells in the rudiment [48] and maintained throughout thymus development, detectable in numerous TECs in the adult thymus [49]. The function of FoxN1 in the adult steady-state thymus is less well understood, but thought to be involved in the maintenance of the epithelium and homeostasis [50]. Wnt and bone morphogenetic protein (BMP) signalling are responsible for initiating [51] and maintaining FoxN1 expression [52].

The initial stages of TEC formation occur independently of thymocytes [53], while the later stages rely on specific interactions between the TECs and thymocytes [54]. Studies in mice have indicated that signals delivered by thymocytes are crucial for the maturation of cTEC and mTEC subsets from a common precursor, as well as the support and maintenance of the thymic architecture. In the mouse, thymic epithelial progenitor cells (TEPC) have been phenotypically identified as $MTS24^+$, keratin (K) 5^+ and $K8^+$ [55], with differentiation into mature cTEC subsets phenotypically marked by the loss of K5 and the retention of K8, whereas mTECs can be marked by the expression of K5 and the loss of K8 [53]. As development proceeds the thymus increases in size and compartmentalises into discrete specialised areas, which include an outer cortex housing the cTECs, and an inner medulla housing the mTECs, with the two regions being separated by the cortico-medullary junction [43].

11.3.2 Thymopoiesis and Central Tolerance

The thymus is responsible for providing T cells throughout adult life. To do so the thymus must recruit hematopoietic progenitors from the BM via the blood, a process known as thymus seeding (Fig. 11.1a). Thymopoiesis is initiated by thymic chemokines, attracting BM-derived progenitor cells expressing the receptors CCR7 and CCR9 [56], as well as recognition of P-selectin on thymic endothelium through P-selectin glycoprotein ligand (PSGL)-1 [57].

Upon entry, the blood-borne progenitor cells rapidly commence thymic commitment, following a number of well-defined differentiation steps, which occur

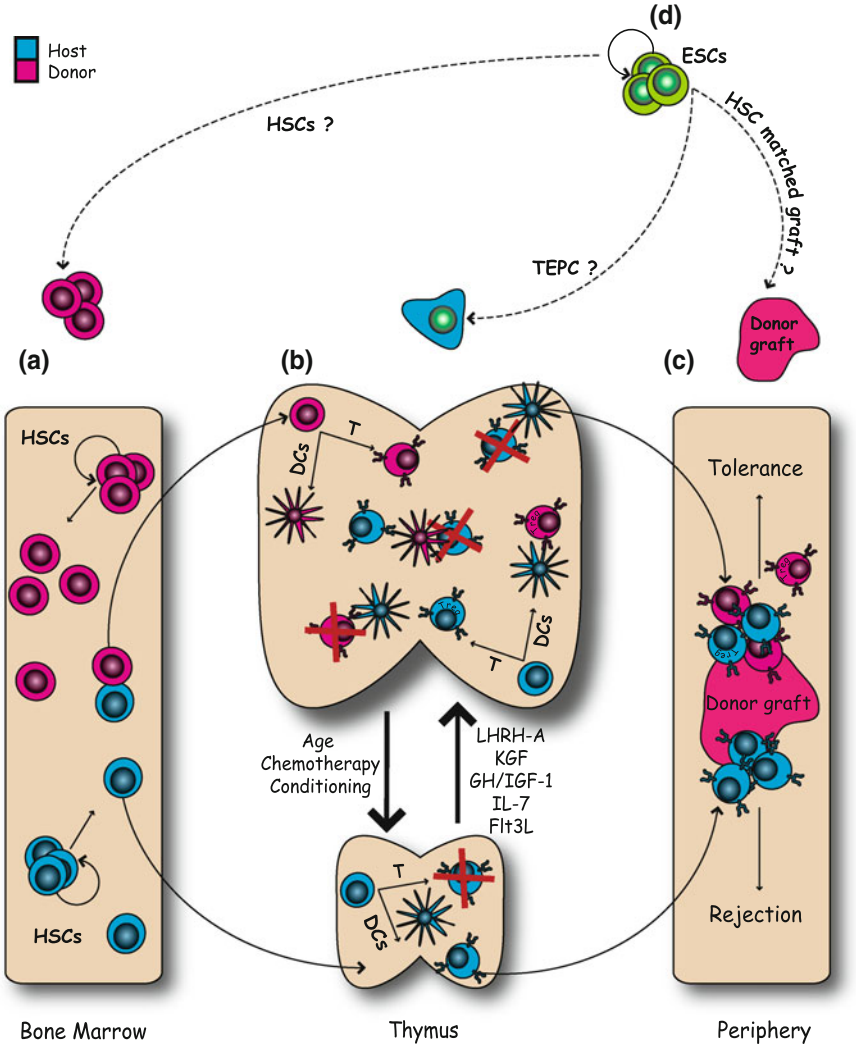

Fig. 11.1 Tolerance to an allogeneic graft can be induced by generating hematopoietic chimerism. Host cells are shown in *blue* and donor cells are shown in *pink*. **a** Following appropriate *BM* conditioning, transplanted donor *HSCs* engraft in the host *BM*, producing T cell and *DC* precursors, which migrate to the thymus. **b** In the thymus, precursors differentiate into T cells, *Tregs* and *DCs*. Reactive cells are deleted upon encounter with their cognate self-antigen presented by *TECs* as well as host and donor-derived *DCs*, a process known as negative selection. T cell output is significantly hindered in the atrophied thymus, which occurs through ageing, chemotherapy and *BM* conditioning. Thymic atrophy can be reversed through the ablation of sex steroids and/or the provision of growth factors. Educated T cells, along with both host and donor-derived *Tregs*, migrate to the periphery, **c** where tolerogenic host and donor-derived *Tregs* induce anergy in any reactive T cells that have escaped negative selection, promoting tolerance and graft acceptance. **d** Stem cell technologies may enable the generation of *HSCs*, *TEPCs* and tissue grafts from the same pluripotent stem cells for transplantation

within discrete thymic regions. Progenitors progress from immature $CD3^{-}CD4^{-}CD8^{-}$ (triple negative, TN) stage to $CD4^{+}CD8^{+}$ (double positive, DP) thymocytes, which, if able to recognise their respective peptide-MHC complexes with appropriate level of low affinity, receive a survival signal, in a process termed positive selection, to become mature $CD3^{+}CD4^{+}CD8^{-}$ or $CD3^{+}CD4^{-}CD8^{+}$ (single positive, SP) thymocytes. SP thymocytes are subject to a further developmental checkpoint whereby potentially auto-reactive cells binding with high affinity to MHC-peptide complexes are deleted from the repertoire (negative selection). The end result is a pool of naive T cells tolerant to self-antigens and capable of recognising a plethora of foreign antigens. This thymic, or "central" tolerance is mediated predominantly by DCs [58]. As dedicated antigen-presenting cells, DCs provide thymocytes with the optimal means of responding to self-peptides many of which they may encounter in the periphery [59]. Thymocytes expressing T cell receptors (TCRs) with high affinity for self-peptides presented by DCs undergo apoptosis [60], are functionally inactivated (anergy) [61], lose their auto-reactive TCR (editing) [62], or are directed into a mature T cell lineage such as regulatory T cells (agonist selection) [63]. In this way, potentially auto-reactive T cells are purged from the nascent repertoire.

It is now known that, in addition to DCs, mTECs play a major role in the induction of self-tolerance through their unique ability to promiscuously express a diverse range of genes usually constrained to peripheral tissues, such as insulin, thyroglobulin, myelin oligodendrocyte glycoprotein and the acetylcholine receptor [64]. The expression of these peripheral tissue antigens (PTAs) by mTECs is under the control of the autoimmune regulator (AIRE), which is therefore crucial for preventing the development of autoimmunity [65].

11.3.3 T Regulatory Cells

Although highly efficient, central tolerance is not foolproof. Consequently, other peripheral mechanisms are present, ensuring auto-reactive T cells that have escaped negative selection, do not cause autoimmunity. $CD4^{+}CD25^{+}$ FoxP3-expressing T regulatory cells (Tregs) play an essential role in maintaining self tolerance and preventing T cell mediated autoimmune diseases [66]. "Natural" Tregs, like other T cells, are generated in the thymus through encounter with TCR-agonist ligands expressed on thymic epithelium with an intermediate level of activation signalling [67]. However, unlike conventional $CD4^{+}$ and $CD8^{+}$ T cells, the majority of Tregs are specific for self [68] and are continuously activated [69]. Following thymic emigration, Tregs, along with naïve T cells, home to draining lymph nodes. Here encounter with antigen leads to Treg activation, inducing proliferation and enhancing suppressor function [70]. Activated Tregs regulate neighbouring $CD4^{+}$ and $CD8^{+}$ T cell responses, via cytokine production, and cell-to-cell contact [71], preventing their proliferation [70] and ability to function as effector cells [72].

Thymic selection is not the only means of producing Tregs, since circulating naïve $CD4^+$ T cells can be selected to form so-called "induced" $CD4^+CD25^+$ Treg cells in the periphery via encounter with organ-specific agonist ligands [73]. Furthermore, such Tregs can be intentionally generated by the application of specific agonists under sub-immunogenic conditions (low dose and/or lack of co-stimulation) [74].

11.4 Manipulating the Thymus for Transplantation Tolerance

Given the thymus relies on continual seeding by BM precursors [75], it is possible to supply donor-derived hematopoietic progenitors and induce a state of mixed chimerism; the co-existence of donor and host hematopoietic cells in the same tissue (Fig. 11.1c). Advances in stem cell technologies may one day enable the generation of both HSCs and tissue graft from the same pluripotent stem cell (Fig. 11.1d). If so, the thymus provides all the necessary attributes to "teach" the body to accept the donor graft, essentially re-programming the immune system for the life of the recipient.

11.4.1 Chimerism

Mixed chimerism has been used successfully in many rodent models for the specific induction of allogeneic tolerance. Owen first observed this process over 60 years ago, demonstrating that a mixture of two distinct types of erythrocytes can be found long after birth in fraternal bovine twins that had shared a common placental circulation [76]. Importantly, this was induced in the neonatal period. Shortly afterward, Medawar and colleagues demonstrated that skin grafts between these bovine chimeric twins were accepted indefinitely, indicating that each had acquired a tolerance to the other's tissue [77]. Ten years later, Billingham and colleagues showed that this form of tolerance could be actively induced between MHC-disparate mice, provided the skin graft recipient mice had been exposed to donor antigen in the neonatal stages of development [78].

The principle is simple and can be applied to adults with appropriate conditions, providing successful HSC engraftment and the existence of an active thymus. Recipients are given a BMT or HSCT from an allogeneic donor that is MHC-matched to the tissue (such as skin) to be transplanted. These cells engraft in the BM and differentiate into lymphoid and myeloid progenitors. T cells and DC precursors from both host and donor migrate to the thymus whereby the process of negative selection purges the emerging T cell pool of host-reactive as well as donor-reactive T cells [79] (Fig. 11.1b). When the appropriate immunosuppressive regimes have been applied to eliminate pre-existing mature allo-reactive T cells in

the periphery, tolerance to fully MHC-mismatched skin grafts can be consistently achieved [80]. This has also been observed in clinical settings where patients who had received BM transplants for haematological malignancies subsequently became tolerant to skin [81] and kidney [82] grafts originating from the same donor, with the additional contribution of newly produced thymus derived donor-specific Tregs [83].

Donor-specific Tregs are involved in the induction and maintenance of allogeneic tolerance, through their ability to promote donor cell engraftment [84] and prevent both allogeneic graft rejection [85] and graft versus host disease (GvHD) [86, 87], indicating that they play an important role in the long-term survival of the graft, by providing a natural, more-specific means of immunosuppression [88]. Furthermore, Tregs have recently been shown to play a crucial role in preventing the rejection of allogeneic ES cell-derived grafts, indicating a possible mechanism behind the initial idea that ES cells were "immune privileged" [89].

To allow donor BM cells or HSCs to engraft within the recipient, existing mature alloreactive T cells must be eliminated and "space" must be created within the recipient BM, in a process referred to as "conditioning". To induce a permanent state of immunological tolerance, cells that have engrafted in the BM must have a lifelong multilineage repopulating ability in order to provide the thymus with a constant source of donor antigens. The simplest and most reliable method for creating "space" in the BM is total body irradiation (TBI) prior to transplantation of T cell-depleted donor marrow. The dose is both myeloablative, to create "space" for donor cells, and immunosuppressive, to eliminate the potential for developing host-versus-graft disease (HvGD). Using this approach successfully should induce fully MHC-mismatched allogeneic and xenogeneic (across species) graft tolerance [90].

11.4.2 Thymic Atrophy

A problem that is frequently overlooked with the above approach, is that the thymus, the principal organ responsible for generating a pool of T cells tolerant to both donor and host tissue, undergoes a profound atrophy with age (Fig. 11.1b). Thymic function is most active during the fetal and perinatal stages of development, with a decline in function evident from as early as the first year of human life. Thymic degeneration is progressive and most apparent at puberty, with approximately 95 % of thymus function lost by 50 years of age [91].

This age-related thymic involution is characterised by gradual changes in the thymic microenvironment, including a loss of distinction between cortical and medullary regions, extensive vacuolisation of epithelial cells, and the replacement of thymopoietin tissue with perivascular spaces and adipose deposits [91]. Detailed analysis of thymic stromal subsets reveals an increase in proportion of non-TECs such as fibroblasts, while a decrease in TEC number and proportion of particularly mTECs is evident within the TEC compartment [92]. In addition to thymic stromal

changes, there is a decline in early T cell progenitors, which also display a reduced capacity for differentiation [93].

Together these processes result in decreased production and export of naïve T cells from the thymus, leaving homeostatic proliferation of T cells in the periphery to compensate for this loss. As T cell maintenance in the elderly relies on the expansion of mature T cell subsets rather than naïve T cell emigrants, the diversity of the T cell pool undergoes a bias towards antigens that have already been encountered by the immune system. Within this constricted TCR repertoire, the likelihood of matching the appropriate TCR to novel antigenic epitopes decreases, ultimately limiting the immune system's ability to recognise and respond to unfamiliar challenges [94].

The mechanisms behind thymic atrophy are not clear and several factors have been implicated (Fig. 11.1b). Of these, sex steroid production has been the subject of numerous studies as puberty coincides with the greatest period of thymic involution [91]. Studies have also suggested reduced production of immunostimulatory growth factors and cytokines such as growth hormone (GH) [95] and interleukin (IL) 7 [96], as well as down-regulation of adhesion molecules required to facilitate thymocyte entry to the thymus [97]. Conversely, up-regulation of atrophic factors such as transforming growth factor (TGF) β may also contribute to the involution process. BM progenitors undergo a reduced lymphoid potential and self-renewal capacity with age [98]: since the thymus relies on the BM for continual seeding, age-related BM dysfunction may also play a role in the loss of thymic function with age.

11.5 Thymic Regeneration

One of the fundamental requirements for the induction of tolerance is a functional thymus, which can produce naïve T cells. Age-related thymic atrophy therefore presents a significant challenge for the development of chimerism-based approaches to the induction of tolerance to stem cell grafts in adult patients. Any strategies that manipulate central tolerance for transplantation therapies in the adult should, therefore, be coupled to the restoration of thymic function. Several preclinical and clinical approaches to restore thymic function have been proposed, including hormone blocking therapies and administration of growth factors to regenerate the ageing thymus (Fig. 11.1b). Furthermore, the identification of a putative TEPC may aid the de novo generation of thymic tissue.

11.5.1 Thymic Epithelial Progenitor Cells

The existence of a putative TEPC was demonstrated in the mouse embryo when $MTS24^+$ TECs engrafted under the kidney capsule of nude mice produced both mTECs and cTECs and gave rise to a fully functioning thymus capable of

supporting T cell development [55]. These MTS24$^+$ cells are abundant in the embryo, but become increasingly less frequent as the thymus develops, localising to the medulla and cortico-medullary junction in the adult mouse thymus. These progenitors also co-express both mTEC and cTEC markers K5 and K8 [55], supporting the hypothesis that the thymus develops from a bipotent TEC progenitor. More recent work has demonstrated that the MTS24$^-$ TEC population was also able to give rise to an ectopic thymus graft, but only when a significantly higher number of cells were reaggregated [99]. In the adult thymus, the existence of a TEPC is supported by the ability of both mTEC and cTEC to regenerate after both injury-induced and age-related thymic damage [100]. However, an adult TEPC phenotype has yet to be elucidated.

While identification of a resident adult TEPC should allow in situ manipulation of the thymus to enhance regeneration, it may be possible to generate a TEPC ex vivo from ES cells or even iPS cells. Factors that direct the differentiation of ES cells into the endodermal lineage are still relatively novel [1] and protocols to guide the development of thymus-specific tissue have yet to be established. Candidate pathways include the Wnt and BMP signalling families as both play a role in regulation of the FoxN1 transcription factor required for both TEC formation and maintenance [101]. Once established, ES cell-derived TEPCs could be directly injected into the atrophic thymus or grafted as reaggregate cultures under the kidney capsule, re-establishing function [55].

11.5.2 Sex Steroid Ablation

Evidence for residual thymic function, albeit very limited, in ageing individuals gives credence to the possibility of inducing thymus regeneration in vivo via the removal of inhibitory factors or administration of stimulatory factors. Of the former, sex steroids have been strongly linked to thymic atrophy, since they have considerable inhibitory effects on both lymphoid development and immune function [91]. Sex steroids exert direct effects on the thymic stromal cells, which express sex steroid receptors on the cell surface [102]. Consequently, removal of sex steroids through castration (chemical or surgical) is associated with marked rejuvenation of the thymic compartment in aged mice and following chemically induced thymic damage [100]. This is evident in both thymic architecture [92] and thymic cellularity [100, 103]. Specifically, regeneration is demonstrated by the restoration of cortical and medullary regions, TEC and fibroblast ratios, as well as TEC and thymocyte numbers. Importantly, thymic export of naïve T cells is increased, resulting in enhanced cytolytic activity upon viral infection [104].

Additionally, these improvements correlate with an increase in BM lymphopoiesis, in particular, an increase in the number of IL-7-responsive progenitor cells, an increase in B cell export, as well as enhanced B cell function [105], therefore contributing to an overall improvement in immune competence [106, 107]. The clinical relevance of sex steroid ablation is further demonstrated by accelerated

recovery from chemotherapy and irradiation-induced damage [102, 103, 108]. Of particular relevance to donor-derived tolerance induction, the removal of sex steroids has also been shown to improve engraftment in the BM and peripheral reconstitution following allogeneic BMT, without exacerbating GvHD [107]. If sex steroid ablation can increase thymic seeding by donor progenitor cells and thymic output of donor-tolerant naïve T cells, this strategy could complement BMT protocols for the generation of hematopoietic chimerism for tolerance.

Sex steroid inhibition can be achieved in a reversible manner with the use of a luteinising hormone releasing hormone (LHRH) analogues. LHRH is normally produced by the hypothalamus to stimulate pituitary secretion of luteinising hormone and follicle stimulating hormone, which in turn trigger sex steroid production by the gonads. LHRH agonists (LHRH-A) cause sensitisation and down-regulation of LHRH receptors, resulting in the shutdown of sex steroid production [97]. This process is reversed upon cessation of treatment. Clinical application of sex steroid inhibition in the context of immune regeneration has been demonstrated by improved thymic and immune recovery of LHRH-A-treated patients from autologous or allogeneic HSCT for hematological malignancies [104].

11.5.3 Keratinocyte Growth Factor

Keratinocyte growth factor (KGF) is a fibroblast growth factor that stimulates the proliferation of epithelial cells in a number of tissues. In the thymus, KGF is produced by mesenchymal cells and mature SP thymocytes and plays a significant role in regulating thymic epithelium development and function [109]. Although a deficiency in KGF does not accelerate thymic involution, mice deficient in KGF are unable to reconstitute the peripheral T cell compartment following BMT [110]. By stimulating TEC proliferation [111], exogenous KGF protects these cells from damage induced by cytoablative conditioning [110] and GvHD [112]. In mice receiving allogeneic BMT, KGF treatment enhances recovery of thymic cellularity, thymic function and peripheral T cell reconstitution [110]. Furthermore, KGF supports immune recovery in an additive manner when used in combination with LHRH-A [113].

11.5.4 Growth Hormone

Another key factor that has been associated with thymic involution is GH. GH is known to stimulate thymopoeisis and regulate a number of immunological events in the periphery [114]. Serum levels of GH, as well as expression of gherlin, a GH secretagogue, and its receptor in thymic stromal cells, decrease progressively with age [115]. In old mice, ghrelin infusion improves thymic architecture and increases

T cell output and diversity [116]. Similarly, high-dose GH treatment of HIV-infected patients has been shown to increase thymic export and naïve $CD4^+$ T cell numbers [117]. The effects of GH treatment are not restricted to the thymus as recombinant GH can also reverse irradiation-induced loss of BM progenitor function in mice [118].

GH mediates its immunostimulatory effects primarily through local insulin-like growth factor (IGF)-1 [119], expression of which also decreases with age [120]. In the context of immune regeneration, IGF-1 administration to mice receiving allogeneic BMT has been shown to support both lymphoid and myeloid reconstitution, without exacerbating the development of GvHD [121]. Recently, IGF-1 has been shown to exert its effects in a tissue-specific manner, with neutralisation of local IGF-1 to specific BM stem cell niches reversing the age-related decline in progenitor function [122]. Clinically, however, GH has many side effects including increased susceptibility to diabetes.

11.5.5 IL-7

IL-7 is a growth factor essential for T and B cell development [123]. In the thymus, IL-7 is produced by TECs and decreased IL-7 production with age has been associated with a reduction in IL-7^+ TECs [124]. While it remains unclear whether the decline in IL-7 production is the causative factor in thymic atrophy, IL-7 treatment has been shown to reverse involution-associated changes [125] and enhance peripheral T cell reconstitution in mice after BMT [126, 127]. Interestingly, combination therapy with IL-7 and IGF-1 has an additive effect on B cell but not T cell reconstitution in mice receiving allogeneic BMT [121]. In contrast, concomitant use of IL-7 and sex steroid ablation exerts profound additive effects in the thymus after allogeneic BMT [107]. Importantly, IL-7-treated patients with refractory cancer exhibit preferential expansion of naïve T cells and a more diverse T cell repertoire [128].

11.5.6 Flt3L

Fms-like tyrosine kinase 3 ligand (Flt3L) is another growth factor that can support thymic function and immune reconstitution. Flt3L is recognised by cells that express Fms-like tyrosine kinase 3 (Flt3) which include BM progenitors and immature thymocytes [129]. Unlike IL-7 that primarily acts to enhance peripheral T cell expansion, Flt3L exerts its effects by promoting BM progenitor expansion and downstream thymopoiesis and peripheral T cell reconstitution [129]. In the steady-state thymus, interactions between $Flt3^+$ T cell progenitors and thymic fibroblasts expressing Flt3L are important for maintaining T cell development [130]. Importantly, thymocyte recovery from irradiation-induced damage appears to require Flt3 ligand-receptor interactions [131].

11.6 Conclusion

Given that it is now clear that stem cells are immunogenic (even if some also have immunosuppressive or anti-inflammatory properties), the challenge of overcoming immunological rejection must be addressed before "made-to-order" stem cell transplantation can become a reality. Current methods focussing on long-term immunosuppression are associated with many adverse side effects and, in some circumstances, can, ironically, ultimately lead to graft rejection triggered through infection. Newer approaches which utilise graft-matched HSCT, manipulate the body's own mechanisms to induce tolerance, relying on the thymus to teach the immune system to accept the graft and produce graft specific Tregs for peripheral tolerance. This should provide long-term, low morbidity graft acceptance. Without a functionally active thymus, however, this process becomes severely limited. Hence, to achieve lasting donor-specific immunological tolerance, donor HSCT should be coupled to thymus regeneration.

This goal is clinically feasible, considering a number of therapies that can potentially restore thymic function already have Food and Drug Administration (FDA) approval for use in the clinic, albeit for other conditions. LHRH-A has been used for many years now to treat prostate cancer, endometriosis, fibroids and precocious puberty. KGF has recently been approved for the prevention of chemotherapy-induced mucositis in patients undergoing HSCT. While GH is used routinely to treat conditions caused by GH deficiency, it has a short half-life and supraphysiological doses are often required to achieve efficacy. This raises concerns associated with side effects and toxicity of GH. Hence, a safer alternative to improve immune recovery following HSCT, enhance uptake of donor HSC and subsequent development of donor antigen tolerance, may be temporary sex steroid blockade in combination with KGF or IL-7. These options may present the ideal platform for inducing long-lasting tolerance to stem cell-derived therapies.

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