Hematopoietic stem and progenitor cell recovery in sex steroid ablation-mediated immune regeneration

Danika Khong Mei Po

B.Bms (hons)

Monash Immunology and Stem Cell Laboratories

Faculty of Medicine, Nursing and Health Sciences

Monash University

Melbourne, Australia

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

2012

Copyright Notices

Notice 1

Notice 2

"You know, sometimes all you need is twenty seconds of insane courage. Just literally twenty seconds of just embarrassing bravery. And I promise you, something great will come of it…"

~Benjamin Mee (We Bought A Zoo)

SUMMARY

Lifelong hematopoiesis is supported by hematopoietic stem cells (HSCs) differentiating within the bone marrow (BM) down all blood cell lineages including erythrocytes, platelets, myeloid and lymphoid cells. Although most blood cells are generated entirely within the BM, T cells complete their development in the thymus, a primary lymphoid organ within the mediastinal cavity. Together, B and T cells represent the lymphoid arm of the immune system and are vital in the adaptive protection against foreign pathogens.

While hematopoiesis is continuous for the lifespan of the organism, it is currently well established that, paradoxical to its fundamental importance for establishing and maintaining good health, the adaptive immune system degenerates very early with age; a phenomenon temporally linked to sex steroid exposure. Beginning from birth, but accelerated at the onset of puberty, both the primary lymphoid organs – the BM and thymus, gradually deteriorate, resulting in a decline in the generation of new naïve B and T cells. In healthy adults this does not pose a major threat, however in patients who are immunocompromised following cytoreductive treatments, such as chemo- or radiation-therapy or chronic infections best exemplified by acquired immunodeficiency syndrome (AIDS), immune recovery is considerably delayed leaving these individuals susceptible to opportunistic infections and malignant relapses. As a corollary to the hypothesized underlying cause of immune atrophy, our laboratory and others have previously demonstrated that removing the negative effects of sex steroids by either surgical or chemical (reversible) castration (sex steroid ablation; SSA) facilitates significant immune regeneration in aged mice and can enhance lymphoid recovery post HSC transplantation (HSCT) or treatment with chemotherapy.

This thesis aimed to elucidate the mechanisms underlying SSA-mediated lymphoid recovery by comprehensively assessing the impact of SSA on (1) the function of hematopoietic stem and progenitor cells in the BM, (2) the ability of the supporting BM stromal microenvironment to support hematopoiesis, and (3) the function of the earliest T-lineage progenitors in the thymus.

Consistent with previous reports, we observed an age-associated accumulation of HSCs that demonstrated inferior self-renewal capacity and lymphoid differentiation potential when compared to young HSCs. We further demonstrated both a numerical and functional enhancement of the primitive long-term HSC (LT-HSC) population following SSA. Not only did these LT-HSCs show enhanced self-renewal, they were also more efficient at differentiating into downstream lymphoid cells with SSA. Detailed molecular analysis revealed important cell intrinsic changes pertaining to quiescence, self-renewal, lymphoid differentiation and DNA replication processes occurring within these primitive HSCs, collectively suggesting their role in establishing SSA-mediated immune regeneration.

Since HSCs rely heavily on their stromal cell-based microenvironmental "niches" for signals governing their differentiation and survival, the effects of SSA on the the endosteal and vascular (central marrow) compartments were profiled. Interestingly, a population of osteoblasts (OBLs) with high *Runx2* expression was revealed within the vascular niche of the BM that numerically increased with age; this correlated with an accumulation of LT-HSCs seen with age in the BM. These *Runx2* –rich OBLs also expressed hematopoietic supporting molecules, albeit to a lesser degree than the canonical LT-HSC-associated endosteal osteoblasts. These age-related changes were not observed within the endosteal osteoblasts compartment however. While SSA was unable to reverse the age-associated increase in the vascular OBL population, there was increased expression of genes linked to promoting HSC quiescence, self-renewal, lymphoid differentiation and cell adhesion. This

clearly indicates the important synergisms between the hematopoietic and stromal compartments of the bone marrow – with both contributing to the SSA-induced rejuvenation of the blood system.

With this evidence suggesting that enhanced HSC and BM niche function led to downstream improvements in lymphopoiesis, the final section of this thesis focused on the effects of SSA on the lymphoid progenitors: their ability to seed the thymus and subsequently differentiate into T cells. While there has been considerable insight into the developmental hierarchy of HSCs and the downstream progenitors within the BM, little is known about the identity of the thymic-bound progenitor that egresses from the BM.

Increases in the $CD27^{+}CD62L^{+}$ lymphoid primed multipotent progenitors (LMPP) and common lymphoid progenitors (CLPs) within the BM, and the early thymic progenitor (ETP) and CLP-2 within the thymus were evident following SSA. The earliest T cell progenitor within the thymus is the ETP, which has proficient T differentiation potential. However the earliest cellular increase in the thymus post SSA was by the CLP2 population at day 2. The CLP and its downstream CLP-2 produced in the BM, are by default a B cell progenitors, yet have a very efficient capacity for thymic entry and the propensity to form T cells once influenced by Notch signaling within the thymus. Several thymic homing molecules, CCR-7, CCR-9 and PSGL-1 have been identified on various circulating thymic progenitors (CTPs), however the expression of these molecules was unaltered following SSA. This would suggest the increased ability of these BM derived CLP-2s to migrate into the aged regenerating thymus, is possibly due to a noncanonical “stress” or “damage” associated pathway. The data are thus consistent with the downstream improvements observed in lymphopoiesis being the result of an increased supply of lymphoid progenitors from the BM, subsequently entering the thymus coupled with an enhanced ability of the

intrathymic microenvironment in supporting thymopoiesis, rather than an enhanced intrinsic ability of these progenitors to differentiate into T cells, on a per cell level.

Collectively, the work presented here provides a tantalizing glimpse into the mechanisms underlying SSA-mediated lymphoid regeneration, and hence a means of improving transplant outcomes, perhaps by either conditioning donor HSCs or the recipient BM niche, or by targeted acceleration of the regeneration process to reduce the time required for immune recovery.

PART A: General Declaration

Monash University
Monash Research Graduate School

Declaration for thesis based or partially based on conjointly published or unpublished work

General Declaration

In accordance with Monash University Doctorate Regulation 17/ Doctor of Philosophy and Master of Philosophy (MPhil) 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 0 original papers published in peer reviewed journals and 3 unpublished publications. The core theme of the thesis is hematopoietic stem and progenitor cells in sex steroid-ablation mediated immune regeneration. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the Monash Immunology and Stem Cell Laboratories under the supervision of Professor Richard Boyd, Ass.Professor Ann Chidge and Dr Jarrod Dudakov.

[The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.]

In the case of chapters 2,3 and 4, my contribution to the work involved the following:

[If this is a laboratory-based discipline, a paragraph outlining the assistance given during the experiments, the nature of the experiments and an attribution to the contributors could follow.]

Thesis chapter	Publication title	Publication status*	Nature and extent of candidate's contribution
2	Immune regeneration mediated by enhanced hematopoietic stem and progenitor cell function	Submitted	Writing and drafting manuscript, experimental design, execution and analysis of experiments and interpretations of results
3	Rejuvenation of the hematopoietic niche following sex steroid ablation contributes to immune regeneration	Manuscript in preparation	Writing and drafting manuscript, experimental design, execution and analysis of experiments and interpretations of results
4	Withdrawal of sex steroids enhances bone marrow production, release and thymic uptake of canonical and noncanonical T-lineage progenitors	Manuscript in preparation	Writing and drafting manuscript, experimental design, execution and analysis of experiments and interpretations of results

[* For example, 'published'/ 'in press'/ 'accepted'/ 'returned for revision']

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

Signed: .....[redacted].....

Date: 27/08/2012

General declaration

In accordance with Monash University Doctor of Philosophy regulations, the following are made:

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

This thesis comprises 1 primary manuscript submitted for publication and 2 primary manuscripts being prepared for submission. The proportional contributions of the co-authors are described in the declarations of authorship on the following pages.

Thesis Chapter Declarations

Declaration for Thesis Chapter 2

Name	% Contribution	Nature of contribution
Danika Khong	70	Writing and drafting manuscript, experimental design, execution and analysis of experiments and interpretation of results
Jarrod Dudakov		Supervisory role – intellectual input, experimental design and interpretation and drafting of manuscript
Maree Hammett		Technical assistance
Lisa Spyroglou		Technical assistance
Richard Boyd		Supervisory role – intellectual input, experimental design and interpretation and drafting of manuscript
Ann Chidgey		Supervisory role – intellectual input, experimental design and interpretation and drafting of manuscript

Declaration by co-authors

The undersigned hereby certify that:

1. They meet the criteria for authorship in that thy have participated in the conception, execution, or interpretation, of at least part of the publication in their field of expertise;
2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
3. There are no other authors for the publication according to these criteria;
4. 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
5. The original data are stored at the following location(s) and will be held for at leaest five years from the date indicated below

Location(s) | Monash Immunology and Stem Cell Laboratories (MISCL)
Monash University- Clayton Campus

Signature#	Name and Signature	Date
1	Danika Khong [redacted]	24/07/2012
2	Jarrod Dudakov [redacted]	24/07/2012
3	Maree Hammett [redacted]	24/07/2012
4	Lisa Spyroglou [redacted]	24/07/2012
5	Richard Boyd [redacted]	24/07/2012
6	Ann Chidgey [redacted]	24/07/2012

Thesis Chapter Declarations

Declaration for Thesis Chapter 3

Name	% Contribution	Nature of contribution
Danika Khong	70	Writing and drafting manuscript, experimental design, execution and analysis of experiments and interpretation of results
Jarrod Dudakov		Supervisory role – intellectual input, experimental design and interpretation and drafting of manuscript
Maree Hammett		Technical assistance
Lisa Spyroglou		Technical assistance
Kahlia Wong	5	Technical assistance
Ann Chidgey		Supervisory role – intellectual input, experimental design and interpretation and drafting of manuscript
Richard Boyd		Supervisory role – intellectual input, experimental design and interpretation and drafting of manuscript

Declaration by co-authors

The undersigned hereby certify that:

1. They meet the criteria for authorship in that thy have participated in the conception, execution, or interpretation, of at least part of the publication in their field of expertise;
2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
3. There are no other authors for the publication according to these criteria;
4. 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
5. The original data are stored at the following location(s) and will be held for at leaest five years from the date indicated below

Location(s)	Monash Immunology and Stem Cell Laboratories (MISCL) Monash University- Clayton Campus

Signature#	Name and Sig[redacted]	Date
1	Danika Khong	24/07/2012
2	Jarrod Dudakov	24/07/2012
3	Lisa Spyroglou	24/07/2012
4	Kahlia Wong	24/07/2012
5	Richard Boyd	24/07/2012
6	Ann Chidgey	24/07/2012

Thesis Chapter Declarations

Declaration for Thesis Chapter 4

Name	% Contribution	Nature of contribution
Danika Khong	70	Writing and drafting manuscript, experimental design, execution and analysis of experiments and interpretation of results
Jarrod Dudakov		Supervisory role – intellectual input, experimental design and interpretation and drafting of manuscript
Lisa Spyroglou		Technical assistance
Kahlia Wong	5	Technical assistance
Ann Chidgey		Supervisory role – intellectual input, experimental design and interpretation and drafting of manuscript
Richard Boyd		Supervisory role – intellectual input, experimental design and interpretation and drafting of manuscript

Declaration by co-authors

The undersigned hereby certify that:

1. They meet the criteria for authorship in that thy have participated in the conception, execution, or interpretation, of at least part of the publication in their field of expertise;
2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
3. There are no other authors for the publication according to these criteria;
4. 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
5. The original data are stored at the following location(s) and will be held for at leaest five years from the date indicated below

Location(s)	Monash Immunology and Stem Cell Laboratories (MISCL) Monash University- Clayton Campus

Signature#	Name and Signature	Date
1	Danika Khong	24/07/2012
2	Jarrod Dudakov	24/07/2012
3	Maree Hammett	24/07/2012
4	Lisa Spyroglou	24/07/2012
5	Kahlia Wong	24/07/2012
6	Richard Boyd	24/07/2012
7	Ann Chidgey	24/07/2012

PUBLICATIONS AND PRESENTATIONS

PUBLICATIONS

I. Heng SP, Dudakov JA, Khong DMP, Chidgey AP, and Boyd RL (2009) Stem Cells-Meet Immunity. *Journal of Molecular Medicine* 87(11), 1061-1069

II. Dudakov JA, Khong DMP, Chidgey AP, and Boyd RL (2010) Feeding the fire: the role of defective bone marrow function in exacerbating thymic involution. *Trends in Immunology* 31(5), 191-198

III. Khong DMP[1] and Dudakov JA[1] , Hammett MV, Spyroglou L, Boyd RL[2] and Chidgey AP[2] Immune regeneration mediated by enhanced hematopoietic stem and progenitor function. (Manuscript in preparation)

IV. Khong DMP, Dudakov JA, Hammett MV, Spyroglou L, Wong K, Boyd RL and Chidgey AP Rejuvenation of the hematopoietic niche following sex steroid ablation contributes to immune regeneration. (Manuscript in preparation)

V. Khong DMP, Dudakov JA, Spyroglou L, Wong K, Chidgey AP and Boyd RL Withdrawal of sex steroids enhances bone marrow production, release and thymic uptake of canonical and noncanonical T-lineage progenitors. (Manuscript in preparation)

[1,2]These authors contributed equally

PRESENTATIONS

INTERNATIONAL MEETINGS:

- **Khong DMP,** Dudakov JA, Hammett MV, Goldberg GL, Jurblum M, Ueno T, Spyroglou L, Young L, van den Brink M, Chidgey AP and Boyd RL. Immune regeneration mediated by enhanced mouse hematopoietic stem and progenitor function. ISSCR. Toronto, Canada June 15th-18th 2011 (poster presentation)

- **Khong DMP**, Dudakov JA, Chidgey AP and Boyd RL. The role of haematopoietic progenitor cells in sex steroid ablation mediated thymic regeneration. ThymOZ, Heron Island (Queensland) March 17th-22nd 2010 (oral presentation)

- Dudakov JA, **Khong DMP**, Goldberg GL, Chidgey AP and Boyd RL. Enhancing thymic uptake and T cell differentiation of hematopoietic progenitors. ThymUS:

Conference on Lymphopoiesis, T cell differentiation and Immune Reconstitution. San Juan, Puerto Rico November 8th-14th 2008 (oral presentation)

- Dudakov JA, Goldberg GL, **Khong DMP**, Chidgey AP and Boyd RL. Withdrawal of sex steroids reverses age-related defects in murine hematopoietic stem cell function. International Society for Experimental Hematology conference, Boston July 9th – 12th 2008 (oral presentation)

- Dudakov JA, Goldberg GL, **Khong DMP**, Chidgey AP and Boyd RL. Sex steroid ablation promotes thymic entry and enhances lymphoid commitment of hematopoietic stem cells in aged mice. International Society of Stem Cell Research conference, Philadelphia June 18th – 22nd 2008 (poster presentation)

NATIONAL MEETINGS:

- **Khong DMP**, Dudakov JA, Chidgey AP and Boyd RL. The role of haematopoietic progenitors in thymic regeneration. Australian Society of Immunology Meeting , Perth (Western Australia) December 9th-13th 2010 (oral presentation)

- **Khong DMP**, Dudakov JA, Chidgey AP and Boyd RL. The role of haematopoietic progenitors in thymic regeneration. ASCC Conference, Kingscliff (New South Wales) June 9th-11th 2010 (invited poster presentation)

- **Khong DMP**, Dudakov JA, Chidgey AP and Boyd RL. The role of haematopoietic progenitors in thymic regeneration. Australian Society of Immunology Meeting, Gold Coast (Queensland) December 6th-10th 2009 (poster presentation)

- **Khong DMP**, Dudakov JA, Chidgey AP and Boyd RL. The role of haematopoietic progenitors in thymic regeneration. Immunology Group Victoria conference, Victoria October 6th to 11th 2009 (oral presentation)

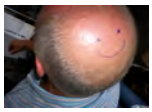
- Hince M, **Khong DMP**, Chidgey A and Boyd RL. Cosudex improves LHRH-agonist mediated thymic recovery following chemotherapy. Infection and Immunity Conference, Gold Coast June 24th-26th 2009 (poster presentation)

- **Khong DMP**, Dudakov JA, Chidgey AP and Boyd RL. The role of haematopoietic progenitors in thymic regeneration. Immunology Group Victoria conference, Victoria October 12th-14th 2008 (oral presentation)

- Dudakov JA, Goldberg GL, **Khong DMP**, Chidgey AP and Boyd RL. Withdrawal of sex steroids reverses age-related defects in murine hematopoietic stem cell function. Australia-China-Australia Center for Excellence Stem Cell workshop, Rye May 30th March– 1st April 2008 (invited oral presentation)

Presenting author is underlined.

Acknowledgements



Richard! I thank you for the opportunity to have been a part of what I can only describe as the Cirque du Soleil of labs...one filled with amazing talent and endless entertainment! I am grateful for your continuous support and encouragement. I have utterly enjoyed our occasional philosophical chats - your insight into life's many mysteries has always intrigued me....and I find you to be highly inspiring! Thank you!

Ann....your tireless support and encouragement particularly over the last few weeks has not gone unnoticed. Thank you for all the opportunities you have given me. Oh...and for future reference, the next time I'm at Monash and we have a tennis tournament against the rest...I'd really like you to pick me to be on your team...because boy do you kickass on the courts!

Jaz!...the backbone behind my PhD! Mentor and confidant... I have thoroughly enjoyed being your minion (you must remember referring to me as your minion my honors year!...and yes..thats honors with no "u"). In all seriousness though, I know (most) of what I know because of you, it has been such an honor to learn from someone so energetic and intelligent as yourself! Your enthusiasm is contagious and I always find myself "rejuvenated" after our conversations, no matter how defeated I would've started off as. You are somone I respect dearly and hold in high regard...the "McGyver" of my scientific journey thus far...able to turn any sour situation into something positive! I look forward to many more Duck Extravaganzas with you!

Lisa...! Coffee buddy, bone-crushing partner in crime, tennis hitting partner (of equal and not greater talent), landlady/housemate, OP9 whisperer...and perhaps the most rewarding of the list....friend (though I have to say...OP9 whisperer was pretty helpful too!). I have enjoyed the book chats we've had, the tv-shows and movies you've "made" me watch - Eclipse was definitely my favorite!, all the times you've "made" me hemacytometer instead of coulter count!...the list goes on...but most of all I am grateful you have always been there for me - you know....when sometimes in life, there just hasnt been enough potatoes... Thank you!

Lovey! A surprising, albeit special friendship indeedy! You have always found a way to lift my spirits up when you've known I've needed it and for that I am grateful. Because of you, I want to be a better mouse-handler...I've had a special time inhaling isofluorane with you and love our gas-induced chats! You are the wackiest person I've ever had the pleasure of knowing...thank you for everything!

Kahlia K (k)Wong: (like your picture? - like Tyrion, you are calm and composed but with the wit of a (insert witty something here)!! Like you said though, Ronan said it best: I do say it best when I say nothing at all.
That is all.

Jade B: Simply thinking about the fun working nights gives me stitches...I have enjoyed getting to know you and I thank you for letting me get your dinners for you - an absolute pleasure its been (seriously)!...and...
May you never get lab piles!

MV Hammett: You ROCK! Thank you for keeping my televsion-education up to scratch! You have been there everytime I've needed a hug and its been such a privelege to have worked with you. I look forward to giggling at your emails (most likely filled with many many funny typsos)!

Acknowledgements

J. Mo: Conference roomie! I have loved travelling with you (I will always gladly give up my aisle seat for you so your hair will never be messed up- you can count on me!) eventhough you are the noisiest quiet person I've ever met!! You will always be my Japanese food person and we will always have depressing ASIs and Rottnest to ponder back upon! Thank you for all the lunches...and I look forwad to many more conferences with you in the future!But for now..we will always have Rottnest! (and the permanent visual of that weirdo in his undies) Much love Bianca-From Holly.

Tracy! I've had so much fun hanging out with you....thank you for all the advice you have given me throughout - I've learnt a lot from you!

Mark-s-million! Ah..soo many memorably crypted immensely deep (and silly) conversations we've had...at the strangest of times! Our friendship...is like a Tim Burton movie...extraordinarily quirky yet rather intriguing!

Louiiissa! I have had so much fun hanging out with you - that lasagna with prosciutto made me want to hang out with you even more! IWMODDDDDDD (I will miss our dinners dinners dinners dinners....)

Kat! My favorite desk buddy (even if this wasnt mutual...damn Lauren and her orange bribes!). Thank you for letting me run to you for advice everytime I needed it....and most of all...thank you for our frappcapades!

Luci: Gosh I've missed our beers on the syringe-filled-beach! I'm still trying to get over missing your housewarming, breaking the tradition of having attended all 3 or your others! Thank you for everything you have done for me in the lab...you have made things that could've been very difficult a lot easier to deal with.

Marco! My fellow illegal immigrant....pity we couldnt be deported off to Bora Bora together eh...would've been a great place to have written up! Thank you for making th e prospect of being deported a little more fun to have dealt with...and yeah yeah we know you move like Jagger!

Adie, Anthony, Chew-Li, Sam,Kiran and Tara: Thank you for making the lab such a warm place to be in!

Gaby and Sacha: My support team outside of the lab! Gaby...you are awesome! I've never felt the distance between us (except when I had to actually travel it to get to you...so far!)..you have always been there for me no matter what the circumstance and to that, I say thank you! Sacha!!! Thank you for letting me annoy the bejesus out of you...it has been very therapeutic! You have been there every step of the way...you have listened to me vent numerous times and you have been my emotional pillar throughout it all!
Thank you!

Acknowledgements

Annou, Kat, Merusha and Dr Neeha: You have made my time in Melbourne extremely special...with your laughs (because I'm that funny) and food...and truly touching chats.
Thank you for everything!

To my family:
Mom! You're the most imspiring peson I've ever met...and though frustrating at times, your support and belief in me has gotten me where I am today! You are AWESOME! Thank you.
Paps: Thank you for all the opportunities you have given me...I owe you a round of golf!

Abbreviations

Ab	Antibody
Ang-1	Angiopoietin-1
APC	Allophycocyanin
AR	Androgen Receptor
BCR	B cell Receptor
BSA	Bovine Serum Albumin
Bio	Biotin
BM	Bone Marrow
BMP	Bone Morphogenic Protein
BMT	Bone Marrow Transplant
BSA	Bovine Serum Albumin
CAR	CXCL12-abundant Reticular Cells
CaR	Calcium-sensing Receptor
CCR9	Chemokine Receptor 9
CD	Cluster of Differentiation
CEP	Cortical Epithelial Progenitor
CLP	Common Lymphoid Progenitor
CMJ	Cortico-medullary Junction
CMP	Common Myeloid Progenitor
cTEC	Cortical Thymic Epithelial Cell
CTP	Circulating Thymic Progenitor
Cx	Castrate/Castration
Cy	Cyclophosphamide
DC	Dendritic Cell
DN	Double Negative Cell
DP	Double Positive Cell
EBF	Early B cell Factor
Egr	Early Growth Responsse gene

ELP	Early Lymphoid Progenitor
EPLM	Early Progenitor of Lymphoid and Myeloid potential
ETP	Early Thymic Progenitor
ER	Estrogen Receptor
FACS	Fluorescence Activated Cell Sorting
FCS	Fetal Calf Serum
FITC	Fluoroscein Isothiocyanate
Flt3	Fms-like tyrosine kinase 3
Flt3L	Fms-like tyrosine kinase 3 Ligand
Fn	Fibronectin
FSH	Follicle Stimulating Hormone
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
GMP	Granlocyte Macrophage Progenitor
GvHD	Graft versus Host Disease
HA	Hyaluronic Acid
HIV	Human Immunodeficiency Virus
HPC	Hematopoietic Progenitor Cell
HSC	Hematopoietic Stem Cell
HSCT	Hematopoietic Stem Cell Transplant
HPC	Hematopoietic Progenitor Cell
IGF-1	Insulin-like Growth Factor-1
IFN	Interferon
IL-7	Interleukin-7
IMN	Intrathymic Microenvironmental Niche
IMV	Intrathymic Microenvascular
KGF	Keratinocyte Growth Factor
LCP	Lymphoid Committed Progenitor
LH	Luteinizing Hormone
LHRH	Luteinizing Hormone Releasing Hormone
Lin	Lineage Markers

LMPP	Lymphoid-primed Multipotent Progenitor
LSP	Lymphoid Specific Precursor
LT-HSC	Long Term Hematopoietic Stem Cell
LSK	Lin^{-} Sca 1^{+} c-Kit^{+} cells
MCP	Myeloid Committed Progenitor
MHC	Major Histocompatibility Complex
MPP	Multipotent Progenitor
mTEC	Medullary Thymic Epithelial Cell
N-cad	N-cadherin
NFκB	Nuclear Factor κB
NKT	Natural Killer T cells
OBL	Osteoblast
OCL	Osteoclast
Opn	Osteopontin
Pax5	Paired box protein 5
PB	Peripheral Blood
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
PerCP	Peridinin Chlorophyll Protein
PPR	PTH/PTHrP Receptor
Pre-Tα	Pre-T Receptor α
PSGL-1	P-selectin Glycoprotein Ligand
PTH	Parathyroid Hormone
PTHrP	Parathyroid Hormone related Protein
PVS	Perivascular Space
RAG	Recombination Activating Gene
RANKL	Receptor Activator of Nuclear Factor κB
RBC	Red Blood Cell
RTE	Recent Thymic Emigrant
SA	Steptavidin

Sca1	Spinocerebellar Ataxia 1
SCF	Stem Cell Factor
SDF-1	Stromal Derived Factor 1
Sh SSA	Sham Sex Steroid Ablation
SLAM	Signalling Lymphocyte Activation Molecule
SNO	Spindle-shaped N-cadherin^{+} Osteoblast
SP	Single Positive
SSA	Sex Steroid Ablation
ST-HSC	Short Term Hematopoietic Stem Cell
TCR	T cell Receptor
TEC	Thymic Epithelial Cell
TESC	Thymic Epithelial Stem Cell
TGFβ	Transforming Growth Factor-β
TN	Triple Negative Thymocyte
TPO	Thrombopoietin
Tregs	Regulatory T cell
VCAM1	Vascular Cell Adhesion Molecule 1
VLA	Very Late Antigen

Chapter 1

Literature Review

Manuscript information

Part of this literature review were adapted for the following manuscript: Dudakov, Khong, Boyd and Chidgey (2010) Feeding the fire: the role of defective bone marrow function in exacerbating thymic involution. *Trends in Immunology*. 5:191-198. The full manuscript is contained in the appendices.

1.1 Abstract

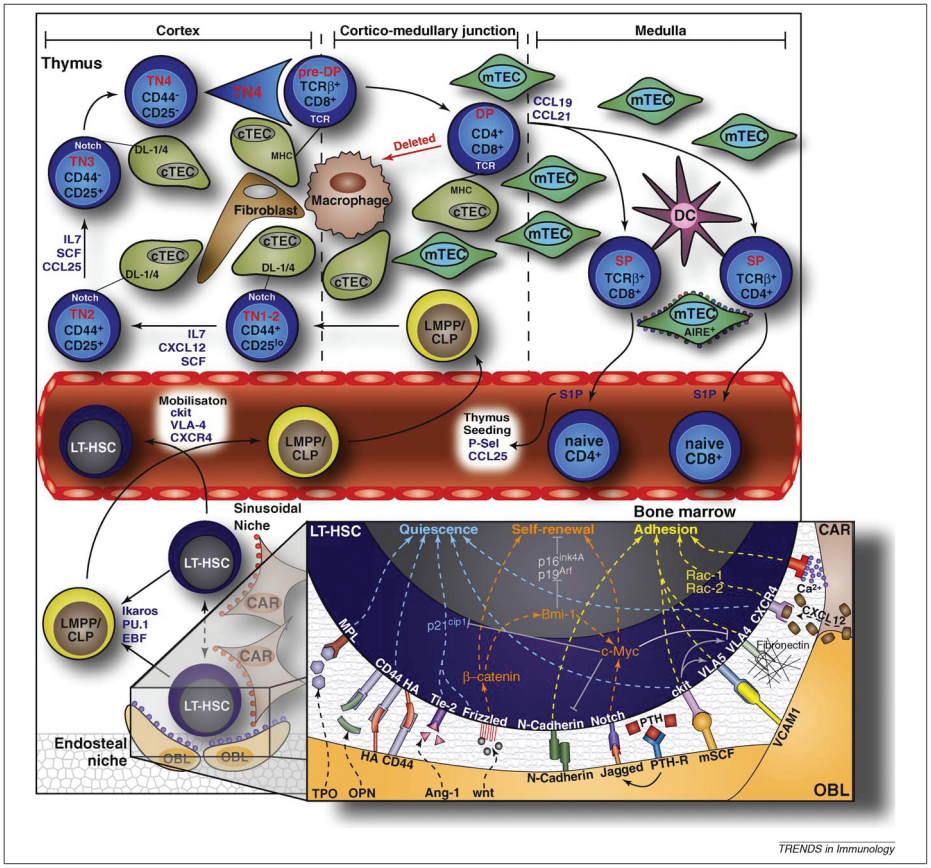
Adaptive immunity is predicated on the continuous production of T cells in the thymus and B cells in the BM from hematopoietic stem cells (HSCs). HSCs differentiate into multiple distinct lineages in addition to maintaining the pool of stem cells and it is this dual function that ensures the support of blood cell production for the lifespan of the organism. This balance between stem cells quiescence and self-renewal, in addition to the differentiation of downstream progenitors, occurs via a network of complex interactions with the supporting microenvironmental niche. Although hematopoiesis is continuous for the duration of life, there is a profound reduction in lymphoid development and function with age that could be at least one etiological basis for the increased incidence of malignant disease. Developing strategies to enhance lymphopoiesis, particularly following cytoablative treatments, is therefore a significant unmet clinical need.

1.2 Introduction

Hematopoiesis is predicated on the complex interactions between hematopoietic stem and progenitor cells (HSPCs) and their supporting stromal microenvironmental niches and remains responsible for maintaining all blood cell lineages throughout an organism's lifespan (Figure 1.1 and (1)). Although most hematopoietic lineages are generated from hematopoietic stem cells (HSCs) in the bone marrow (BM), T cell precursors begin their development in the BM before migrating to the thymus (Figure 1.1 and (2-4)). While there is some debate about the nature of the thymus-seeding BM-derived precursor, there is increasing evidence that there is an intrinsic link between the mobilization of precursors from the BM and their gated entry into the thymus (5). Under the instructive interaction with the highly specialized thymic stromal compartment, precursor T cells differentiate through a series of distinct stages of development, ultimately generating a diverse repertoire of T cells to protect from foreign pathogens.

With age, there is considerable degeneration of both BM and thymus lymphopoiesis and hence a decline in lymphoid cell production and function (6-9). The thymus atrophies severely with age, with profound disruption of functional thymic tissue. Many of these age-related effects can be correlated to sex steroids, and the cessation of sex steroid production through either chemical or surgical castration leads to the regeneration of lymphopoiesis in both the BM and thymus (8, 10). However, the specific mechanisms that underlie sex steroid ablation (SSA)-mediated regeneration are unclear. Several possibilities arise, including 1) intrinsic regeneration of HSPCs in the BM leads to the restoration of their lymphoid lineage capacity, 2) intrinsic impacts on the stromal compartments of the BM and thymus lead to enhanced support of lymphoid lineage commitment sets a new demand for HSPCs from the

Figure 1.1 Maintenance of HSCs in the niche and their differentiation down lymphoid lineages in the BM and thymus. Long-term hematopoietic stem cells (LT-HSC) reside in specialized niches in the endosteum and adjacent to sinusoidal vascular epithelial cells. CAR cells and a subset of hematopoiesis-supporting osteoblasts (OBL) are though to be critical for HSC maintenance in the niche. Adhesion of HSCs to these niches is mediated by signals through the calcium-sensing receptor, CXCR4, VLA-4 and 5, ckit and N-cadherin. The balance between self-renewal and quiescence is maintained through the Notch and β-catenin pathways as well as signalling through Tie-2, hyaluronic acid, CD44 and MPL by their respective ligands Ang-1, CD44, OPN and TPO. Differentiation down the lymphoid lineage towards LMPP and CLP is associated with expression of PU.1 and Ikaros. For T cell development to occur, hematopoietic progenitors periodically egress from the BM, associated with interactions through ckit, VLA-4, Flt3 and CXCR4, and enter the circulation. Expression on the surface of circulating progenitor cells of CCR9 and PSGL-1 are crucial for the entry of hematopoietic progenitors into the thymus. Once in the thymus, commitment to the T-cell lineage is secured by interaction with the Notch ligands delta-like 1 and 4. With appropriate interactions from the supporting stromal microenvironment, comprised primarily of cortical and medullary thymic epithelial cells (cTEC and mTEC respectively) and fibroblasts, T cell development progresses through distinct stages and positive and negative selection, to form self-tolerant single positive T cells. Naïve T cells are exported from the thymus based on an S1P gradient that can influence the expression of P-selectin and CCL25 by thymic stromal cells and thereby influence thymic receptivity to circulating progenitors.


Cortex
Cortico-medullary junction
Medulla
Thymus
TN4
CD44⁻ CD25⁻
pre-DP
TCRβ⁺ CD8⁺
TCR
DP
CD4⁺ CD8⁺
TCR
mTEC
CCL19
CCL21
cTEC
MHC
Deleted
Notch
TN3
CD44⁻ CD25⁺
DL-1/4
Fibroblast
Macrophage
DC
IL7
SCF
CCL25
Notch
TN2
CD44⁺ CD25⁺
Notch
TN1-2
CD44⁺ CD25lo
IL7
CXCL12
SCF
LMPP/ CLP
SP
TCRβ⁺ CD8⁺
SP
TCRβ⁺ CD4⁺
AIRE⁺
S1P
S1P
LT-HSC
Mobilisaton
ckit
VLA-4
CXCR4
LMPP/ CLP
Thymus Seeding
P-Sel
CCL25
naive CD4⁺
naive CD8⁺
Sinusoidal Niche
Bone marrow
LT-HSC
Quiescence
Self-renewal
Adhesion
CAR
p16Ink4A
p19Arf
Rac-1
Rac-2
Ca2+
CXCL12
Fibronectin
Mpl
p21clp1
Bmi-1
c-Myc
β-catenin
CD44 HA
Tie-2
Frizzled
N-Cadherin
Notch
ckit
VLA5
VLA4
CXCR4
PTH
VCAM1
HA CD44
TPO
OPN
Ang-1
wnt
N-Cadherin
Jagged
PTH-R
mSCF
OBL
LMPP/ CLP
Ikaros
PU.1
EBF
LT-HSC
CAR
CAR
LT-HSC
Endosteal niche
OBL
OBL
TRENDS in Immunology

BM, or 3) a combination of these two alternative. Since lymphoid regeneration can severely improve the clinical outcomes for patients who have undergone myeloablative treatments, elucidating the mechanisms surrounding SSA-mediated immune regeneration will likely prove clinically beneficial through targeted approaches.

1.3 Hematopoiesis in the Bone Marrow

1.3.1 Hematopoietic Stem Cells

1.3.1.1 Phenotype

HSCs are multipotent stem cells able to generate all blood lineages. In the mouse, all HSC activity has been attributed to a phenotype known as "LSK": that is they are phenotypically defined as being negative for all lineage-associated markers but positive for Sca1 and cKit (11-16).

The LSK population is a very broad phenotypic definition of HSCs with only approximately 1 in 10 LSK cells containing true HSC capacity (17). The LSK fraction can be further subdivided into LT-HSCs (long term HSCs), ST-HSCs (short term HSCs) and MPPs (multipotent progenitors) based on their reconstitution capacity and expression of the markers CD34, Flt3, CD150 and CD48 or through the use of the supravital Hoechst-33342 dye (17-19) (Figure 1.1).

The upregulation of Flt3 has been associated with a decline in the self-renewal capacity of LSK cells (20, 21). Consistent with this notion, LT-HSCs lack expression of CD34 and Flt3 (17, 22, 23). The remaining LSK Flt3- population is divided into the $CD34^{+}$ (MPP) and $CD34^{-}$ (ST-HSC) fractions.

More recently another method of phenotyping the three subsets of HSCs have been introduced based on the expression of the SLAM family markers. The SLAM family is comprised of 10 to 11 cell surface receptors that are thought to regulate the proliferation

and activation of lymphocytes (24, 25). Based on these set of markers, it has been found that the CD150+ CD48- population represents a highly purified LT-HSC pool (26, 27). These cells also uniformly lack CD244 expression (25). The utility of these markers in conjunction with the canonical HSC markers and the exclusion dye (Hoechst dye) provides us with a highly purified LT-HSC population (25, 28).

1.3.2 The supporting hematopoietic niche

The BM HSC niche is a highly organized 3-dimensional microenvironment comprised of fibroblasts, macrophages, megakaryocytes, endothelium, adipocytes and osteogenic cells (osteoblasts - OBLs and osteoclasts). Moreover, recent evidence also suggests a role for upstream mesenchymal stem cells (MSCs) in the hematopoietic niche (29). In addition to these cellular components, the BM niche also relies on the extracellular matrix (ECM) and various hematopoietic growth factors.

The spatial organization of these niche components is itself highly strategic and correlates closely to both the lineage and function of the supported HSCs, although there is some contention about this. Although one hypothesis suggests the existence of two distinct HSC niches within the BM, residing at the endosteal surface and adjoining sinusoidal endothelium, another suggests that only the endosteal niche supports true HSCs (30, 31). The endosteum is arbitrarily defined as the width of 12 cells between the bone-core marrow interface and has been described as home to the most primitive quiescent HSCs (31). However, recent findings suggest the sinusoidal niche can also support primitive HSCs and that quiescence is regulated by the hypoxia of the environment rather than its spatial localization (32, 33). These two niches are comprised of different cells with the endosteum enriched for hematopoiesis supporting OBLs while the vascular niche comprised of endothelium and MSCs. In contrast to these two-niche hypotheses, Lo Celso and colleagues propose a one-niche hypothesis, which lacks spatial distinction between these two regions

and argues that since OBLs are perivascularly located, the endosteal and sinusoidal niches are not, in fact different to each other (34).

1.3.2.1 Osteoclasts and OBLs at the endosteal surface

Osteoclasts and OBLs work primarily in bone remodelling, with the phagocytic osteoclasts responsible for bone resorption and the OBLs for bone formation (35). Apart from their primary role in osteogenesis, these cells also play a role in hematopoiesis.

Osteoclasts are monocyte-derived cells that are thought to play a crucial role in hematopoiesis by breaking down the bone matrix and releasing growth factors such as TGF-β (transforming growth factor β), IGFs (insulin like growth factors) and BMP (bone morphogenesis protein), all of which have been implicated in hematopoiesis (36-39). TGF-β has been shown to be involved in the maintenance of HSC quiescence (40) while IGF-1 promoted survival of progenitor cells in the BM (37). BMPs represent a large family of secreted signalling molecules that play various roles in different stem cell systems (41). BMP4 is one such example and is vital in the maintenance of HSC long term repopulating activity (36). Osteoclasts are also thought to release high concentrations of hydroxyapatite-bound calcium, a regulator of HSC accumulation at the endosteal interface (42). Additionally, recent evidence suggests a role for osteoclasts in the mobilization of hematopoietic progenitors from the BM into the circulation; mediated by osteoclast-derived matrix metalloproteinase 9 (MMP) and cathepsin K-mediated cleavage of SDF-1 (43). In a study by Lymperi and colleagues, a resorption inhibitor; bisphosphonate alendronate (ALN), was used to repress osteoclast-mediated bone resorption. This resulted in a reduction in the number and reconstitution capacity of primitive HSCs with a subsequent expansion of the HPCs (38).

Of the numerous cell types implicated in supporting hematopoiesis, OBLs are perhaps the most widely studied (1, 44-47). OBLs are bone-lining cells that have dual functions:

regulating bone formation; by both producing bone matrix proteins and initiating the differentiation of osteoclasts, and supporting hematopoiesis (48). OBLs are derived from MSCs (49), which are $CD34^-$ $CD73^+CD105^+CD166^+CD90^+$ and generally HLA $(A,B,C)^+$ cells found in the BM that can differentiate into adipocytes, chondrocytes and osteogenic cells (49, 50) (Figure 1.2).

1.3.2.2 The role of endothelium in BM hematopoiesis

BM endothelial cells are also of critical importance in supporting hematopoiesis. In fact, endothelial cells are one of the few cells able to successfully support HSCs *in vitro* (51, 52). In the study by Ohneda and colleagues, two different endothelial cell lines were derived from the $CD34^+$ population in the aorta-gonad-mesonephros (AGM) region of an embryo. Both these cell lines were then tested for their ability to support hematopoiesis *in vitro*. Interestingly, while the DAS 104-8 endothelial cell line promoted differentiation of co-cultured fetal liver HSCs into erythroid, myeloid
and B lymphoid pathways, the DAS 104-4 line was only able to support expansion and self-renewal of the co-cultured HSCs (51). Similarly in another study by *Li et al.*, primary endothelial cells isolated from the yolk sac of an embryo was capable of supporting the expansion of adult HSPCs *ex vivo* (52).

1.3.2.3 *The role of fibroblasts and MSCs in the HSC niche*

MSCs are multipotent cells capable of differentiating down adipogenic, chondrogenic and osteogenic pathways. MSCs commit to an osteoblastic lineage and develop to pre-OBLs through expression of Runx2, which inhibits MSC progression down adipogenic and

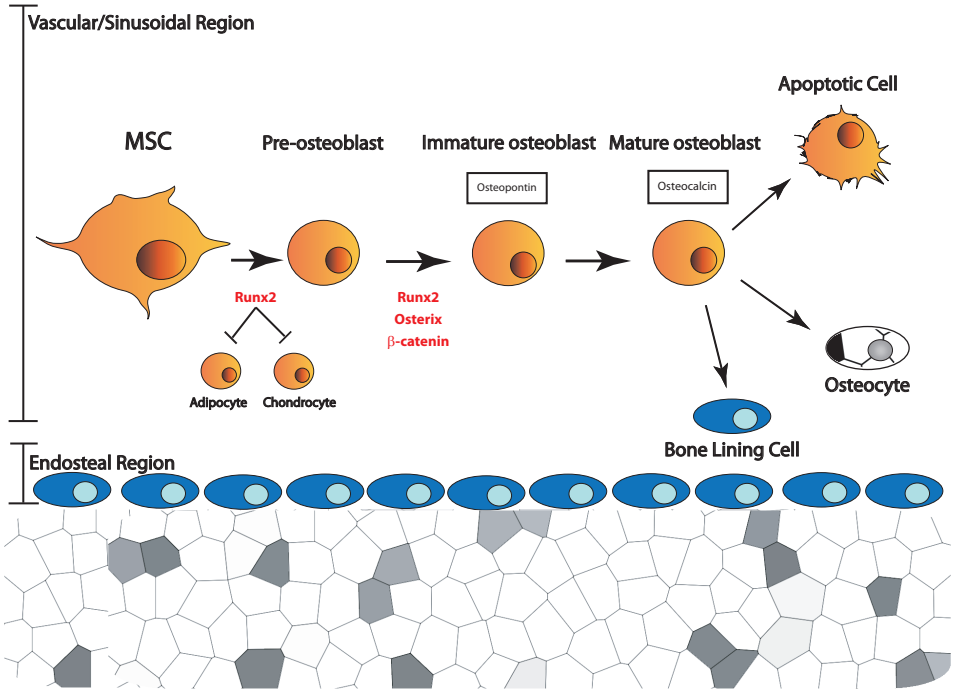
chondrogenic lineages (53). Osterix and β-catenin are transcription factors that act downstream to Runx2 and are vital for pre-OBLs becoming immature OBLs (54, 55). Immature OBLs express matrix protein genes and high levels of osteopontin. They then differentiate into mature OBLs, expressing high levels of osteocalcin (56). These osteocalcin-expressing mature OBLs now have the propensity towards one of three fates: becoming quiescent bone lining cells or differentiate into osteocytes and embed into the bone matrix or to die by apoptosis (57) (Figure 1.2).

Moreover, there is increasing evidence that have also suggested a role for MSCs in directly supporting hematopoiesis (29). Mendez-Ferrer *et al*. have described a role for $CD45^-$ $nestin^+$ MSCs in the homing of HSCs into the BM; since they have been shown to be perivascularly located in both the endosteum and sinusoidal niches and in close proximity to HSCs as well as sympathetic nerve fibres. It is thought that the sympathetic nervous system controls HSC mobilization and homing through regulating the expression of CXCL12 via the β_3-adrenergic receptor (29). They were also found to express the hematopoietic associated genes CXCL12, stem cell factor (SCF), angiopoieitin, IL7, VCAM1 and osteopontin at levels higher than that found in OBLs (29).

1.3.3 Crosstalk in the hematopoietic niche

Stem cells can be defined by two fundamental characteristics, 1) the ability to differentiate into multiple distinct downstream cells, that is differentiation, and 2) the ability to generate new stem cells, that is, self-renewal. The size of the stem cell pool is maintained by the balance between HSC quiescence and self-renewal and is tightly regulated through interactions with the surrounding BM microenvironment. This regulation, known as the niche concept,was first proposed by Schofield in the late 70s and has gained wide acceptance since (58).

Figure 1.2 Mesenchymcal stem cell differentiation into the osteoblast lineage. Osteoblasts differentiate from mesenchymal stem cells (MSCs). MSCs are BM-resident cells that have the propensity to differentiate into adipocytes, chondrocytes and osteogenic cells. Runx2 expression inhibits MSC progression down the adipogenic and chondrogenic lineages and promotes preosteoblast formation. Preosteoblasts then differentiate into immature osteoblasts upon osterix and β-catenin transcription control. It is at the immature osteoblast stage that osteopontin expression begins. Immature osteoblasts also begin expressing extracellular matrix proteins and become mature osteoblasts, which express high levels of osteocalcin. Mature osteoblasts now become quiescent bone lining cells, differentiate into osteocytes within the bone matrix or die by apoptosis.


Vascular/Sinusoidal Region
Apoptotic Cell
MSC
Pre-osteoblast
Immature osteoblast
Mature osteoblast
Osteopontin
Osteocalcin
Runx2
Runx2
Osterix
β-catenin
Adipocyte
Chondrocyte
Osteocyte
Bone Lining Cell
Endosteal Region

1.3.3.1 HSC quiescence and self-renewal

Stem cell quiescence and self-renewal are critical for hematopoietic maintenance. Of several significant factors involved in regulating HSC quiescence, one of the most significant has been the hypoxic environment, either in endosteum or sinusoidal niches (59). Winkler *et al*. were able to show through the use of Hoescht dye and flow cytometry that the more primitive HSCs primarily resided in avascular regions of the BM. Only LSK $CD41^{-}$ $CD48^{-}$ $CD150^{+}$ cells that excluded the Hoechst dye (Ho^{neg}) and hence that were more distally located to blood vessels, were able to serially reconstitute lethally irradiated congenic recipients (59). Interestingly, HSCs with the same phenotype but found in vascularised regions of the BM (Ho^{bright}) were only able to reconstitute a single host mice upon transplantation (59). Similarly, the Hoechst method of measuring perfusion was applied to BM stromal cells and it was found that the OBLs were more avascularly situated when compared to both endothelial and mesenchymal stromal cells (59). It is hypothesized that low oxygen and blood-borne nutrient concentrations resulting from being located near low velocity sinusoidal vessels, as well as a rich localized secretion of trophic niche factors favors more primitive long-term hematopoietic stem cells within the BM (59). Along with the physical environment, several trophic factors have also been implicated in maintaining HSC quiescence. These include angiopoietin, thrombopoietin, CXCL-12, Wnt signalling, transforming growth factor β (TGF-β) and osteopontin.

The angiopoieitin-1 (Ang-1)/Tie2 signaling pathway is thought to play a crucial role in mediating quiescence. Ang-1 is expressed on OBLs while Tie2 is a receptor tyrosine kinase expressed in slow cycling HSCs that localized at the endosteal surface (60). Through an elegant transplantation assay involving BM cells transfected with a retrovirus containing Ang-1, Arai and colleagues were able to successfully demonstrate that Tie2/Ang-1 signalling played a direct role in promoting HSC quiescence (60). Arai *et al*. also showed that

Tie2/Ang-1 signalling led to the activation of β1-integrins and hence firm adhesion to the endosteal surface (60, 61).

In a similar fashion, the interaction between thrombopoieitin (Tpo) and its receptor Myeloproliferative leukemia virus oncogene (Mpl) is another pathway found to be vital for quiescence, at least partially via the upregulation of β1-integrins (62). Mpl is expressed on quiescent HSCs while Tpo is produced by OBLs. Using neutralizing antibodies against Mpl or Tpo-deficient mouse models, it was found that there was upregulation of the G_1-phase CDK inhibitor $p57^{Kip2}$, which has been found to be specifically expressed in LT-HSCs, leading to HSC quiescence (63, 64). Interestingly, Tpo has a variety of functions; in addition to playing a role in maintaining HSC quiescence and in platelet production; it also acts on its receptor Mpl to facilitate HSC expansion in a transplantation setting (64).

HSC localization in the niche is important for quiescence and the cell adhesion molecules that are associated with this, such as N-cadherin, β1-integrin and osteopontin are also thought to have a direct influence on quiescence (65). As mentioned previously, both the Tie2/Ang-1 and Tpo/Mpl pathways lead to downstream activation of β1-integrin. These signalling cascades also upregulate N-cadherin. N-cadherin is expressed on endothelial cells, HSPCs as well as a subpopulation of OBLs termed spindle-shaped N-cadherin$^+$ OBLs (SNO cells) (66). It is involved in strong homotypic interactions that hold the primitive HSC onto the endosteal surface, preventing them from differentiating into downstream progenitors (30).

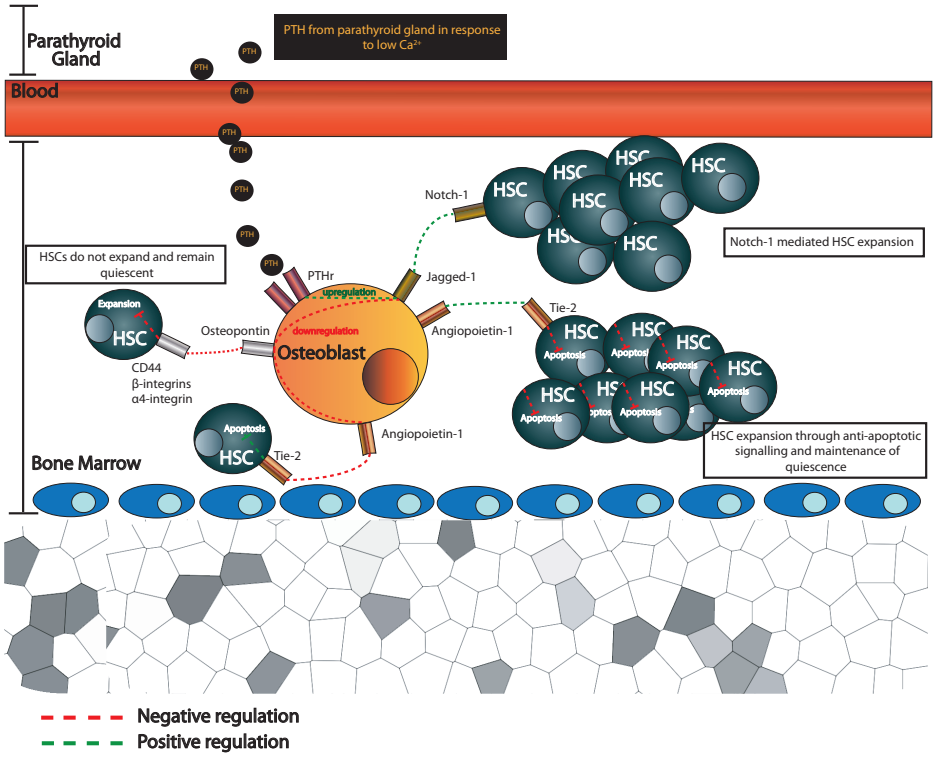
TGF-β is perhaps one of the most effective inhibitors of HSC expansion as shown in numerous *in vitro* assays and like activins and bone morphogenic proteins (BMPs) mediates its downstream effects via Smad pathways (67). It has been suggested that TGF-β signalling alters cytokine receptor expression, ckit expression and up-regulates cyclin-dependent kinase inhibitors like $p21^{cip1}$, $p27^{kip1}$and $p57^{kip2}$ (68, 69). Bmps on the other hand play a more ambiguous role in hematopoiesis with its effects being dose dependent. At high

concentrations of Bmp 2 and 7 and low concentrations of Bmp 4, $CD34^+$ $CD38^-$ HSCs were more quiescent while at low concentrations of Bmp 2 and 7 and high concentrations of Bmp 4, there was a greater degree of expansion and differentiation (70, 71).

Osteopontin (Opn) is a multidomain phosphorylated glycoprotein that negatively regulates HSC expansion, thereby influencing HSC quiescence. It is synthesized by many cell types including endothelial cells and OBLs. Its interaction to primitive HSCs is thought to be mediated by β1-integrins, α4-intergrin and CD44 (72) (Figure 1.3). Opn has well-defined roles in chemotaxis, adhesion and proliferation both within the blood and bone systems, however its precise role in hematopoiesis was only explored as recently as the mid 2000s. In $Opn^{-/-}$ mice, hematopoiesis, as measured by total lymphoid and myeloid output, appeared unaltered; however upon further dissection, these Opn null mice possessed a higher frequency of LSK cells (73). Iwata *et al.* were able to establish a link between Opn and Notch-1 in human $CD34^+$ cells, which led to Calvi and colleagues finding a similar relationship in mice (47, 74).

Jagged-1, a Notch-1 ligand, is produced by OBLs and endothelial cells and acts inversely to Opn to control the stem cell pool size (Figure 1.3). $Opn^{-/-}$ show an upregulation of both Jagged-1 and Ang-1; and hence quiescence as well as self-renewal, and reciprocally, when BM stromal cells were co-cultured with that from $Opn^{-/-}$, there was downregulation of Jagged-1 and therefore control over HSC population size (47, 73). In one respect, Parathyroid hormone (PTH) works antagonistically to Opn to increase the HSC pool through activation of Jagged1 expression on OBLs (Figure 1.3). Produced by the parathyroid gland and sharing its receptor with the parathyroid related protein (PTHrP); PTH receptor (PTHR), PTH also acts on OBLs to activate and induce production of Opn as

Figure 1.3 Maintenance of HSC quiescence. Osteopontin (Opn) and Jagged-1 act antagonistically to each other to control the stem cell pool size. Opn, expressed on osteoblasts binds to CD44, β-integrins and α4-integrins on HSCs to prevent HSC expansion and promote quiescence. Jagged-1 signaling via Notch-1 on HSCs causes expansion of the HSCs. Similarly, Angiopoietin-1 on osteoblasts bind to Tie-2 on HSCs to downregulate apoptosis hence promoting HSC expansion but also whilst maintaing quiescence. Opn expression appears to downregulate Jagged-1 and Angiopoietin-1 expression thereby inhibiting Ang-1/Tie-2 mediated apoptosis as well as Jagged-1/Notch-1 mediated expansion and maintaining the HSC pool size once again. Parathyroid hormone is secreted from the parathyroid gland in response to low Ca^{2+} in the blood. It binds to the parathyroid hormone receptor on osteoblasts and upregulates the Jagged-1/Notch-1 signaling pathway as well as activates osteoblasts to produce Opn to regulate the stem cell pool size.


Parathyroid Gland
PTH from parathyroid gland in response to low Ca2+
Blood
PTH
HSCs do not expand and remain quiescent
Notch-1
HSC
Notch-1 mediated HSC expansion
PTHr
upregulation
Jagged-1
Tie-2
Expansion
Osteopontin
downregulation
Angiopoietin-1
Osteoblast
CD44
β-integrins
α4-integrin
Apoptosis
Angiopoietin-1
Tie-2
Bone Marrow
HSC expansion through anti-apoptotic signalling and maintenance of quiescence
Negative regulation
Positive regulation

a means of regulating stem cell pool size (73). PTH responds to low Ca^{2+} levels and primarily acts on the kidneys and bone to regulate Ca^{2+} (47).

Stem cell fate is thought to be governed by three major signalling pathways: Notch, Wnt and Smad.

The Wnt/β-catenin signaling pathway promotes self-renewal and negatively regulates quiescence and HSC differentiation (75). *In vitro* studies indicate that Wnt5a expands the fetal HSC pool while Wnt3a increases self-renewal of adult HSCs (76). The activation of β-catenin within HSCs by Wnt leads to the upregulation of other genes previously implicated in the self-renewal function of HSCs, namely HoxB4 and Notch1 (75, 77, 78). The role of Notch-1 in self-renewal is dose dependent; with constitutive Notch-1 signalling in *in vitro* experiments able to generate immortalized HSC cell lines with the Notch-1/Jagged-1 signalling capable of expanding primitive HSPCs (78, 79).

1.4 Lymphopoiesis

1.4.1 The thymus

The thymus, which is the primary site of T cell development (thymopoiesis), is a multi-lobulated bi-lobed structure situated in the central anterior compartment of the mediastinum. However, despite its anatomic prominence it was not until the discoveries of the Australian scientist Jacques Miller in the early 1960's that its function was recognized (80). The internal structure of the thymus is divided into the functionally distinct regions of subcapsule (internal region near the capsule), cortex and medulla. Each of these distinct areas supports a specific stage of thymocyte development.

Like all leukocytes, T cells are direct progeny of BM-resident HSCs. However, the thymus contains no self-renewing progenitors, instead relying on the importation of progenitor cells from the circulation, previously released from the BM in a process known as mobilization,

While there are multiple progenitors with the ability to either generate T cells and/or contain the ability to seed the thymus, the canonical pathway for T cell development appears to be a progenitor immediately downstream of the HSC which is skewed towards the lymphoid lineage but maintains myeloid potential (81) (Figure 1.1). This circulating T cell progenitor (CTP) egresses from the BM and, in an active process mediated by chemokines and adhesion molecules, homes to the thymus. Once seeded in the thymus and through close interactions with the thymic stromal microenvironment, passes through a series of developmentally distinct stages of T cell development (Figure 1.1). The thymic stromal microenvironment is comprised of many different cells including fibroblasts, endothelium, macrophages, dendritic cells and highly specialized thymic epithelial cells (TECs) that together with extracellular, form a three-dimensional network of cells inside the thymic capsule (65, 66). It is within this three-dimensional structure that imported CTPs initially become committed to the T lineage through Notch signalling and progressively develop into the various defined stages of thymopoiesis and, ultimately, to leave the thymus and circulate as a naïve T cell (82)(Figure 1.1).

One of the hallmarks of thymopoiesis is that it is severely abrogated with age (8). Thymic involution is a gradual process that is exacerbated from puberty, leading to the generally accepted role of sex steroids in thymic involution (8). While the proportion of thymocyte subsets remains remarkably unchanged with age, emphasizing the homeostatic regulation of T cell development pathways, there is a considerable decline in total thymopoiesis and significant disruption to thymic architecture (8), resulting in a reduction in emigration of naïve T cells into the periphery (83). This leads to a situation where the numbers of circulating T cells are maintained by homeostatic expansion of pre-existing memory T cells (reviewed in (84)). The subsequent decline in T cell receptor diversity leads to compromised responsiveness to foreign pathogens (reviewed in (84)).

While there is little impact of thymic involution on healthy individuals, there is considerable delay in recovery following immune depletion such as that caused by chemotherapy, radiation therapy, infection and stress. As a consequence, these individuals are left in a severely immunocompromised state and susceptible to infecting pathogens. In the case of patients undergoing hematopoietic stem cell transplants this prolonged immune deficiency leads to significantly higher rates of morbidity and mortality.

1.4.2 BM derived T cell Progenitors

The contains no self-renewing T-lineage stem cells, instead relying on the importation of BM-derived progenitors from the circulation. This process is maintained by the periodic egress of these progenitors from the BM (85) (Figure 1.1). However, the precise identity of the thymus-seeding progenitor cell remains elusive and, in fact, several cell types have been postulated all with thymus-seeding potential.

Considerable work has recently called into question the classic model of hematopoiesis in which there is strict delineation between myeloid and lymphoid lineages. The classic model proposed only true multipotent stem cells within the HSC compartment and several lineage-committed downstream progenitors such as common lymphoid (CLPs) and myeloid progenitors (CMPs). However, recent work has found that within the HSC compartment there are lineage-biased precursor cells (86, 87). Furthermore, two recent papers have also recently suggested that the progenitor that gains entry into the thymus also retains myeloid potential (88, 89). Within the stem cell compartment, this is dependent upon Flt3, PU.1 and GATA-1 expression. Flt3 (Flk2) expression as well as PU.1 activation and GATA-1 repression prevent a MPP from having megarkaryocyte or erythroid potential (90, 91), however this remains controversial (86).

To date, there are a multitude of phenotypically distinct progenitors with lymphoid potential, although their specific relationships to each other and physiological relevance

remain poorly understood. Historically, the classic model proposed a CLP that gave rise to all lymphoid lineages T, B and NK cells, and was described as $Lin^{-}cKit^{lo}Sca1^{lo}Thy1^{-}IL7R\alpha^{+}B220^{-}CD19^{-}$ [(2)]. However, recent findings suggest that lymphopoiesis from HSCs involve passage through a lymphoid-biased LSK cell known as the lymphoid-primed multipotent progenitors (LMPPs) (81) (Figure 1.1). These cells are characterized by the upregulation of lymphoid associated genes such as PU.1 and Ikaros and the downregulation of GATA-1 and Mpl (90, 92-94) (Figure 1.1). Within this LMPP population lie subsets of progenitors distinguished by their RAG1 and VCAM1 expression (95). RAG1 and VCAM1 expressing LMPPs are termed ELPs (early lymphoid progenitors) and represent the earliest committed lymphoid progenitor in the hierarchy of HSPCs with minimal myeloid and erythroid potential. Lymphoid specific progenitor (LSPs) however are $RAG1^{-}$ LMPPs and are thought to give rise to the more lymphoid-restricted common lymphoid progenitors (CLPs) (75). Furthermore, consistent with the identification of LMPPs was that its phenotype mirrored that of the earliest intrathymic T-lineage progenitor (ETP) however the thymus-seeding progenitors retained myeloid potential (88, 89) (Figure 1.1).

Utilizing a transgenic mouse engineered to contain a human CD25 (huCD25) gene tag under the control of the pre-TCR α promoter, Martin and colleagues were able to describe a new progenitor with the capacity for efficient thymus-seeding (2). These cells were phenotypically similar to CLP-1 and termed CLP-2: $Lin^{-}ckit^{-}B220^{+}CD19^{+}huCD25^{+}IL7R\alpha^{+}$. Furthermore, in an *in vitro* differentiation assay it was found that CLP-2 are descendants of CLP-1 (2)(Figure 1.2). Balciunaite and colleagues in fact suggest that this transition occurs via another intermediate progenitor, the early progenitor with lymphoid and myeloid potential (EPLM) (96) (Figure 1.2). Similar to CLP-1 however, they are able to differentiate into both T and B lineages but show no myeloid potential *in vivo* (96). Through short-term transplantation assays and subsequent thymic organ cultures, the authors effectively showed that CLP-2s posses both the ability to home to the thymus as well as differentiate

into CD4 and CD8 T cells. However, it is currently unclear the physiological role of CLP-2 in generating T cells is currently unclear as *in vivo* they appear to be primarily B cell precursors (2).

Of the aforementioned progenitors, only few are found in the blood stream and even fewer have been detected in the thymus, although this latter fact could be due to the extremely rapid differentiation that occurs immediately upon contact with the thymic microenvironment (mediated primarily by Notch). HSCs, MPPs, LMPPs, LSPs, ELPs, CLPs, CTPs have all been found in the circulation, however CLP-2 has yet to be detected in the bloodstream (3, 81, 97-99).

1.4.3 BM Egress and Thymic Entry

1.4.3.1 BM Egress

Mobilization of HSPCs out of the marrow is a multistage process beginning with the uncoupling from adhesion molecules followed by their transmigration across specialized BM sinusoidal endothelium (100). Of the many specific interactions that have been identified in this process of mobilization, including VLA-4/VCAM-1, VLA-5/Fibronectin, ckit/mSCF and CaR/Ca, the chemokine receptor CXCR4 on HSPCs and its ligand SDF-1α (CXCL12), expressed by the BM stroma, play a crucial role in regulating the localization of HPSCs within the BM niche (8, 101-105). Disruption of this axis results in rapid mobilization of HSCs into the periphery.

An additional mechanism for mobilization involves activation of the parathyroid hormone (PTH) receptor, which is primarily expressed by OBLs as previously mentioned (106). In conjunction with its ability to increase the size of the HSC pool, PTH is also thought to positively influence both engraftment and mobilization of HSCs to and from the BM niche (106). Brunner *et al.* showed that while not as effective as G-CSF, PTH was indeed capable of mobilizing HSCs from the BM into peripheral blood (107). They propose that PTH acts via

endogenous G-CSF to mobilize HSCs and appears to replenish and maintain the HSC pool within the BM as it promotes mobilization – G-CSF on the other hand depletes its HSC supply as it mobilizes them from the BM (107).

Recent studies have suggested, that low oxygen levels can also impact on mobilization through modulation of SDF-1α levels, much like maintenance of HSC quiescence. Hypoxia in peripheral tissue can also lead to the upregulation of SDF-1α expression on endothelial cells and hence the recruitment of HSPCs from the circulation into the tissue (108). Furthermore, nitric oxide (NO) promotes mobilization of $CD34^{+}Flk\text{-}1^{+}$ progenitor cells; which includes ST-HSCs and MPPs but not LT-HSCs (109).

Apart from the paracrine factors mentioned above, another method of mobilization involves disrupting cell adhesion molecules that are involved in homing and adhesion of HSPCs to the stroma. The integrins very late antigen 4 and 5 (VLA-4 and VLA-5) are cell adhesion molecules found on the surface of HSPCs (110). VLA-4 is responsible for both cell-cell and cell-matrix interactions and binds to several ligands expressed on stromal cells, the most important being vascular cell adhesion molecule 1 (VCAM-1) and fibronectin. VLA-5 on the other hand is primarily responsible for cell-matrix interactions and binds to fibronectin. Anti-VLA-4 and anti-VCAM-1 antibody pre-transplantation treatment resulted in an abrogation of HSC homing into the BM and an accumulation of HSCs in the blood (110).

The exploitation of these phenomena have been extremely successful in the clinic to enable and enhance mobilization of HSPCs in donors of HSCT - a far less invasive procedure than extracting BM. This is extremely important as one of the major obstacles for successful transplantation is ensuring there is a sufficient number of HSCs infused for functional reconstitution of the recipient (111) .While several next generation mobilization agents are under development, granulocyte colony stimulating factor (G-CSF) has been widely used to promote mobilization. G-CSF promotes mobilization through modulating SDF-1 and CXCR4

expression and creating a proteolytic environment where release of elastase and cathepsin G lead to cleavage and degradation of CXCL12 as well as adhesion molecules like ckit (112). Recently it has been shown that G-CSF also mobilizes HSCs by stimulating neutrophils to release elastase and cathepsin G, both of which degrade CXCL12 (SDF-1) (112). Specifically targeting the CXCR4-CXCL12 axis, AMD3100, a CXCR4 antagonist, is extremely effective at mobilizing HSPCs into the periphery (111).

1.4.3.2 CTP thymic Entry

While the mobilization of HSPCs into the periphery, both in the steady-state and enforced mobilization, has been a widely studied phenomenon, the specific mechanisms governing the entry of T-lineage progenitors into the thymus are only beginning to be resolved. Although several molecules have been implicated in influencing the entry of progenitors into the thymus, recent work has clearly established that expression of chemokine receptors 7 and 9 (CCR7 and CCR9) and P-selectin glycoprotein ligand-1 (PSGL-1) by circulating progenitors and their respective ligands/receptors on thymic stromal cells is crucial for entry of progenitors into the thymus (113-116). Indeed only CCR9-expressing MPPs or CLPs could seed the thymus and, moreover, expression of CCR9 was dependent on Flt3 signaling in the BM, which is also associated with lymphoid priming suggesting an intrinsic link between the processes of lymphoid commitment and thymic entry (91).

Thymic entry is a multistep event initiated by the chemokine gradient formed with CCL-25 and proceeding to the interaction of P-selectin, found on thymic endothelium, and P-selectin glycoprotein ligand 1 (PSGL-1) expressed on thymic-bound HSPCs (113, 117). This allows the thymic-bound HSPC to respond to local chemokine gradients and interact with both intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) found on endothelial cells. This interaction occurs via integrins found on the CTP and allows the CTP to be firmly attached to the thymic endothelium (117).

Through the use of elegant adoptive experiments, it was demonstrated that free intrathymic microenvironmental niches control the importation of thymic progenitors via the opening or closing of so-called intrathymic microvascular gates (118). This process is thought to occur in coordination with the periodic release of progenitors from the BM, suggesting a feedback loop exists between the BM and thymus (5). While elucidation of this periodic link between the BM and thymus has remained difficult, a recent study found that both niche availability, possible 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 naïve T cells from the thymus, can control expression of P-selectin and the chemokine CCL25 (ligands for PSGL-1 and CCR9 respectively), thereby directing thymic receptivity to circulating progenitors (119, 120).

1.4.3.3 Intrathymic T cell Development

Once in the thymus, thymic progenitors form an intimate interaction with the supporting stromal microenvironment to pass through several distinct and well-defined stages, ultimately to generate naïve MHC-restricted and self-tolerant T cells. The ETP is the earliest known thymic progenitor to be found within the thymus. While the CTP retains myeloid potential (88), once in the thymus Notch signalling quickly leads to commitment to the T cell lineage (82). cTECs surrounding TN1 cells have the highest level of Delta-like 1, a ligand for Notch mediated signalling (121). ETPs fall within what is known as a triple negative (TN) phenotype – that is they lack the expression of the mature T cell markers CD3, CD4 and CD8. TN cells themselves can be subdivided based on expression of CD25 and CD44. Within the T cell lineage, TN populations remain uncommitted and retain the potential for both the γδ and αβ T cell receptor (TCR) expressing T cells (122). ETPs remain in the TN1 state for approximately 10 days and receive signals such as stem cell factor (SCF), Hedgehog

and low levels of IL7 (to some subsets of TN1) from thymic cortical TECs (cTECs) (123-125).

Definitive T cell commitment occurs at the onset of TCR expression. Upregulation of CD25 (IL2R-α) promotes expression of the pre-TCR signalling complex, which can be directly regulated by Notch signalling (126, 127). αβ-TCR rearrangement begins at the TN3 stage, where the pTα couples with the TCRβ chain and CD3/ζ forming the pre-TCR complex. This requires recombination-activating gene-1 and 2 (RAG-1 and RAG-2), which allow DNA rearrangements of the TCRβ chain (128, 129). Pre-TCR signalling is tested at this point in a process known as β-selection. If deemed non-functional, maturation will be arrested at the DN3 stage. Thymocytes that pass β-selection progress to the DN4 stage where rearrangement of the TCRα chain leads to formation of the mature αβ TCR. This correlates with upregulation of CD4 and CD8 and thus progression to the DP stage of development.

Positive selection by MHC (Major Histocompatibility Complex) restriction occurs at DP stage ($CD4^+CD8^+$) in the cortex. DPs that show strong affinities for self-MHC molecules are deleted from the pool of TCR repertoire as part of central tolerance (reviewed in (130)). It is estimated that approximately 90% of the DPs fail to progress through positive selection (131). DPs that have passed positive selection migrate to the thymic medulla where they downregulate either CD4 or CD8 and undergo further tolerance checkpoints. Negative selection is predominantly mediated by dendritic cells (DC) as well as mTECs. DCs present self-peptides synthesized by medullary TECs to SP cells (reviewed in (101)). SPs that show strong affinity towards these self-peptide:MHC complexes are deleted, preventing auto-reactive T cells from being released into the circulation. Much like several other lymphocyte migration processes, naïve SP T cells exit the thymus in a SIP_1 dependent manner (102).

1.5 Immunodepletion

1.5.1 Immunosenescence

It is well established that the adaptive immune system deteriorates considerably with age. The concept of immune aging is poorly understood, with many studies assuming that it is simply the result of wear and tear (103). The notion that aging is perhaps an active and regulated process remains foreign to many. Through studies involving elderly individuals it was observed that whilst there were differences in the lymphoid population with age, there was no difference in natural killer (NK) cell activity suggesting that the impacts of aging were not a system-wide deterioration (105).

1.5.1.1 The Thymus with Age

Paradoxical with its pivotal role in establishing and sustaining a healthy immune system, the thymus atrophies with age. This process begins shortly after birth but is heightened from the onset of puberty (8, 132). With age there is severe accumulation of adipose tissue in the thymus accompanied by a dramatic loss of functional thymic tissue (133). Consequently there is an age-related decline in thymic cellularity and thymic output of naïve T cells leading to a reduced TCR repertoire in the periphery, although the absolute number of circulating T cells is maintained by homeostatic expansion of pre-existing memory T cells (134). Aging also alters the balance of T helper 1 (TH1) and T helper 2 (TH2) cells as well as functional changes in the ability of these T cells to produce cytokines , possibly as a result of impaired signal transduction pathways (135-137). Under normal circumstances, this decline in thymic function is not detrimental to the individual as most of the naïve T cell repertoire has been established and maintained in the periphery from an early age (8, 138). However, many patients who could benefit from a rejuvenated thymus to regenerate their adaptive immune system following depletion caused by chronic infection or standard of care cytoreductive cancer treatments (such as chemotherapy or radiotherapy), as a consequence of their reduced thymic function with age, there is a severely delayed ability to restore peripheral immune function (139). These individuals are subsequently impaired in their

ability to combat opportunistic infections and, as such, the morbidity and mortality associated with these patients is high (8, 139).

The production of IL-7, which is crucial for proliferation and differentiation of thymocytes, is reduced significantly with age and has thus been implicated in age-related thymic involution (140). However, exogenous administration of recombinant IL-7 does not reverse thymic atrophy significantly, suggesting the decline in IL-7 production might only partially explain thymic involution (140, 141).

The underlying cause of thymic atrophy has largely been attributed to increased levels of sex steroids (8, 142). Androgen receptors (AR) are expressed by both thymocytes and TECs, however in the thymus, TECS are the most affected by sex steroids (143), with testosterone treatment inducing TEC apoptosis(142, 144, 145, 146).

Whilst ARs are also expressed on stem cells, few studies have looked at expression throughout the different stages of lymphoid differentiation. Primitive lymphoid progenitors within the BM, specifically the CLPs, appear to be selectively depleted in mice treated with estrogen (147). Interestingly, estrogen receptors (ERs) are found to be expressed on adult human BM $CD34^+$ cells but not cord blood HSCs indicating that sex steroid-mediated regulation of hematopoiesis occurs post-natally (148). In a similar fashion, androgens also exhibit an inibitory effect on B and T lymphopoiesis (149). The effects of sex steroids on myelopoiesis are in contrast to those seen in lymphopoiesis. It has even been proposed that sex steroids influence the branching of hematopoietic lineages from common precursors (150, 151). Studies using *in vitro* organ cultures have shown that treatment of thymic tissue from both female and male mice results in AR-mediated apoptosis (152). Estrogen on the other hand appears to negatively affect the thymus by depleting the supply of progenitors that seed it. While there was significant loss of thymocytes upon estrogen administration, it is possible this could be caused by depletion of the LSK population within the BM (153). The effects of estrogen on the thymus are more

complex than those of androgens. Estrogen signalling via ERα is actually vital for normal thymic development but ERβ has been shown to mediate thymic atrophy (154).

Using androgen resistant testicular-feminization mice (Tfm), Olsen *et al.* were able to show that ARs were expressed on the thymic epithelium and that this was important in the context of thymic development (155, 156), possibly through the upregulation of TGFβ and downregulation of IL7 production (146, 157, 158).

1.5.1.2 HSPCs with Age

While the direct effects of sex steroids on thymocytes as well as on the thymic microenvironment contribute significantly to thymic involution, the role of the BM and its progenitors in thymic atrophy is unclear. Considering the direct effects of sex steroids on HSCs and in particular, LMPPs (148, 159, 160), it is conceivable that a decline in the supply of lymphoid progenitors (161, 162), together with a decline in their release into the circulation, contributes towards age-related thymic involution. While it has been shown that the decline in thymopoiesis with age is intrinsically linked to the degradation of the thymic stroma by the action of sex steroids, the interdependence in development of both hematopoietic and stromal elements (163) implies that a decline in one would lead to a decline in the other. As such, declines in the export of BM progenitors into the circulation, along with the reduced ability of the thymus to accept these progenitors for seeding, could contribute to a degeneration of both the thymic stroma and developing thymocytes with age.

While there is thought to be a shift in the clonal composition within the HSC population, there is also increasing evidence that supports the notion that HSCs are intrinsically altered in an aged setting (164, 165). Previous studies using assays such as colony forming unit (CFU) activity, cobblestone area-forming cell (CAFC) activity, BM transplantation (BMT) and serial transplantation assays on aged HSCs have offered conflicting results depending

on the assay as well as strain of mice used (166-168). Current techniques however have provided a means of assessing purified aged HSCs at a single cell level thus allowing a more accurate investigation (138).

In C57Bl/6 mice, the most widely used strain for hematopoiesis research, and arguably one of the most similar strains with respect to hematopoieisis, an accumulation of LT-HSCs with age, assessed by phenotype (170). However, these cells display a decreased ability to differentiate into lymphoid progeny (165). Upon transplantation, HSCs were also found to be deficient in homing and engraftment in recipient BM (171). Moreover, in competitive repopulation assays, aged HSCs are at least 2 to 4 times less efficient at long-term reconstitution than young HSCs (172, 173). Interestingly, aged HSCs exhibit a greater propensity towards myeloid reconstitution. Consistent with this, genome-wide analysis demonstrated significant upregulation with myeloid- and downregulation of lymphoid-associated genes with age (165, 174, 172, 175).

Interestingly there is a significant age-related decline in the expression of the B-lineage transcription factors Pax-5 and E2A as well as the general lymphoid factor Ikaros (176, 177). While reduction in Ikaros expression is likely to impact on lymphoid lineage differentiation, other T lineage factors such as GATA-3, similar to Pax-5 and E2A, might underlie the reduced generation of T cells with age. One possible mechanism by which Ikaros may influence this bias is explained by the age-associated decline in IL-7R expression in HSCs (178, 165). IL-7R signaling is vital for both the formation of lymphoid cells as well as their maintenance and survival and hence the absence of it during early HSPC development may provide a more myeloid conducive pathway. Furthermore, the number of CLPs in aged mice is significantly less than in young mice of the same strain (179). In contrast, myeloid progenitors (MEP, CMP and GMP) were found to be either at normal or elevated levels within an aged mouse (165).

Along with these age-related differences in lineage commitment and reconstitution potential, there has also been identified an intrinsic decline in the ability of HSCs to self-renew (180). Consistent with this, Bmi-1 which has an integral role in self-renewal, has been implicated in the senescence and aging of HSCs, predominantly through its downstream targets, $p16^{ink4A}$ and $p19^{Arf}$ (173, 181-185). It is unlikely that these age-associated functional changes are initiated exclusively intrinsic to the HSC. Rather, influences of the surrounding BM microenvironment are likely to contribute significantly towards these age-related alterations in HSC function. In fact, Geiger and colleagues have demonstrated that the adhesion between an aged HSC and its surrounding stroma is compromised (186). They suggest that this is mediated by Cdc42, a small RhoGTPase that has been previously implicated in adhesion signalling in HSCs (186). Aged HSCs upregulate Cdc42 which correlates to a reduction in the adhesiveness to fibronectin; an ECM component of the BM HSC niche. Apart from the intrinsic changes in genes associated with lineage-commitment, self-renewal and localization, there also appears to be downregulation of genes that promote epigenetic maintenance in HSCs (187). However, it is currently unclear if this is a consequence or trigger of HSC aging.

While supply of blood-borne hematopoietic or thymic progenitors is crucial for maintaining thymopoiesis throughout life, a reduced ability for their importation does not appear to underlie the initiation of thymic involution. Although the aged thymus does not appear to be impacted significantly in its ability to import hematopoietic progenitors from the circulation, intrathymic T cell precursors, along with their upstream BM-resident lymphoid progenitors, deteriorate with age in frequency, number and their ability to produce T cells (161, 188-191). Expression of Flt3 on LSK cells is also significantly reduced with age and might be associated with a decline in the absolute number of BM-resident lymphoid progenitors through the link to CCR9 (165, 192).

It has recently been suggested that plasma levels of S1P, which is involved with regulating the export of naïve SP thymocytes, also regulates the expression of P-selectin, CCL25 and subsequently thymic receptivity (193). In the aged, where there is a decline in thymic export, curiously there is no apparent corresponding change in the expression of P-selectin or CCL25 and subsequently receptivity of the thymus to the entry of circulating progenitors (190). The direct relationship between age, circulating S1P levels and thymic expression of P-selectin and CCL25 is still unresolved. However, given the considerable role for S1P not only in the thymic importation of TCP, but also in HSC migration, homing and mobilization, this is a potentially fruitful avenue of research to pursue in aiding thymic regeneration (194-196).

HSC aging is a combination of intrinsic, extrinsic and systemic factors that, combined, renders the HSC functionally inferior to young HSCs. Understanding the processes underlying these defects will be important when trying to enhance BM transplantation outcomes in a clinical setting.

1.5.2 Clinically Induced Immunodepletion

One of the drawbacks of common standard-of care strategies for treating both hematopoietic and solid tumour malignancies is the resulting immunodepletion that occurs. This induced immunodepletion is achieved through the use of chemotherapeutic cytoablative agents such as cyclophosphamide (Cy) or ionizing irradiation. It is used clinically when 1) diseased hematopoietic cells or cancer cells need to be eradicated, 2) as part of the conditioning regime for successful HSC transplantation (HSCT) or 3) as a means of immunosuppresion following solid organ transplantation (197). This is particularly devastating to the lymphoid compartment, which is composed of highly proliferative cells, and can result in high morbidity and mortality if immune recovery is delayed by complications such as an aging immune system (197-200). Efficient T cell recovery is one of

the most challenging obstacles post BMT. As mentioned previously, the aged thymus is extremely inefficient at producing T cells and immune recovery in aged individuals poses a major threat to their prognosis. B cell recovery is also impaired in aged mice following irradiation (201).

Primitive HSCs are less susceptible to cytoablative chemotherapy due to their quiescence (202). Primitive HSC frequency is unaffected by low dose Cy treatment in mice, however functionally they do decline in repopulation ability. Busulfan, which does target HSCs, induced elevated levels of senescence-associated markers $p16^{INK4A}$ and $p19^{Arf}$ (203). Ionizing radiation on the other hand appears to induce apoptosis in HSCs via the p53-$p21^{Cip1/Waf1}$ – dependent pathway (203).

One area that has been far less studied but is of great clinical concern, is the damage to the BM stromal microenvironment. Ionizing radiation and chemotherapy severely compromise the microvasculature that comprises the sinusoidal niche, suggesting that HSC function will be significantly altered by the niche (204, 205). While very few studies have assessed the effects of cytoablative therapies on the function of OBLs in a hematopoietic setting, many have investigated this in the context of bone formation. In an *in vitro* assay involving human OBLs cultured with varying combinations of chemotherapeutic agents, chemotherapy was found to cause a reduction in osteoblast numbers by inducing apoptosis (206). In a separate study, also with human BM cells, high-dose chemotherapy negatively affected the ability of BM stromal cells to form confluent layers when compared to unablated controls (207).

1.5.3 Immune Regeneration

1.5.3.1 Thymic Rejuvenation with Growth Factors

Several strategies have been described to enhance thymic function. These include cytokines, growth factors and hormone strategies. Most have been studied in the context of recovery from allogeneic HSCT.

IL-7 is an important lymphopoietic factor and is essential for thymopoiesis. It is expressed in a subset of TECs that reside in the cortico-medullary junction of the thymus as well as in specialized BM stromal cells (208). Interestingly, the number of TECs that express IL-7 diminishes with age contributing to thymic involution (209). In a study by Bhatia *et al.*, the administration of anti-IL-7 antibodies was able to induce thymic atrophy and this was reversible upon cessation of the antibody treatment (210). Pre-clinical studies in mice and primates have shown remarkable increases in both the peripheral expansion of T cells as well as output of T cells from the thymus after administration of exogenous IL-7, possibly through reduced apoptosis and promotion of T cell expansion (211-213). More recently, clinical trials in humans using recombinant human IL-7 have shown similar results to that seen in mice and primates and report an upregulation of the anti-apoptotic marker bcl-2 followed by increases in both peripheral $CD4^+$ and $CD8^+$ T cells (214).

Preclinical studies have shown that exogenous Flt3L enhances both thymus-dependent and independent T cell reconstitution. The effects of Flt3L are predominantly due to an expansion of Flt3+ progenitors in the BM (215). However, increases in T cell reconstitution can be at the expense of B-lymphopoiesis which is significantly declined with exogenous Flt3L administration and, in particular, its effects on the EPLM subset of BM progenitors (216, 217).

The cytokines discussed thus far exert their regenerative effects on the lymphoid compartment. Keratinocyte growth factor (KGF or FGF-7) enhances expansion of both immature and mature TECs and also promotes the differentiation of mature TECs and activates pathways within the TEC that support thymopoiesis (218). KGF treatment in a mouse aging model was able to enhance thymic micro-architecture, enhance thymic

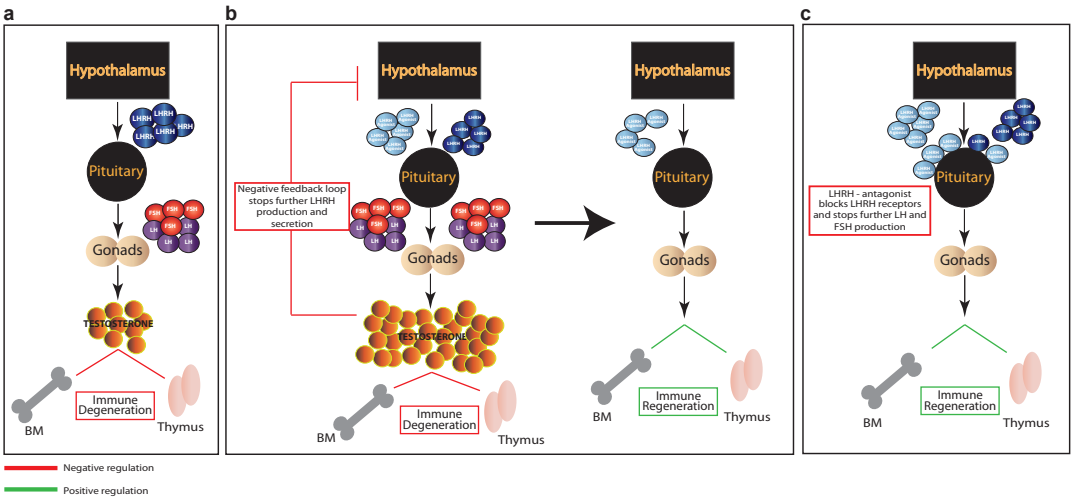
cellularity to untreated young levels and restore both naïve T cells in the periphery as well as restore their ability to mediate effector function through T cell-dependent antibody production levels (219).

Recombinant growth hormone (GH) has also been found to have beneficial effects in the thymus. GH was able to enhance both age and irradiation associated depletion of HSPCs within the BM (220). GH is thought to operate through insulin growth factor-1 (IGF-1), however, the precise mechanism through which immune regeneration occurs remains vague. Upon administration of exogenous IGF-1 however, while there was no apparent enlargement of the thymus, there were larger TN2 and TN3 populations within the thymus as well as increased peripheral $CD3^+$ T cells, B cells and myeloid cells within the spleen and blood circulation (221). When used in combination with exogenous IL-7, there was also synergistic improvement in B cell lymphopoiesis with no difference observed in thymopoiesis (221).

1.5.3.2 Sex Steroid Ablation

The removal of sex steroids through either surgical or chemical castration results in regeneration of lymphopoiesis in both the BM and thymus as well as a complete restoration of the stromal architecture in the thymus (8, 222, 223). Chemical castration can be pharmacologically achieved through the use of luteinizing hormone releasing hormone (LHRH) analogues or antagonists (Figure 1.4). Synthesized and released within the hypothalamus, LHRH stimulates release of follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary gland. These hormones subsequently act on the gonads to regulate the release of sex steroids through a negative feedback loop mechanism. Both forms of pharmacological castration involve reversible cessation of testosterone from the testes, albeit in moderately different ways: while the LHRH-agonist results in overstimulation of the LHRH receptors, the LHRH-antagonist blocks signalling of

Figure 1.4 The hypothalamo-pituitary-gonadal axis and its pharmaceutical manipulation to induce lymphoid regeneration. (Panel A) The hypothalamus produces and releases the luteinizing hormone releasing hormone (LHRH), which acts on the pituitary gland to secrete follicle-stimulating hormone (FSH) and luteinizing hormone (LH). FSH and LH in turn bind to receptors on the gonads (in this case testes) and stimulate sex steroid (testosterone) production and release by the gonads. Testosterone negatively acts on both the hematopoietic and stromal compartments of the bone marrow and thymus causing immune degeneration. **(Panel B)** The LHRH agonist binds to the LHRH receptors in the pituitary gland causing an oversecretion of LH and FSH which in turn causes an oversecretion of sex steroids (in this case testosterone) from the gonads (testes). This oversecretion feeds back to the hypothalamus and eventually secretion of sex steroids from the gonads ceases leading to immune regeneration. **(Panel C)** The LHRH antagonist blocks the LHRH receptor from binding to LHRH. As a result, there is little or no production and release of LH and FSH and therefore cessation of sex steroid (testosterone) production once again leading to immune regeneration.


a
b
c
Hypothalamus
Pituitary
Gonads
TESTOSTERONE
Immune Degeneration
BM
Thymus
Negative feedback loop stops further LHRH production and secretion
Immune Regeneration
LHRH - antagonist blocks LHRH receptors and stops further LH and FSH production
Negative regulation
Positive regulation

the LHRH receptor. The mechanism by which the agonist, is by initial hyperstimulation of the pituitary LHRH-R inducing an initial temporary surge of LHRH and subsequent testosterone production, before downregulation of LHRH-R and the associated negative feedback loop takes effect (Figure 1.4).

Sex steroid ablation results in both BM and thymic rejuvenation and hence immune enhancement. Previous studies in our laboratory have also shown enhanced immune recovery following both autologous and allogeneic BMT (8, 223-228),. Interestingly, following thymic regeneration mediated by sex steroid ablation (SSA) there is both an increase in thymic export and enhanced thymic CCL25 expression and receptivity, suggesting enhanced thymic activity. An improvement in HSC engraftment, BM function and an increase in B cell output is also observed with SSA. With an overall improvement in lymphoid output and function, the clinical implications that result from temporary SSA are numerous, particularly in situations of severe immunodepletion.

1.6 CONCLUSION

The thymus contains no self-renewing stem cell for T cell development and relies heavily on the supply of T cell precursors from the BM. Through sex steroid exposure, with age, there is considerable decline in lymphopoiesis as the BM and thymus degenerate. While not so dramatic in the BM, the thymus deteriorates profoundly from puberty, with severe disruption of its stromal microenvironment and hence T cell producing function. It remains unclear how degeneration is initiated, whether intrinsic hematopoietic defects trigger stromal degeneration within both organs or vice versa, since the development of both the hematopoietic and stromal compartments are interdependent. HSPCs within both the BM and thymus from aged mice models have been found to be both numerically and functionally inferior to their young counterparts. There appears to be a more myeloid skewing within aged HSCs than in young HSCs and this is thought to affect the prognosis of patients undergoing cytoablative therapies.

Immunosenescence is therefore a major clinical issue in individuals who are severely immunocompromized, either due to immunodepleting diseases or cytoreductive treatments. Current research has shown that the ablation of sex steroids, through either chemical or surgical castration, can lead to the rejuvenation of the immune system. There is considerable enhancement in both lymphoid output and function with sex steroid ablation post bone marrow transplantation. The mechanism that leads to this regeneration is however unclear. Whether intrinsic changes within the hematopoietic compartment in the BM and thymus initiate the enhancement of the stromal compartments or the rejuvenation of the molecular signalling and growth factor production by the stromal cells improve HSPC function remains unanswered to date. It will be highly beneficial from a clinical standpoint to elucidate these mechanisms, as it will improve the approaches for immune regeneration following cytoreductive therapies on adult patients.

1.7 References

1. Wilson, A., and A. Trumpp. 2006. Bone-marrow haematopoietic-stem-cell niches. Nat Rev Immunol 6:93-106.
2. Martin, C. H., I. Aifantis, M. L. Scimone, U. H. von Andrian, B. Reizis, H. von Boehmer, and F. Gounari. 2003. Efficient thymic immigration of B220+ lymphoid-restricted bone marrow cells with T precursor potential. Nat Immunol 4:866-873.
3. Krueger, A., and H. von Boehmer. 2007. Identification of a T lineage-committed progenitor in adult blood. Immunity 26:105-116.
4. Kondo, M., I. Weissman, and K. Akashi. 1997. Identification of clonogenic common lymphoid progenitors in mouse bone marrow. Cell 91:661-672.
5. Goldschneider, I. 2006. Cyclical mobilization and gated importation of thymocyte progenitors in the adult mouse: evidence for a thymus-bone marrow feedback loop. Immunological Reviews 209:58-75.
6. Rossi, D. J., D. Bryder, J. M. Zahn, H. Ahlenius, R. Sonu, A. J. Wagers, and I. L. Weissman. 2005. Cell intrinsic alterations underlie hematopoietic stem cell aging. Proc Natl Acad Sci U S A 102:9194-9199.
7. Dudakov, J. A., G. L. Goldberg, J. J. Reiseger, A. P. Chidgey, and R. L. Boyd. 2009. Withdrawal of Sex Steroids Reverses Age- and Chemotherapy-Related Defects in Bone Marrow Lymphopoiesis. The Journal of Immunology 182:6247-6260.
8. Sutherland, J. S., G. L. Goldberg, M. V. Hammett, A. P. Uldrich, S. P. Berzins, T. S. Heng, B. R. Blazar, J. L. Millar, M. A. Malin, A. P. Chidgey, and R. L. Boyd. 2005. Activation of thymic regeneration in mice and humans following androgen blockade. J Immunol 175:2741-2753.
9. Griffith, A. V., M. Fallahi, T. Venables, and H. T. Petrie. 2012. Persistent degenerative changes in thymic organ function revealed by an inducible model of organ regrowth. Aging Cell 11:169-177.
10. Olsen, N. J., X. Gu, and W. J. Kovacs. 2001. Bone marrow stromal cells mediate androgenic suppression of B lymphocyte development. J Clin Invest 108:1697-1704.
11. Spangrude, G. G. J., S. S. Heimfeld, and I. I. L. Weissman. 1988. Purification and characterization of mouse hematopoietic stem cells. Science 241:58-62.
12. Spangrude, G. J., and R. Scollay. 1990. Differentiation of hematopoietic stem cells in irradiated mouse thymic lobes. Kinetics and phenotype of progeny. J Immunol 145:3661-3668.

13. Okada, S., H. Nakauchi, K. Nagayoshi, S. Nishikawa, S. Nishikawa, Y. Miura, and T. Suda. 1991. Enrichment and characterization of murine hematopoietic stem cells that express c-kit molecule. Blood 78:1706-1712.
14. Okada, S., H. Nakauchi, K. Nagayoshi, S. Nishikawa, Y. Miura, and T. Suda. 1992. In vivo and in vitro stem cell function of c-kit- and Sca-1-positive murine hematopoietic cells. Blood 80:3044-3050.
15. Ikuta, K., and I. L. Weissman. 1992. Evidence that hematopoietic stem cells express mouse c-kit but do not depend on steel factor for their generation. Proc Natl Acad Sci U S A 89:1502-1506.
16. Li, C. L., and G. R. Johnson. 1995. Murine hematopoietic stem and progenitor cells: I. Enrichment and biologic characterization. Blood 85:1472-1479.
17. Osawa, M., K. Hanada, H. Hamada, and H. Nakauchi. 1996. Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. Science 273:242-245.
18. Goodell, M., K. Brose, G. Paradis, A. S. Conner, and R. Mulligan. 1996. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. The Journal of Experimental Medicine 183:1797-1806.
19. Yang, L., D. Bryder, J. Adolfsson, J. Nygren, R. Månsson, M. Sigvardsson, and S. E. W. Jacobsen. 2005. Identification of Lin–Sca1+kit+CD34+Flt3– short-term hematopoietic stem cells capable of rapidly reconstituting and rescuing myeloablated transplant recipients. Blood 105:2717-2723.
20. Adolfsson, J., O. J. Borge, D. Bryder, K. Theilgaard-Monch, I. Astrand-Grundstrom, E. Sitnicka, Y. Sasaki, and S. E. Jacobsen. 2001. Upregulation of Flt3 expression within the bone marrow Lin(-)Sca1(+)c-kit(+) stem cell compartment is accompanied by loss of self-renewal capacity. Immunity 15:659-669.
21. Wiesmann, A., R. L. Phillips, M. Mojica, L. J. Pierce, A. E. Searles, G. J. Spangrude, and I. Lemischka. 2000. Expression of CD27 on murine hematopoietic stem and progenitor cells. Immunity 12:193-199.
22. Christensen, J. L., and I. L. Weissman. 2001. Flk-2 is a marker in hematopoietic stem cell differentiation: a simple method to isolate long-term stem cells. Proceedings of the National Academy of Science USA 98:14541-14546.
23. Morrison, S. J., and I. L. Weissman. 1994. The long-term repopulating subset of hematopoietic stem cells is deterministic and isolatable by phenotype. Immunity 1:661-673.

24. Yilmaz, O., M. Kiel, and S. Morrison. 2006. SLAM family markers are conserved among hematopoietic stem cells from old and reconstituted mice and markedly increase their purity. Blood 107:924-930.
25. Kiel, M. J., O. H. Yilmaz, T. Iwashita, O. H. Yilmaz, C. Terhorst, and S. J. Morrison. 2005. SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. Cell 121:1109-1121.
26. Wang, N., A. Satoskar, W. Faubion, D. Howie, S. Okamoto, S. Feske, C. Gullo, K. Clarke, M. R. Sosa, A. H. Sharpe, and C. Terhost. 2004. The cell surface receptor SLAM controls T cell and macrophage functions. The Journal of Experimental Medicine 199:1255-1264.
27. Howie, D., S. Okamoto, S. Rietdijk, K. Clarke, N. Wang, C. Gullo, J. P. Bruggeman, S. Manning, A. J. Coyle, E. Greenfield, V. Kuchroo, and C. Terhorst. 2002. The role of SAP in murine CD150 (SLAM)-mediated T-cell proliferation and interferon gamma production. Blood 100:2899-2907.
28. Weksberg, D. C., S. M. Chambers, N. C. Boles, and M. A. Goodell. 2008. CD150- side population cells represent a functionally distinct population of long-term hematopoietic stem cells. Blood 111:2444-2451.
29. Méndez-Ferrer, S., T. V. Michurina, F. Ferraro, A. R. Mazloom, B. D. Macarthur, S. A. Lira, D. T. Scadden, A. Ma'ayan, G. N. Enikolopov, and P. S. Frenette. 2010. Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. Nature 466:829-834.
30. Nilsson, S. K. 2001. Spatial localization of transplanted hemopoietic stem cells: inferences for the localization of stem cell niches. Blood 97:2293-2299.
31. Haylock, D. N., B. Williams, H. M. Johnston, M. C. P. Liu, K. E. Rutherford, G. A. Whitty, P. J. Simmons, I. Bertoncello, and S. K. Nilsson. 2007. Hemopoietic stem cells with higher hemopoietic potential reside at the bone marrow endosteum. Stem Cells 25:1062-1069.
32. Parmar, K., P. Mauch, J.-A. Vergilio, R. Sackstein, and J. D. Down. 2007. Distribution of hematopoietic stem cells in the bone marrow according to regional hypoxia. Proc Natl Acad Sci USA 104:5431-5436.
33. Jang, Y.-Y., and S. J. Sharkis. 2007. A low level of reactive oxygen species selects for primitive hematopoietic stem cells that may reside in the low-oxygenic niche. Blood 110:3056-3063.

34. Lo Celso, C., H. E. Fleming, J. W. Wu, C. X. Zhao, S. Miake-Lye, J. Fujisaki, D. Côté, D. W. Rowe, C. P. Lin, and D. T. Scadden. 2009. Live-animal tracking of individual haematopoietic stem/progenitor cells in their niche. Nature 457:92-96.
35. Matsuo, K., and N. Irie. 2008. Osteoclast–osteoblast communication. Archives of Biochemistry and Biophysics 473:201-209.
36. Goldman, D. C., A. S. Bailey, D. L. Pfaffle, A. Al Masri, J. L. Christian, and W. H. Fleming. 2009. BMP4 regulates the hematopoietic stem cell niche. Blood 114:4393-4401.
37. Burgess, W., K. Jesse, Q. Tang, S. R. Broussard, R. Dantzer, and K. W. Kelley. 2003. Insulin-like growth factor-I and the cytokines IL-3 and IL-4 promote survival of progenitor myeloid cells by different mechanisms. Journal of Neuroimmunology 135:82-90.
38. Lymperi, S., A. Ersek, F. Ferraro, F. Dazzi, and N. J. Horwood. 2010. Inhibition of osteoclast function reduces hematopoietic stem cell numbers in vivo. Blood 117:1540-1549.
39. Batard, P., M. N. Monier, N. Fortunel, K. Ducos, P. Sansilvestri-Morel, T. Phan, A. Hatzfeld, and J. A. Hatzfeld. 2000. TGF-(beta)1 maintains hematopoietic immaturity by a reversible negative control of cell cycle and induces CD34 antigen up-modulation. J Cell Sci 113 (Pt 3):383-390.
40. Sitnicka, E., F. Ruscetti, G. V. Priestley, N. S. Wolf, and S. H. Bartelmez. 1996. Transforming growth factor beta 1 directly and reversibly inhibits the initial cell divisions of long-term repopulating hematopoietic stem cells. Blood 88:82-88.
41. Zhang, J., and L. Li. 2005. BMP signaling and stem cell regulation. Developmental Biology 284:1-11.
42. Adams, G. B., K. T. Chabner, I. R. Alley, D. P. Olson, Z. M. Szczepiorkowski, M. C. Poznansky, C. H. Kos, M. R. Pollak, E. M. Brown, and D. T. Scadden. 2006. Stem cell engraftment at the endosteal niche is specified by the calcium-sensing receptor. Nature 439:599-603.
43. Kollet, O., A. Dar, S. Shivtiel, A. Kalinkovich, K. Lapid, Y. Sztainberg, M. Tesio, R. M. Samstein, P. Goichberg, A. Spiegel, A. Elson, and T. Lapidot. 2006. Osteoclasts degrade endosteal components and promote mobilization of hematopoietic progenitor cells. Nat Med 12:657-664.
44. Kiel, M. J., and S. J. Morrison. 2008. Uncertainty in the niches that maintain haematopoietic stem cells. Nat Rev Immunol 8:290-301.

45. Wu, J. Y., D. T. Scadden, and H. M. Kronenberg. 2009. Role of the osteoblast lineage in the bone marrow hematopoietic niches. J Bone Miner Res 24:759-764.
46. Visnjic, D., Z. Kalajzic, D. W. Rowe, V. Katavic, J. Lorenzo, and H. L. Aguila. 2004. Hematopoiesis is severely altered in mice with an induced osteoblast deficiency. Blood 103:3258 - 3264.
47. Calvi, L. M., G. B. Adams, K. W. Weibrecht, J. M. Weber, D. P. Olson, M. C. Knight, R. P. Martin, E. Schipani, P. Divieti, F. R. Bringhurst, L. A. Milner, H. M. Kronenberg, and D. T. Scadden. 2003. Osteoblastic cells regulate the haematopoietic stem cell niche. Nature 425:841-846.
48. Taichman, R. 2005. Blood and bone: two tissues whose fates are intertwined to create the hematopoietic stem-cell niche. Blood 105:2631 - 2639.
49. Pittenger, M. F., A. M. Mackay, S. C. Beck, R. K. Jaiswal, R. Douglas, J. D. Mosca, M. A. Moorman, D. W. Simonetti, S. Craig, and D. R. Marshak. 1999. Multilineage Potential of Adult Human Mesenchymal Stem Cells. Science 284:143-147.
50. Huss, R. 2000. Isolation of primary and immortalized CD34-hematopoietic and mesenchymal stem cells from various sources. Stem Cells 18:1-9.
51. Ohneda, O., C. Fennie, Z. Zheng, C. Donahue, H. La, R. Villacorta, B. Cairns, and L. A. Lasky. 1998. Hematopoietic Stem Cell Maintenance and Differentiation Are Supported by Embryonic Aorta-Gonad-Mesonephros Region–Derived Endothelium. Blood 92:908-919.
52. Li, W., S. A. Johnson, W. C. Shelley, M. Ferkowicz, P. Morrison, Y. Li, and M. C. Yoder. 2003. Primary endothelial cells isolated from the yolk sac and para-aortic splanchnopleura support the expansion of adult marrow stem cells in vitro. Blood 102:4345-4353.
53. Geoffroy, V., M. Kneissel, B. Fournier, A. Boyde, and P. Matthias. 2002. High bone resorption in adult aging transgenic mice overexpressing cbfa1/runx2 in cells of the osteoblastic lineage. Molecular and cellular biology 22:6222-6233.
54. Nakashima, K., X. Zhou, G. Kunkel, Z. Zhang, J. Deng, R. Behringer, and B. de Crombrugghe. 2002. The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. Cell 108:17-29.
55. Hu, H., M. Hilton, X. Tu, K. Yu, D. Ornitz, and F. Long. 2005. Sequential roles of Hedgehog and Wnt signaling in osteoblast development. Development 132:49-60.
56. Komori, T. 2006. Regulation of osteoblast differentiation by transcription factors. Journal of Cellular Biochemistry 99:1233-1239.

57. Manolagas, S. 2000. Birth and death of bone cells: basic regulatory mechanisms and implications for the pathogenesis and treatment of osteoporosis. Endocr Rev 21:115-137.
58. Schofield, R. 1978. The relationsihp between the spleen colony-forming cell and the hematopoietic stem cell. Blood Cells 4:7-25.
59. Winkler, I. G., V. Barbier, R. Wadley, A. C. W. Zannettino, S. Williams, and J.-P. Lévesque. 2010. Positioning of bone marrow hematopoietic and stromal cells relative to blood flow in vivo: serially reconstituting hematopoietic stem cells reside in distinct nonperfused niches. Blood 116:375-385.
60. Arai, F., A. Hirao, M. Ohmura, H. Sato, S. Matsuoka, K. Takubo, K. Ito, G. Y. Koh, and T. Suda. 2004. Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. Cell 118:149-161.
61. Takakura, N., X. L. Huang, T. Naruse, I. Hamaguchi, D. J. Dumont, G. D. Yancopoulos, and T. Suda. 1998. Critical role of the TIE2 endothelial cell receptor in the development of definitive hematopoiesis. Immunity 9:677-686.
62. Yoshihara, H., F. Arai, K. Hosokawa, T. Hagiwara, K. Takubo, Y. Nakamura, Y. Gomei, H. Iwasaki, S. Matsuoka, K. Miyamoto, H. Miyazaki, T. Takahashi, and T. Suda. 2007. Thrombopoietin/MPL Signaling Regulates Hematopoietic Stem Cell Quiescence and Interaction with the Osteoblastic Niche. Cell Stem Cell 1:685-697.
63. Yamazaki, S., A. Iwama, S. Takayanagi, Y. Morita, K. Eto, H. Ema, and H. Nakauchi. 2006. Cytokine signals modulated via lipid rafts mimic niche signals and induce hibernation in hematopoietic stem cells. The EMBO Journal 25:3515-3523.
64. Qian, H., N. Buza-Vidas, C. Hyland, C. Jensen, J. Antonchuk, R. Mansson, L. Thoren, M. Ekblom, W. S. Alexander, and S. Jacobsen. 2007. Critical role of thrombopoietin in maintaining adult quiescent hematopoietic stem cells. Cell Stem Cell 1:671-684.
65. Suda. 2008. Quiescent stem cells in the niche. StemBook:1-11.
66. Puch, S., S. Armeanu, C. Kibler, K. R. Johnson, C. A. Müller, M. J. Wheelock, and G. Klein. 2001. N-cadherin is developmentally regulated and functionally involved in early hematopoietic cell differentiation. J Cell Sci 114:1567-1577.
67. Blank, U., G. Karlsson, and S. Karlsson. 2008. Signaling pathways governing stem-cell fate. Blood 111:492-503.
68. Ducos, K., B. Panterne, N. Fortunel, A. Hatzfeld, M.-N. Monier, and J. Hatzfeld. 2000. p21cip1 mRNA is controlled by endogenous transforming growth factor-β1 in

quiescent human hematopoietic stem/progenitor cells. Journal of Cellular Physiology 184:80-85.

69. Dubois, C. M., F. W. Ruscetti, J. Stankova, and J. R. Keller. 1994. Transforming growth factor-beta regulates c-kit message stability and cell-surface protein expression in hematopoietic progenitors. Blood 83:3138-3145.

70. Bhardwaj, G., B. Murdoch, D. Wu, D. P. Baker, K. P. Williams, K. Chadwick, L. E. Ling, F. N. Karanu, and M. Bhatia. 2001. Sonic hedgehog induces the proliferation of primitive human hematopoietic cells via BMP regulation. Nature Immunology 2:172-180.

71. Bhatia, M., D. Bonnet, D. Wu, B. Murdoch, J. Wrana, L. Gallacher, and J. E. Dick. 1999. Bone morphogenetic proteins regulate the developmental program of human hematopoietic stem cells. J Exp Med 189:1139-1148.

72. Nilsson, S. K., H. M. Johnston, G. A. Whitty, B. Williams, R. J. Webb, D. T. Denhardt, I. Bertoncello, L. J. Bendall, P. J. Simmons, and D. N. Haylock. 2005. Osteopontin, a key component of the hematopoietic stem cell niche and regulator of primitive hematopoietic progenitor cells. Blood 106:1232-1239.

73. Stier, S., Y. Ko, R. Forkert, C. Lutz, T. Neuhaus, E. Grunewald, T. Cheng, D. Dombkowski, L. M. Calvi, S. R. Rittling, and D. T. Scadden. 2005. Osteopontin is a hematopoietic stem cell niche component that negatively regulates stem cell pool size. The Journal of Experimental Medicine 201:1781-1791.

74. Iwata, M., N. Awaya, L. Graf, C. Kahl, and B. Torok-Storb. 2004. Human marrow stromal cells activate monocytes to secrete osteopontin, which down-regulates Notch1 gene expression in CD34+ cells. Blood 103:4496-4502.

75. Reya, T., A. W. Duncan, L. Ailles, J. Domen, D. C. Scherer, K. Willert, L. Hintz, R. Nusse, and I. L. Weissman. 2003. A role for Wnt signalling in self-renewal of haematopoietic stem cells. Nature 423:409-414.

76. Fleming, H. E., V. Janzen, C. Lo Celso, J. Guo, K. M. Leahy, H. M. Kronenberg, and D. T. Scadden. 2008. Wnt signaling in the niche enforces hematopoietic stem cell quiescence and is necessary to preserve self-renewal in vivo. Cell Stem Cell 2:274-283.

77. Antonchuk, J., G. Sauvageau, and R. K. Humphries. 2002. HOXB4-induced expansion of adult hematopoietic stem cells ex vivo. Cell 109:39-45.

78. Varnum-Finney, B., L. Xu, C. Brashem-Stein, C. Nourigat, D. Flowers, S. Bakkour, W. S. Pear, and I. D. Bernstein. 2000. Pluripotent, cytokine-dependent, hematopoietic stem cells are immortalized by constitutive Notch1 signaling. Nat Med 6:1278-1281.

79. Varnum-Finney, B., L. E. Purton, M. Yu, C. Brashem-Stein, D. Flowers, S. Staats, K. A. Moore, I. Le Roux, R. Mann, G. Gray, S. Artavanis-Tsakonas, and I. D. Bernstein. 1998. The Notch ligand, Jagged-1, influences the development of primitive hematopoietic precursor cells. Blood 91:4084-4091.
80. Miller, J. F. 1961. Immunological function of the thymus. Lancet 2:748-749.
81. Perry, S. S., R. S. Welner, T. Kouro, P. W. Kincade, and X.-H. Sun. 2006. Primitive lymphoid progenitors in bone marrow with T lineage reconstituting potential. J Immunol 177:2880-2887.
82. Sambandam, A., I. Maillard, V. P. Zediak, L. Xu, R. M. Gerstein, J. C. Aster, W. S. Pear, and A. Bhandoola. 2005. Notch signaling controls the generation and differentiation of early T lineage progenitors. Nature Immunology 6:663-670.
83. Sutherland, J. S., G. L. Goldberg, M. V. Hammett, A. P. Uldrich, S. P. Berzins, T. S. Heng, B. B.R., J. L. Millar, M. A. Malin, A. P. Chidgey, and R. L. Boyd. 2005. Activation of thymic regeneration in mice and humans following androgen blockade. The Journal of Immunology 175:2741 - 2753.
84. Goronzy, J. J., and C. M. Weyand. 2005. T cell development and receptor diversity during aging. Current Opinion in Immunology 17:468-475.
85. Donskoy, E., D. Foss, and I. Goldschneider. 2003. Gated importation of prothymocytes by adult mouse thymus is coordinated with their periodic mobilization from bone marrow. The Journal of Immunology 171:3568-3575.
86. Forsberg, E. C., T. Serwold, S. Kogan, I. L. Weissman, and E. Passegué. 2006. New evidence supporting megakaryocyte-erythrocyte potential of flk2/flt3+ multipotent hematopoietic progenitors. Cell 126:415-426.
87. Muller-Sieburg, C. E., R. H. Cho, L. Karlsson, J.-F. Huang, and H. B. Sieburg. 2004. Myeloid-biased hematopoietic stem cells have extensive self-renewal capacity but generate diminished lymphoid progeny with impaired IL-7 responsiveness. Blood 103:4111-4118.
88. Bell, J. J., and A. Bhandoola. 2008. The earliest thymic progenitors for T cells possess myeloid lineage potential. Nature 452:764-767.
89. Wada, H., K. Masuda, R. Satoh, K. Kakugawa, T. Ikawa, Y. Katsura, and H. Kawamoto. 2008. Adult T-cell progenitors retain myeloid potential. Nature 452:768-772.
90. Yoshida, T., S. Y.-M. Ng, J. C. Zuniga-Pflucker, and K. Georgopoulos. 2006. Early hematopoietic lineage restrictions directed by Ikaros. Nature Immunology 7:382-391.

91. Adolfsson, J., R. Månsson, N. Buza-Vidas, A. Hultquist, K. Liuba, C. T. Jensen, D. Bryder, L. Yang, O.-J. Borge, L. A. M. Thoren, K. Anderson, E. Sitnicka, Y. Sasaki, M. Sigvardsson, and S. E. W. Jacobsen. 2005. Identification of Flt3+ lympho-myeloid stem cells lacking erythro-megakaryocytic potential a revised road map for adult blood lineage commitment. Cell 121:295-306.

92. Luc, S., K. Anderson, S. Kharazi, N. Buza-Vidas, C. Boiers, C. T. Jensen, Z. Ma, L. Wittmann, and S. E. W. Jacobsen. 2008. Down-regulation of Mpl marks the transition to lymphoid-primed multipotent progenitors with gradual loss of granulocyte-monocyte potential. Blood 111:3424-3434.

93. Scott, E., M. Simon, J. Anastasi, and H. Singh. 1994. Requirement of transcription factor PU. 1 in the development of multiple hematopoietic lineages. Science 265:1573-1577.

94. Orkin, S., R. Shivdasani, M. Mcdevitt, Y. Fujiwara, and M. Mcdevitt. 1998. Transcription factor GATA-1 in megakaryocyte development. Stem Cells 16 Suppl 2:79-83.

95. Igarashi, H., S. Gregory, T. Yokota, N. Sakaguchi, and P. Kincade. 2002. Transcription from the RAG1 locus marks the earliest lymphocyte progenitors in bone marrow. Immunity 17:117-130.

96. Balciunaite, G., R. Ceredig, S. Massa, and A. G. Rolink. 2005. A B220+ CD117+ CD19±hematopoietic progenitor with potent lymphoid and myeloid developmental potential. European Journal of Immunology 35:2019-2030.

97. Goodman, J., and G. S. HODGSON. 1962. Evidence for stem cells in the peripheral blood of mice. Blood 19:702-714.

98. Wright, D. E., A. J. Wagers, A. P. Gulati, F. L. Johnson, and I. L. Weissman. 2001. Physiological migration of hematopoietic stem and progenitor cells. Science 294:1933-1936.

99. Schwarz, B. A., and A. Bhandoola. 2004. Circulating hematopoietic progenitors with T lineage potential. Nat Immunol 5:953-960.

100. Schulz, C., U. H. von Andrian, and S. Massberg. 2009. Hematopoietic stem and progenitor cells: their mobilization and homing to bone marrow and peripheral tissue. Immunol Res 44:160-168.

101. Petrie, H. T., and J. C. Zuniga-Pflucker. 2007. Zoned out: functional mapping of stromal signaling microenvironments in the thymus. Annu Rev Immunol 25:649-679.

102. Matloubian, M., C. Lo, G. Cinamon, M. Lesneski, Y. Xu, V. Brinkmann, M. Allende, R. Proia, and J. Cyster. 2004. Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. Nature 427:355-360.
103. Franceschi, C., D. Monti, P. Sansoni, and A. Cossarizza. 1995. The immunology of exceptional individuals: the lesson of centenarians. Immunology Today 16:12-16.
104. Ligthart, G. J., J. X. Corberand, H. G. Geertzen, A. E. Meinders, D. L. Knook, and W. Hijmans. 1990. Necessity of the assessment of health status in human immunogerontological studies: evaluation of the SENIEUR protocol. Mech Ageing Dev 55:89-105.
105. Aspinall, R., and D. Andrew. 2000. Thymic involution in aging. Journal of Clinical Immunology 20:250-256.
106. Adams, G. B., R. P. Martin, I. R. Alley, K. T. Chabner, K. S. Cohen, L. M. Calvi, H. M. Kronenberg, and D. T. Scadden. 2007. Therapeutic targeting of a stem cell niche. Nat Biotechnol 25:238-243.
107. Brunner, S., M.-M. Zaruba, B. Huber, R. David, M. Vallaster, G. Assmann, J. Mueller-Hoecker, and W.-M. Franz. 2008. Parathyroid hormone effectively induces mobilization of progenitor cells without depletion of bone marrow. Exp Hematol 36:1157-1166.
108. Ceradini, D. J., and G. C. Gurtner. 2005. Homing to hypoxia: HIF-1 as a mediator of progenitor cell recruitment to injured tissue. Trends Cardiovasc Med 15:57-63.
109. Ozüyaman, B., P. Ebner, U. Niesler, J. Ziemann, P. Kleinbongard, T. Jax, A. Gödecke, M. Kelm, and C. Kalka. 2005. Nitric oxide differentially regulates proliferation and mobilization of endothelial progenitor cells but not of hematopoietic stem cells. Thromb Haemost 94:770-772.
110. Papayannopoulou, T., C. Craddock, B. Nakamoto, G. V. Priestley, and N. S. Wolf. 1995. The VLA4/VCAM-1 adhesion pathway defines contrasting mechanisms of lodgement of transplanted murine hemopoietic progenitors between bone marrow and spleen. Proceedings of the National Academy of Sciences 92:9647-9651.
111. Liles, W. C., E. Rodger, H. E. Broxmeyer, C. Dehner, K. Badel, G. Calandra, J. Christensen, B. Wood, T. H. Price, and D. C. Dale. 2005. Augmented mobilization and collection of CD34+ hematopoietic cells from normal human volunteers stimulated with granulocyte–colony-stimulating factor by single-dose administration of AMD3100, a CXCR4 antagonist. Transfusion 45:295-300.

112. Levesque, J.-P. 2003. Disruption of the CXCR4/CXCL12 chemotactic interaction during hematopoietic stem cell mobilization induced by GCSF or cyclophosphamide. Journal of Clinical Investigation 111:187-196.

113. Rossi, F. M. V., S. Y. Corbel, J. S. Merzaban, D. A. Carlow, K. Gossens, J. Duenas, L. So, L. Yi, and H. J. Ziltener. 2005. Recruitment of adult thymic progenitors is regulated by P-selectin and its ligand PSGL-1. Nature Immunology 6:626-634.

114. Schwarz, B. A., A. Sambandam, I. Maillard, B. C. Harman, P. E. Love, and A. Bhandoola. 2007. Selective thymus settling regulated by cytokine and chemokine receptors. The Journal of Immunology 178:2008-2017.

115. Zlotoff, D. A., A. Sambandam, T. D. Logan, J. J. Bell, B. A. Schwarz, and A. Bhandoola. 2010. CCR7 and CCR9 together recruit hematopoietic progenitors to the adult thymus. Blood 115:1897-1905.

116. Krueger, A., S. Willenzon, M. Lyszkiewicz, E. Kremmer, and R. Forster. 2010. CC chemokine receptor 7 and 9 double-deficient hematopoietic progenitors are severely impaired in seeding the adult thymus. Blood 115:1906-1912.

117. Scimone, M. L., I. Aifantis, I. Apostolou, H. von Boehmer, and U. H. von Andrian. 2006. A multistep adhesion cascade for lymphoid progenitor cell homing to the thymus. Proc Natl Acad Sci USA 103:7006-7011.

118. Prockop, S. E., and H. T. Petrie. 2004. Regulation of thymus size by competition for stromal niches among early T cell progenitors. The Journal of Immunology 173:1604-1611.

119. Gossens, K., S. Naus, S. Y. Corbel, S. Lin, F. M. V. Rossi, J. Kast, and H. J. Ziltener. 2009. Thymic progenitor homing and lymphocyte homeostasis are linked via S1P-controlled expression of thymic P-selectin/CCL25. Journal of Experimental Medicine 206:761-778.

120. Schnell, F. J., A. L. Zoller, S. R. Patel, I. R. Williams, and G. J. Kersh. 2006. Early growth response gene 1 provides negative feedback to inhibit entry of progenitor cells into the thymus. The Journal of Immunology 176:4740-4747.

121. Schmitt, T. M., M. Ciofani, H. T. Petrie, and J. C. Zuniga-Pflucker. 2004. Maintenance of T cell specification and differentiation requires recurrent notch receptor–ligand interactions. The Journal of Experimental Medicine 200:469-479.

122. Robey, E., and B. J. Fowlkes. 1994. Selective events in T cell development. Annual Review of Immunology 12:675-705.

123. El Andaloussi, A., S. Graves, F. Meng, M. Mandal, M. Mashayekhi, and I. Aifantis. 2006. Hedgehog signaling controls thymocyte progenitor homeostasis and differentiation in the thymus. Nature Immunology 7:418-426.
124. Massa, S., G. Balciunaite, R. Ceredig, and A. G. Rolink. 2006. Critical role for c-kit (CD117) in T cell lineage commitment and early thymocyte development in vitro. European Journal of Immunology 36:526-532.
125. Rodewald, H., M. Ogawa, C. Haller, C. Waskow, and J. DiSanto. 1997. Pro-thymocyte expansion by c-kit and the common cytokine receptor γ chain is essential for repertoire formation. Immunity 6:265-272.
126. Gounari, F., I. Aifantis, C. Martin, H. J. Fehling, S. Hoeflinger, P. Leder, H. von Boehmer, and B. Reizis. 2002. Tracing lymphopoiesis with the aid of a pTα-controlled reporter gene. Nature Immunology 3:489-496.
127. Saint-Ruf, C., K. Ungewiss, M. Groettrup, L. Bruno, H. Fehling, and H. Von Boehmer. 1994. Analysis and expression of a cloned pre-T cell receptor gene. Science 266:1208-1212.
128. Mombaerts, P., J. Iacomini, R. Johnson, K. Herrup, S. Tonegawa, and V. Papaioannou. 1992. RAG-1-deficient mice have no mature B and T lymphocytes. Cell 68:869-877.
129. Shinkai, Y., S. Koyasu, K. Nakayama, K. Murphy, D. Loh, E. Reinherz, and F. Alt. 1993. Restoration of T cell development in RAG-2-deficient mice by functional TCR transgenes. Science 259:822-825.
130. Jameson, S. C., K. A. Hogquist, and M. J. Bevan. 1995. Positive selection of thymocytes. Annual review of immunology 13:93-126.
131. Germain, R. N. 2002. T-cell development and the CD4-CD8 lineage decision. Nat Rev Immunol 2:309-322.
132. Mackall, C. C. L., and R. R. E. Gress. 1997. Thymic aging and T-cell regeneration. Immunological Reviews 160:91-102.
133. Francis, I. R., G. M. Glazer, F. L. Bookstein, and B. H. Gross. 1985. The thymus: reexamination of age-related changes in size and shape. AJR Am J Roentgenol 145:249-254.
134. Hale, J. S., T. E. Boursalian, G. L. Turk, and P. J. Fink. 2006. Thymic output in aged mice. Proceedings of the National Academy of Science USA 103:8447-8452.

135. Utsuyama, M., Z. Varga, K. Fukami, Y. Homma, T. Takenawa, and K. Hirokawa. 1993. Influence of age on the signal transduction of T cells in mice. International Immunology 5:1177-1182.
136. Hirokawa, K. 1992. Understanding the mechanism of the age-related decline in immune function. Nutrition Reviews 50:361-366.
137. Hobbs, M. V., W. O. Weigle, D. J. Noonan, B. E. Torbett, R. J. McEvilly, R. J. Koch, G. J. Cardenas, and D. N. Ernst. 1993. Patterns of cytokine gene expression by CD4+ T cells from young and old mice. The Journal of Immunology 150:3602-3614.
138. Semino, C. E. 2003. Can we build artificial stem cell compartments. Journal of Biomedicine and Biotechnology 3:164 - 169.
139. Haynes, B. F., M. L. Markert, G. D. Sempowski, D. D. Patel, and L. P. Hale. 2000. The role of the thymus in immune reconstitution in aging, bone marrow transplantation, and HIV-1 infection. Annual Reviews Immunology 18:529 - 560.
140. Aspinall, R. 2006. T cell development, ageing and Interleukin-7. Mech Ageing Dev 127:572-578.
141. Chu, Y. W., S. A. Memon, S. O. Sharrow, F. T. Hakim, M. Eckhaus, P. J. Lucas, and R. E. Gress. 2004. Exogenous IL-7 increases recent thymic emigrants in peripheral lymphoid tissue without enhanced thymic function. Blood 104:1110-1119.
142. Olsen, N., S. Viselli, J. Fan, and W. Kovacs. 1998. Androgens accelerate thymocyte apoptosis. Endocrinology 139:748-752.
143. Olsen, N. J., G. Olson, S. M. Viselli, X. Gu, and W. J. Kovacs. 2001. Androgen receptors in thymic epithelium modulate thymus size and thymocyte development. Endocrinology 142:1278-1283.
144. Viselli, S. M., N. J. Olsen, K. Shults, G. Steizer, and W. J. Kovacs. 1995. Immunochemical and flow cytometric analysis of androgen receptor expression in thymocytes. Mol Cell Endocrinol 109:19-26.
145. Savino, W., D. M. Villa-Verde, L. A. Alves, and M. Dardenne. 1998. Neuroendocrine control of the thymus. Ann N Y Acad Sci 840:470-479.
146. Kumar, N., L. X. Shan, M. P. Hardy, C. W. Bardin, and K. Sundaram. 1995. Mechanism of androgen-induced thymolysis in rats. Endocrinology 136:4887-4893.
147. Medina, K. L., K. P. Garrett, L. F. Thompson, M. I. D. Rossi, K. J. Payne, and P. W. Kincade. 2001. Identification of very early lymphoid precursors in bone marrow and their regulation by estrogen. Nat Immunol 2:718-724.

148. Igarashi, H., T. Kouro, T. Yokota, P. C. Comp, and P. Kincade. 2001. Age and stage dependency of estrogen receptor expression by lymphocyte precursors. Proceedings of the National Academy of Science USA 98:15131-15136.

149. Viselli, S., K. Reese, J. Fan, W. Kovacs, and N. Olsen. 1997. Androgens alter B cell development in normal male mice. Cell Immunol 182:99-104.

150. Harman, B. C., J. P. Miller, N. Nikbakht, R. Gerstein, and D. Allman. 2006. Mouse plasmacytoid dendritic cells derive exclusively from estrogen-resistant myeloid progenitors. Blood 108:878-885.

151. Kincade, P. W., H. Igarashi, K. L. Medina, T. Kouro, T. Yokota, M. I. Rossi, J. J. Owen, K. P. Garrett, X. H. Sun, and N. Sakaguchi. 2002. Lymphoid lineage cells in adult murine bone marrow diverge from those of other blood cells at an early, hormone-sensitive stage. Seminars in immunology 14:385-394.

152. Olsen, N. N. J., S. S. M. Viselli, J. J. Fan, and W. W. J. Kovacs. 1998. Androgens accelerate thymocyte apoptosis. Endocrinology 139:748-752.

153. Zoller, A. L., and G. J. Kersh. 2006. Estrogen induces thymic atrophy by eliminating early thymic progenitors and inhibiting proliferation of beta-selected thymocytes. J Immunol 176:7371-7378.

154. Erlandsson, M. C., C. Ohlsson, J. A. Gustafsson, and H. Carlsten. 2001. Role of oestrogen receptors α and β in immune organ development and in oestrogen-mediated effects on thymus. Immunology 103:17-25.

155. Olsen, N. N. J., G. G. Olson, S. S. M. Viselli, X. X. Gu, and W. W. J. Kovacs. 2001. Androgen receptors in thymic epithelium modulate thymus size and thymocyte development. Endocrinology 142:1278-1283.

156. Viselli, S. S. M., N. N. J. Olsen, K. K. Shults, G. G. Steizer, and W. W. J. Kovacs. 1995. Immunochemical and flow cytometric analysis of androgen receptor expression in thymocytes. Molecular and cellular endocrinology 109:19-26.

157. Andrew, D., and R. Aspinall. 2002. Age-associated thymic atrophy is linked to a decline in IL-7 production. Experimental Gerontology 37:455-463.

158. Olsen, N. J., P. Zhou, H. Ong, and W. J. Kovacs. 1993. Testosterone induces expression of transforming growth factor-beta1 in the murine thymus. J Steroid Biochem Mol Biol 45:327-332.

159. Igarashi, H., K. L. Medina, T. Yokota, M. I. Rossi, N. Sakaguchi, P. C. Comp, and P. W. Kincade. 2005. Early lymphoid progenitors in mouse and man are highly sensitive to glucocorticoids. Int Immunol 17:501-511.

160. Kincade, P. W., K. L. Medina, and G. Smithson. 1994. Sex hormones as negative regulators of lymphopoiesis. Immunol Rev 137:119-134.
161. Min, H., E. Montecino-Rodriguez, and K. Dorshkind. 2004. Reduction in the developmental potential of intrathymic T cell progenitors with age. Journal of Immunology 173:245-250.
162. Min, H., E. Montecino-Rodriguez, and K. Dorshkind. 2006. Effects of aging on the common lymphoid progenitor to pro-B cell transition. J Immunol 176:1007-1012.
163. Petrie, H. T., and J. C. Zuniga-Pflucker. 2007. Zoned out: functional mapping of stromal signaling microenvironments in the thymus. Annu Rev Immunol 25:649-679.
164. Cho, R. H., H. B. Sieburg, and C. E. Muller-Sieburg. 2008. A new mechanism for the aging of hematopoietic stem cells: aging changes the clonal composition of the stem cell compartment but not individual stem cells. Blood 111:5553-5561.
165. Rossi, D. J., D. Bryder, J. M. Zahn, H. Ahlenius, R. Sonu, A. J. Wagers, and I. L. Weissman. 2005. Cell intrinsic alterations underlie hematopoietic stem cell aging. Proceedings of the National Academy of Science USA 102.
166. Makinodan, T. 1976. Decline in the growth potential of spleen-colonizing bone marrow stem cells of long-lived aging mice. J Exp Med.
167. Micklem, H. S., C. E. Ford, E. P. Evans, D. A. Ogden, and D. S. Papworth. 1972. Competitive in vivo proliferation of foetal and adult haematopoietic cells in lethally irradiated mice. Journal of Cellular Physiology 79:293-298.
168. de Haan, G., W. Nijhof, and G. Van Zant. 1997. Mouse strain-dependent changes in frequency and proliferation of hematopoietic stem cells during aging: correlation between lifespan and cycling activity. Blood 89:1543-1550.
169. Spangrude, G. G. J., S. S. Heimfeld, and I. I. L. Weissman. 1988. Purification and characterization of mouse hematopoietic stem cells. Science 241:58-62.
170. Sudo, K., H. Ema, Y. Morita, and H. Nakauchi. 2000. Age-associated characteristics of murine hematopoietic stem cells. Journal of Experimental Medicine 9:1273-1280.
171. Liang, Y., G. Van Zant, and S. J. Szilvassy. 2005. Effects of aging on the homing and engraftment of murine hematopoietic stem and progenitor cells. Blood 106:1479-1487.
172. Morrison, S. J., A. M. Wandycz, K. Akashi, A. Globerson, and I. L. Weissman. 1996. The aging of hematopoietic stem cells. Nat Med 2:1011-1016.

173. Yilmaz, O., M. Kiel, and S. Morrison. 2006. SLAM family markers are conserved among hematopoietic stem cells from old and reconstituted mice and markedly increase their purity. Blood 107:924-930.
174. Chen, J., C. M. Astle, and D. E. Harrison. 2000. Genetic regulation of primitive hematopoietic stem cell senescence. Exp Hematol 28:442-450.
175. Harrison, D., C. Astle, and M. Stone. 1989. Numbers and functions of transplantable primitive immunohematopoietic stem cells. Effects of age. The Journal of Immunology 142:3833-3840.
176. Van der Put, E., D. Frasca, A. M. King, B. B. Blomberg, and R. L. Riley. 2004. Decreased E47 in senescent B cell precursors is stage specific and regulated posttranslationally by protein turnover. The Journal of Immunology 173:818-827.
177. Riley, R. L., E. Van der Put, A. M. King, D. Frasca, and B. B. CBlomberg. 2005. Deficient B lymphopoiesis in murine senescence: potential roles for dysregulation of E2A, Pax-5, and STAT5. Seminars in Immunology 17:330-336.
178. Yoshida, T., S. Y.-M. Ng, J. C. Zuniga-Pflucker, and K. Georgopoulos. 2006. Early hematopoietic lineage restrictions directed by Ikaros. Nature Immunology 7:382-391.
179. Miller, J. P., and D. Allman. 2003. The decline in B lymphopoiesis in aged mice reflects loss of very early B-lineage precursors. The Journal of Immunology 171:2326-2330.
180. Harrison, D. E. 1983. Long-term erythropoietic repopulating ability of old, young, and fetal stem cells. J Exp Med 157:1496-1504.
181. Rossi, D., D. Bryder, J. Seita, A. Nussenzweig, J. Hoeijmakers, and I. Weissman. 2007. Deficiencies in DNA damage repair limit the function of haematopoietic stem cells with age. Nature 447:725-729.
182. Park, I.-k., D. Qian, M. Kiel, M. W. Becker, M. Pihalja, I. L. Weissman, S. J. Morrison, and M. F. Clarke. 2003. Bmi-1 is required for maintenance of adult self-renewing haematopoietic stem cells. Nature 423:302-305.
183. Krishnamurthy, J., C. Torrice, M. R. Ramsey, G. I. Kovalev, K. Al-Regaiey, L. Su, and N. E. Sharpless. 2004. Ink4a/Arf expression is a biomarker of aging. J Clin Invest 114:1299-1307.
184. Signer, R. A., E. Montecino-Rodriguez, O. N. Witte, and K. Dorshkind. 2008. Aging and cancer resistance in lymphoid progenitors are linked processes conferred by p16Ink4a and Arf. Genes & Development 22:3115-3120.

185. Shanley, D., D. Aw, N. Manley, and D. Palmer. 2009. An evolutionary perspective on the mechanisms of immunosenescence. Trends in Immunology 30:374-381.
186. Geiger, H., A. Koehler, and M. Gunzer. 2007. Stem cells, aging, niche, adhesion and Cdc42: a model for changes in cell-cell interactions and hematopoietic stem cell aging. Cell cycle 6:884-887.
187. Chambers, S. M., C. A. Shaw, C. Gatza, C. J. Fisk, L. A. Donehower, and M. A. Goodell. 2007. Aging Hematopoietic Stem Cells Decline in Function and Exhibit Epigenetic Dysregulation. Plos Biol 5:e201.
188. Mackall, C. C. L., and R. R. E. Gress. 1997. Thymic aging and T-cell regeneration. Immunological Reviews 160:91-102.
189. Zediak, V., I. Maillard, and A. Bhandoola. 2007. Multiple prethymic defects underlie age-related loss of T progenitor competence. Blood 110:1161-1167.
190. Gui, J., X. Zhu, J. Dohkan, L. Cheng, P. F. Barnes, and D. M. Su. 2007. The aged thymus shows normal recruitment of lymphohematopoietic progenitors but has defects in thymic epithelial cells. International Immunology 19:1201-1211.
191. Zhu, X., J. Gui, J. Dohkan, L. Cheng, P. Barnes, and D. Su. 2007. Lymphohematopoietic progenitors do not have a synchronized defect with age-related thymic involution. Aging Cell 6:663-672.
192. Schwarz, B. A. 2007. Selective thymus settling regulated by cytokine and chemokine receptors. The Journal of Immunology 178:2008.
193. Gossens, K., S. Naus, S. Y. Corbel, S. Lin, F. M. V. Rossi, J. Kast, and H. J. Ziltener. 2009. Thymic progenitor homing and lymphocyte homeostasis are linked via S1P-controlled expression of thymic P-selectin/CCL25. Journal of Experimental Medicine 206:761-778.
194. Chidgey, A., J. Dudakov, N. Seach, and R. Boyd. 2007. Impact of niche aging on thymic regeneration and immune reconstitution. Semin Immunol 19:331-340.
195. Kimura, T., A. M. Boehmler, G. Seitz, S. Kuci, T. Wiesner, V. Brinkmann, L. Kanz, and R. Mohler. 2004. The sphingosine 1-phosphate receptor agonist FTY720 supports CXCR4-dependent migration and bone marrow homing of human CD34+ progenitor cells. Blood 103:4478-4486.
196. Massberg, S., P. Schaerli, I. Knezevic-Maramica, M. Kollngerger, N. Tubo, E. Moseman, I. Huff, T. Junt, A. Wagers, I. Mazo, and U. von Andrian. 2007. Immunosurveillance by hematopoietic progenitor cells trafficking through blood, lymph, and peripheral tissues. Cell 131:994-1008.

197. Mauch, P., L. Constine, J. Greenberger, W. Knospe, J. Sullivan, J. Liesveld, and H. Deeg. 1995. Hematopoietic stem cell compartment: acute and late effects of radiation therapy and chemotherapy. Int J Radiat Oncol Biol Phys 31:1319-1339.

198. Mackall, C. L., T. A. Fleisher, M. R. Brown, I. T. Magrath, A. T. Shad, M. E. Horowitz, L. H. Wexler, M. A. Adde, L. L. McClure, and R. E. Gress. 1994. Lymphocyte depletion during treatment with intensive chemotherapy for cancer. Blood 84:2221-2228.

199. Mackall, C. L., T. A. Fleisher, M. R. Brown, M. P. Andrich, C. C. Chen, I. M. Feuerstein, M. E. Horowitz, I. T. Magrath, A. T. Shad, and S. M. Steinberg. 1995. Age, thymopoiesis, and CD4+ T-lymphocyte regeneration after intensive chemotherapy. New England Journal of Medicine 332:143-149.

200. Hakim, F. T., S. A. Memon, R. Cepeda, E. C. Jones, C. K. Chow, C. Kasten-Sportes, J. Odom, B. A. Vance, B. L. Christensen, C. L. Mackall, and R. E. Gress. 2005. Age-dependent incidence, time course, and consequence of thymic renewal in adults. Journal of Clinical Investigation 115:930 - 939.

201. Li, F., F. Jin, A. A. Freitas, P. Szabo, and M. E. Weksler. 2001. Impaired regeneration of the peripheral B cell repertoire from bone marrow following lymphopenia in old mice. European Journal of Immunology 31:500-505.

202. Gardner, R. V., E. McKinnon, and C. M. Astle. 2001. Analysis of the stem cell sparing properties of cyclophosphamide. European Journal of Haematology 67:14-22.

203. Meng, A., Y. Wang, G. Van Zant, and D. Zhou. 2003. Ionizing radiation and busulfan induce premature senescence in murine bone marrow hematopoietic cells. Cancer Research 63:5414-5419.

204. Fliedner, T. M., D. Graessle, C. Paulsen, and K. Reimers. 2002. Structure and function of bone marrow hemopoiesis: mechanisms of response to ionizing radiation exposure. Cancer Biother Radiopharm 17:405-426.

205. Kopp, H. G., A. T. Hooper, S. T. Avecilla, and S. Rafii. 2009. Functional heterogeneity of the bone marrow vascular niche. Annals of the New York Academy of Sciences 1176:47-54.

206. Davies, J. H., B. A. J. Evans, M. E. M. Jenney, and J. W. Gregory. 2002. In vitro effects of combination chemotherapy on osteoblasts: implications for osteopenia in childhood malignancy. Bone 31:319-326.

207. Domenech, J., E. Gihana, A. Dayan, D. Truglio, C. Linassier, I. Desbois, J. P. Lamagnere, P. Colombat, and C. Binet. 1994. Haemopoiesis of transplanted patients

with autologous marrows assessed by long-term marrow culture. British journal of haematology 88:488-496.

208. Nagasawa, T. 2006. Microenvironmental niches in the bone marrow required for B-cell development. Nat Rev Immunol 6:107-116.

209. Alves, N. L., O. Richard-Le Goff, N. D. Huntington, A. P. Sousa, V. S. Ribeiro, A. Bordack, F. L. Vives, L. Peduto, A. P. Chidgey, A. Cumano, R. Boyd, G. Eberl, and J. P. Di Santo. 2009. Characterization of the thymic IL-7 niche in vivo. Proceedings of the National Academy of Science USA 106:1512-1517.

210. Bhatia, S. K., L. T. Tygrett, K. H. Grabstein, and T. J. Waldschmidt. 1995. The effect of in vivo IL-7 deprivation on T cell maturation. The Journal of Experimental Medicine 181:1399-1409.

211. Beq, S., M. Nugeyre, R. Ho Tsong Fang, D. Gautier, R. Legrand, N. Schmitt, J. Estaquier, F. Barre-Sinoussi, B. Hurtrel, R. Cheynier, and N. Israel. 2006. IL-7 induces immunological improvement in SIV-infected rhesus macaques under antiviral therapy. Journal of Immunology 176:914-922.

212. Mackall, C. L., T. J. Fry, C. Bare, P. Morgan, A. Galbraith, and R. E. Gress. 2001. IL-7 increases both thymic-dependent and thymic-independent T-cell regeneration after bone marrow transplantation. Blood 97:1491-1497.

213. Fry, T. J., M. Moniuszko, S. Creekmore, S. J. Donohue, D. C. Douek, S. Giardina, T. T. Hecht, B. J. Hill, K. L. Komschlies, J. Tomaszewski, G. Franchini, and C. L. Mackall. 2003. IL-7 therapy dramatically alters peripheral T-cell homeostasis in normal and SIV-infected nonhuman primates. Blood 101:2294-2299.

214. Sportès, C., F. T. Hakim, S. A. Memon, H. Zhang, K. S. Chua, M. R. Brown, T. A. Fleisher, M. C. Krumlauf, R. R. Babb, C. K. Chow, T. J. Fry, J. Engels, R. Buffet, M. Morre, R. J. Amato, D. J. Venzon, R. Korngold, A. Pecora, R. E. Gress, and C. L. Mackall. 2008. Administration of rhIL-7 in humans increases in vivo TCR repertoire diversity by preferential expansion of naive T cell subsets. The Journal of Experimental Medicine 205:1701-1714.

215. Wils, E. J., E. Braakman, G. M. Verjans, E. J. Rombouts, A. E. Broers, H. G. Niesters, G. Wagemaker, F. J. Staal, B. Lowenberg, H. Spits, and J. J. Cornelissen. 2007. Flt3 ligand expands lymphoid progenitors prior to recovery of thymopoiesis and accelerates T cell reconstitution after bone marrow transplantation. J Immunol 178:3551-3557.

216. Balciunaite, G., R. Ceredig, S. Massa, and A. G. Rolink. 2005. A B220+ CD117+ CD19±hematopoietic progenitor with potent lymphoid and myeloid developmental potential. European Journal of Immunology 35:2019-2030.

217. Ceredig, R., M. Rauch, G. Balciunaite, and A. G. Rolink. 2006. Increasing Flt3L availability alters composition of a novel bone marrow lymphoid progenitor compartment. Blood 108:1216-1222.

218. Rossi, S. W., L. T. Jeker, T. Ueno, S. Kuse, M. P. Keller, S. Zuklys, A. V. Gudkov, Y. Takahama, W. Krenger, B. R. Blazar, and G. A. Hollander. 2007. Keratinocyte growth factor (KGF) enhances postnatal T-cell development via enhancements in proliferation and function of thymic epithelial cells. Blood 109:3803-3811.

219. Min, D., A. Panoskaltsis-Mortari, M. Kuro-O, G. A. Hollander, B. R. Blazar, and K. I. Weinberg. 2007. Sustained thymopoiesis and improvement in functional immunity induced by exogenous KGF administration in murine models of aging. Blood 109:2529-2537.

220. Carlo-Stella, C., M. Di Nicola, R. Milani, P. Longoni, M. Milanesi, C. Bifulco, C. Stucchi, A. Guidetti, L. Cleris, F. Formelli, G. Garotta, and A. M. Gianni. 2004. Age- and irradiation-associated loss of bone marrow hematopoietic function in mice is reversed by recombinant human growth hormone. Experimental Hematology 32:171-178.

221. Alpdogan, O., S. J. Muriglan, B. J. Kappel, E. Doubrovina, C. Schmaltz, R. Schiro, J. M. Eng, A. S. Greenberg, L. M. Willis, J. A. Rotolo, R. J. O'Reilly, and M. R. M. van den Brink. 2003. Insulin-like growth factor-I enhances lymphoid and myeloid reconstitution after allogeneic bone marrow transplantation. Transplantation 75:1977-1983.

222. Moretti, R. M., M. Monagnani Marelli, J. C. van Groeninghen, M. Motta, and P. Limonta. 2003. Inhibitory activity of luteinizing hormone-releasing hormone on tumor growth and progression. Endocr Relat Cancer 10:161-167.

223. Dudakov, J. A., G. L. Goldberg, J. J. Reiseger, A. P. Chidgey, and R. L. Boyd. 2009. Withdrawal of Sex Steroids Reverses Age- and Chemotherapy-Related Defects in Bone Marrow Lymphopoiesis. The Journal of Immunology 182:6247-6260.

224. Dudakov, J. A., G. L. Goldberg, J. J. Reiseger, K. Vlahos, A. P. Chidgey, and R. L. Boyd. 2009. Sex Steroid Ablation Enhances Hematopoietic Recovery following Cytotoxic Antineoplastic Therapy in Aged Mice. The Journal of Immunology 183:7084-7094.

225. Goldberg, G. L., J. A. Dudakov, J. J. Reiseger, N. Seach, T. Ueno, K. Vlahos, M. V. Hammett, L. F. Young, T. S. Heng, R. L. Boyd, and A. P. Chidgey. 2010. Sex Steroid Ablation Enhances Immune Reconstitution Following Cytotoxic Antineoplastic Therapy in Young Mice. The Journal of Immunology 184:6014-6024.

226. Goldberg, G. L., J. S. Sutherland, M. V. Hammett, M. K. Milton, T. S. Heng, A. P. Chidgey, and R. L. Boyd. 2005. Sex steroid ablation enhances lymphoid recovery following autologous hematopoietic stem cell transplantation. Transplantation 80:1604-1613.

227. Heng, T. S. P., G. L. Goldberg, D. H. D. Gray, J. S. Sutherland, A. P. Chidgey, and R. L. Boyd. 2005. Effects of castration on thymocyte development in two different models of thymic involution. J Immunol 175:2982-2993.

228. Goldberg, G. L., C. G. King, R. A. Nejat, D. Y. Suh, O. M. Smith, J. C. Bretz, R. M. Samstein, J. A. Dudakov, A. P. Chidgey, S. Chen-Kiang, R. L. Boyd, and M. R. M. van den Brink. 2009. Luteinizing hormone-releasing hormone enhances T cell recovery following allogeneic bone marrow transplantation. J Immunol 182:5846-5854.

Chapter 2

Immune regeneration mediated by enhanced hematopoietic stem and progenitor cell function

Manuscript information

The following manuscript is being prepared to submit to the journal *Cell Stem Cell*. The candidate, Danika Khong, was responsible for planning, performing and analysing experiments as well as writing and editing the manuscript. The co-authors Jarrod Dudakov, Maree Hammett, Lisa Spyroglou, Richard Boyd and Ann Chidgey assisted with the experimental design, technical assistance, interpretation of results and drafting of the manuscript. Proportional contributions explained in the signed declaration.

MANUSCRIPT

Immune regeneration mediated by enhanced hematopoietic stem and progenitor cell function

Danika M.P. Khong[1,3,4] and Jarrod A. Dudakov[1,2,4*], Maree V. Hammett[1], Lisa Spyroglou[1], Richard L. Boyd[1,4*] and Ann P. Chidgey[1,4]

[1] Immune Regeneration Laboratory, Monash Immunology and Stem Cell Laboratories (MISCL), Monash University, Wellington Rd, Clayton, Australia, 3800

[2] Current Address: Department of Immunology, Memorial Sloan-Kettering Cancer Center, 1275 York Ave, New York, NY, USA 10065

[3] Current Address: Harvard Stem Cell Institute, Holyoke Center, Suite 727W, 1350 Massachusetts Avenue, Cambridge, MA, USA 02138

[4] These authors contributed equally to this work.

* Prof. Richard L. Boyd, P: [redacted] F: +[redacted]

Abbreviations used in this paper: Ang, Angiopoietin-1; BM, bone marrow; BMP, bone morphogenic protein; BMT, Bone Marrow Transplant; CLP, Common Lymphoid Progenitor; Cy, cyclophosphamide; ETP, early T-lineage progenitor; HSC, Haematopoietic Stem Cell; IGF, insulin-like growth factor, LMPP, lymphoid-primed multipotent progenitor; LT-HSC, long-term hematopoietic stem cell; LSK, $Lin^{-}Sca1^{+}ckit^{+}$; MPP, multipotent progenitor; Opn, osteopontin; OBL, osteoblast; shSSA, sham sex steroid ablation; SSA, Sex Steroid Ablation; ST-HSC, short-term hematopoietic stem cell; WBM, whole bone marrow

Keywords – BM, lymphopoiesis, hematopoiesis, hematopoietic stem cell

2.1 ABSTRACT

There is clearly a major unmet clinical need to restore lymphopoiesis as a prelude to overcoming the clinical manifestations of chemotherapy-, radiation- and age-related immune system degeneration. Of the many potential aetiological factors, fundamental intrinsic defects within lymphoid progenitors and upstream hematopoietic stem cells may be contributing. We have previously shown that sex steroid ablation (SSA) rejuvenates thymic and BM lymphopoiesis and enhances recovery from HSC transplantation. In the present study we examined the impact of SSA on the earliest hematopoietic stem and progenitor cells. SSA increased the number of long-term HSCs and functionally enhanced their differentiation and self-renewal potential at the population level and per cell basis. Underlying this were intrinsic molecular changes in long-term hematopoietic stem cells (LT-HSCs), most significantly upregulation of molecules associated with HSC quiescence. Together these findings demonstrate a role for SSA in boosting HSC function and lay the groundwork for improving outcomes in HSC transplantation.

Highlights

- Withdrawal of sex steroids enhances the number of hematopoietic stem and progenitor cells, primarily restricted to the vascular niche
- Prior to expanding in number, functionally these cells have significantly enhanced lymphoid differentiation, self-renewal and hematopoietic repopulation potential.

2.2 Introduction

With age there is a widely accepted chronic progressive decline in immune system function (Dorshkind et al., 2009). This deterioration is most evident through increased levels of opportunistic infections and poor responsiveness to vaccines. At its origin is a reduction in naïve T cells, in turn derived from waning lymphopoiesis in both the thymus (Rodewald, 1998) and ultimately bone marrow (BM) (Miller and Allman, 2003). There is also a reduction with age in the number and function of bone marrow (Min et al., 2006) and intrathymic lymphoid progenitors (Min et al., 2004) and, moreover intrinsic changes to hematopoietic stem cells (HSCs) contribute towards immune degeneration with age (Guerrettaz et al., 2008).

Paradoxically, there are up to five times the number of HSCs in aged mice (Rossi et al., 2005; Sudo et al., 2000), however, functionally they exhibit only one quarter the homing and engraftment capacity as HSCs from young mice (Liang et al., 2005) and are less able to reconstitute the immune system (Kim et al., 2003; Morrison et al., 1996; Rossi et al., 2005; Sudo et al., 2000). In particular, these studies highlighted defects in B cell reconstitution, suggesting that age-related lymphoid deterioration may be, at least in part, due to decline in HSC function (Guerrettaz et al., 2008). The mechanisms behind these intrinsic HSC changes with age are poorly understood. However, an upregulation of myeloid-, at the expense of lymphoid-, associated genes (Rossi et al., 2005), accumulation of mitochondrial and genomic DNA mutations (Geiger et al., 2005; Yao et al., 2007), deficiency in DNA repair (Rossi et al., 2007) and altered p53 expression (Dumble et al., 2007), all contribute to altered HSC function with age. These studies support the hypothesis that aged HSCs are functionally biased with predominantly myeloid lineage capability (Muller-Sieburg et al.,

2004; Signer et al., 2007). HSCs from aged patients also have a reduced ability to form T cells *in vitro* (Offner et al., 1999), albeit in an artificial environment, and exhibit a reduced proliferative potential (Vaziri et al., 1994). Furthermore, patients receiving HSCs from young donors had a better chance at disease-free survival than patients who received HSCs from older donors (Carreras et al., 2006).

Previous studies have demonstrated that sex steroids play a major role in the degeneration of lymphopoiesis in the thymus (Olsen et al., 2001b) and BM (Olsen et al., 2001a), at least partially through effects on the supporting microenvironment. These studies, however, failed to examine the effect on hematopoietic progenitor cells, likely to be pertinent considering other work has shown that exogenously administered estrogen is a potent negative regulator of lymphoid commitment at the level of the HSC (Thurmond et al., 2000) and lymphoid-primed multipotent progenitor (LMPP) cells (Medina et al., 2001). We, and others, have previously shown that SSA is able to regenerate aged and immunodepleted BM and thymus, enhance peripheral T and B cell function and promote recovery from autologous and allogeneic HSC transplant (Chidgey et al., 2007; Chidgey et al., 2008; Dudakov et al., 2009a; Dudakov et al., 2009b; Goldberg et al., 2010). The mechanisms behind this regeneration have yet to be fully elucidated.

While age-induced reduction in HSC function does not seem to reach its nadir until at least 24 months of age in mice, it is clear that significant defects in the capacity for T cell differentiation at least, are already evident by 9-mo due to the significant thymic atrophy observed with age (Chidgey et al., 2007; Dudakov et al., 2010). B cell genesis is also compromised at this age (Dudakov et al., 2009a). We sought to examine the events upstream of SSA-mediated thymic and BM lymphopoietic regeneration. We report here that

SSA induces a significant numerical increase in BM LT-HSCs and LMPPs as well as functionally enhancing their capacity for lymphoid differentiation, self-renewal and hematopoietic repopulation potential. These findings suggest that SSA-mediated regeneration of lymphopoiesis is predicated on enhanced HSC function.

2.3 Materials and Methods

2.3.1 Animals

Inbred C57Bl/6 or Ly5.1 mice were obtained from the Animal Resources Centre (Perth, Australia), Monash Animal Services (Clayton, Australia) or the Baker Institute Precinct Animal Centre (Prahran, Australia). IL7R$\alpha^{-/-}$ animals were a gift from A. Strasser (Walter and Eliza Hall Institute, Melbourne). Young mice were between 6-8 weeks old and aged mice were 9-12 months old in this study. Mice were maintained at the Precinct Animal Centre (Prahran, Australia), Mouseworks (Clayton, Australia) or Monash Animal Services (Clayton, Australia). Animals were allowed to acclimatize for at least 2 days before experimentation, which was performed according to Monash University animal experiment ethics committee guidelines and which approved these studies.

2.3.2 Surgical Castration

Mice were anaesthetized and a small incision was made in the scrotum. The testes were exposed, ligated with dissolvable sutures, and removed. The wound was closed with dissolvable sutures. For surgical stress control, shSSA was performed as above, but without removal of the testes.

2.3.3 Cell Suspensions

2.3.3.1 Vascular (central marrow) Fraction

Individual or pooled single cell suspensions of freshly dissected BM were obtained by flushing tibias and femurs with cold FACS buffer (PBS supplemented with 0.2% v/v BSA and 0.5mM EDTA). Bone ends were crushed in a mortar and pestle with FACS buffer, the

crushed ends washed to remove contaminating marrow and the wash added to the flushed vascular cells.

2.3.3.2 Endosteal Fraction

Flushed bones were crushed and added to the washed crushed ends with 0.3% (w/v) Collagenase/Dispase (Roche, Germany) and 0.1% (w/v) DNAse I (Roche, Germany) in RPMI-1640 for digestion. Bones were incubated for 40 minutes total at 37°C in a water bath, mixing the cells at 20 minutes.

Both fractions were lysed of red blood cells in pre-warmed ammonium chloride (0.15M) in 0.01M Tris-EDTA in RO-water for 1-3 minutes at 37°C. Cell counts were determined by gating viable cells based on cell size using a Z2 Coulter Counter (Beckman Coulter).

2.3.4 Flow Cytometric Analysis

The following fluorochrome labelled antibodies against Murine antigens (as well as appropriate isotype controls) were used: FITC-conjugated anti-CD34 (RAM34), PE-conjugated anti-CD27 (LG.3A10), PE-Cy5-conjugated anti-CD135 (A2F10.1), APC-conjugated anti-CD117 (2B8), PE or PE-Cy7-conjugated anti-Sca1 (D7), APC-Alexa Fluor 750-conjugated anti-CD62L (MEL-14), Pacific Blue-conjugated CD44 (IM7), biotin-conjugated anti-CD184 (2B11/CXCR4), biotin-conjugated anti-CD49d (9C10), biotin-conjugated anti-CD49e (5H10-27), FITC, eFluor450, PE-Cy7 or biotin-conjugated anti-CD45.1 (A20), FITC or biotin-conjugated anti-CD45.2 (104). Secondary reagents used were Fitc, PercP, PE-Cy7 or Pacific Orange-conjugated to streptavidin. Lineage cocktail consisted of the following

antibodies conjugated to Biotin, FITC or PE: anti-CD3 (145-2C11), anti-CD4 (GK1.5), anti-CD8α (53-6.7), anti-CD19 (1D3), anti-CD11b (M1/70), anti-CD11c (HL3), anti-Gr-1 (RB6-8C5) and anti-B220 (RA3-6B2). B220 was not added for CLP stains. BM stromal populations were analysed using Percp-Cy5.5 or APC-conjugated anti-CD45 (30-F11), Fitc, PE, Percp-Cy5.5 or APC-conjugated anti-TER119 (TER119), PE-conjugated anti-CD51 (RMU-7), Fitc-conjugated anti-CD61 (2C9.G2), APC-conjugated anti-CD31 (MEC 13.3). All conjugates were purchased from eBiosciences or BD Biosciences except streptavidin-Pacific Orange, which was purchased from Molecular Probes. All cells were analysed or sorted on multiparameter FACSCalibur, FACSCanto II, FACSVantage, FACSAria or Influx (BD Biosciences) cytometers and cell sorters using CellQuest Pro (BD Biosciences) or FlowJo software (Treestar Software).

2.3.5 Competitive Repopulation Analysis

Donor cells were harvested and pooled from untreated CD45.1 2-mo; 9-12mo $CD45.1^+$ animals 7 days following surgical shSSA (d7shSSA); or 9-12mo $CD45.1^+$ animals 7 days following surgical SSA (d7SSA). Competitor cells were harvested from untreated 2-mo $CD45.2^+$ congenic animals. 2.5 x 10^6 donor whole bone marrow (WBM) cells or 2000 purified LSK cells were mixed with 2 or 2.5 x 10^6 competitor cells and injected into each lethally irradiated recipient mouse.

For serial transplantation donor mice were sacrificed and their BM individually suspended and transferred so that there were two recipients/individual donor at a dose of 2.5 x 10^6 WBM cells along with an equal dose of untreated $CD45.2^+$ 2-mo competitor cells. Primary recipients were sacrificed at 12 weeks after transplant and their BM individually suspended and transferred so that there was one recipient/individual donor at a dose of 2.5 x 10^6 WBM

cells along with an equal dose of untreated 2-mo CD45.2^{+} competitor cells. Secondary recipients were sacrificed at 12 weeks following transplant. Recipient C57Bl/6J (CD45.2) mice were lethally irradiated with a dose of 1100cGy total body irradiation (^{137}Cs source) as a split dose (2 x 550cGy) separated by 3 hours. At least 1 hour after the final irradiation, 100μL, comprising 4-5 x 10^{6} total BM cells in RPMI, was injected into the tail vein of recipient mice. Recipients were provided with antibiotics (Baytril) in water for 2 weeks after irradiation. At staged timepoints after transplant, recipients were humanely killed and thymus, spleen and BM were harvested for flow cytometric analysis.

Limit-dilution competitive repopulation analysis was performed. Cell doses of 10, 100 or 1000 Lin^{-}Sca1^{+}ckit^{+} (LSK) cells were FACS purified from vascular (central marrow) fractions of untreated CD45.2^{+} 2-mo; 9-12mo CD45.2^{+} animals 7 days following surgical shSSA (d7shSSA); or 9-12mo CD45.2^{+} animals 7 days following surgical SSA (d7SSA) were competed against 5 x 10^{5} CD45.1^{+} WBM cells were transplanted into six lethally irradiated (2 x 550cGy) CD45.1 recipients/cell dose/treatment. Peripheral blood and spleen were analysed 12 weeks after transplant and competitive repopulation unit (CRU) frequencies were calculated by L-Calc software (Stem Cell Technologies, Vancouver) using Poisson statistical analysis. Multilineage reconstitution was taken as greater than 1% donor derived B cells, T cells, granulocytes and macrophages as measured by expression of B220, TCRβ, Gr-1 and Mac-1. Bipotent progenitor frequency was determined as greater than 1% reconstitution in T or B cells and either granulocytes or macrophages.

2.3.6 Short-term Homing Assays

Donor cells were individually harvested from untreated CD45.1 2-mo; 9-12mo CD45.1^{+} animals 7 days following surgical shSSA (d7shSSA); or 9-12mo CD45.1^{+} animals 7 days following surgical SSA (d7SSA) and a saturating dose of 20 x 10^{6} WBM cells were transferred into untreated (no myeloablation) 2-mo C57Bl/6J (CD45.2^{+}); 9-12mo C57Bl/6J (CD45.2^{+}); or 2-mo IL7R$\alpha^{-/-}$ (CD45.2^{+}) recipient animals such that one recipient/donor. Recipient animals were sacrificed 40 hours after transfer and engraftment in the BM of donor CD34^{-}Flt3^{-} LSK cells (LT-HSCs) was examined by flow cytometry.

2.3.7 OP9-DL1 Cultures

OP9-DL1 were maintained and co-cultured as per the protocol described previously (Schmitt and Zúñiga-Pflücker, 2006). LT-HSCs (8X10^{3} to 1X10^{4}) were sorted from d7ShSSA and d7SSA mice and cultured on GFP OP9-DL1 for 12 days in the presence of 5ng/mL of each mouse Flt3 ligand and mouse IL7 purchased from Miltenyi Biotec. Cells were analyzed by flow cytometry based on CD44 and CD25 expression after removal of all lineage (CD4, CD8, CD3, B220,Gr1, CD11b, TER119, NK1.1) and GFP positive cells.

2.3.8 Microarray Analysis

30000-50000 LT-HSCs were purified by lineage depletion of whole BM from 9mo ShSSA and SSA mice 2 days post surgical castration, using the Miltenyi Lineage Depletion Kit (Miltenyi Biotechnology, Germany). Depleted cells were then enriched by FACS for LT-HSCs (LSK CD34^{-}Flt3^{-}).

Mouse total RNA was quality ascertained using the Agilent Bioanalyser 2100 using the NanoChip protocol. A total of 500ng was labelled using the Ambion Total Prep RNA amplification kit (Cat. No. – IL1791). A total of 1.5ug of labelled cRNA was then prepared for hybridisation to the Mouse WG-6_V2 array (Illumina, www.illumina.com) by preparing a probe cocktail (cRNA @ 0.05ug/ul) that includes GEX-HYB Hybridisation Buffer (supplied with the beadchip).

A total hybridisation volume of 30ul is prepared for each sample and 30ul loaded into a single array on the Mouse WG-6_V2 array. A total of 6 different labelled samples can be loaded into 6 individual arrays per beadchip. The chip is hybridised at 58 °C for 16 hours in an oven with a rocking platform. After hybridisation, the chip is washed using the appropriate protocols as outlined in the Illumina manual. Upon completion of the washing, the chips are then coupled with Cy3 and scanned on Illumina iScan scanner. The scanner operating software, GenomeStudio, converts the signal on the array into a TXT file for analysis.

2.3.8.1 Gene Expression and Pathway Analysis:

Raw data were imported into Partek v 6.5 (www.Partek.com). Data below a value of 50 were floored to an expression level of 50, which was about 50% of background signal intensity. Thereafter, data were filtered by coefficient of variance, which had to be at least 20%. Then, data were log2 transformed, and quantile normalized. Testing for statistical significance was performed by applying ANOVA. Probe sets with unadjusted p-value of less than 0.05 and a fold change of at least 1.5 were considered to be differentially expressed genes (DEG). DEGs

were used for hierarchical cluster analysis, using squared Euclidean and single linkage as distance measure.

Pathway analysis on the set of differentially expressed genes was performed with GO ANOVA (Gene Ontology ANOVA) (Partek v 6.5). In the present analysis we restricted our focus on pathways with p-values smaller than 0.05. Those pathways were then clustered using Euclidean distance and average linkage.

Microarray processing and initial bioinformatics analyses using Partek Genomic Suite and R/Bioconductor, were conducted by the Australian Genome Research Facility Ltd (AGRF; Melbourne).

2.3.9 Statistical Analysis

Statistical analysis was performed with the nonparametric, unpaired Mann-Whitney *U* test. Limiting dilution CRU frequencies were calculated by L-calc software (Stem Cell Technologies, Vancouver), using Poisson statistics (2-tailed test). Comparative analysis of relative gene expression in SSA animals was performed using the students t test. All figures follow the convention */^$p < 0.05$, **/^^$p < 0.01$, ***/^^^$p < 0.001$.

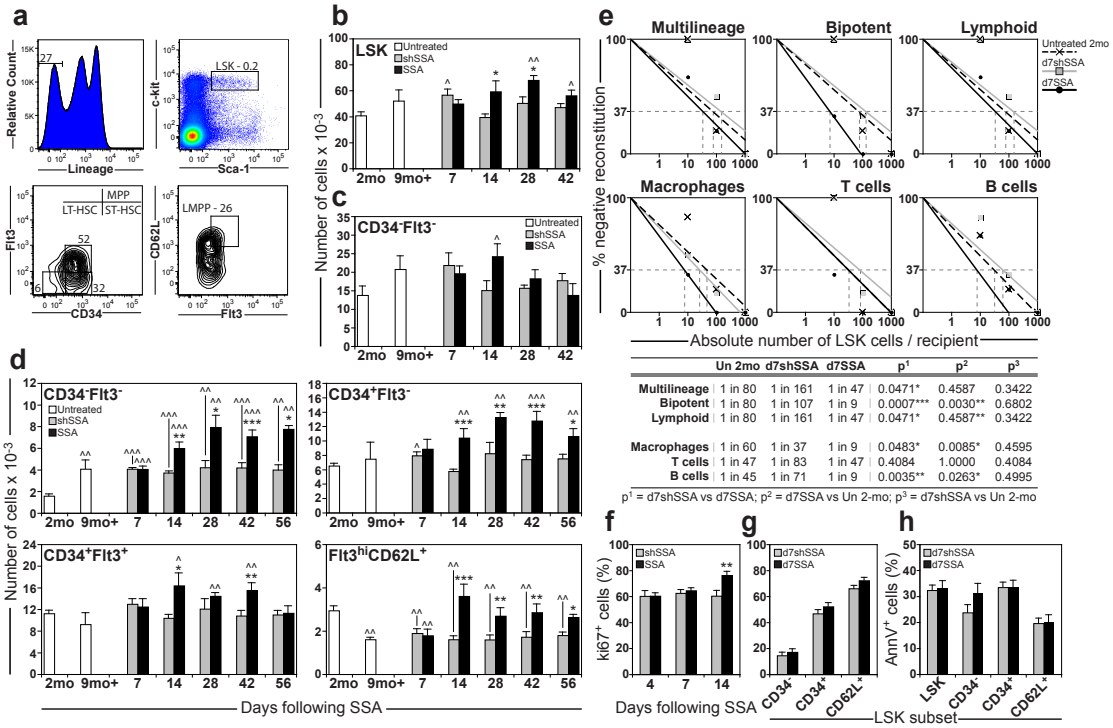
2.4 RESULTS

2.4.1 *SSA Increases the Number of Hematopoietic Stem and Progenitor Cells by Phenotype and Lineage Potential*

Mice (9-12mo) were surgically castrated and the endosteum and central marrow (vascular fraction) enumerated for HSCs at multiple timepoints up to 8 weeks following SSA, by multiparameter flow cytometry (Figure 2.1 a). Pooling both endosteal and vascular fractions there was no change in the total number of LSK cells by 9-12mo (Figure 1B) and only minimal impact of SSA. However, significant changes were found when the subsets were analysed. While there was no change in the number of $CD34^{-}Flt3^{-}$ LT-HSCs located in the endosteal region (Figure 2.1 c), following SSA there was a significant increase in LT-HSCs in the vascular niche from day 14 and maintained through to day 56 compared to sham-SSA (shSSA) control animals (Figure 2.1 d). SSA also caused a significant and sustained increase in $CD34^{+}Flt3^{-}$ ST-HSCs restricted to the vascular niche. While there was no observable impact of age or SSA on the total number of $CD34^{+}Flt3^{+}$ MPP cells in the vascular niche, there was a selective decrease in $Flt3^{hi}CD62L^{+}$ LMPPs by 9-12mo, which was reversed following SSA.

A defining characteristic of HSC function is the ability to differentiate into multiple lineages. The frequency of multilineage repopulating cells was enumerated using a limiting-dilution competitive repopulation assay. 10, 100 or 1000 FACS sorted vascular LSK cells from untreated 2-mo, 9-12mo animals 7 days following surgical shSSA (d7shSSA), or 9-12mo animals 7 days following surgical SSA (d7SSA) were transferred along with 5×10^5 supporting BM cells into lethally irradiated congenic recipients and peripheral reconstitution measured 12 weeks after transplant. Mice were considered reconstituted by a single HSC if there was ≥1% contribution of donor cells (Figure 2.1 e). While there was no

Figure 2.1 SSA increases the number of LT-HSCs. (a) $Lin^{-}Sca1^{+}ckit^{+}$ (LSK) BM can be subdivided into populations of LT-HSCs ($CD34^{-}Flt3^{-}$), ST-HSCs ($CD34^{+}Flt3^{-}$) and MPPs ($CD34^{+}Flt3^{+}$). The MPP population was further fractionated based on Flt3 and CD62L expression for analysis of LMPPs ($Flt3^{hi}CD62L^{+}$). Shown are representative flow cytometry profiles from an untreated 2-mo mouse to illustrate gating strategies. **(b)** Absolute number of LSK cells in both endosteal and vascular fractions of BM from 2-mo and 9-mo untreated (□), 9mo-sham-SSA (▢) or 9-mo SSA (■) mice at various timepoints after surgical SSA (n=5/group/timepoint). **(c)** Absolute number of endosteal $CD34^{-}Flt3^{-}$ LT-HSCs (n=5/group/timepoint). **(d)** Absolute number of $CD34^{-}Flt3^{-}$ LT-HSCs, $CD34^{+}Flt3^{-}$ ST-HSCs, $CD34^{+}Flt3^{+}$ MPPs and $CD34^{+}Flt3^{hi}CD62L^{+}$ LMPPs in vascular BM (n=10/group/timepoint). **(e)** LSK cells from untreated $CD45.2^{+}$ 2-mo; $CD45.2^{+}$ 9-12mo animals 7 days following surgical shSSA (d7shSSA); or $CD45.2^{+}$ 9-12mo animals 7 days following surgical SSA (d7SSA) (n=6 recipients/group/dose) were FACS purified and graded doses of cells were transferred into lethally irradiated congenic CD45.1 recipients along with 5 x 10^{5} $CD45.1^{+}$ supporting BM cells. Reconstitution was analysed 12 weeks after transplant and the frequency of repopulating cells was calculated by Poisson statistics using L-Calc software (Stem Cell Technologies). **(f)** Proportion of $Ki67^{+}$ LSK cells 4, 7 and 14 days after surgical SSA in 9-12mo shSSA or SSA mice (n=10/treatment/timepoint). **(g)** Proportion of $Ki67^{+}$ cells 7 days after SSA in $CD34^{-}$ HSCs, $CD34^{+}CD62L^{-}$ MPPs and $CD34^{+}CD62L^{+}$ LMPPs (n=10/treatment). (H) Proportion of $AnnV^{+}$ cells 7 days after SSA in $CD34^{-}$ HSCs, $CD34^{+}CD62L^{-}$ MPPs and $CD34^{+}CD62L^{+}$ LMPPs (n=10/treatment). All data are expressed as mean ± SEM. Data are cumulative of at least two independent experiments. * compared with shSSA 9-12mo mice. ^ compared with 2-mo mice.



	Un 2mo	d7shSSA	d7SSA	p1	p2	p3
Multilineage	1 in 80	1 in 161	1 in 47	0.0471*	0.4587	0.3422
Bipotent	1 in 80	1 in 107	1 in 9	0.0007***	0.0030**	0.6802
Lymphoid	1 in 80	1 in 161	1 in 47	0.0471*	0.4587**	0.3422
Macrophages	1 in 60	1 in 37	1 in 9	0.0483*	0.0085*	0.4595
T cells	1 in 47	1 in 83	1 in 47	0.4084	1.0000	0.4084
B cells	1 in 45	1 in 71	1 in 9	0.0035**	0.0263*	0.4995

p1 = d7shSSA vs d7SSA; p2 = d7SSA vs Un 2-mo; p3 = d7shSSA vs Un 2-mo

statistical decrease in the frequency of multi-lineage repopulating cells with age, at day 7 following SSA there was a significant increase in the frequency of these cells compared to d7shSSA control mice. The frequency of bipotent LSK, or those restricted to the lymphoid lineage was similarly increased from d7SSA. Accordingly, when individual lineages were analysed for reconstitution, there was no change as a result of age, in the frequency of B, T or macrophage reconstituting cells, however, d7SSA had an increased frequency of B-lineage and macrophage reconstituting cells to 1 in 9 (compared to 1 in 71 and 1 in 37 respectively in d7shSSA). There was no change in the frequency of T-lineage or granulocyte reconstituting cells and, in fact, granulopoiesis was reconstituted in 100% of mice thereby precluding limited-dilution analysis (data not shown).

Ki67 and AnnV were used to determine if the increases in the number of HSCs in the vascular niche was due to a change in their cycling status and apoptosis respectively. Following SSA there was little change in Ki67 expression on total LSK cells at days 4 and 7, however, it was significantly increased by day 14 (Figure 2.1 f) in accord with increases in the total number of LSK cells. Within LSK subsets there was no change in Ki67 expression on $CD34^-$, $CD34^+$ or $CD34^+CD62L^+$ subsets at day 7 following SSA (Figure 2.1 g). Similarly, at day 7 there was no change in expression of AnnV (Figure 2.1 h).

2.4.2 Long-term Competitive Repopulation Reveals 9-mo BM is Significantly Enhanced Following SSA

Vascular BM (2.5×10^6 cells) from $CD45.1^+$ d7shSSA or d7SSA were co-transplanted at a 1:1 ratio with congenic untreated 2mo competitor BM into lethally irradiated $CD45.2^+$ recipients. Over the 17 weeks analysed, total $CD45.1^+$ donor-derived peripheral leukocyte reconstitution of animals transplanted with d7SSA BM was significantly higher than those

reconstituted by d7shSSA animals (Figures 2.2 a and b). Reflecting this, d7SSA-derived BM significantly improved B cell engraftment, with T cell engraftment transiently increased at 4

weeks after transplant compared to d7shSSA controls (Figure 2.2 c). Enhanced reconstitution by d7SSA was not restricted to the lymphoid compartment, with a significant improvement in myeloid engraftment also observed.

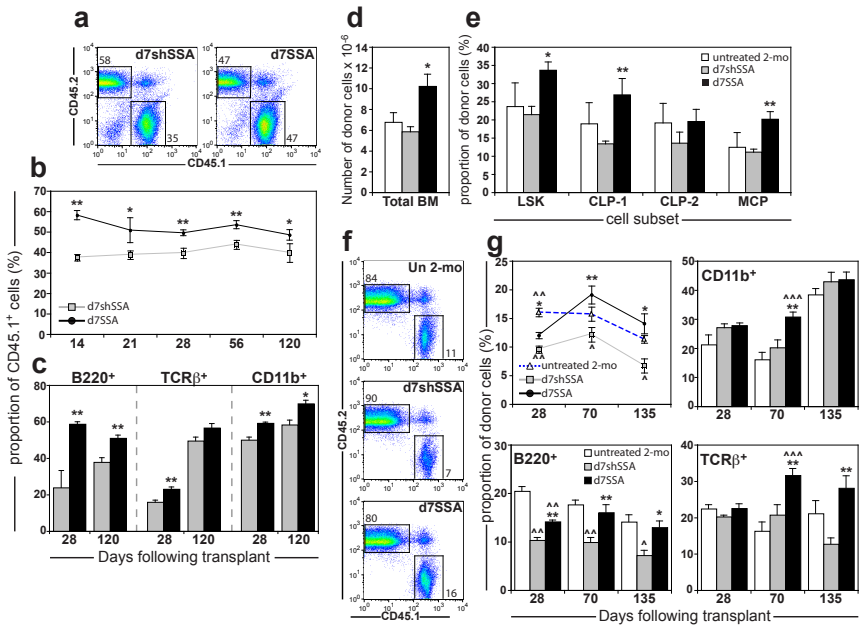
2.4.3 *Enhanced Long-term Repopulation Ability of BM Following SSA is Intrinsic to LSK Fraction of the Vascular Niche*

To assess the intrinsic impact on stem cells by SSA, 2000 purified LSK cells from untreated 2-mo, d7shSSA, or d7SSA (9-12mo) Ly5.1$^+$ mice were co-transplanted with 2 x 10^6 BM cells from untreated congenic competitors into lethally irradiated recipients. Reconstitution was measured up to 19 weeks after transplant. There was no age-induced impact at 9-12mo in the ability of LSK to engraft the BM 28 days after transplant; however, d7SSA-LSK significantly increased the number of CD45.1$^+$ cells in the marrow (Figure 2.2 d) including

LSK cells and downstream Lin$^-$Sca1$^-$ckit$^+$ myeloid progenitors and Lin$^-$IL7Rα^+ckit$^+$ CLP-1 populations (Figure 2.2 e).

Long-term peripheral repopulation was significantly impaired with 9-12mo d7shSSA compared to untreated 2-mo LSK (Figures 2.2 f and g). Over the same timeframe there was a significant increase in the repopulation potential of LSK cells derived from d7SSA mice compared to d7shSSA mice, restoring LSK repopulation potential to 2mo levels. Principally these consisted of improved B cell engraftment, but also improved T cell repopulation from day 70 after transplant. Although myeloid engraftment increased with age, we found that

Figure 2.2 Sex steroid ablation improves the repopulation potential of HSCs. 2.5 x 10^6 CD45.1 BM cells were harvested from 2-mo untreated CD45.1 (□), 9-12mo CD45.1 d7shSSA (▨), (n=5 recipients/donor group) or 9-12mo CD45.1 d7SSA (■) (n=5 recipients/donor group) and transferred with an equal dose of untreated 2-mo CD45.2 BM cells into lethally irradiated CD45.2 recipients. Reconstitution was analysed at the designated timepoints. **(a)** Representative donor (CD45.1) versus competitor (CD45.2) flow cytometry profiles of spleen 17 weeks after transplant. **(b)** Total peripheral reconstitution measured by CD45.1 in spleen or peripheral blood over 120 days. **(c)** Lineage specific reconstitution of B cells, T cells, granulocytes and macrophages in the spleen at 28 and 120 days after transplant. To assess the intrinsic functional effect on HSCs, 2000 CD45.1 LSK cells from untreated CD45.1$^+$ 2-mo; 9-12mo CD45.1$^+$ d7shSSA; or 9-12mo CD45.1$^+$ d7SSA were transferred along with 2 x 10^6 BM cells from untreated 2-mo CD45.2 competitors into lethally irradiated CD45.2 recipients (n=5-7 / donor group). **(d)** Total number of CD45.1 donor-derived cells in the BM of recipients 28 days after transplant. **(e**) Proportion of CD45.1$^+$ whole BM, LSK, CLP-1, CLP-2 and MCP cells in the BM 28 days after transplant. **(f)** Representative donor (CD45.1) versus competitor (CD45.2) flow cytometry profiles of spleen 19 weeks after transplant. **(g)** Peripheral reconstitution in 28, 70 and 135 days after transplant. Lineage repopulation was measured amongst CD11b$^+$ myeloid cells, B220$^+$ B cells and TCRβ^+ T cells. Results are expressed as mean ± SEM of five to seven mice for each group at each time point. * compared with shSSA 9-12mo mice. ^ compared with 2-mo mice.


a
d7shSSA
d7SSA
CD45.2
CD45.1
58
35
47
47
b
proportion of CD45.1+ cells (%)
d7shSSA
d7SSA
14
21
28
56
120
c
B220+
TCRβ+
CD11b+
28
120
Days following transplant
d
Number of donor cells x 10-6
Total BM
e
proportion of donor cells (%)
untreated 2-mo
d7shSSA
d7SSA
LSK
CLP-1
CLP-2
MCP
cell subset
f
Un 2-mo
d7shSSA
d7SSA
84
11
90
7
80
16
CD45.2
CD45.1
g
proportion of donor cells (%)
untreated 2-mo
d7shSSA
d7SSA
CD11b+
B220+
TCRβ+
28
70
135
Days following transplant

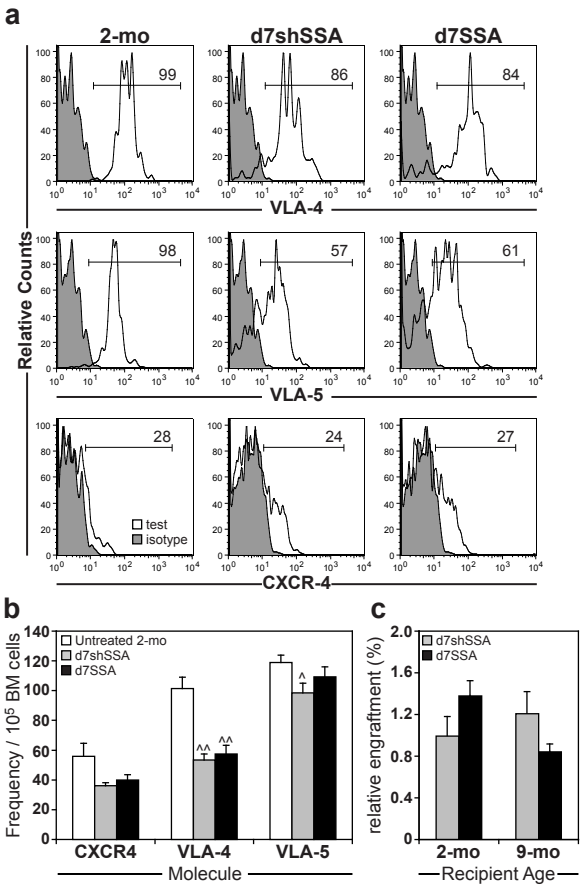
d7SSA further increased this (as indicated by $CD11b^+$ cells), albeit transiently at 70 weeks after transplant (Figure 2.2 g).

2.4.4 Enhanced Repopulation Ability Following SSA is Not Due to the Altered Homing of HSCs to the Bone Marrow or Altered Expression of Migration-related Markers

To assess if improved homing and engraftment could explain SSA-mediated improvements to HSC repopulation, LSK cells were first analysed 7 days post-SSA for expression of the homing and adhesion molecules VLA-4, VLA-5 and CXCR4. With age, there was a decrease in the proportion of LSK cells expressing VLA-4, VLA-5 and CXCR4 (Figure 2.3 a) and also a decrease in the mean fluorescence intensity of VLA-4 (150±6 to 101±5, p=0.008) and VLA-5 (56±1 to 37±0.9, p=0.008). Following SSA there was a slight increase in the proportion of CXCR4 expressing LSK cells (23.5±0.8 to 26.1± 0.8, p=0.036), however there was no change in the expression of VLA-4 or VLA-5. There was also no change in the frequency of CXCR4, VLA-4 or VLA-5 expressing LSK cells following SSA (Figure 2.3 b).

To directly examine the homing ability of LT-HSCs *in vivo*, BM was harvested from individual d7shSSA or d7SSA animals and transplanted into unablated congenic 2-mo or 9mo recipients. BM was harvested 40 hours later and analysed for short-term homing and engraftment of $CD34^-Flt3^-$ LT-HSCs relative to the number of LT-HSCs transferred (Figure 2.3 c). Consistent with the minimal FACS differences, there was no change in homing and engraftment between groups transplanted into 2-mo or 9-mo recipients. The same proportion homed to BM irrespective of the age of the recipient, or whether donors had undergone SSA. This result held true for downstream ST-HSCs and MPPs (data not shown).

Figure 2.3 Withdrawal of sex steroids does not improve the homing efficiency of HSCs. (a) Representative FACS plots showing expression of VLA-4, VLA_5 and CXCR-4 on LSK cells from untreated 2-mo; 9-12mo d7shSSA; or 9-12mo d7SSA. Shaded plots represent isotype controls. **(b)** Frequency of LSK cells expressing CXCR4 (n=10/treatment), VLA-4 (n=5/treatment) or VLA-5 (n=5/treatment) in vascular BM from 2-mo untreated (□),9mo-sham-SSA (▨) or 9-mo SSA (■) mice. **(c)** 20 x 10^6 BM cells from individual $CD45.1^+$ 9-12mo d7shSSA; or $CD45.1^+$ 9-12mo d7SSA were transferred into unablated 2-mo (n=5/donor group) or 9-12mo (n=5/donor group) C57Bl/6 mice. The proportion of $CD45.1^+$ $CD34^-Flt3^-$ LT-HSCs was measured in vascular BM 40 hours after transplant and engraftment was calculated relative to the number of $CD34^-Flt3^-$ LT-HSCs transferred. Results are expressed as mean ± SEM. ^ compared with untreated 2-mo mice.


a
2-mo
d7shSSA
d7SSA
99
86
84
VLA-4
98
57
61
VLA-5
28
24
27
test
isotype
CXCR-4
Relative Counts
b
Untreated 2-mo
d7shSSA
d7SSA
Frequency / 10^5 BM cells
CXCR4
VLA-4
VLA-5
Molecule
c
d7shSSA
d7SSA
relative engraftment (%)
2-mo
9-mo
Recipient Age

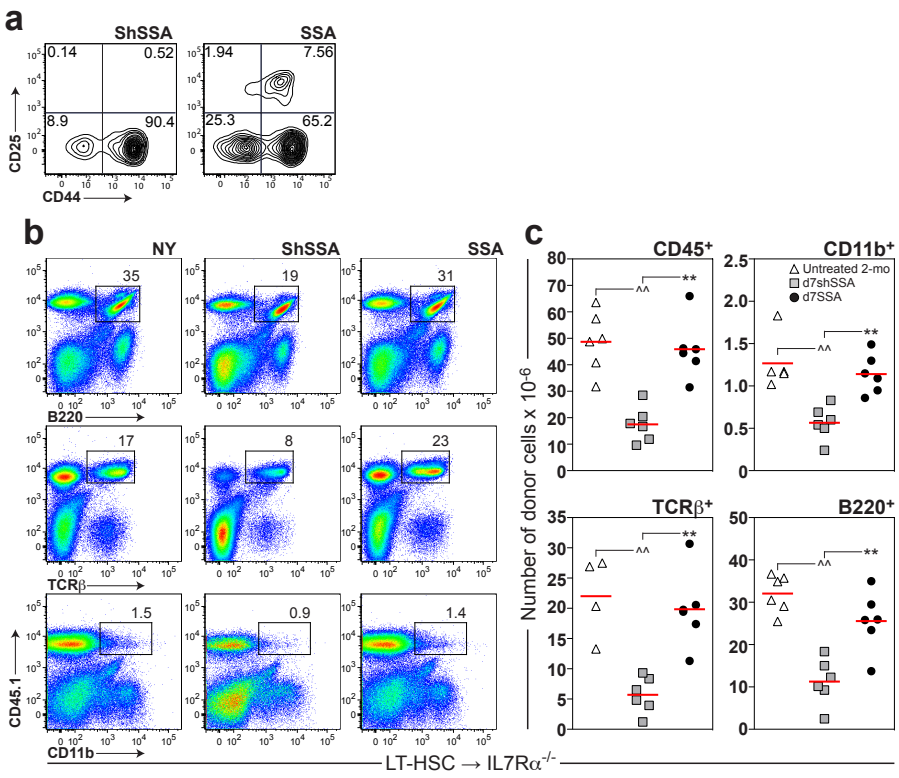
2.4.5 SSA Increases Lymphoid Differentiation Capacity of LT-HSCs

To assess the direct effect of SSA on the lymphoid differentiation capacity of HSCs, we seeded 8000 to 10000 LT-HSCs from d7ShSSA or d7SSA mice onto OP9-DL1 cells and co-cultured these cells for 12 days. OP9-DL1 cells are a BM stromal cell line transduced to express the Notch ligand delta-like 1 (DL1), enabling them to support T cell differentiation.

Importantly, LT-HSCs derived from d7SSA mice progressed through triple negative (TN) stages more rapidly when compared to LT-HSCs derived from d7ShSSA mice (Figure 2.4 a). There were also more TN3 cells in the d7SSA (1.94% of Lin^- GFP^-) than in d7ShSSA (0.14% of Lin^- GFP^-).

To assess these functional effects *in vivo*, we developed an assay to test lymphoid differentiation without the confounding impacts of lethal TBI. Mice lacking expression of IL7Rα, which have a block in lymphoid development, exhibit high levels of donor HSC engraftment, even if recipients are unablated (Gossens et al., 2009). This receptivity makes them an ideal model to assess lymphoid differentiation in the absence of confounding factors such as myeloablation. To specifically assess the lymphoid differentiation capacity of HSCs, 2000 $CD34^-Flt3^-$ LSK cells were FACS purified from untreated 2-mo, 9-12mo d7shSSA or d7SSA vascular niche BM and transferred into unablated $IL7R\alpha^{-/-}$ recipients and donor-induced hematopoiesis examined 70 days after transplant (Figure 2.4 b and c). d7shSSA-derived LT-HSCs exhibited reduced engraftment capacity compared to untreated 2-mo HSCs in all lineages examined, including in this model a reduction in $CD11b^+$ myeloid engraftment. Recipients reconstituted with d7SSA HSCs, on the other hand, had increased levels of $CD45^+$, $CD11b^+$, $TCR\beta^+$ and $B220^+$ donor engraftment compared to d7shSSA, restoring their differentiation capacity to that of untreated 2-mo.

Figure 2.4 Sex steroid ablation improves lymphoid differentiation capacity of hematopoietic progenitors. (a) 8000-10000 LT-HSCs from d7shSSA or d7SSA mice were purified and seeded onto OP9-DL1 cells *in vitro*. Representative TN differentiation profiles of LT-HSCs 12 days after being seeded onto OP9-DL1 T cell differentiation system. **(b)** 2000 $CD34^{-}Flt3^{-}$ LSK cells from untreated $CD45.1^{+}$ 2-mo (□); $CD45.1^{+}$ 9-12mo 7shSSA (▨); or $CD45.1^{+}$ 9-12mo d7SSA (■) mice were transferred into unablated 2-mo $IL7R\alpha^{-/-}$ recipients (n=6 recipients/donor group, one recipient/donor) and donor-derived hematopoiesis measured in the spleen 70 days after transfer. Representative profiles showing donor-derived $B220^{+}$ B cells, $TCR\beta^{+}$ T cells and $CD11b^{+}$ myeloid cells in $IL7R\alpha^{-/-}$ recipients. **(c)** Number of donor derived cells in the periphery of $IL7R\alpha^{-/-}$ recipients. Results are expressed as mean ± SEM. * compared with shSSA 9-12mo mice. ^ compared with 2-mo mice.


a
ShSSA
SSA
0.14
0.52
8.9
90.4
1.94
7.56
25.3
65.2
CD25
CD44
b
NY
ShSSA
SSA
35
19
31
B220
17
8
23
TCRβ
1.5
0.9
1.4
CD45.1
CD11b
c
CD45+
CD11b+
TCRβ+
B220+
Untreated 2-mo
d7shSSA
d7SSA
Number of donor cells x 10-6
LT-HSC → IL7Rα-/-

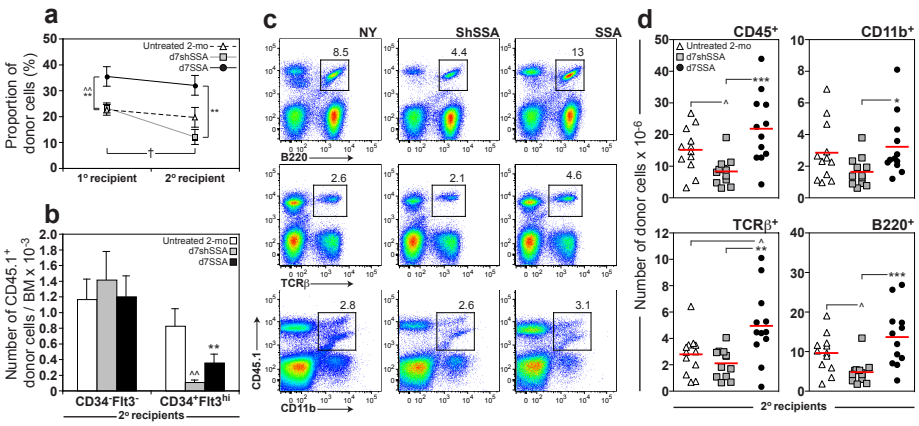
2.4.6 Improved HSC Self-renewal Following SSA

Lethally irradiated congenic mice were initially transplanted with 2 x 10^6 CD45.1 BM cells from untreated 2-mo, d7shSSA or d7SSA mice at a 1:1 ratio with untreated 2-mo CD45.2 BM. 12 weeks after transplantation, BM from primary recipients were transferred into secondary recipients. In both primary and secondary recipients, no change in BM

engraftment of d7shSSA cells was evident compared to those from untreated 2-mo animals (Figure 2.5 a). However, with age there was a significant decline in the proportion of donor-derived cells upon secondary transplant (Figure 2.5 a). In both primary and secondary recipients there was increased engraftment of d7SSA cells compared to both d7shSSA and 2-mo BM. BM from d7SSA animals showed no appreciable loss in donor engraftment in secondary recipients and was comparable to BM of untreated 2-mo, if not better. Decline in

reconstitution of secondary recipients was not due to a defect in the engraftment of donor-derived LT-HSCs as there was no change as a consequence of age or SSA (Figure 2.5 b). There were significantly decreased donor-derived LMPPs from aged d7shSSA BM, which was reversed when d7SSA BM was transplanted, confirming defective lymphoid differentiation with age. In the periphery of secondary recipients we found that there was no loss in the proportion of donor-derived T cells or $CD11b^+$ myeloid cells with age. There were, however, declines in B cell engraftment, which contributed towards a global decline in $CD45^+$ cells (Figure 2.5 c). d7SSA-derived BM significantly increased the engraftment of all leukocyte subsets, reflected in the number of donor-derived cells (Figure 2.5 d).

Figure 2.5 SSA enhances self-renewal in HSCs. 2.5 x 10^6 CD45.1 BM cells from CD45.1 2-mo untreated (□); 9-12mo d7shSSA (▒); or 9-12mo d7SSA (■) were transferred along with 2.5 x 10^6 untreated 2-mo CD45.2 BM cells into lethally irradiated CD45.2 primary recipients (n=6 recipients/donor group, one individual recipient/individual donor). 12 weeks after transplant primary recipient BM was harvested and 2.5 x 10^6 BM cells were transplanted along with an equal dose of untreated 2-mo CD45.2 BM cells into lethally irradiated CD45.2 secondary recipients (n=12 recipients/donor group, two recipients/individual donor). Reconstitution of secondary recipients was measured 12 weeks after transplant. **(a)** Proportion of $CD45.1^+$ donor cells in BM 12 weeks following transplant in primary and secondary recipients (*, compared with 9-12mo d7shSSA mice within 1° or 2° recipients. ^, compared to untreated 2-mo mice within either 1° or 2° recipients. †, compared to d-7shSSA between 1° and 2° recipients). **(b)** Frequency of donor-derived $CD34^-Flt3^-$ LT-HSCs and $CD34^+Flt3^{hi}CD62L^+$ LMPPs in the BM of secondary recipients 12 weeks after transplant. **(c)** Representative flow cytometric profiles of donor reconstitution among $B220^+$ B cells, $TCR\beta^+$ T cells and $CD11b^+$ myeloid cells in the spleen of secondary recipients 12 weeks after transplant. **(d)** Absolute number of $CD45.1^+$ donor cells in the spleen of secondary recipients 12 weeks after transplant. Results are expressed as mean ± SEM. * compared with d7shSSA 9-12mo mice. ^ compared with untreated 2-mo.


a
Proportion of donor cells (%)
Untreated 2-mo
d7shSSA
d7SSA
1° recipient
2° recipient
b
Number of CD45.1+ donor cells / BM x 10-3
CD34-Flt3-
CD34+Flt3hi
2° recipients
c
NY
ShSSA
SSA
B220
TCRβ
CD45.1
CD11b
d
CD45+
CD11b+
TCRβ+
B220+
Number of donor cells x 10-6
2° recipients

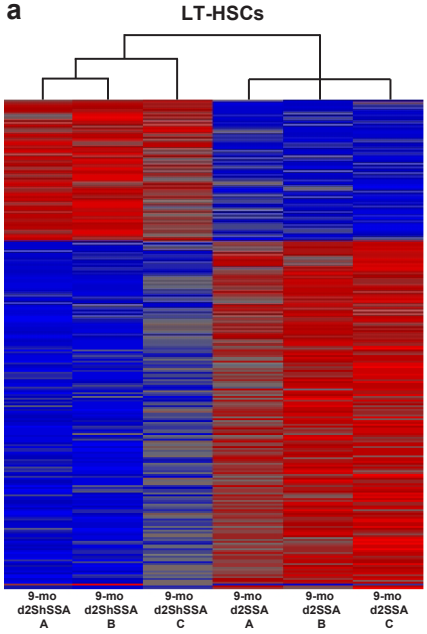
2.4.7 Intrinsic Hematopoietic Alterations in 9mo-SSA LT-HSCs Begin by Day 2 Post Surgical Castration

To determine the earliest intrinsic changes to the stem cell compartment after SSA, LT-HSCs from 9mo-d2ShSSA and d2SSA mice were FACS purified and transcriptome analysis performed.

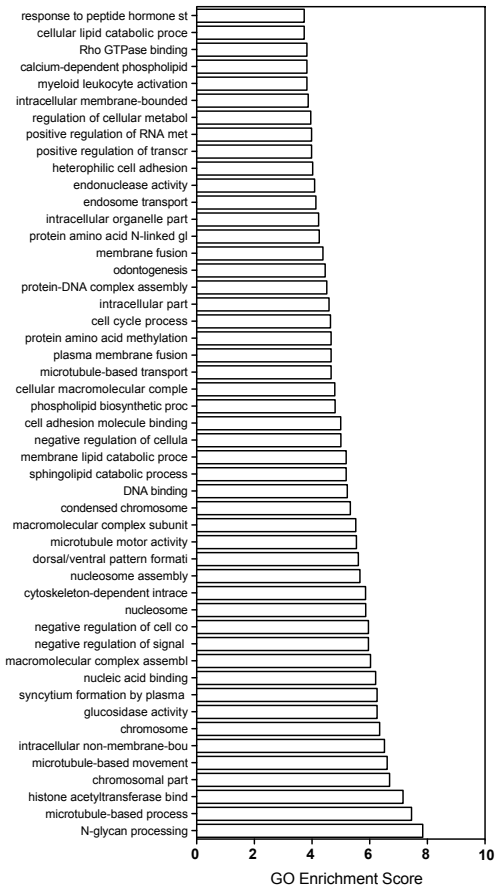
We found 770 genes significantly altered by day 2 post SSA (upregulated or downregulated Figure 2.6 a). Pathway analysis revealed an overall upregulation of genes associated with DNA replication processes as well as intercellular and cell-matrix interactions (Figure 2.6 b). Importantly, of the 770 differentially expressed genes, several key hematopoietic-associated genes were altered by day 2 post surgical castration (Figure 2.6 c), including downregulation of early stem cell genes (*Socs4, kit*), myeloid associated genes (*Mpo*) and genes associated with aging (*Igf1r*) while lymphoid associated genes were significantly upregulated (*Bcl10, Zbtb5, Zbtb7a, Tcf712, Rag1ap1*) (Figure 2.6 c).

Figure 2.6 Hematopoietic-associated molecular alterations in LT-HSCs at the early timepoint following SSA. 30000-50000 LT-HSCs from 9-mo d2shSSA or 9-mo d2SSA mice BM were purified by FACS and analysed by microarray using the Partek 6.6 software. **(a)** Heat map showing the differential gene expression between 9-mo d2shSSA and 9-mo d2SSA LT-HSCs. Gene upregulation is presented in red (—) and downregulation in blue (—) **(b)** Pathway analysis GO enrichment scores based on genes that were significantly altered (GO ANOVA p-value ≤ 0.05) between 9-mo d2shSSA vs 9-mo d2SSA. **(c)** Fold-change and function of selected hematopoietic-associated genes that are significantly (GO ANOVA p-value ≤ 0.05) altered with SSA in LT-HSCs.

a



b



c

Gene	FC[1]	p-value	Function	References
Mpo	-2.4	0.02	Myelopoiesis	(Yoshida et al., 2006)
Rgs1	-2.1	0.04	Inhibition of cell signaling	(Druey et al., 1996)
Ireb2	-1.9	0.004	Erythropoiesis	(Kerenyi et al. 2008)
Anxa3	-1.6	0.03	Cell growth regulation	(Chong et al., 2010)
Socs4	-1.6	0.01	Cytokine inhibition	(Starr et al., 1997)
Zbtb7a	3.9	0.03	B-cell development	(Maeda *et al.* 2007)
Socs2	2.1	0.02	IGF signaling inhibition	(Dey et al., 1998)
Sox18	2.0	0.02	Proliferation of HSCs	(Serrano *et al.* 2010)
Bmp4	1.8	0.008	Self-renewal	(Goldman *et al.* 2009)
Vegfc	1.8	0.04	Angiogenesis	(Kukk *et al.* 1996)
Elmo3	1.8	0.04	Cell migration	(Gumienny et al., 2001)
Fgfbp3	1.8	0.03	Cytokine enhancer	(Ilkin et al., 2012)
Rag1ap1	1.7	0.05	Lymphopoiesis	(Mombaerts et al., 1992)
Hoxb2	1.6	0.04	Erythropoiesis	(Giampaolo *et al.* 1994)
E2f5	1.6	0.007	Proliferation	(Chen et al., 2004)
Tcf712	1.6	0.02	Wnt-signaling mediator	(Faro et al., 2009)

[1]FC = fold-change d2shSSA vs. d2SSA

2.5 Discussion

There is growing evidence for intrinsic age-related changes in HSC function (Dumble et al., 2007; Geiger et al., 2005; Rossi et al., 2007; Rossi et al., 2005; Sudo et al., 2000). We have previously demonstrated that SSA regenerates age-related declines in lymphopoiesis and enhances recovery following HSCT (Chidgey et al., 2007; Chidgey et al., 2008; Dudakov et al., 2009a; Dudakov et al., 2009b; Goldberg et al., 2010). We hypothesised that age-related declines in lymphopoiesis correlate with defects in HSC function. Conversely, we sought to determine if lymphoid regeneration following SSA is mediated by enhanced hematopoietic stem and progenitor cell function – even in middle-aged mice that by consensus do not yet have considerable stem cell defects, but exhibit all the hallmarks of immune degeneration.

We found that despite the previously reported accumulation of LT-HSCs with age (Rossi et al., 2005; Sudo et al., 2000) there was a further increase in the number of these cells following SSA. These increases were restricted to the vascular niche with little impact observed in the endosteum. Long-term reconstitution studies revealed d7SSA-derived HSCs had a significant increase in the frequency of multi-lineage repopulating cells as well as an increased repopulation potential on a per cell basis. In serial transplant experiments there were no differences between groups in the number of donor-derived LT-HSCs but there was a significant defect in their differentiation into LMPPs when transplanted with d7shSSA BM. SSA reversed this cell-intrinsic block in differentiation. Transplantation of d7shSSA $CD34^{-}Flt3^{-}$ LT-HSCs into $IL7R^{-/-}$ recipients confirmed this defect in lymphoid differentiation, which was again reversed by SSA. Collectively these findings highlight the important role of SSA in enhancing HSC function and capacity for lymphoid regeneration.

While 9-12mo mice showed a decreased expression of Flt3 on LSK cells, this was not accompanied by reduced numbers of MPPs, which are observed in older mice (Rossi et al., 2005). Within this subset we found in 9-12mo mice a selective depletion of $CD62L^{+}Flt3^{hi}$ LMPPs, a subset analogous to intrathymic early T-lineage progenitors (ETPs) (Perry et al., 2004) - potentially explaining the profoundly reduced number and function of ETPs with age (Heng et al., 2005; Min et al., 2004). Taken together with the previously reported sensitivity of early lymphoid progenitors to negative regulators such as sex steroids and glucocorticoids (Igarashi et al., 2005; Medina et al., 2001), as well as our own data showing significant increases in downstream lymphoid progenitors following SSA (Dudakov et al., 2009a), it is consistent that SSA also significantly increased LMPPs herein. The kinetics of regeneration of ETPs following SSA, which increase in number from day 7-10 (Heng et al., 2005), are similar to those we report here amongst BM $Flt3^{hi}CD62L^{+}$ LMPPs – further linking these two populations. *In vivo* differentiation in $IL7R\alpha^{-/-}$ animals showed that twelve weeks after transfer of LT-HSCs from 9-12mo d7shSSA was significantly reduced in comparison to untreated 2-mo BM. This decline in HSC function was restored to untreated 2mo levels by d7SSA implying that SSA-mediated lymphoid regeneration, and consequently age-related lymphoid degeneration, is predicated on the altered function of BM-derived hematopoietic stem and progenitors. Moreover, our findings suggest that these improved repopulation and differentiation abilities of HSCs following SSA are not due to changes in the homing, engraftment and retention of HSCs in the BM.

Clinically, HSCs from aged, or chemotherapy treated patients exhibit a reduced proliferative potential and ability to form T cells *in vitro* (Offner et al., 1999; Vaziri et al., 1994). Moreover, patients receiving HSCs from young donors fare better than those receiving HSCs from older patients (Carreras et al., 2006). Previous findings, which identified myeloid-biased HSCs (Muller-Sieburg et al., 2004; Sudo et al., 2000) and showed increased

myeloid gene usage in aged LT-HSCs (Rossi et al., 2005), implied an intrinsic age-related lineage skewing away from lymphopoiesis and towards myelopoiesis. The accumulation of these cells with age is also consistent with the age-associated increased incidence in myelogenous leukemias (Muller-Sieburg et al., 2004) and has led to the clinical hypothesis that myeloid-biased HSCs are the targets for transforming into myelogenous leukemia (Appelbaum et al., 2006; Bell and Van Zant, 2004; Muller-Sieburg et al., 2004; Satoh and Ogata, 2006; Sudo et al., 2000). At least some of the defects observed in HSCs with age can be attributed to an accumulation of damage to mitochondrial DNA (Yao et al., 2007) as well as defects in p53 activity (Dumble et al., 2007) and DNA damage repair (Geiger et al., 2005). Importantly, our data revealing the upregulation of lymphoid associated genes by day 2 suggests that SSA directly promotes the regeneration of lymphoid lineage potential within LT-HSCs (Maeda et al., 2007; McLeod et al., 2011; Pridans et al., 2008; Xue et al., 2003). It is unclear whether this is evident within a defined subset of newly formed cells or generic to the total population.

The data presented here suggest that withdrawal of sex steroids can dramatically improve HSC function, particularly with respect to their lymphoid differentiation potential - possibly even ameliorating myeloid dysplasia. These findings suggest that enhanced lymphopoiesis, in both the BM and thymus can be explained by the impacts of SSA on improving the function of HSCs. This is further supported by their exposure to a "rejuvenated" microenvironment, sufficient to restore function of aged satellite progenitor cells (Conboy et al., 2005). The improvement of BM function and enhanced HSC function by SSA is a novel and rational approach to the unmet clinical need of lymphoid regeneration following cytoreductive therapy for haematological malignancies, particularly given that this can be achieved clinically and reversibly using the hormone LHRH (Chidgey et al., 2007). Moreover, demonstrating enhanced HSC function lays a solid foundation for improving

transplantation outcomes in patients undergoing high dose cytoreductive therapy, by ensuring effective engraftment and subsequent immune reconstitution.

2.6 Acknowledgements

We gratefully acknowledge Jade Homann, Luciana Thompson and Jade Barbuto for surgery and animal handling; Andrew Fryga, Darren Ellemor, Karen Yuen, Kathryn Flanagan (FlowCore) and Geza Paukovics (AMREP) for expert cell sorting; Dr Andreas Strasser for IL7R$\alpha^{-/-}$ mice; Dr Andreas Scherer (AGRF) for expert bioinformatics analysis; and Mark Malin, Marcel van den Brink and Hanasha Lan for helpful discussions. This study was funded by grants from the Australian Stem Cell Centre, the Australian National Health & Medical Research Council, and Norwood Immunology. J.A.D. was supported by fellowships from the Australian National Health and Medical Research Council, the Leukemia and Lymphoma Society and a Sydney Parker Smith fellowship from the Cancer Council of Victoria.

2.7 References

Appelbaum, F.R., Gundacker, H., Head, D.R., Slovak, M.L., Willman, C.L., Godwin, J.E., Anderson, J.E., and Petersdorf, S.H. (2006). Age and acute myeloid leukemia. Blood 107, 3481-3485.

Bell, D.R., and Van Zant, G. (2004). Stem cells, aging, and cancer: inevitabilities and outcomes. Oncogene 23, 7290-7296.

Carreras, E., Jimenez, M., Gomez-Garcia, V., de la Camara, R., Martin, C., Martinez, F., Iriondo, A., Sanz, G., Canizo, C., Cabrera, R., et al. (2006). Donor age and degree of HLA matching have a major impact on the outcome of unrelated donor haematopoietic cell transplantation for chronic myeloid leukaemia. Bone marrow transplantation 37, 33-40.

Chen, Q., Liang, D., Yang, T., Leone, G., and Overbeek, P.A. (2004). Distinct capacities of individual E2Fs to induce cell cycle re-entry in postmitotic lens fiber cells of transgenic mice. Dev Neurosci 26, 435-445.

Chidgey, A., Dudakov, J., Seach, N., and Boyd, R. (2007). Impact of niche aging on thymic regeneration and immune reconstitution. Semin Immunol 19, 331-340.

Chidgey, A.P., Seach, N., Dudakov, J., Hammett, M.V., and Boyd, R.L. (2008). Strategies for reconstituting and boosting T cell-based immunity following haematopoietic stem cell transplantation: pre-clinical and clinical approaches. Semin Immunopathol 30, 457-477.

Chong, K.W.Y., Chen, M.J., Koay, E.S.C., Wong, B.S., Lee, A.Y.-W., Russo-Marie, F., and Cheung, N.S. (2010). Annexin A3 is associated with cell death in lactacystin-mediated neuronal injury. Neurosci Lett 485, 129-133.

Conboy, I.M., Conboy, M.J., Wagers, A.J., Girma, E.R., Weissman, I.L., and Rando, T.A. (2005). Rejuvenation of aged progenitor cells by exposure to a young systemic environment. Nature 433, 760-764.

Dey, B.R., Spence, S.L., Nissley, P., and Furlanetto, R.W. (1998). Interaction of human suppressor of cytokine signaling (SOCS)-2 with the insulin-like growth factor-I receptor. J Biol Chem 273, 24095-24101.

Dorshkind, K., Montecino-Rodriguez, E., and Signer, R.A.J. (2009). The ageing immune system: is it ever too old to become young again? Nat Rev Immunol 9, 57-62.

Druey, K.M., Blumer, K.J., Kang, V.H., and Kehrl, J.H. (1996). Inhibition of G-protein-mediated MAP kinase activation by a new mammalian gene family. Nature 379, 742-746.

Dudakov, J.A., Goldberg, G.L., Reiseger, J.J., Chidgey, A.P., and Boyd, R.L. (2009a). Withdrawal of Sex Steroids Reverses Age- and Chemotherapy-Related Defects in Bone Marrow Lymphopoiesis. J. Immunol. 182, 6247-6260.

Dudakov, J.A., Goldberg, G.L., Reiseger, J.J., Vlahos, K., Chidgey, A.P., and Boyd, R.L. (2009b). Sex Steroid Ablation Enhances Hematopoietic Recovery following Cytotoxic Antineoplastic Therapy in Aged Mice. J. Immunol. 183, 7084-7094.

Dudakov, J.A., Khong, D.M.P., Boyd, R.L., and Chidgey, A.P. (2010). Feeding the fire: the role of defective bone marrow function in exacerbating thymic involution. Trends Immunol. 31, 191-198.

Dumble, M., Moore, L., Chambers, S.M., Geiger, H., Van Zant, G., Goodell, M.A., and Donehower, L.A. (2007). The impact of altered p53 dosage on hematopoietic stem cell dynamics during aging. Blood 109, 1736-1742.

Faro, A., Boj, S.F., Ambrósio, R., van den Broek, O., Korving, J., and Clevers, H. (2009). T-cell factor 4 (tcf7l2) is the main effector of Wnt signaling during zebrafish intestine organogenesis. Zebrafish 6, 59-68.

Geiger, H., Rennebeck, G., and Van Zant, G. (2005). Regulation of hematopoietic stem cell aging in vivo by a distinct genetic element. Proc Natl Acad Sci U S A 102, 5102-5107.

Giampaolo, A., Sterpetti, P., Bulgarini, D., Samoggia, P., Pelosi, E., Valtieri, M., and Peschle, C. (1994). Key functional role and lineage-specific expression of selected HOXB genes in purified hematopoietic progenitor differentiation. Blood 84, 3637-3647.

Goldberg, G.L., Dudakov, J.A., Reiseger, J.J., Seach, N., Ueno, T., Vlahos, K., Hammett, M.V., Young, L.F., Heng, T.S.P., Boyd, R.L., and Chidgey, A.P. (2010). Sex Steroid Ablation Enhances Immune Reconstitution Following Cytotoxic Antineoplastic Therapy in Young Mice. J. Immunol. 184, 6014-6024.

Goldman, D.C., Bailey, A.S., Pfaffle, D.L., Al Masri, A., Christian, J.L., and Fleming, W.H. (2009). BMP4 regulates the hematopoietic stem cell niche. Blood 114, 4393-4401.

Gossens, K., Naus, S., Corbel, S.Y., Lin, S., Rossi, F.M.V., Kast, J., and Ziltener, H.J. (2009). Thymic progenitor homing and lymphocyte homeostasis are linked via S1P-controlled expression of thymic P-selectin/CCL25. J. Exp. Med. 206, 761-778.

Guerrettaz, L.M., Johnson, S.A., and Cambier, J.C. (2008). Acquired hematopoietic stem cell defects determine B-cell repertoire changes associated with aging. Proc Natl Acad Sci U S A 105, 11898-11902.

Gumienny, T.L., Brugnera, E., Tosello-Trampont, A.C., Kinchen, J.M., Haney, L.B., Nishiwaki, K., Walk, S.F., Nemergut, M.E., Macara, I.G., Francis, R., et al. (2001). CED-12/ELMO, a novel member of the CrkII/Dock180/Rac pathway, is required for phagocytosis and cell migration. Cell 107, 27-41.

Heng, T.S., Goldberg, G.L., Gray, D.H., Sutherland, J.S., Chidgey, A.P., and Boyd, R.L. (2005). Effects of castration on thymocyte development in two different models of thymic involution. J. Immunol. 175, 2982-2993.

Igarashi, H., Medina, K.L., Yokota, T., Rossi, M.I., Sakaguchi, N., Comp, P.C., and Kincade, P.W. (2005). Early lymphoid progenitors in mouse and man are highly sensitive to glucocorticoids. Int Immunol 17, 501-511.

Itkin, T., Ludin, A., Gradus, B., Gur-Cohen, S., Kalinkovich, A., Schajnovitz, A., Ovadya, Y., Kollet, O., Canaani, J., Shezen, E., et al. (2012). FGF-2 expands murine hematopoietic stem and progenitor cells via proliferation of stromal cells, c-Kit activation and CXCL12 downregulation. Blood.

Kerenyi, M.A., Grebien, F., Gehart, H., Schifrer, M., Artaker, M., Kovacic, B., Beug, H., Moriggl, R., and Müllner, E.W. (2008). Stat5 regulates cellular iron uptake of erythroid cells via IRP-2 and TfR-1. Blood 112, 3878-3888.

Kim, M., Moon, H.B., and Spangrude, G.J. (2003). Major age-related changes of mouse hematopoietic stem/progenitor cells. Ann N Y Acad Sci 996, 195-208.

Kukk, E., Lymboussaki, A., Taira, S., Kaipainen, A., Jeltsch, M., Joukov, V., and Alitalo, K. (1996). VEGF-C receptor binding and pattern of expression with VEGFR-3 suggests a role in lymphatic vascular development. Development 122, 3829-3837.

Liang, Y., Van Zant, G., and Szilvassy, S.J. (2005). Effects of aging on the homing and engraftment of murine hematopoietic stem and progenitor cells. Blood 106, 1479-1487.

Maeda, T., Merghoub, T., Hobbs, R.M., Dong, L., Maeda, M., Zakrzewski, J., van den Brink, M.R.M., Zelent, A., Shigematsu, H., Akashi, K., et al. (2007). Regulation of B versus T lymphoid lineage fate decision by the proto-oncogene LRF. Science 316, 860-866.

McLeod, I.X., Zhou, X., Li, Q.-J., Wang, F., and He, Y.-W. (2011). The class III kinase Vps34 promotes T lymphocyte survival through regulating IL-7Rα surface expression. J Immunol 187, 5051-5061.

Medina, K.L., Garrett, K.P., Thompson, L.F., Rossi, M.I., Payne, K.J., and Kincade, P.W. (2001). Identification of very early lymphoid precursors in bone marrow and their regulation by estrogen. Nat Immunol 2, 718-724.

Miller, J.P., and Allman, D. (2003). The decline in B lymphopoiesis in aged mice reflects loss of very early B-lineage precursors. J. Immunol. 171, 2326-2330.

Min, H., Montecino-Rodriguez, E., and Dorshkind, K. (2004). Reduction in the developmental potential of intrathymic T cell progenitors with age. J. Immunol. 173, 245-250.

Min, H., Montecino-Rodriguez, E., and Dorshkind, K. (2006). Effects of aging on the common lymphoid progenitor to pro-B cell transition. J Immunol 176, 1007-1012.

Mombaerts, P., Iacomini, J., Johnson, R.S., Herrup, K., Tonegawa, S., and Papaioannou, V.E. (1992). RAG-1-deficient mice have no mature B and T lymphocytes. Cell 68, 869-877.

Morrison, S.J., Wandycz, A.M., Akashi, K., Globerson, A., and Weissman, I.L. (1996). The aging of hematopoietic stem cells. Nat Med 2, 1011-1016.

Muller-Sieburg, C.E., Cho, R.H., Karlsson, L., Huang, J.F., and Sieburg, H.B. (2004). Myeloid-biased hematopoietic stem cells have extensive self-renewal capacity but generate diminished lymphoid progeny with impaired IL-7 responsiveness. Blood 103, 4111-4118.

Offner, F., Kerre, T., De Smedt, M., and Plum, J. (1999). Bone marrow CD34 cells generate fewer T cells in vitro with increasing age and following chemotherapy. Br J Haematol 104, 801-808.

Olsen, N.J., Gu, X., and Kovacs, W.J. (2001a). Bone marrow stromal cells mediate androgenic suppression of B lymphocyte development. J Clin Invest 108, 1697-1704.

Olsen, N.J., Olson, G., Viselli, S.M., Gu, X., and Kovacs, W.J. (2001b). Androgen receptors in thymic epithelium modulate thymus size and thymocyte development. Endocrinology 142, 1278-1283.

Perry, S.S., Wang, H., Pierce, L.J., Yang, A.M., Tsai, S., and Spangrude, G.J. (2004). L-selectin defines a bone marrow analog to the thymic early T-lineage progenitor. Blood 103, 2990-2996.

Pridans, C., Holmes, M.L., Polli, M., Wettenhall, J.M., Dakic, A., Corcoran, L.M., Smyth, G.K., and Nutt, S.L. (2008). Identification of Pax5 target genes in early B cell differentiation. J Immunol 180, 1719-1728.

Rodewald, H.R. (1998). The thymus in the age of retirement. Nature 396, 630-631.

Rossi, D.J., Bryder, D., Seita, J., Nussenzweig, A., Hoeijmakers, J., and Weissman, I.L. (2007). Deficiencies in DNA damage repair limit the function of haematopoietic stem cells with age. Nature 447, 725-729.

Rossi, D.J., Bryder, D., Zahn, J.M., Ahlenius, H., Sonu, R., Wagers, A.J., and Weissman, I.L. (2005). Cell intrinsic alterations underlie hematopoietic stem cell aging. Proc Natl Acad Sci U S A 102, 9194-9199.

Satoh, C., and Ogata, K. (2006). Hypothesis: myeloid-restricted hematopoietic stem cells with self-renewal capacity may be the transformation site in acute myeloid leukemia. Leuk Res 30, 491-495.

Schmitt, T.M., and Zúñiga-Pflücker, J.C. (2006). T-cell development, doing it in a dish. Immunol Rev 209, 95-102.

Serrano, A.G., Gandillet, A., Pearson, S., Lacaud, G., and Kouskoff, V. (2010). Contrasting effects of Sox17- and Sox18-sustained expression at the onset of blood specification. Blood 115, 3895-3898.

Signer, R.A.J., Montecino-Rodriguez, E., Witte, O.N., McLaughlin, J., and Dorshkind, K. (2007). Age-related defects in B lymphopoiesis underlie the myeloid dominance of adult leukemia. Blood 110, 1831-1839.

Starr, R., Willson, T.A., Viney, E.M., Murray, L.J., Rayner, J.R., Jenkins, B.J., Gonda, T.J., Alexander, W.S., Metcalf, D., Nicola, N.A., and Hilton, D.J. (1997). A family of cytokine-inducible inhibitors of signalling. Nature 387, 917-921.

Sudo, K., Ema, H., Morita, Y., and Nakauchi, H. (2000). Age-associated characteristics of murine hematopoietic stem cells. J Exp Med 192, 1273-1280.

Thurmond, T.S., Murante, F.G., Staples, J.E., Silverstone, A.E., Korach, K.S., and Gasiewicz, T.A. (2000). Role of estrogen receptor alpha in hematopoietic stem cell development and B lymphocyte maturation in the male mouse. Endocrinology 141, 2309-2318.

Vaziri, H., Dragowska, W., Allsopp, R.C., Thomas, T.E., Harley, C.B., and Lansdorp, P.M. (1994). Evidence for a mitotic clock in human hematopoietic stem cells: loss of telomeric DNA with age. Proc Natl Acad Sci U S A 91, 9857-9860.

Xue, L., Morris, S.W., Orihuela, C., Tuomanen, E., Cui, X., Wen, R., and Wang, D. (2003). Defective development and function of Bcl10-deficient follicular, marginal zone and B1 B cells. Nature Immunology 4, 857-865.

Yao, Y.G., Ellison, F.M., McCoy, J.P., Chen, J., and Young, N.S. (2007). Age-dependent accumulation of mtDNA mutations in murine hematopoietic stem cells is modulated by the nuclear genetic background. Hum Mol Genet 16, 286-294.

Yoshida, T., Ng, S.Y.-M., Zuniga-Pflucker, J.C., and Georgopoulos, K. (2006). Early hematopoietic lineage restrictions directed by Ikaros. Nature Immunology 7, 382-391.

Chapter 3

Rejuvenation of the hematopoietic niche following sex steroid ablation contributes to immune regeneration

Manuscript information

The following manuscript is being prepared to submit to the *Journal of Immunology*. The candidate, Danika Khong, was responsible for planning, performing and analysing experiments as well as writing and editing the manuscript. The co-authors Jarrod Dudakov, Maree Hammett, Lisa Spyroglou, Kahlia Wong, Ann Chidgey and Richard Boyd assisted with the experimental design, technical assistance, interpretation of results and drafting of the manuscript. Proportional contributions explained in the signed declaration.

Rejuvenation of the hematopoietic niche following sex steroid ablation contributes to immune regeneration

Danika M.P. Khong[1,3], Jarrod A. Dudakov[1,2], Maree V. Hammett[1], Lisa Spyroglou[1], Kahlia Wong[1] , Richard L. Boyd[1,4] and Ann P. Chidgey[1,4,*]

[1] Immune Regeneration Laboratory, Monash Immunology and Stem Cell Laboratories (MISCL), Monash University, Wellington Rd, Clayton, Australia, 3800

[2] Current Address: Department of Immunology, Memorial Sloan-Kettering Cancer Center, 1275 York Ave, New York, NY, USA 10065

[3] Current Address: Harvard Stem Cell Institute, Holyoke Center, Suite 727W, 1350 Massachusetts Avenue, Cambridge, MA, USA 02138

[4] These authors contributed equally to this work.

* A/Prof. Ann P. Chidgey, P: [redacted] F: [redacted]

Abbreviations used in this paper: Ang, Angiopoietin-1; BM, bone marrow; BMP, bone morphogenic protein; BMT, Bone Marrow Transplant; CLP, Common Lymphoid Progenitor; Cy, cyclophosphamide; ETP, early T-lineage progenitor; HSC, Hematopoietic Stem Cell; IGF, insulin-like growth factor, LMPP, lymphoid-primed multipotent progenitor; LT-HSC, long-term hematopoietic stem cell; LSK, $Lin^{-}Sca1^{+}ckit^{+}$; MPP, multipotent progenitor; Opn, osteopontin; OBL, osteoblast; shSSA, sham sex steroid ablation; SSA, Sex Steroid Ablation; ST-HSC, short-term hematopoietic stem cell; WBM, whole bone marrow

Keywords – BM, osteoblasts, hematopoiesis, hematopoietic stem cell

3.1 ABSTRACT

Severe immune degeneration is a consequence of aging. Architectural deterioration of the two primary lymphoid organs, the bone marrow (BM) and thymus, lead to functional impairments such as reduced B and T cell output as well as peripheral function leading to increased susceptibility to opportunistic infections, recurring malignancies as well as a reduced ability to recover from cytoablation (chemo- and radio-therapies) in those who are immune-compromised. We have previously described the rejuvenation of lymphopoiesis following sex steroid ablation (SSA) and in particular, the contribution of SSA hematopoietic stem cells (HSCs) in SSA-mediated lymphoid regeneration. This study focuses on the impact of SSA on the BM hematopoietic niche, particularly the $CD51^+$ osteoblasts (OBLs) and $CD31^+$ endothelial cells, and their role in immune regeneration. Vascular $CD51^+$ OBLs represent a more immature population compared to the endosteally located OBLs and are seen to accumulate with age. While numerically unaffected by SSA, these cells showed enhanced expression of hematopoietic-associated molecules at an earlier timepoint when compared to the more mature endosteal OBLs and prior to any observed functional changes within their associated LT-HSCs. Collectively however, the 9-mo SSA HSC niche appears to alter progressively towards the younger 2-mo BM phenotype suggesting their involvement in supporting enhanced hematopoiesis noticed with SSA-mediated immune regeneration. In conclusion, SSA improves the hematopoietic-associated molecular profile of both the endosteal and vascular OBLs and hence their ability to support hematopoiesis.

3.2 Introduction

One of the hallmarks of mammalian immunity is its degeneration with age, a phenomenon referred to as immune-senescence. The two primary lymphoid organs; thymus and bone marrow, progressively become less efficient at producing T and B cells respectively. These functional declines begin primarily at puberty. In healthy individuals immune-senescence does not normally pose a significant problem as most of the adaptive repertoire is already established. However in those adults whose immune system is severely depleted by high dose chemo- and radio- therapy, stress or chronic infection, the severely compromised function of the thymus means these individuals are often left immune-compromised for prolonged periods and hence are extremely susceptible to infections and malignant relapse. As a corollary, immune recovery in adults has been intrinsically linked with the need for regeneration of thymic function. The key question is – how can this be achieved? In turn this requires an understanding of what causes thymic decline. Several previous studies have reported that the removal of sex steroids through either chemical or surgical castration rejuvenates the immune system and lymphopoiesis in particular (1-8). But how does this happen? Is it a consequence of overcoming endogenous thymic defects?

Alternatively, given that the thymus has a continual need for T cell progenitors since it does not contain any self-renewing HSC or equivalent progenitor cell (HSPC) for the T cell lineage, is thymic decline a consequence of BM deficiency? Indeed HSC and HSPC are functionally altered with age manifesting as a progressive bias towards the myeloid lineages and away from the lymphoid lineages (9-13). This skewing could well explain the increased incidence in myelopathologies and anemia with age (12). Through comparative microarray analysis, studies have identified an obvious upregulation of myeloid associated genes and

downregulation of lymphoid associated genes in aged mice (13). Moreover, previous reports from our laboratory and others have reported a quantitative decline in lymphoid progenitors in the BM with age (Chapters 2 and 4 (13)).

In addition to this myeloid skewing, there is also a well-described age-associated accumulation of functionally deficient HSCs within the BM. These phenotypically identical HSCs show reduced self-renewal potential, as seen in long term repopulating assays, as well as diminished lymphoid differentiation capacities; all of which improve upon sex steroid ablation (Chapter 2 (14)). Hence, similar to the thymus, sex steroid ablation reverses the impacts of age on BM function.

Although the impacts of age on HSC function are well described, it is unclear how this arises. As for the thymus, is it a direct, cell intrinsic feature or rather attributed to defects in the microenvironmental niches, which nurture HSC development and maintenance? Although the latter potential niche involvement was first postulated by Schofield and colleagues in 1978, it is only recently that satisfactory methods have been developed for the cellular and hence molecular dissection of the BM HSC niche (15). The cells include fibroblasts, adipocytes, macrophages, endothelial cells, osteoblasts (OBLs), osteoclasts and specialized mesenchymal stromal cells (MSCs) (16, 17). These are arranged into complex 3-dimensional structures held together by the extracellular matrix (ECM) and spatially arranged at the endosteal surface (endosteal HSC niche) and adjoining sinusoidal endothelium within the central marrow (vascular HSC niche), although there is some uncertainty whether this represents just one or more distinct HSC niches (17-19).

OBLs, which are of mesenchymal origin, have a dual function within the BM: to synthesize bone matrix and to maintain the HSC pool size (20, 21). While mature osteocalcin expressing OBLs are primarily found lining the bone in highly vascular regions of the endosteum, more primitive *Runx2* expressing pre- and immature OBLs is proposed to be further away from the bone (reviewed in 22). The vascular endothelium includes arteries that penetrate through dense bone structures and form capillaries that converge into a central sinus (23, 24). These sinusoidal blood vessels are highly fenestrated and form a network often in the close proximity to endosteal surfaces (24).

These niche components are able to regulate HSC function through several molecular interactions including osteopontin (Opn), which maintains HSC quiescence; Parathyroid Hormone Receptors (PTHrs), which activate OBLs and causes a Notch- mediated increase in HSC proliferation (21, 25, 26); Angiopoietin-1 (Ang-1), which promotes stem cell quiescence and regulates HSC numbers via its anti-apoptotic effects; CXCL-12, which regulates HSC localization and promotes their quiescence (27, 28); and Notch activation which promote HSC self-renewal (29).

Recent studies suggest a decline in cell-to-cell adhesion within the niche with age, a finding supported by enhanced mobilization of HSCs as a result of granulocyte-colony stimulating factor (G-CSF) treatment (30, 31). Moreover, with age, HSCs were also found to be further away from the endosteum (32). While the aging of OBLs within the context of bone synthesis has shown declining proliferation and bone synthesizing abilities, its role in the aging HSC microenvironment is less well understood (33, 34). This study will assess how aging affects the ability of the hematopoietic niche to support hematopoiesis, particularly within the hematopoietic niche. Furthermore, given the impact of sex steroid ablation (SSA)

on hematopoiesis and lymphoid development (described in Chapter 2), we sought to assess the impact of SSA on the BM microenvironment.

3.3 Materials and Methods

3.3.1 Animals

Inbred male C57BL/6 or congenic CD45.1 mice were obtained from the Animal Resources Centre (Perth, Australia) or Monash Animal Services (Clayton, Australia). Young mice were between the ages of 6-8 weeks and aged mice were more than 8 months of age in this study. Mice were maintained at the Monash Animal Services (Clayton, Australia) and were allowed to acclimatize for 7 days before experimentation, which was performed according to animal experiment ethics committee guidelines and approval.

3.3.2 Surgical Castration

Mice were anaesthetized by inhalation of a gaseous mixture of 2.5% isofluorane and 2% oxygen. A small incision was made in the scrotum. The testes were exposed, ligated with dissolvable sutures and removed. The wound was closed with dissolvable sutures. For surgical stress control, sham SSA (ShSSA) was performed as above, but without the removal of the testes.

3.3.3 Cell Suspensions

Single cell suspensions of the hematopoietic vascular niche of bone marrow (BM) were obtained by flushing freshly dissected tibias and femurs with cold PBS supplemented with 0.2%BSA and 0.02% sodium azide (FACS buffer) with a 22G needle (Becton Dickinson, USA). Bone ends were crushed in a mortar and pestle with FACS buffer, the crushed ends washed to remove marrow and the wash added to the flushed vascular niche cells. The resulting marrow was then made into a single cell suspension by gentle pipetting. Marrow

preparations for the hematopoietic content involved flushing the aforementioned bones with cold FACS buffer using a 26G needle (Becton Dickinson, USA) and then made into single cell suspension using a 22G needle (Becton Dickinson, USA). In the case of endosteal niche preparations, the flushed tibias and femurs were crushed with a mortar and pestle in the presence of 0.3% Collagenase/Dispase and 0.1% DNAse I (Roche, USA). The bones were incubated with the enzymes at 37^0C for 2 x 30 minute digests. Cells were recovered by centrifugation at 470 x g_{max} for 5 minutes at 4^0C. Cell counts were determined by gating viable cells based on cell size using a Z2 Coulter Counter (Beckman Coulter, U.S.A.).

3.3.4 Red Blood Cell Lysis

Red blood cells in the bone marrow preparation (hematopoietic niche) were lysed by adding 1mL of 8.3g/L ammonium chloride in 0.01M Tris-HCL buffer per a bone marrow suspension for 2 minutes at room temperature. Cells were then washed with 9mL FACS buffer and recovered by centrifugation at 470 x g_{max} for 5 minutes at 4^0C.

3.3.5 BM Transplantation Assay with Lethal Irradiation

3.3.5.1 LT-HSC Transplant Assay

Donor cells were harvested and pooled from untreated CD45.2$^+$ 2-mo; 9-mo CD45.2$^+$ animals 2 days following surgical shSSA (d2shSSA); or 9-mo CD45.2$^+$ animals 2 days following surgical SSA (d2SSA). Supporting cells were harvested from untreated 2-mo CD45.1$^+$ congenic animals. 1 x 10^6 CD45.1$^+$ cells whole bone marrow (WBM) cells or 2000 purified CD45.2$^+$ LT-HSCs were mixed and injected into each recipient mouse, intravenously via the tail vein. Recipient 2-mo CD45.1$^+$ mice were conditioned with lethal 1100 rad split

dose 2 hours before transplantation. Spleens were harvested 6 weeks post transplantation and FACS analysed to assess peripheral reconstitution.

3.3.5.2 BM Cell Dose-response Assay

Donor cells were harvested and pooled from untreated CD45.1$^+$ 2-mo; 9-mo CD45.1$^+$ animals 7 days following surgical shSSA (d7shSSA); or 9-mo CD45.2$^+$ animals 7 days following surgical SSA (d7SSA). Recipient 2-mo CD45.2$^+$ mice were conditioned as mentioned above and injected with either 5 x 10^5 or 5 x 10^6 BM cells intravenously via the tail vein. BM, spleen and thymus were analysed via FACS to assess peripheral reconstitution.

3.3.6 Flow Cytometric Analysis

The following fluorochrome labelled antibodies against murine antigens (as well as appropriate isotype controls) were used: FITC-conjugated anti-CD34 (RAM34), anti-CD45 (30-F11), anti-CD61 (2C9.G2), anti-TER119 (TER-119), PE-conjugated anti-CD135 (A2F10.1) and anti-CD51 (RMV-7), APC-conjugated anti-CD117 (2B8), anti-CD45 (30-F11) and anti-TER119 (TER-119), PECy7-conjugated anti-Sca1 (D7), biotin (MEC 13.3) or APC (MEC 13.3) -conjugated anti-CD31, APC eFluor 780-conjugated anti-CD25, and Pacific Blue-conjugated anti-CD44 (IM7) and anti-CD45.1 (A20). Lineage cocktail consisted of the following antibodies conjugated to Biotin: anti-CD3 (145-2C11), anti-CD4 (GK1.5), anti-CD8α (53-6.7), anti-CD19 (1D3), anti-CD11b (M1/70), anti-CD11c (HL3), anti-Gr-1 (RB6-8C5) and anti-B220 (RA3-6B2). Secondary reagents used were APC Cy7, Pacific Orange or PercPCy5.5 conjugated streptavidin.

All conjugates were purchased from BD Biosciences except streptavidin- APC Cy7 and PerCPCy5.5, which were purchased from eBiosciences and Pacific Orange from Molecular Probes.

All cells were analyzed on the multiparameter FACSCanto II (BD Biosciences, U.S.A.) using Flow Jo software (Flow Jo, USA).

3.3.7 Quantitative PCR Analysis

Bone marrow stromal cells purified by cell sorting from pooled vascular or endosteal fractions (at least 10 animals/treatment) snap frozen in liquid nitrogen and stored at -80°C prior to RNA extraction. Total RNA was isolated using the Qiagen RNEasy Micro- or Mini-Kit according to manufacturer's instructions (Qiagen, USA). cDNA was produced using Superscript III First-strand Synthesis System (Invitrogen, USA) with oligo$(dT)_{20}$ oligonucleotides (Invitrogen, USA) as per manufactures instructions. Q-PCR was performed using Platinum SYBR Green Supermix-UDG (Invitrogen, USA) on a Corbett Rotor-Gene 3000 (Corbett Research, Australia). The $2^{-\Delta\Delta Ct}$ method was used to calculate relative levels of target mRNA compared to GAPDH. Relative expression of d7SSA animals was compared to d7shSSA control animals. The pre-validated primers for *CXCL12* (QT00161112); *SCF* (QT00199430); *Osteopontin* (QT00157724); *Angiopoietin* (QT00166859); *Jagged-1* (QT00115703); *VCAM-1* (QT00128793); *BMP-4* (QT00111174); *IL-7* (QT00101318); *TGF-b1* (QT00145250), *Osterix, Osteopontin, Osteocalcin, Osteoactivin, Alkaine phosphatase*; were purchased from Qiagen.

3.3.8 Microarray Analysis

BM niche (CD45$^-$, OBLs and endothelial cells): 500,000-1,000,000 CD45$^-$Ter119$^-$, OBLs or endothelial cells were isolated from either the vascular or endosteal niche bone marrow of 2-mo untreated, 9-mo untreated, 9-mo ShSSA and 9-mo SSA mice 2, 4, 7, 10 days post-surgical castration, using Miltenyi CD45 Depletion Kit (Miltenyi Biotechnology, Germany) and FACS (CD45$^-$Ter119$^-$ with either CD31 or CD51 for endothelium and OBLs respectively). Total RNA was isolated using the Qiagen RNEasy Micro- or Mini-Kit according to manufacturer's instructions (Qiagen, USA). Mouse total RNA was quality ascertained using the Agilent Bioanalyser 2100 using the NanoChip protocol. A total of 500ng was labelled using the Ambion Total Prep RNA amplification kit (Cat. No. – IL1791). A total of 1.5μg of labelled cDNA was then prepared for hybridisation to the Mouse WG-6_V2 array (illumina, www.illumina.com) by preparing a probe cocktail (cDNA at 0.05μg/μl) that includes GEX-HYB Hybridisation Buffer (supplied with the beadchip).

A total hybridisation volume of 30μl is prepared for each sample and 30μl loaded into a single array on the Mouse WG-6_V2 array. A total of 6 different labelled samples can be loaded into 6 individual arrays per beadchip. The chip is hybridised at 58°C for 16 hours in an oven with a rocking platform. After hybridisation, the chip was washed using the appropriate protocols as outlined in the illumina manual. Upon completion of the washing, the chips were then coupled with Cy3 and scanned in the illumina iScan scanner. The scanner operating software, GenomeStudio, was used to convert the signal on the array into a TXT file for analysis.

3.3.8.1 Gene Expression and Pathway Analysis:

Raw data was imported into Partek v 6.5 (www.Partek.com). Data below a value of 50 were floored to an expression level of 50, which was approximately 50% of background signal intensity. Thereafter, data was filtered by coefficient of variance, which had to be at least 20%. Then, data was log2 transformed, and quantile normalized. Testing for statistical significance was performed by applying ANOVA. Probe sets with unadjusted p-value of less than 0.05 and a fold change of at least 1.5 were considered to be differentially expressed genes (DEG). DEGs were used for hierarchical cluster analysis, using squared Euclidean and single linkage as distance measure.

Microarray processing and initial bioinformatics analyses using Partek Genomic Suite and R/Bioconductor, were conducted by the Australian Genome Research Facility Ltd (AGRF; Melbourne).

3.3.9 Statistical Analysis

Statistical analysis was performed with the nonparametric, unpaired Mann-Whitney *U* test (when n≥ 4) or student *T* test (when n=3) using Graphpad Prism software (Graphpad Software, USA). A *p* value less than or equal to 0.05 was considered to be statistically significant.

3.4 RESULTS

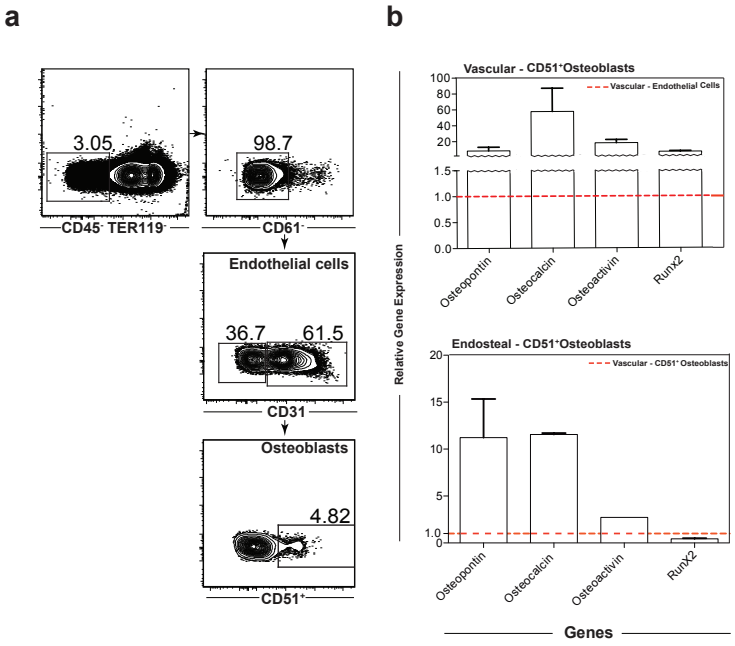
3.4.1 OBLs can be Phenotypically Isolated from Vascular BM

In order to assess the hematopoietic supporting capacity of the BM, the BM niche was further characterized by analysing both the endosteal and vascular compartments. Given the importance of OBLs in LT-HSC maintenance and development, we focussed our analysis on this population. Interestingly, we identified $CD51^+$ putative OBLs in both vascular and endosteal fractions of the BM (Figure 3.1 a). Endothelial and osteoclast lineage cells were excluded from the analysed population by lack of expression of CD31 and CD61, respectively. To test the osteolineage potential of $CD51^+$cells in these fractions, we isolated mRNA from the $CD45^-TER119^-CD61^-CD31^-CD51^+$ population and analysed for expression of OBL specific genes (*osteopontin, osteocalcin, osteoactivin* and *Runx2*) (35, 36). Whilst OBLs have been primarily identified within the endosteum, vascular $CD51^+$ OBLs clearly expressed these markers, which include the early factor *Runx2* (Figure 3.1 b), suggesting a precursor osteoblastic lineage. Consistent with this interpretation, endosteal $CD51^+$ OBLs had greater expression of the mature marker osteocalcin and lower expression of the immature marker *Runx2* when compared to vascular OBLs (Figure 3.1 b).

3.4.2 Age-associated Decrease in Endothelial Cells is Reversed by SSA but Does Not Alter the Age-associated Increase in OBLs

SSA has been shown to enhance the function of HSPCs within the BM (Chapter 2,4 and (14)). To assess the effects of SSA on cellular components of the BM niche, mice were surgically ShSSA or SSA (reduces serum testosterone levels to ~1% of normal levels within 6 hours) and the hematopoietic niche analysed by flow cytometry. Analysis was restricted to the non-hematopoietic compartment of the BM ($TER119^-$ $CD45^-$ $CD61^-$).

Figure 3.1 Phenotypic characterization CD51$^+$ cells in the vascular niche reveal an immature OBL population (a) The TER119$^-$ CD45$^-$ stromal population can be further subdivided into CD31$^+$ endothelial cells and CD31$^-$ CD51$^+$ osteoblast cells. Shown are representative flow cytometry profiles from an untreated 2-mo mouse to illustrate gating strategies. **(b)** Expression of key OBL-associated genes in vascular CD51$^+$ OBLs (▭) relative to CD31$^+$ endothelial cells (---) and endosteal OBLs(---) in 2-mo mice (n=10).


a
b
3.05
98.7
CD45- TER119-
CD61-
Endothelial cells
36.7
61.5
CD31
Osteoblasts
4.82
CD51+
Vascular - CD51+Osteoblasts
Vascular - Endothelial Cells
Relative Gene Expression
Osteopontin
Osteocalcin
Osteoactivin
Runx2
Endosteal - CD51+Osteoblasts
Vascular - CD51+ Osteoblasts
Genes

Following SSA, there were no changes in total BM cellularity except at the early timepoint, day 7, whereby there was a decline in total vascular niche cells with surgery compared to 2-mo untreated BM (Figure 3.2 a). Further dissecting the $CD45^-$ fraction, whilst there were no changes with age in endosteal OBLs cell numbers (Figure 3.2 c) we found an age-associated increase in the number of $CD51^+$ OBLs in the vascular hematopoietic niche; (Figure 3.2 b), consistent with the increase in HSC numbers by phenotype (11, 13) (Figure 2.1). However,

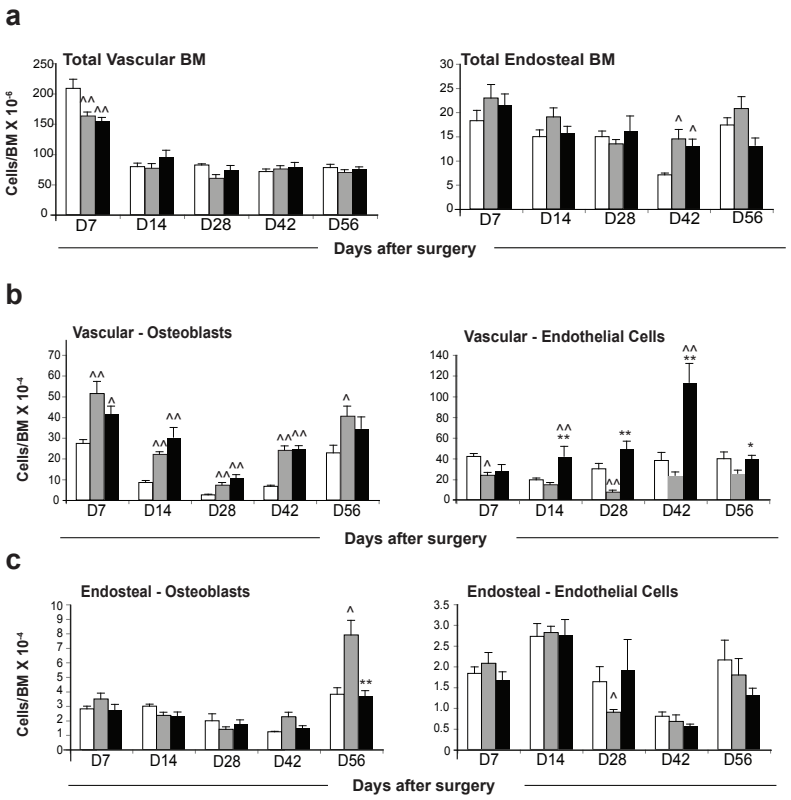
despite its impact on HSC numbers after day 14, SSA (Figure 2.1) did not alter the number of the vascular OBLs (Figure 3.2 b).

In contrast to our findings with $CD51^+$ OBLs, the number of vascular endothelial cells declines with age (Figure 3.2 b). SSA restored the number of endothelial cells back to levels beyond untreated young mice from day 14 post SSA and remained significantly elevated through to at least day 56 (Figure 3.2 b). This was not seen with the endosteal endothelial cells (Figure 3.2 c).

3.4.3 SSA Causes Profound Shifts in the Molecular Profile of the Hematopoietic Niche to Promote Lymphopoiesis

To better understand the molecular alterations associated with SSA-mediated hematopoietic rejuvenation; and in particular the role of hematopoietic niches in SSA-mediated lymphoid regeneration, we performed transcriptome analysis on the $CD45^-$ cells from the flushed marrow fraction (vascular niche) from untreated 2-mo and 9-mo, and 9-mo ShSSA and SSA mice at days 2, 4, 7 and 10 post surgery. Using the statistical analysis of variants (ANOVA), 232 genes had an absolute fold change greater than 2, between 2-mo and 9-mo mice, with 160 of these genes being downregulated in 9-mo samples. A heat map with expression of

Figure 3.2 SSA does not affect age-associated increase in OBL proportion. (a) Absolute number of total BM cells in the vascular and endosteal hematopoietic niche from 2-mo untreated (□), 9mo-sham-SSA (▨) or 9-mo SSA (■) mice at various timepoints after surgical SSA (n=5/group/timepoint). **(b)** Number of vascular and endosteal $CD51^+$ OBLs and $CD31^+$ endothelial cells (n=5/group/timepoint). All data are expressed as mean ± SEM. Data are cumulative of at least two independent experiments. * compared with shSSA 9-mo mice. ^ compared with 2-mo mice.


a
Total Vascular BM
Cells/BM X 10⁻⁶
250
200
150
100
50
0
D7
D14
D28
D42
D56
Total Endosteal BM
30
25
20
15
10
5
0
Days after surgery
b
Vascular - Osteoblasts
Cells/BM X 10⁻⁴
70
60
50
40
30
20
10
Vascular - Endothelial Cells
140
120
100
80
60
40
20
Days after surgery
c
Endosteal - Osteoblasts
Cells/BM X 10⁻⁴
10
9
8
7
6
5
4
3
2
1
0
Endosteal - Endothelial Cells
3.5
3.0
2.5
2.0
1.5
1.0
0.5
Days after surgery

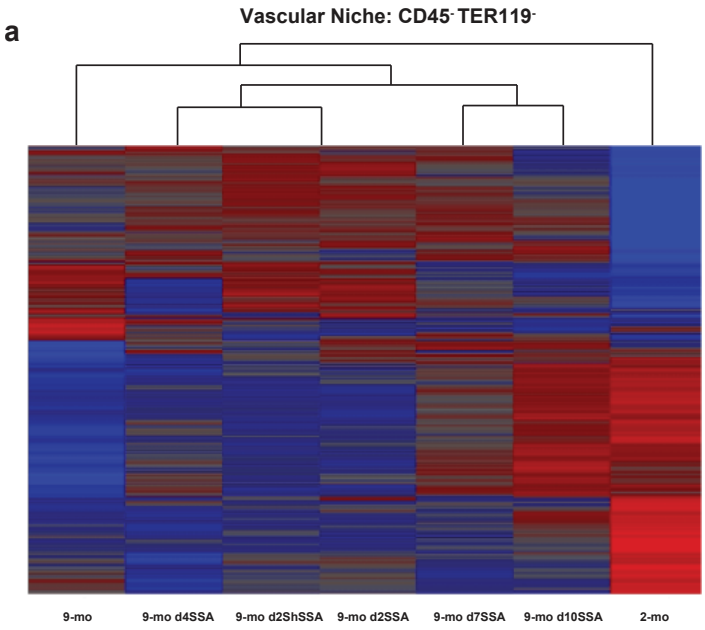
these genes across all timepoints is shown in Figure 3.3 a. Genes of particular interest that were downregulated in the aged include molecules associated with the extracellular matrix – collagens (15 fold decrease); osteoblast differentiation, such as secreted acidic cysteine rich glycoprotein (*Sparc; osteonectin*) (12 fold decrease) and bone carboxyglutamate proteins (*Bglap: Osteocalcin*), a promoter in pre-OBLs (10 fold decrease). Pattern discovery (correlation >0.7) identified 127 genes that demonstrated an increasing gene expression trend towards young profiles following castration of 9-mo old mice (Figure 3.3 b). This suggests that 80% of the genes downregulated with age in BM stromal cells were upregulated following SSA and reached close to young expression levels by day 10 (Figure 3.3 b).

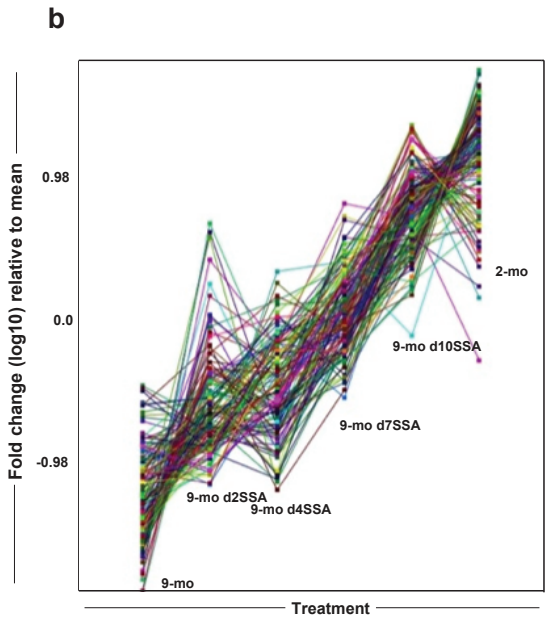
Further analysis using quantitative PCR of known hematopoiesis associated genes within $CD51^+$ OBLs and $CD31^+$ endothelial cells in the vascular and endosteal niche (Figure 3.4 a-b), revealed that while by d2SSA, trends suggest enhancement in the expression of key hematopoietic supporting molecules in vascular OBLs (Figure 3.4 a), this was only seen in

d7SSA endosteal OBLs when compared to ShSSA mice. In particular osteopontin and angiopoietin, two key regulators of HSC quiescence and self-renewal, were expressed at significantly greater levels compared to the d7ShSSA within the $CD51^+$ OBLs from both BM niche compartments (29, 37). Within the endosteal $CD51^+$ OBLs, other vital hematopoietic-associated signals involved in HSC homing (*CXCL-12* and *VCAM-1* (28, 38)), expansion (*Jagged-1* (39)), survival (*IGF-1* (40)) and quiescence (*Bmp4* and *TGF-*β (41, 42)) were significantly increased at d7SSA (Figure 3.4 b)

Also of importance, stem cell factor (*SCF*) and *IL-7* were also increased within vascular $CD31^+$ endothelial cells, indicating enhanced ability to support and promote lymphoid differentiation (Figure 3.4 b) (43, 44).

Figure 3.3 Progressive rejuvenation of the BM hematopoietic niche with SSA towards a 2-mo niche phenotype. 500,000-1,000,000 TER119$^-$ CD45$^-$ BM vascular cells were purified from 9-mo- d2,d4,d7,d10 following SSA and compared to sham-SSA and untreated 9-mo and 2-mo BM through microarray analysis using the Partek 6.6 software. **(a)** Heat map showing the differential gene expression between 9-mo and 2-mo CD45$^-$ TER119$^-$ hematopoietic stromal cells Gene upregulation is presented in red (—) and downregulation in blue (—). **(b)** Microarray pattern matching analysis based on a pattern discovery coefficient of >0.7 reveal 127 genes with progressive upregulation towards 2-mo levels with SSA.


a
Vascular Niche: CD45- TER119-
9-mo
9-mo d4SSA
9-mo d2ShSSA
9-mo d2SSA
9-mo d7SSA
9-mo d10SSA
2-mo


b
Fold change (log10) relative to mean
0.98
0.0
-0.98
2-mo
9-mo d10SSA
9-mo d7SSA
9-mo d2SSA
9-mo d4SSA
9-mo
Treatment

To assess whether the early molecular rejuvenation (d2SSA) that was observed within the vascular niche had any functional hematopoietic implications, we transplanted 2000 d2SSA and d2ShSSA CD45.2^{+} LT-HSCs together with 1 x 10^{6} supporting CD45.1^{+} whole BM cells into lethally irradiated (1100 rads) CD45.1^{+} 2-mo mice. Recipient spleens were harvested 6 weeks post transplantation and analysed by flow cytometry. d2SSA LT-HSCs exhibited no functional difference to d2ShSSA LT-HSCs indicating that these observed molecular changes within the vascular niche preceded any functional changes seen within the LT-HSC population at that timepoint (Figures 2.4 e and 3.4 c).

3.4.4 Sex Steroid Ablation Reduces the Number of Cells Required for Hematopoietic Engraftment Following HSCT

In order to measure the functional implications of these observed molecular rejuvenation in the CD45^{-} stromal population, we postulated that fewer of these cells would be required for similar levels of engraftment – such cell dose sparing has important clinical implications because one of the greatest challenges is obtaining sufficient HSC/kg body weight for the transplant. To test this, 2-mo recipient C57Bl/6 mice were given sham-SSA or SSA and 1 day later, lethally irradiated and reconstituted with either 5 x 10^{6} or 5 x 10^{5} BMCs from untreated congenic CD45.1 donors. At 28 days after transplant, primary organs were measured for lymphoid engraftment and at 84 days peripheral reconstitution was measured.

In the BM, while there was no significant difference in the total number of CD45.1^{+} LSK cells regardless of BMC dose transplanted (Figure 3.5 a), suggesting that even with the lower cell dose all BM niches had been filled, there was a significant increase in the number of donor-derived LSK cells in both dosage groups after SSA reflecting an increase in available BM niches. However, the number of cells transferred significantly affected the

Figure 3.4 Hematopoietic-associated molecular alterations precedes functional changes within LT-HSCs with SSA. $CD51^+$ OBLs and $CD31^+$endothelial cells were FACS purified from the $CD45^-TER119^-CD61^-$ stromal compartment of both the vascular and endosteal niche 2 **(a)** or 7 **(b)** days after SSA or sham-SSA (n=10) and relative expression of (a) CXCL-12, (b) SCF, (c) Osteopontin (Opn), (d) Angipoietin-1 (Ang), (e) Jagged-1 (Jag), (f) VCAM-1, (g) BMP-4, (h) IL-7, (i) TGFβ and (j) IGF-1 determined by quantitative PCR. Expression is represented as d7SSA (□) relative to d7shSSA control mice (---), n=3-11 independent experiments. * compared with d7shSSA 9-12mo mice.**(c)** 2000 $CD45.2^+$ LT-HSCs were FACS purified from d2SSA and d2sham-SSA mice and injected into 2-mo lethally irradiated $CD45.1^+$ mice. Donor derived hematopoiesis was measured in the spleen 6 weeks following intravenous injection. Representative profiles showing proportion of donor derived $TCR\beta^+$ T cells, $B220^+$ B cells and $CD11b^+$ myeloid cells in the spleen (n=6/group/treatment). All data are expressed as mean ± SEM. Data are cumulative of at least two independent experiments. * compared with shSSA 9-mo mice. ^ compared with 2-mo mice.

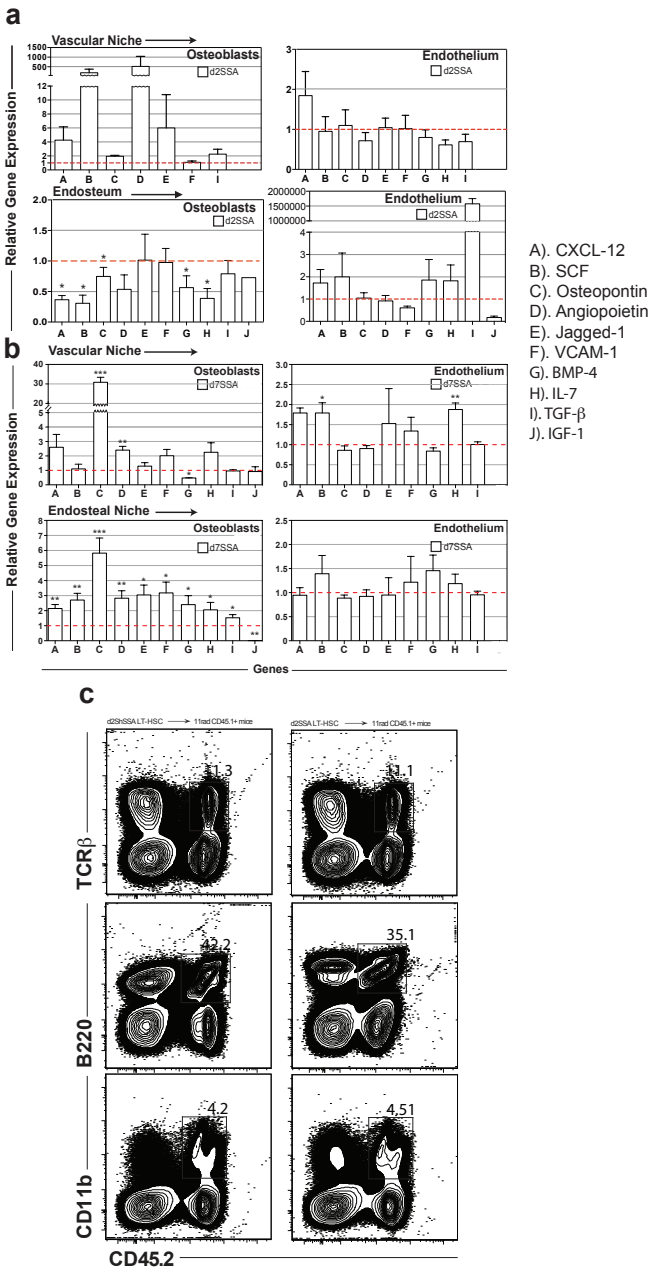
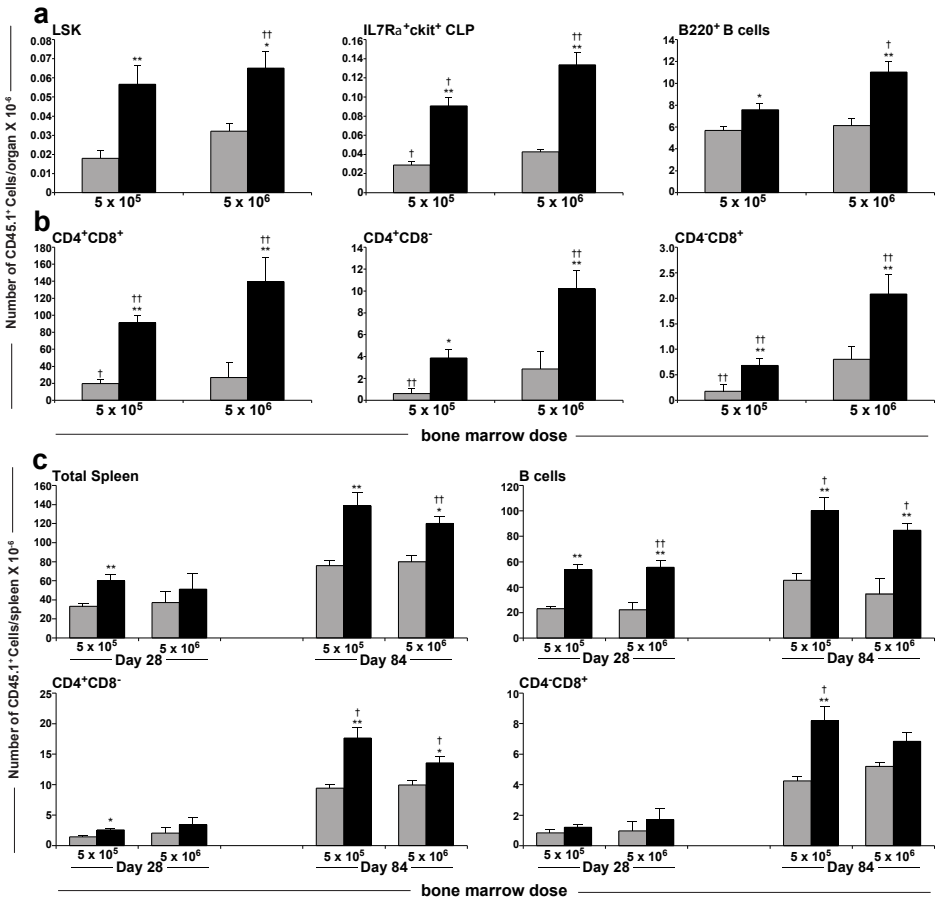

a
Vascular Niche
Osteoblasts
d2SSA
Endothelium
d2SSA
Endosteum
Osteoblasts
d2SSA
Endothelium
d2SSA
Relative Gene Expression
b
Vascular Niche
Osteoblasts
d7SSA
Endothelium
d7SSA
Endosteal Niche
Osteoblasts
d7SSA
Endothelium
d7SSA
Relative Gene Expression
Genes
A). CXCL-12
B). SCF
C). Osteopontin
D). Angiopoietin
E). Jagged-1
F). VCAM-1
G). BMP-4
H). IL-7
I). TGF-β
J). IGF-1
c
d2SHSSA LT-HSC → 11rad CD45.1+ mice
d2SSA LT-HSC → 11rad CD45.1+ mice
TCRβ
B220
CD11b
CD45.2
11.3
11.1
42.2
35.1
4.2
4.51

Figure 3.5 Withdrawal of sex steroids acts on both progenitors and the microenvironment to promote entry of circulating progenitors. Lethally irradiated 2-mo sham-SSA (□) or SSA (■) were reconstituted with either 5 x 10^5 or 5 x 10^6 BMCs from untreated 2-mo CD45.1$^+$ donors. **(a)** Number of donor-derived LSK, CLP-1 (Lin$^-$IL7Rα$^+$ckit$^+$) and B220$^+$ B cells in the BM 28 days after transplant. **(b)** Number of donor-derived intrathymic DP (CD4$^+$CD8$^+$) and SP (CD4$^+$TCRβ$^+$ or CD8$^+$TCRβ$^+$) thymocytes 28 days after transplant. **(c)** Number of donor derived peripheral leukocytes and lymphoid subsets 28 and 84 days after transplant. Results are expressed as mean ± SEM of five to seven mice for each group. Data are representative of two or more independent experiments. *, ($p \leq 0.05$); **, ($p \leq 0.01$); compared with sham-SSA mice receiving 5 x 10^5 BMCs. †, ($p \leq 0.05$); ††, ($p \leq 0.01$); compared sham-SSA mice receiving 5 x 10^6 BMCs.


a
LSK
IL7Ra+ckit+ CLP
B220+ B cells
b
CD4+CD8+
CD4+CD8-
CD4-CD8+
Number of CD45.1+ Cells/organ X 10-6
5 x 10^5
5 x 10^6
bone marrow dose
c
Total Spleen
B cells
CD4+CD8-
CD4-CD8+
Number of CD45.1+ Cells/spleen X 10-6
Day 28
Day 84
bone marrow dose

number of donor-derived CLPs ($Lin^{-}ckit^{+}IL7R\alpha^{+}$): SSA mice receiving 5 x 10^5 cells had an increased number of CLP cells compared to sham-SSA mice receiving 5 x 10^6 cells.

Downstream of CLPs, SSA improved engraftment of B cells in both dosage groups. In the thymus, there was also a direct relationship between the number of BMCs transplanted and the level of engraftment of T cells with a decrease in the number of donor derived DP, $CD4^{+}$ SP and $CD8^{+}$ SP cells in mice transplanted with 5 x 10^5 BMCs compared to 5 x 10^6, as expected (Figure 3.4 b). Following SSA there was a significant increase in the number of donor-derived thymocytes in both doses and for B cells in the BM.

In the periphery, while there was no precursor cell dose-dependent link to the number of donor-derived $CD45^{+}$ leukocytes at either 28 or 84 days after transplant in sham-SSA mice (Figure 3.4 c), following SSA there was a significant increase in both dosage groups compared to sham-SSA controls. This was reflected in both circulating B and T cell compartments. While the number of donor-derived B cells was significantly increased in both dosage groups and at both timepoints, $CD4^{-}CD8^{+}$ T cells were only increased in the SSA group receiving 5 x 10^5 cells at day 84.

3.5 Discussion

The functional aging of hematopoietic stem and progenitor cells is a significant clinical problem, particularly in the realm of HSC transplantation. Although several studies have demonstrated HSC-intrinsic defects as a result of aging, few have addressed extrinsic factors, particularly the impact of age on the supporting microenvironmental niche (9, 11, 45, 46). We have previously found SSA causes lymphoid regeneration and rejuvenation of HSPC function (2, 7, 14, 47, 48). Therefore we hypothesized that immunosenescence stemming from age-associated HSC defects are a consequence of both HSC-intrinsic and –extrinsic factors.

Several studies have made a correlation between the number of OBLs and the number of HSCs (21, 49). Consistent with this – and the well described age-associated increase in the number of HSCs – we found an accumulation of OBLs within the BM with age (Chapter 2 and (11, 13)). Interestingly, this age-associated increase was only found within the more vascularly located *Runx2* expressing pre-OBLs which also expressed hematopoiesis-supporting factors (50). The reduction in Osteocalcin promoter with age in these cells may be a contributing factor, although there was no obvious parallel reduction in osteoblast numbers in the endosteal niche, at least at 9-mo. Despite the numerical increase of HSCs after SSA, there was no corresponding change in the number of these OBLs. Transcriptome analysis however did reveal molecular changes in BM stromal cells after SSA, strongly indicative of functional improvement: these changes transformed the molecular "fingerprint" after SSA towards that of an untreated young mouse. Moreover, PCR revealed enhanced hematopoiesis-associated molecules within specific endothelial and OBL populations. Importantly, and consistent with our observed changes in HSC function after

SSA, both *Ang-1* and *Opn*; (regulators of HSC self-renewal and quiescence), were significantly increased by day 7 after SSA (26, 29, 37, 49, 51).

Interestingly, increases in *Opn* expression were observed as early as day 2 after SSA in vascular but not endosteal OBLs. Together with our findings that reveal early changes in gene expression within LT-HSCs after SSA (Chapter 2), this suggests that the effects of SSA can manifest in both hematopoietic stem cells as well as the stromal microenvironment that supports them. Although the suggestion is that SSA impacts on hematopoiesis by simultaneously and intrinsically altering the function of HSCs and their supporting stromal microenvironment, further studies are needed to elucidate the specific sequence of triggering events of SSA-mediated immune regeneration.

We have previously demonstrated that SSA can be used to condition recipients of autologous and allogeneic HSCT to enhance lymphoid reconstitution (4, 52). Strikingly, these studies also suggest that the effects of SSA on the hematopoietic niche can be exploited to achieve successful hematopoietic engraftment using fewer HSCs. This should not only improve all currently performed HSCT but may also contribute towards the use of cord blood HSCs for adult recipients, which is currently limited by the low yield and delayed reconstitution kinetics of these cells (53, 54). Thus, these data offer proof-of-principle for the clinical use of fewer HSCs for equivalent reconstitution outcomes in adults (46).

This study provides evidence for decline in the hematopoietic niche that underlies the degeneration in HSC function with age. While it is paradoxical that puberty and increasing age collectively depress the production and function of lymphoid cells, the ability of temporary sex steroid reduction to intrinsically rejuvenate both the HSC and BM stromal

function with age, provides a fundamentally new paradigm for the treatment of major clinical disorders. In particular, these findings suggest that SSA impacts intrinsically on the hematopoietic niche and this could be of immense benefit for the use of cord blood as a standard source of HSCs for transplants in older patients where the number of HSC per Kilogram of body weight is directly linked to survival outcome.

3.6 Acknowledgements

We gratefully acknowledge Jade Homann, Luciana Thompson and Jade Barbuto for surgery and animal handling; Andrew Fryga, Darren Ellemor, Karen Yuen, Kathryn Flanagan (FlowCore) and Geza Paukovics (AMREP) for expert cell sorting; This study was funded by grants from the Australian Stem Cell Centre, the Australian National Health & Medical Research Council, and Norwood Immunology. J.A.D. was supported by fellowships from the Australian National Health and Medical Research Council, the Leukemia and Lymphoma Society and a Sydney Parker Smith fellowship from the Cancer Council of Victoria.

3.7 REFERENCES

1. Dudakov, J. A., D. M. P. Khong, R. L. Boyd, and A. P. Chidgey. 2010. Feeding the fire: the role of defective bone marrow function in exacerbating thymic involution. *Trends in Immunology* 31:191-198.

2. Dudakov, J. A., G. L. Goldberg, J. J. Reiseger, K. Vlahos, A. P. Chidgey, and R. L. Boyd. 2009. Sex Steroid Ablation Enhances Hematopoietic Recovery following Cytotoxic Antineoplastic Therapy in Aged Mice. *The Journal of Immunology* 183:7084-7094.

3. Goldberg, G. L., C. G. King, R. A. Nejat, D. Y. Suh, O. M. Smith, J. C. Bretz, R. M. Samstein, J. A. Dudakov, A. P. Chidgey, S. Chen-Kiang, R. L. Boyd, and M. R. M. van den Brink. 2009. Luteinizing hormone-releasing hormone enhances T cell recovery following allogeneic bone marrow transplantation. *J Immunol* 182:5846-5854.

4. Goldberg, G. L., J. S. Sutherland, M. V. Hammett, M. K. Milton, T. S. Heng, A. P. Chidgey, and R. L. Boyd. 2005. Sex steroid ablation enhances lymphoid recovery following autologous hematopoietic stem cell transplantation. *Transplantation* 80:1604-1613.

5. Heng, T. S. P., G. L. Goldberg, D. H. D. Gray, J. S. Sutherland, A. P. Chidgey, and R. L. Boyd. 2005. Effects of castration on thymocyte development in two different models of thymic involution. *J Immunol* 175:2982-2993.

6. Sutherland, J. S., G. L. Goldberg, M. V. Hammett, A. P. Uldrich, S. P. Berzins, T. S. Heng, B. B.R., J. L. Millar, M. A. Malin, A. P. Chidgey, and R. L. Boyd. 2005. Activation of thymic regeneration in mice and humans following androgen blockade. *The Journal of Immunology* 175:2741 - 2753.

7. Chidgey, A., J. Dudakov, N. Seach, and R. Boyd. 2007. Impact of niche aging on thymic regeneration and immune reconstitution. *Semin Immunol* 19:331-340.

8. Nikolich-Zugich, J. 2008. Ageing and life-long maintenance of T-cell subsets in the face of latent persistent infections. *Nat Rev Immunol* 8:512-522.

9. Rossi, D., D. Bryder, J. Seita, and A. Nussenzweig.... 2007. Deficiencies in DNA damage repair limit the function of haematopoietic stem cells with age. *Nature*.

10. Geiger, H., and K. L. Rudolph. 2009. Aging in the lympho-hematopoietic stem cell compartment. *Trends in Immunology* 30:360-365.

11. Sudo, K., H. Ema, Y. Morita, and H. Nakauchi. 2000. Age-associated characteristics of murine hematopoietic stem cells. *J Exp Med* 192:1273-1280.

12. Kim, M., H. B. Moon, and G. J. Spangrude. 2003. Major age-related changes of mouse hematopoietic stem/progenitor cells. *Ann N Y Acad Sci* 996:195-208.

13. Rossi, D. J., D. Bryder, J. M. Zahn, H. Ahlenius, R. Sonu, A. J. Wagers, and I. L. Weissman. 2005. Cell intrinsic alterations underlie hematopoietic stem cell aging. *Proc Natl Acad Sci U S A* 102:9194-9199.

14. Dudakov, J. A., G. L. Goldberg, J. J. Reiseger, A. P. Chidgey, and R. L. Boyd. 2009. Withdrawal of Sex Steroids Reverses Age- and Chemotherapy-Related Defects in Bone Marrow Lymphopoiesis. *The Journal of Immunology* 182:6247-6260.

15. Schofield, R. 1978. The relationsihp between the spleen colony-forming cell and the hematopoietic stem cell. *Blood Cells* 4:7-25.

16. Méndez-Ferrer, S., T. V. Michurina, F. Ferraro, A. R. Mazloom, B. D. Macarthur, S. A. Lira, D. T. Scadden, A. Ma'ayan, G. N. Enikolopov, and P. S. Frenette. 2010. Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. *Nature* 466:829-834.

17. Ellis, S. L., and S. K. Nilsson. 2011. The location and cellular composition of the hemopoietic stem cell niche. *Cytotherapy* 14:135-143.

18. Nilsson, S. K. 2001. Spatial localization of transplanted hemopoietic stem cells: inferences for the localization of stem cell niches. *Blood* 97:2293-2299.

19. Wilson, A. A., and A. A. Trumpp. 2006. Bone-marrow haematopoietic-stem-cell niches. *Nature reviews. Immunology* 6:93-106.

20. Rashidi, N., and G. B. Adams. 2009. The influence of parathyroid hormone on the adult hematopoietic stem cell niche. *Curr Osteoporos Rep* 7:53-57.

21. Calvi, L. M., G. B. Adams, K. W. Weibrecht, J. M. Weber, D. P. Olson, M. C. Knight, R. P. Martin, E. Schipani, P. Divieti, F. R. Bringhurst, L. A. Milner, H. M. Kronenberg, and D. T. Scadden. 2003. Osteoblastic cells regulate the haematopoietic stem cell niche. *Nature* 425:841-846.

22. Komori, T. 2006. Regulation of osteoblast differentiation by transcription factors. *Journal of Cellular Biochemistry* 99:1233-1239.

23. Winkler, I. G., V. Barbier, R. Wadley, A. C. W. Zannettino, S. Williams, and J.-P. Lévesque. 2010. Positioning of bone marrow hematopoietic and stromal cells relative to blood flow in vivo: serially reconstituting hematopoietic stem cells reside in distinct nonperfused niches. *Blood* 116:375-385.

24. Lo Celso, C., H. E. Fleming, J. W. Wu, C. X. Zhao, S. Miake-Lye, J. Fujisaki, D. Côté, D. W. Rowe, C. P. Lin, and D. T. Scadden. 2009. Live-animal tracking of individual haematopoietic stem/progenitor cells in their niche. *Nature* 457:92-96.

25. Iwata, M., N. Awaya, L. Graf, C. Kahl, and B. Torok-Storb. 2004. Human marrow stromal cells activate monocytes to secrete osteopontin, which down-regulates Notch1 gene expression in CD34+ cells. *Blood* 103:4496-4502.

26. Stier, S., Y. Ko, R. Forkert, and C. Lutz.... 2005. Osteopontin is a hematopoietic stem cell niche component that negatively regulates stem cell pool size. *The Journal of* ....

27. Nie, Y., Y.-C. Han, and Y.-R. Zou. 2008. CXCR4 is required for the quiescence of primitive hematopoietic cells. *J. Exp. Med.* 205:777-783.

28. Christopher, M. J., F. Liu, M. J. Hilton, F. Long, and D. C. Link. 2009. Suppression of CXCL12 production by bone marrow osteoblasts is a common and critical pathway for cytokine-induced mobilization. *Blood* 114:1331-1339.

29. Arai, F., A. Hirao, M. Ohmura, H. Sato, S. Matsuoka, K. Takubo, K. Ito, G. Y. Koh, and T. Suda. 2004. Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. *Cell* 118:149-161.

30. Geiger, H., A. Koehler, and M. Gunzer. 2007. Stem cells, aging, niche, adhesion and Cdc42: a model for changes in cell-cell interactions and hematopoietic stem cell aging. *Cell cycle* 6:884-887.

31. Xing, Z., M. A. Ryan, D. Daria, K. J. Nattamai, G. van Zant, L. Wang, Y. Zheng, and H. Geiger. 2006. Increased hematopoietic stem cell mobilization in aged mice. *Blood* 108:2190-2197.

32. Köhler, A., V. Schmithorst, M.-D. Filippi, M. A. Ryan, D. Daria, M. Gunzer, and H. Geiger. 2009. Altered cellular dynamics and endosteal location of aged early hematopoietic progenitor cells revealed by time-lapse intravital imaging in long bones. *Blood* 114:290-298.

33. Kassem, M., and P. J. Marie. 2011. Senescence-associated intrinsic mechanisms of osteoblast dysfunctions. *Aging Cell* 10:191-197.

34. Pignolo, R. J., R. K. Suda, E. A. McMillan, J. Shen, S.-H. Lee, Y. Choi, A. C. Wright, and F. B. Johnson. 2008. Defects in telomere maintenance molecules impair osteoblast differentiation and promote osteoporosis. *Aging Cell* 7:23-31.

35. Geoffroy, V., M. Kneissel, B. Fournier, A. Boyde, and P. Matthias. 2002. High bone resorption in adult aging transgenic mice overexpressing cbfa1/runx2 in cells of the osteoblastic lineage. *Molecular and Cellular Biology* 22:6222-6233.

36. Manolagas, S. 2000. Birth and death of bone cells: basic regulatory mechanisms and implications for the pathogenesis and treatment of osteoporosis. *Endocr Rev* 21:115-137.

37. Nilsson, S. K., H. M. Johnston, G. A. Whitty, B. Williams, R. J. Webb, D. T. Denhardt, I. Bertoncello, L. J. Bendall, P. J. Simmons, and D. N. Haylock. 2005. Osteopontin, a key component of the hematopoietic stem cell niche and regulator of primitive hematopoietic progenitor cells. *Blood* 106:1232-1239.

38. Papayannopoulou, T., C. Craddock, B. Nakamoto, G. V. Priestley, and N. S. Wolf. 1995. The VLA4/VCAM-1 adhesion pathway defines contrasting mechanisms of lodgement of transplanted murine hemopoietic progenitors between bone marrow and spleen. *Proceedings of the National Academy of Sciences* 92:9647-9651.

39. Varnum-Finney, B., L. E. Purton, M. Yu, C. Brashem-Stein, D. Flowers, S. Staats, K. A. Moore, I. Le Roux, R. Mann, G. Gray, S. Artavanis-Tsakonas, and I. D. Bernstein. 1998. The Notch ligand, Jagged-1, influences the development of primitive hematopoietic precursor cells. *Blood* 91:4084-4091.

40. Burgess, W., K. Jesse, Q. Tang, S. R. Broussard, R. Dantzer, and K. W. Kelley. 2003. Insulin-like growth factor-I and the cytokines IL-3 and IL-4 promote survival of progenitor myeloid cells by different mechanisms. *Journal of Neuroimmunology* 135:82-90.

41. Goldman, D. C., A. S. Bailey, D. L. Pfaffle, A. Al Masri, J. L. Christian, and W. H. Fleming. 2009. BMP4 regulates the hematopoietic stem cell niche. *Blood* 114:4393-4401.

42. Batard, P., M. N. Monier, N. Fortunel, K. Ducos, P. Sansilvestri-Morel, T. Phan, A. Hatzfeld, and J. A. Hatzfeld. 2000. TGF-(beta)1 maintains hematopoietic immaturity by a reversible negative control of cell cycle and induces CD34 antigen up-modulation. *J Cell Sci* 113 (Pt 3):383-390.

43. Massa, S., G. Balciunaite, R. Ceredig, and A. G. Rolink. 2006. Critical role for c-kit (CD117) in T cell lineage commitment and early thymocyte development in vitro. *European Journal of Immunology* 36:526-532.

44. Bhatia, S. K., L. T. Tygrett, K. H. Grabstein, and T. J. Waldschmidt. 1995. The effect of in vivo IL-7 deprivation on T cell maturation. *The Journal of Experimental Medicine* 181:1399-1409.

45. Rossi, D. J., D. Bryder, J. M. Zahn, H. Ahlenius, R. Sonu, A. J. Wagers, and I. L. Weissman. 2005. Cell intrinsic alterations underlie hematopoietic stem cell aging. *Proceedings of the National Academy of Science USA* 102.

46. Donnini, A., F. Re, F. Orlando, and M. Provinciali. 2007. Intrinsic and microenvironmental defects are involved in the age-related changes of Lin - c-kit+ hematopoietic progenitor cells. *Rejuvenation research* 10:459-472.

47. Chidgey, A. P., N. Seach, J. Dudakov, M. V. Hammett, and R. L. Boyd. 2008. Strategies for reconstituting and boosting T cell-based immunity following haematopoietic stem cell transplantation: pre-clinical and clinical approaches. *Semin Immunopathol* 30:457-477.

48. Goldberg, G. L., J. A. Dudakov, J. J. Reiseger, N. Seach, T. Ueno, K. Vlahos, M. V. Hammett, L. F. Young, T. S. Heng, R. L. Boyd, and A. P. Chidgey. 2010. Sex Steroid Ablation Enhances Immune Reconstitution Following Cytotoxic Antineoplastic Therapy in Young Mice. *The Journal of Immunology* 184:6014-6024.

49. Zhang, J., C. Niu, L. Ye, H. Huang, X. He, W.-G. Tong, J. Ross, J. Haug, T. Johnson, J. Q. Feng, S. Harris, L. M. Wiedemann, Y. Mishina, and L. Li. 2003. Identification of the haematopoietic stem cell niche and control of the niche size. *Nature* 425:836-841.

50. Nakashima, K., X. Zhou, G. Kunkel, Z. Zhang, J. Deng, R. Behringer, and B. de Crombrugghe. 2002. The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. *Cell* 108:17-29.

51. Takakura, N., X. L. Huang, T. Naruse, I. Hamaguchi, D. J. Dumont, G. D. Yancopoulos, and T. Suda. 1998. Critical role of the TIE2 endothelial cell receptor in the development of definitive hematopoiesis. *Immunity* 9:677-686.

52. Goldberg, G. L., J. L. Zakrzewski, M. A. Perales, and M. R. M. van den Brink. 2007. Clinical strategies to enhance T cell reconstitution. *Semin Immunol* 19:289-296.

53. Gluckman, E. 2000. Current status of umbilical cord blood hematopoietic stem cell transplantation. *Exp Hematol* 28:1197-1205.

54. Wagner, J. E., J. N. Barker, T. E. DeFor, K. S. Baker, B. R. Blazar, C. Eide, A. Goldman, J. Kersey, W. Krivit, M. L. MacMillan, P. J. Orchard, C. Peters, D. J. Weisdorf, N. K. C. Ramsay, and S. M. Davies. 2002. Transplantation of unrelated donor umbilical cord blood in 102 patients with malignant and nonmalignant diseases: influence of CD34 cell dose and HLA disparity on treatment-related mortality and survival. *Blood* 100:1611-1618.

Chapter 4

Withdrawal of sex steroids enhances bone marrow production, release and thymic uptake of canonical and non-canonical T-lineage progenitors

Manuscript information

The following manuscript is being prepared to submit to the *Journal of Immunology*. The candidate, Danika Khong, was responsible for planning, performing and analysing experiments as well as writing and editing the manuscript. The co-authors Jarrod Dudakov, Lisa Spyroglou, Kahlia Wong, Ann Chidgey and Richard Boyd assisted with the experimental design, technical assistance, interpretation of results and drafting of the manuscript. Proportional contributions explained in the signed declaration.

MANUSCRIPT

Withdrawal of sex steroids enhances bone marrow production, release and thymic uptake of canonical and non-canonical T-lineage progenitors

Danika M.P. Khong[1,3], Jarrod A. Dudakov[1,2], Lisa Spyroglou[1], Kahlia Wong[1] , Ann P. Chidgey[1] and Richard L. Boyd[1,]

This work was supported by grants from Norwood Immunology, the Australian Stem Cell Centre and the National Health and Medical Research Council

[1] Immune Regeneration Laboratory, Monash Immunology and Stem Cell Laboratories (MISCL), Monash University, Wellington Rd, Clayton, Australia, 3800

[2] Current Address: Department of Immunology, Memorial Sloan-Kettering Cancer Center, 1275 York Ave, New York, NY, USA 10065

[3] Current Address: Harvard Stem Cell Institute, Holyoke Center, Suite 727W, 1350 Massachusetts Avenue, Cambridge, MA, USA 02138

* Prof. Richard L. Boyd, P: +[redacted] F: [redacted]

[redacted]

Abbreviations used in this paper: BM, Bone marrow; BMT, Bone marrow transplant; CLP, Common lymphoid progenitor; ELP, Early lymphoid progenitor; EPLM, Early progenitor of lymphoid and myeloid potential; ETP, early T-lineage progenitor; HSC, Hematopoietic stem cell; LMPP, lymphoid-primed multipotent progenitor; LT-HSC, long-term hematopoietic stem cell; LSK, $Lin^{-}Sca1^{+}ckit^{+}$; LSP, lymphoid specific precursor; MPP, multipotent progenitor; shSSA, sham sex steroid ablation; SSA, Sex steroid ablation; ST-HSC, short-term hematopoietic stem cell; WBM, whole bone marrow

Keywords – BM, lymphopoiesis, hematopoiesis, hematopoietic stem cell

4.1 Abstract

With age there is a significant decline in thymic function, which results in a decrease in the adaptive immune response. The thymus maintains T cell development through adult life by importing progenitors released from the BM into the circulation. We, and others, have previously shown that sex steroid ablation (SSA) regenerates the aged atrophic thymus and restores the number of intrathymic precursors to untreated young levels. Furthermore, BM hematopoietic stem cells (HSCs), lymphoid-primed multipotent progenitors (LMPPs) and common lymphoid progenitors (CLPs) are all increased indicating that SSA influences the production and, presumably, export of hematopoietic progenitors from the BM and uptake into the thymus. It is not known, however, if an increase in progenitors is sufficient to initiate the expansion of the thymus or whether triggers within the thymic stromal cells drive the increased import of progenitors into the thymus. Here we show that following SSA there is an increase in intrathymic canonical and non-canonical progenitors and BM content of LSK cells indicating an increased potential to develop into T cells. In a series of adoptive transfer experiments, we found that SSA improved the outcome of BMT at both the recipient and donor levels: SSA-recipient mice imported higher levels of both canonical and noncanonical progenitors into the thymus, while cells derived from SSA-treated donors resulted in increased entry of noncanonical progenitors into the thymus, but only in a damage model. Finally, SSA significantly reduced the number of BM cells required for efficient reconstitution of the immune system. These findings suggest that SSA impacts on both the hematopoietic microenvironment as well as hematopoietic progenitors cells to enhance lymphopoiesis in aged mice. This indicates that SSA can be clinically used to enhance lymphopoiesis following immunodepletion.

4.2 Introduction

The thymus, which is the primary site of T cell development, contains no self-renewing progenitors to maintain thymopoiesis, instead relying on the uptake of circulating bone marrow (BM) - derived progenitors (1) . The importation of thymus-bound progenitors is thought to be a gated event that coincides with their periodic release from the BM, and in the freeing of intrathymic microenvironmental niches (2-4). Although the specific mechanisms governing this axis remain unclear, expression of several specific molecules on circulating progenitors are important for mediating thymic entry by binding to their appropriate ligand on vascular endothelial cells lining the post-capillary venules, primarily within the cortico-medullary junction of the thymus (5-9). These include P-selectin glycosylated ligand (PSGL-1) on the progenitors, binding to P- selectin on the endothelium and the chemokine receptors CCR-9 and -7 which bind CCL-25 and CCL-19/ CC-21 respectively (7, 10, 11).

In addition to the specific mechanisms underlying thymic entry, the precise identity of the thymus-seeding progenitor also remains unclear. Long-term HSCs (LT-HSCs), which are capable of self-renewal and T cell development, are found in circulation but cannot be found within the thymus suggesting a lack of thymic homing capacity or immediate differentiation upon thymic endothelial binding and entry. Several studies have proposed that downstream CLP cells have the ability to both differentiate into T cells and are efficient at entering the thymus, however while experimentally these capacities can be demonstrated, their physiological role in steady-state T cell development is still unclear (12).

There is increasing evidence that lymphopoiesis involves passage through a lymphoid-biased Lineage$^-$ cKit$^+$ Sca1$^+$ (LSK) cell known as the lymphoid-primed multipotent progenitor (LMPP) (13, 14). Lymphoid associated PU.1, Ikaros, Flt3 and CD27 are upregulated within LMPPs while the megakarocyte/erythroid genes (GATA-1) and thrombopoietin as well as

the myeloid-associated myeloproliferative leukemia virus oncogene (Mpl) are both downregulated (15-21). LMPPs phenotypically and functionally mirror the earliest thymic progenitors (ETPs), which are cKit^{hi} and found within the early intrathymic triple negative 1 (TN1) CD44^{+} CD25^{-} population, specifically TN1a-c, making the LMPP a prime candidate for the putative thymus-seeding precursor (22, 23). Whilst ETPs retain some myeloid potential, they are very efficient at differentiating into T cells (24).

Interestingly, a recent study found that while populations of BM-derived and circulating MPPs passed through an ETP stage in the thymus, no such transition was noted for CLP-2 (Lineage^{-} IL7Rα^{+} B220^{+}cKit^{-} pre-Tα^{+}), suggesting a route of T cell development separate from the canonical ETP pathway (25).

With age there is a profound decline in lymphopoiesis in both the BM and thymus leading to a decrease in export of naïve lymphocytes to the periphery (26-29). Upstream there is a decrease in the number of lymphoid progenitors supporting T and B lymphopoiesis as well as a decline in the lymphoid potential of HSCs (30-35). These effects of age are largely due to the influence of sex steroids, which directly impacts both the microenvironment supporting lymphopoiesis, as well as primitive lymphoid progenitors (36-40). We, and others, have previously shown that sex steroid ablation (SSA), either surgically or chemically using, for example, lutenizing hormone releasing hormone (LHRH) analogues, profoundly reverses age-related thymic atrophy and increasing the number of all thymocytes (41, 42). Furthermore, we have shown that SSA can intrinsically improve the function of HSCs, particularly in their ability to generate lymphopoiesis and that there is a selective increase in the previously mentioned LMPPs; which are the CD62L^{+}CD27^{+} population of CD34^{+}Flt3^{+} MPPs, a putative circulating cell capable of seeding the thymus (Chapter 2). The present study aimed to characterise the impact of SSA on the earliest intrathymic progenitors as well as to assess the importation of canonical (LMPP and ETP) and noncanonical (CLP-2) BM-derived thymus-seeding progenitors following SSA.

4.3 Materials and Methods

4.3.1 Animals

Inbred male C57BL/6, IL7R$\alpha^{-/-}$ or congenic CD45.1 mice were obtained from the Animal Resources Centre (Perth, Australia) or Monash Animal Services (Clayton, Australia). Young mice were between the ages of 6-8 weeks and aged mice were 8-9 months of age in this study. Mice were maintained at the Monash Animal Services (Clayton, Australia) and were allowed to acclimatize for 7 days before experimentation, which was performed according to animal experiment ethics committee guidelines and approval.

4.3.2 Surgical Castration

Mice were anaesthetized by inhalation of a gaseous mixture of 2.5% isofluorane and 2% oxygen. A small incision was made in the scrotum. The testes were exposed, ligated with dissolvable sutures and removed. The wound was closed with dissolvable sutures. For surgical stress control, sham SSA was performed as above, but without the removal of the testes.

4.3.3 Cell Suspensions

Single cell suspensions of the vascular niche of bone marrow (BM) were obtained by flushing freshly dissected tibias and femurs with cold PBS supplemented with 0.2% BSA and 0.02% sodium azide (FACS buffer) with a 22G needle (Becton Dickinson, USA). The resulting central marrow was then made into a single cell suspension by gentle pipetting. Marrow preparations for the hematopoietic content involved flushing the aforementioned bones with cold FACS buffer using a 26G needle (Becton Dickinson, USA) and then made into single cell suspension using a 22G needle (Becton Dickinson, USA). Single cell suspensions of spleen and thymus were obtained by mechanical digestion using two frosted glass slides. Cells were recovered by centrifugation at 470 x g_{max} for 5 minutes at 4^0C. Cell counts were

determined by gating viable cells based on cell size using a Z2 Coulter Counter (Beckman Coulter, U.S.A.).

4.3.4 Short-term Homing Assays

Femurs and tibias were harvested from untreated young, aged ShSSA and aged SSA from Ly5.1 (CD45.1) mice 7 days after surgery. A saturating transplant dose of 2 X 10^7 BM cells were transferred into unablated untreated young, aged ShSSA and aged SSA C57 Bl/6 (CD45.2) mice 7 days after surgery. Thymuses were harvested 40 hours after transplant and analyzed for donor reconstitution.

4.3.5 Intrathymic Injection of LSK Cells

Femurs and tibias were harvested from untreated young, aged ShSSA and aged SSA Ly5.1 (CD45.1) mice 7 days after surgery and 200 Lineage$^-$Sca1$^+$cKit$^+$ cells were purified and transferred into each thymic lobe of unablated young C57 Bl/6 (CD45.2) recipients. Recipient thymuses were harvested 14 days after transplant and analyzed for donor reconstitution.

4.3.6 OP9-DL1 Cultures

OP9-DL1 were maintained and co-cultured as per the protocol described previously (43). 3000 LMPPs (Lineage$^-$ Sca1$^+$ cKit$^+$ CD34$^+$ Flt3$^+$ CD62L$^+$) were sorted from 9-mo ShSSA and SSA mice and cultured on GFP OP9-DL1 for 11 days in the presence of 5ng/mL of each mouse Flt3 ligand and mouse IL7 purchased from Miltenyi Biotec. Cells were analyzed by flow cytometry based on CD44 and CD25 expression after removal of all lineage (CD4, CD8, CD3, B220, Gr1, CD11b, TER119, NK1.1) and GFP positive cells.

4.3.7 LMPP Transplantation into IL7Rα-/-

3000 purified LMPPs (Lineage$^-$ Sca1$^+$ cKit$^+$ CD34$^+$ Flt3$^+$ CD62L$^+$) were isolated from the BM of untreated 2-mo, 9-mo sham-SSA or 9-mo Ly5.1 (CD45.1$^+$) mice SSA 7 days after surgery and intravenously injected into IL7Rα$^{-/-}$ (CD45.2$^+$) mice. Donor T cell development (CD45.1) was measured by flow cytometry 28 days post transplantation by harvesting thymus and spleen.

4.3.8 Flow Cytometric Analysis

The following fluorochrome labelled antibodies against murine antigens (as well as appropriate isotype controls) were used: FITC-conjugated anti-CD24 (M1/69), PE-conjugated anti-CD127 (A7R34), PercP-conjugated anti-B220 (RA3-6B2), APC-conjugated anti-CD117 (2B8), PE-Cy7-conjugated anti-Sca1 (D7), APC-Alexa Fluor 750-conjugated anti-CD25 (PC61.5), Pacific Blue-conjugated CD44 (IM7), PE-Cy7 or biotin-conjugated anti-CD45.1 (A20). Secondary reagents used were FITC, PercP or Pacific Orange-conjugated to streptavidin. Lineage cocktail consisted of the following antibodies conjugated to Biotin, FITC or PE: anti-CD3 (145-2C11), anti-CD4 (GK1.5), anti-CD8β (53-6.7), anti-CD19 (1D3), anti-CD11b (M1/70), anti-CD11c (HL3) and anti-Gr-1 (RB6-8C5).

All conjugates were purchased from BD Biosciences except anti-CD127 PE, which was purchased from Chemicon International. All cells were analysed or sorted on multiparameter FACSCanto II (BD Biosciences), FACSVantage (BD Biosciences) or FACSAria (BD Biosciences) cytometers and cell sorters using CellQuest Pro and Diva software (BD Biosciences).

4.3.9 Statistical Analysis

Statistical analysis was performed with the nonparametric, unpaired Mann-Whitney *U* test (when n≥ 4) or student *T* test (when n=3) using Graphpad Prism software (Graphpad Software, USA). A *p* value less than or equal to 0.05 was considered to be statistically significant.

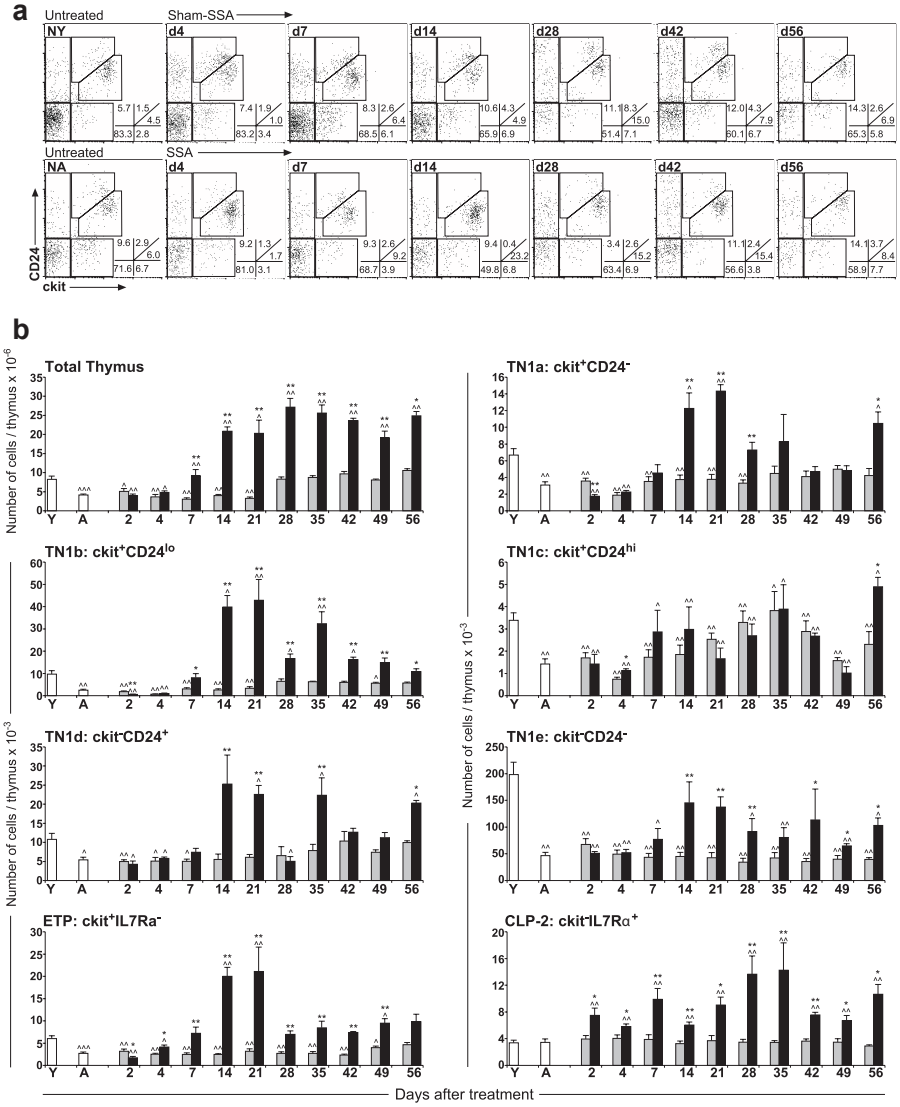
4.4 RESULTS

4.4.1 Withdrawal of Sex Steroids Increases Canonical and Non-Canonical Intrathymic T-lineage Progenitors in Aged Mice

To assess the impact of SSA on the earliest progenitors in the thymus, untreated 2mo and 9mo as well as 9mo sham-SSA and SSA mice were analysed for intrathymic progenitors by multiparameter flow cytometry. The $CD44^{+}CD25^{-}$ TN1 population was separated based on CD24 and ckit (44) as well as intrathymic $IL7R\alpha^{-}$ ETP and $IL7R\alpha^{+}B220^{+}$ CLP-2 populations enumerated. With age there was a shift in the proportion of TN1 subsets with an increase in TN1a-d cells and a decline in the proportion only in TN1e cells (Figure 4.1 a). Following SSA, there was little change in the proportion of most subsets compared to sham-SSA controls; however, at days 7, 14 and 42 there was a skewing away from TN1c cells towards TN1b. There was a significant decrease with age in the number of all TN1 subsets that corresponds with a decline in the total thymic cellularity (Figure 4.1 b). Interestingly, there was no age-associated decrease in the number of intrathymic $B220^{+}ckit^{-}$ cells, which contained the CLP-2 subset. Collectively, these data confirm previous reports, which suggest a decline in the number and function of intrathymic ETPs with age (45, 46).

Following SSA there was an initial decrease in the number of TN1a, b and ETP cells at day 2 (Figure 4.1 b). While there was little change in TN1c cells, there was a significant increase in the number of TN1b cells from day 7 and TN1a, d and e by day 14 after surgery. However, while this increase was maintained for up to 56 days within the TN1b population, increases in other subsets were less consistent (Figure 4.1 b). ETPs and CLP-2s were consistently increased across all timepoints from day 4 and 2 respectively, post-SSA.

Figure 4.1 Sex steroid ablation enhances the number of canonical and noncanonical intrathymic T cell progenitors. (a) Representative FACS plots of expression of CD24 and ckit gated on $Lin^{-}CD25^{-}CD44^{+}$ TN1 cells from untreated 2-mo, 9-mo and sham-SSA or SSA 9-mo mice at selected timepoints up to 56 days after surgery. **(b)** Absolute cellularity of thymus and intrathymic precursors from 2-mo, 9- mo (□) and sham-SSA (▨) or SSA (■) 9-mo mice up to 56 days after surgery. Results are expressed as mean ± SEM of five to ten mice for each group at each time point. Data are representative of two or more independent experiments. *, ($p \leq 0.05$); **, ($p \leq 0.01$); compared with sham-SSA 9-mo mice. ^, ($p \leq 0.05$); ^^, ($p \leq 0.01$); compared with 2-mo mice.


a
Untreated
Sham-SSA
NY
d4
d7
d14
d28
d42
d56
Untreated
SSA
NA
CD24
ckit
b
Total Thymus
TN1a: ckit+CD24-
TN1b: ckit+CD24lo
TN1c: ckit+CD24hi
TN1d: ckit-CD24+
TN1e: ckit-CD24-
ETP: ckit+IL7Ra-
CLP-2: ckit-IL7Rα+
Number of cells / thymus x 10-6
Number of cells / thymus x 10-3
Y A 2 4 7 14 21 28 35 42 49 56
Days after treatment

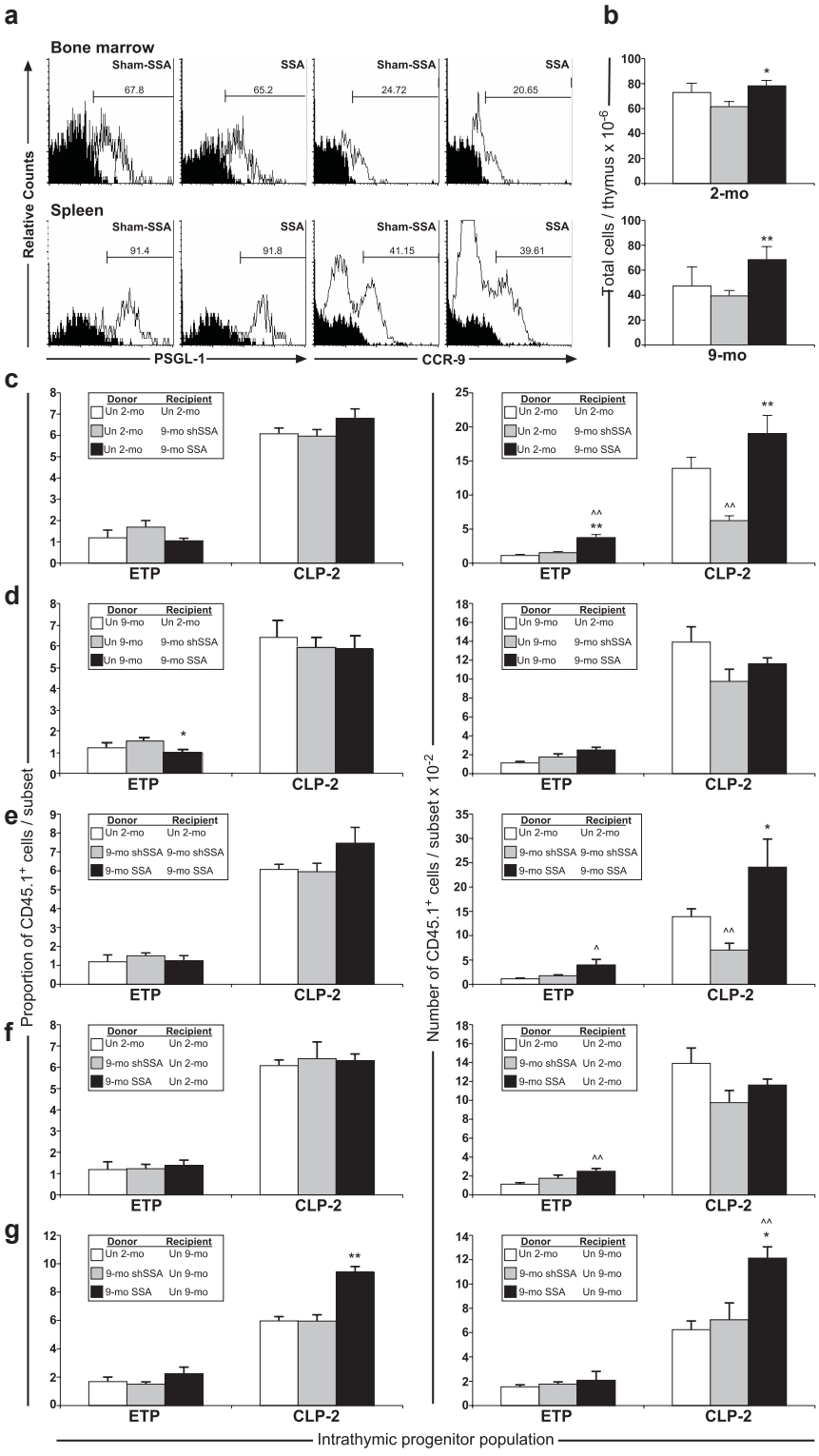
4.4.2 SSA Does Not Alter the Expression of PSGL-1 or CCR9 on BM or Circulating LSK Cells

Several cell surface receptors have been identified as being important for entry of circulating progenitors into the thymus including CD62L (22, 47), CD44 (48) and Flt3 (49) as well as two that are critical: CCR-9 (50) and PSGL-1 (51). To determine if the increase in primitive intrathymic progenitors following SSA was due to an increase in the ability of BM and circulating progenitors to enter the thymus, expression of PSGL-1 and CCR-9 on the surface of LSK cells following SSA (Figure 4.2 a) was analysed. At 7 days following surgery, there was no difference in the proportion of BM-resident or circulating LSK cells expressing CCR-9 or PSGL-1 on the surface.

4.4.3 *SSA Enhances Non-specific Import of T-lineage Progenitors by the Thymic Microenvironment and Directly Promotes the Entry of Non-canonical T-cell Progenitors*

The profound increase in thymic cellularity following SSA is accompanied by co-incident increases in the number of intrathymic progenitors as well as the supporting epithelial cells (46, 52) making identification of the earliest events in thymic regeneration difficult. To address if SSA acts on the thymic microenvironment to increase the uptake of progenitors and/or on the progenitors themselves and their ability to enter the thymus, a series of adoptive transfer experiments were conducted (Table 4.1). Interestingly, when BMCs from SSA mice were transferred into either untreated 2-mo or 9-mo mice, there was a significant increase in thymic cellularity compared to mice receiving BM from sham-SSA mice (Figure 4.2 b).

Figure 4.2 Withdrawal of sex steroids acts on both progenitors and thymic microenvironment to promote entry of circulating progenitors. (a) Representative FACS plots showing expression of PSGL-1 and CCR-9 on BM and splenic $Lin^{-}Sca1^{+}ckit^{+}$ cells from untreated 2-mo, sham-SSA and SSA 9-mo mice 7 days after surgery. **(b)** Total thymic cellularity of 2-mo and 9-mo mice 40 hours after transfer of 2 x 10^7 BMCs from untreated 2-mo and 9-mo sham-SSA or SSA mice 7 days after surgery. **(c)** *and* **(d)**, Proportion and number of donor-derived intrathymic ETP ($Lin^{-}CD25^{-}CD44^{+}IL7R\alpha^{-}ckit^{+}$) and CLP-2-like cells ($Lin^{-}CD25^{-}CD44^{+}IL7R\alpha^{+}ckit^{-}B220^{+}$) 40 hours after 2 x 10^7 untreated 2-mo **(c)** or 9-mo **(d)** BMCs were transplanted into unablated 2-mo and 9-mo sham-SSA or SSA mice 7 days after surgery. **(e)** Proportion and number of donor-derived intrathymic ETPs and CLP-2 cells 40 hours after 2 x 10^7 untreated 2-mo, 9-mo sham-SSA or SSA BMCs 7 days after surgery are transferred into untreated 2-mo, 9-mo sham-SSA or SSA mice 7 days after surgery. ***(f)*** *and* **(g)**, Proportion and number of donor-derived intrathymic ETPs and CLP-2 cells 40 hours after 2 x 10^7 untreated 2-mo, 9-mo sham-SSA or SSA mice 7 days after surgery are transplanted into untreated 2-mo **(f)** and 9-mo **(g)** unablated recipients. Results are expressed as mean ± SEM of five mice for each group. Data are representative of one or more independent experiments. *, ($p \leq 0.05$); **, ($p \leq 0.01$); compared with sham-SSA 9-mo mice. ^, ($p \leq 0.05$); ^^, ($p \leq 0.01$); compared with 2-mo mice.


a
Bone marrow
Sham-SSA
SSA
Sham-SSA
SSA
67.8
65.2
24.72
20.65
Spleen
91.4
91.8
41.15
39.61
Relative Counts
PSGL-1
CCR-9
b
Total cells / thymus x 10-6
2-mo
9-mo
c
d
e
f
g
Donor Recipient
Un 2-mo Un 2-mo
Un 2-mo 9-mo shSSA
Un 2-mo 9-mo SSA
Un 9-mo Un 2-mo
Un 9-mo 9-mo shSSA
Un 9-mo 9-mo SSA
9-mo shSSA 9-mo shSSA
9-mo SSA 9-mo SSA
9-mo shSSA Un 2-mo
9-mo SSA Un 2-mo
9-mo shSSA Un 9-mo
9-mo SSA Un 9-mo
ETP
CLP-2
Proportion of CD45.1+ cells / subset
Number of CD45.1+ cells / subset x 10-2
Intrathymic progenitor population

Donor (CD45.1)	age	n=	Recipient (CD45.2)	age	n*=
			Untreated	2-mo	5
Untreated	2-mo	5	Sham-SSA	9-mo	5
			SSA	9-mo	5
			Untreated	2-mo	5
Sham-SSA	9-mo	5	Sham-SSA	9-mo	5
			SSA	9-mo	5
			Untreated	2-mo	5
SSA	9-mo	5	Sham-SSA	9-mo	5
			SSA	9-mo	5

Table 4.1: Recipient groups for adoptive transfer experiments

* - has been performed at least twice

Untreated 2-mo or 9-mo sham-SSA or SSA mice, 7 days after surgery, were transplanted with 2 x 10^7 BMCs from untreated 2-mo (Figure 4.2 c), untreated 9-mo (Figure 4.2 d) or 9-mo SSA mice 7 days after surgery (Figure 4.2 e). Recipient thymuses were analysed 40 hours after transplant for the proportion and number of canonical and non-canonical progenitors of donor origin, thereby assessing the short-term homing capacity of these cells into untreated young, aged and regenerated thymi.

In mice that received BM cells from untreated 2-mo, there was no change in the proportion of donor-derived ETPs or CLP-2 cells in any of the three recipient groups (Figure 4.2 c). Interestingly, when young donor cells were transferred into 9-mo SSA mice there was a significant increase in both donor ETP and CLP-2 number, presumably reflecting an increase in intrathymic niche availability or very early intrathymic donor cell proliferation. When untreated 9-mo BMCs were transplanted into the same three recipient treatment groups, there was no change in the number of donor ETPs or CLPs, which suggests the quality of BMCs plays an important role in the increase in donor progenitors in SSA mice. This was confirmed by a cohort which received age- and treatment-matched donor BMCs, that is, untreated 2-mo mice received BMCs from untreated 2-mo, 9-mo SSA mice received BMCs from 9-mo SSA mice.

To assess the impact of SSA on the BM progenitors and their ability to enter the thymus, 2 x 10^7 BMCs from untreated 2-mo, 9-mo sham-SSA or 9-mo SSA mice 7 days after surgery were transferred into untreated 2-mo (Figures 4.2 b and f) or 9-mo (Figures 4.2 b and g) mice. Interestingly, while there was no change in total thymus cellularity when BMCs from 9-mo sham-SSA mice compared to 2-mo BMCs transferred, cells taken from SSA mice caused a significant increase in total thymic cellularity when transferred into both 2-mo and 9-mo mice (Figure 4.2 b). In the undamaged young thymus there was no change in the ability of cells from any of the donor groups to enter the thymus with no change in the number or proportion of ETPs or CLP-2 cells (Figure 4.2 f). In the aged, damaged, thymus however, there was a significant increase in the proportion and number of donor-derived CLP-2 cells (Figure 4.2 g) implicating these cells in damage repair.

4.4.4 SSA Enhances the T-cell Developmental Potential of LSK Cells

To ascertain the impact of SSA on the lymphoid developmental potential of HSCs, 200 purified CD45.1 LSK cells from untreated 2-mo or 9-mo sham-SSA or SSA-9-mo mice 7 days

after surgery, were transferred intrathymically into unablated 2-mo congenic recipients. Donor cell development was measured by flow cytometry 21 days after transplant. With age, despite a decline in the frequency of donor-derived ETPs, there is little impact in TN subsets (Figure 4.3). However, by the double positive (DP) stage, there is a significant decrease in the frequency of donor-derived cells from aged mice. This can partly be explained by an accumulation of donor-derived cells at the TN4 stage of thymocyte development. Following SSA there was a significantly enhanced developmental potential of LSK cells as evidenced by an increase in the frequency of donor-derived DP, $CD4^+$ and $CD8^+$ single positive (SP) thymocytes compared to sham-SSA controls. Interestingly, when examining the earliest intrathymic progenitors SSA impacts significantly on the frequency of donor-derived CLP-2 and TN2 cells but not ETPs. This is despite the likelihood that progeny of LSK cells in the thymus would preferentially differentiate down the canonical ETP pathway rather than the non-canonical CLP-2 pathway.

4.4.5 Sex Steroid Ablation Does Not Enhance the T Developmental Potential of LMPPs by Both In vitro and In vivo Assays

To determine the effects of SSA on the T cell progenitors, 3000 purified LMPPs were isolated from the BM of untreated 2-mo, 9-mo sham-SSA or 9-mo SSA 7 days after surgery and co-cultured with the OP9-DL1 BM stromal cell line which has been shown to support T lymphopoiesis *in vitro* (43). T cell development was measured by flow cytometry 11 days after initiation of culture. There were no differences between the 9-mo ShSSA and 9-mo SSA LMPPs in the kinetics of their development into T cells (Figure 4.4 a).

We used the IL7R$\alpha^{-/-}$ mice model as these mice have a block in their lymphocyte differentiation pathways, making their thymus and BM highly receptive to seeding progenitors due to high niche availability (53). Again, 3000 purified LMPPs were isolated from the BM of untreated 2-mo, 9-mo sham-SSA or 9-mo SSA 7 days after surgery and

Figure 4.3 Withdrawal of sex steroids promotes the differentiation of LSK cells down the T-lymphoid lineage. 200 LSK cells from untreated 2-mo (□) and 9-mo sham-SSA (▨) or SSA (■) mice 7 days after surgery, were intrathymically transferred into individual untreated 2-mo thymic lobes and the frequency of donor-derived thymocyte subsets were analysed 14 days after transplant. Results are expressed as mean ± SEM of individual thymic lobes from five mice for each group. *, ($p \leq 0.05$); **, ($p \leq 0.01$); compared with sham-SSA 9-mo mice. ^, ($p \leq 0.05$); ^^, ($p \leq 0.01$); compared with 2-mo mice.

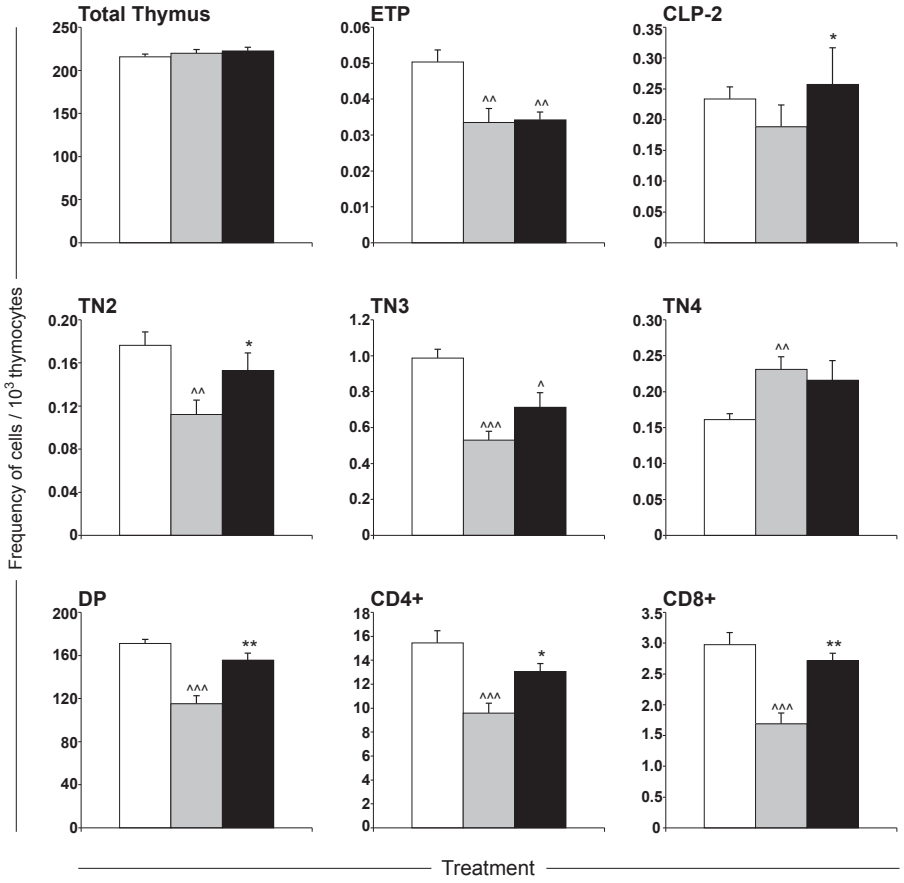
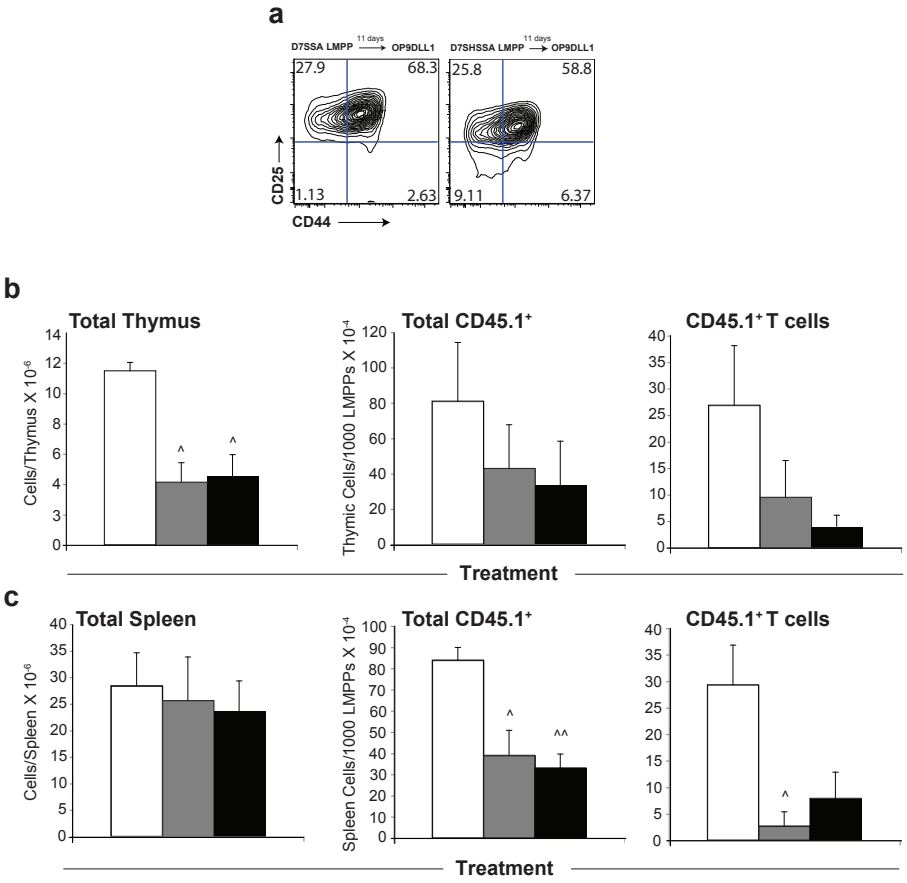

Total Thymus
ETP
CLP-2
TN2
TN3
TN4
DP
CD4+
CD8+
Frequency of cells / 10^3 thymocytes
Treatment

Figure 4.4 Sex steroid ablation does not reverse age-associated deficiencies in LMPP differentiation down the T-lylmphoid lineage. (a) 3000 LMPPs from untreated 2-mo, 9-mo sham-SSA or SSA mice 7 days after surgery were co-cultured with OP9-DL1 cells and analyzed 11 days later. **(b)** *and* **(c)**, 3000 LMPPs from untreated 2-mo (□) and 9-mo sham-SSA (▨) or SSA (■) mice, were intravenously injected into IL7Rα$^{-/-}$ 7 days after surgery. Spleen and thymus were analyzed 28 days after transplant. Results are expressed as mean ± SEM of individual thymic lobes from five mice for each group. *, ($p \leq 0.05$); **, ($p \leq 0.01$); compared with sham-SSA 9-mo mice. ^, ($p \leq 0.05$); ^^, ($p \leq 0.01$); compared with 2-mo mice.


a
D7SSA LMPP
11 days
OP9DLL1
D7SHSSA LMPP
11 days
OP9DLL1
27.9
68.3
1.13
2.63
25.8
58.8
9.11
6.37
CD25
CD44
b
Total Thymus
Cells/Thymus X 10-6
Total CD45.1+
Thymic Cells/1000 LMPPs X 10-4
CD45.1+ T cells
Treatment
c
Total Spleen
Cells/Spleen X 10-6
Total CD45.1+
Spleen Cells/1000 LMPPs X 10-4
CD45.1+ T cells
Treatment

transplanted into IL7Rα$^{-/-}$ mice. Donor T cell development was measured by flow cytometry 28 days post transplantation by harvesting thymus and spleen. There was a significant age-associated reduction in total thymic cellularity, however this was not reversed by d7SSA (Figure 4.4 b). While not statistically significant, the trend followed with reduced total CD45.1^{+} cell and CD45.1^{+} T cell frequencies in the spleen and no reversal using d7SSA cells (Figure 4.4 c).

4.5 Discussion

While the exact identity of the thymus-seeding T cell progenitor remains to be fully determined, it is becoming increasingly clear that multiple progenitors and routes of T cell development exist (13, 22, 24, 54-59). Previous work has indicated shifts within these systems with aging, and that following SSA there is a profound regeneration of the aged and chemically immunodepleted thymus (46, 60) with lymphoid lineage commitment of BM HSCs being restored to young levels (Chapter 2). Furthermore, there is a selective increase in resident BM CD62L^{+} LMPPs, analogous to intrathymic ETPs, suggesting there may be an improved uptake of progenitors into the thymus. However it remains to be seen whether increases in thymic cellularity are due to an increase in the number of progenitor cells able to enter the thymus or due to an increase in the number of intrathymic niches, or their enhanced promotion through intrathymic T cell development pathways. Here we show that following SSA there was an increase in both canonical (TN1a and TN1b) and noncanonical (TN1d and TN1e) subsets - a result confirmed by specific staining identifying IL7Rα^{-} ETPs and IL7Rα^{+}B220^{+} CLP-2-like populations. Interestingly, the earliest changes observed were within the CLP-2 fraction, a population that is unaffected by age.

Using a series of adoptive transfer experiments we showed that SSA-mediated regeneration of the aged thymus causes an increase in both canonical and noncanonical progenitors suggesting that an increase in niche availability, as the thymus enlarges, rather than a selective increase in the uptake of specific progenitor cells. However, as both the thymic microenvironment and the endogenous progenitors were exposed to the effects of SSA, the role of progenitors in triggering thymic regeneration cannot be discounted. Indeed BMCs taken from SSA mice led to enhanced thymic cellularity compared to sham-SSA mice. Interestingly, this increase in donor progenitors into the regenerated thymus only occurred when young or SSA BMCs and not when untreated 9-mo BMCs were transferred.

This suggests that in the young, and following SSA of the old, there is an increase in the number or quality of progenitors able to gain access to the thymus. Furthermore, when BMCs from SSA treated mice are transferred into an aged, but not young, thymus, there is an increase in the proportion and number of donor-derived noncanonical CLP-2 cells. Importantly, this suggests that regeneration following SSA may be, at least in part, mediated or initiated by noncanonical T cell development pathways. This is supported by our findings which show that CLP-2 cells in the thymus are the first precursor cell to be impacted following SSA at 2 days after surgery.

When purified LSK cells were injected directly into the 2-mo thymus, bypassing thymic entry events, there was an age-related decline in the ability of LSK cells to undergo thymopoiesis. This result concurs with previous studies showing an age-related decline in the lymphopoietic function of HSCs (61, 62). Accordingly, when LSK cells from SSA mice were transferred there was a significant increase in the frequency of donor-derived DP and SP cells in the thymus indicating SSA improved their ability to generate T cells.

Co-culture of committed T cell progenitors with the OP9-DLL1 BM stromal cell line indicated that there was a severe age-associated decline in the lymphoid differentiation capacity of LMPPs. Interestingly, this was not reversed with SSA suggesting that, in contrast to its effects on the most primitive LT-HSCs (Chapter 2), SSA promotes an increase in the number of LMPPs and other lymphoid progenitors rather than intrinsically conferring an enhanced lymphoid differentiation capacity on a per cell basis.

The present findings begin to unravel the nature of thymic regeneration following SSA and, in particular, the role of BM-derived progenitor cells in this process. SSA impacts BM and intrathymic progenitors as well as their supporting microenvironments so that, functionally, there is an improvement in the number and functional lymphoid potential of HSCs and LMPPs including their ability to generate T cells. While SSA apparently increases

intrathymic niche availability facilitating a general increase in the uptake of circulating progenitors, the effect of SSA on the progenitors themselves in the BM, seems to induce a noncanonical CLP-2-like precursor bias suggesting an alternate route of T cell recovert is utilized in regenerating a damaged thymus.

4.6 Acknowledgements

and animal handling; Andrew Fryga, Darren Ellemor, Karen Yuen, Kathryn Flanagan (FlowCore) and Geza Paukovics (AMREP) for expert cell sorting; Dr Andreas Strasser for IL7R$\alpha^{-/-}$ mice; Dr Andreas Scherer (AGFR) for bioinformatics analysis; and Mark Malin, Marcel van den Brink and Hanasha Lan for helpful discussions. This study was funded by grants from the Australian Stem Cell Centre, the Australian National Health & Medical Research Council, and Norwood Immunology. J.A.D. was supported by fellowships from the Australian National Health and Medical Research Council, the Leukemia and Lymphoma Society and a Sydney Parker Smith fellowship from the Cancer Council of Victoria.

4.7 References

1. Goldschneider, I., K. L. Komschlies, and D. L. Greiner. 1986. Studies of thymocytopoiesis in rats and mice. I. Kinetics of appearance of thymocytes using a direct intrathymic adoptive transfer assay for thymocyte precursors. *J Exp Med* 163:1-17.
2. Donskoy, E., D. Foss, and I. Goldschneider. 2003. Gated importation of prothymocytes by adult mouse thymus is coordinated with their periodic mobilization from bone marrow. *The Journal of Immunology* 171:3568-3575.
3. Foss, D., E. Donskoy, and I. Goldschneider. 2001. The importation of hematogenous precursors by the thymus is a gated phenomenon in normal adult mice. *Journal of Experimental Medicine* 193:365-374.
4. Prockop, S. E., and H. T. Petrie. 2004. Regulation of thymus size by competition for stromal niches among early T cell progenitors. *Journal of Immunology* 173:1604 - 1611.
5. Schwarz, B. A. 2007. Selective thymus settling regulated by cytokine and chemokine receptors. *The Journal of Immunology* 178:2008.
6. Goldschneider, I. 2006. Cyclical mobilization and gated importation of thymocyte progenitors in the adult mouse: evidence for a thymus-bone marrow feedback loop. *Immunological Reviews* 209:58-75.
7. Rossi, F. M. V., S. Y. Corbel, J. S. Merzaban, D. A. Carlow, K. Gossens, J. Duenas, L. So, L. Yi, and H. J. Ziltener. 2005. Recruitment of adult thymic progenitors is regulated by P-selectin and its ligand PSGL-1. *Nature Immunology* 6:626-634.
8. Wurbel, M. A., J. M. Philippe, C. Nguyen, G. Victorero, T. Freeman, P. Wooding, A. Miazek, M. G. Mattei, M. Malissen, B. R. Jordan, B. Malissen, A. Carrier, and P. Naquet. 2000. The chemokine TECK is expressed by thymic and intestinal epithelial cells and attracts double- and single-positive thymocytes expressing the TECK receptor CCR9. *Eur J Immunol* 30:262-271.
9. Uehara, S., A. Grinberg, J. Farber, and P. E. Love. 2002. A role for CCR9 in T lymphocyte development and migration. *The Journal of Immunology* 168:2811-2819.
10. Zlotoff, D. A., A. Sambandam, T. D. Logan, J. J. Bell, B. A. Schwarz, and A. Bhandoola. 2010. CCR7 and CCR9 together recruit hematopoietic progenitors to the adult thymus. *Blood* 115:1897-1905.
11. Krueger, A., S. Willenzon, M. Lyszkiewicz, E. Kremmer, and R. Forster. 2010. CC chemokine receptor 7 and 9 double-deficient hematopoietic progenitors are severely impaired in seeding the adult thymus. *Blood* 115:1906-1912.
12. Serwold, T. 2009. Reductive isolation from bone marrow and blood implicates common lymphoid progenitors as the major source of thymopoiesis. *Blood* 113:807.
13. Perry, S. S., R. S. Welner, T. Kouro, P. W. Kincade, and X.-H. Sun. 2006. Primitive lymphoid progenitors in bone marrow with T lineage reconstituting potential. *J Immunol* 177:2880-2887.
14. Igarashi, H., S. Gregory, T. Yokota, N. Sakaguchi, and P. Kincade. 2002. Transcription from the RAG1 locus marks the earliest lymphocyte progenitors in bone marrow. *Immunity* 17:117-130.
15. Scott, E., M. Simon, J. Anastasi, and H. Singh. 1994. Requirement of transcription factor PU. 1 in the development of multiple hematopoietic lineages. *Science* 265:1573-1577.
16. Yoshida, T., S. Y.-M. Ng, J. C. Zuniga-Pflucker, and K. Georgopoulos. 2006. Early hematopoietic lineage restrictions directed by Ikaros. *Nature Immunology* 7:382-391.

17. Ferreira, R., K. Ohneda, M. Yamamoto, and S. Philipsen. 2005. GATA1 function, a paradigm for transcription factors in hematopoiesis. *Molecular and Cellular Biology* 25:1215-1227.
18. Adolfsson, J., R. Månsson, N. Buza-Vidas, A. Hultquist, K. Liuba, C. T. Jensen, D. Bryder, L. Yang, O.-J. Borge, L. A. M. Thoren, K. Anderson, E. Sitnicka, Y. Sasaki, M. Sigvardsson, and S. E. W. Jacobsen. 2005. Identification of Flt3+ lympho-myeloid stem cells lacking erythro-megakaryocytic potential a revised road map for adult blood lineage commitment. *Cell* 121:295-306.
19. Luc, S., K. Anderson, S. Kharazi, N. Buza-Vidas, C. Boiers, C. T. Jensen, Z. Ma, L. Wittmann, and S. E. W. Jacobsen. 2008. Down-regulation of Mpl marks the transition to lymphoid-primed multipotent progenitors with gradual loss of granulocyte-monocyte potential. *Blood* 111:3424-3434.
20. Orkin, S., R. Shivdasani, M. Mcdevitt, Y. Fujiwara, and M. Mcdevitt. 1998. Transcription factor GATA-1 in megakaryocyte development. *Stem Cells* 16 Suppl 2:79-83.
21. Serwold, T., L. I. R. Ehrlich, and I. L. Weissman. 2009. Reductive isolation from bone marrow and blood implicates common lymphoid progenitors as the major source of thymopoiesis. *Blood* 113:807-815.
22. Perry, S. S., H. Wang, L. J. Pierce, A. M. Yang, S. Tsai, and G. J. Spangrude. 2004. L-selectin defines a bone marrow analog to the thymic early T-lineage progenitor. *Blood* 103:2990-2996.
23. Allman, D., A. Sambandam, S. Kim, J. P. Miller, A. Pagan, D. Well, A. Meraz, and A. Bhandoola. 2003. Thymopoiesis independent of common lymphoid progenitors. *Nat Immunol* 4:168-174.
24. Bell, J. J., and A. Bhandoola. 2008. The earliest thymic progenitors for T cells possess myeloid lineage potential. *Nature* 452:764-767.
25. Benz, C., V. C. Martins, F. Radtke, and C. C. Bleul. 2008. The stream of precursors that colonizes the thymus proceeds selectively through the early T lineage precursor stage of T cell development. *Journal of Experimental Medicine* 205:1187-1199.
26. LeMaoult, J., P. Szabo, and M. E. Weksler. 1997. Effect of age on humoral immunity, selection of the B-cell repertoire and B-cell development. *Immunol Rev* 160:115-126.
27. Weksler, M. E. 2000. Changes in the B-cell repertoire with age. *Vaccine* 18:1624-1628.
28. Yager, E. J., M. Ahmed, K. Lanzer, T. D. Randall, D. L. Woodland, and M. A. Blackman. 2008. Age-associated decline in T cell repertoire diversity leads to holes in the repertoire and impaired immunity to influenza virus. *J Exp Med* 205:711-723.
29. LeMaoult, J., I. Messaoudi, J. S. Manavalan, H. Potvin, D. Nikolich-Zugich, R. Dyall, P. Szabo, M. E. Weksler, and J. Nikolich-Zugich. 2000. Age-related dysregulation in CD8 T cell homeostasis: kinetics of a diversity loss. *J Immunol* 165:2367-2373.
30. Miller, J., and D. D. Allman. 2003. The decline in B lymphopoiesis in agedm ice reflects loss of very early B-lineage precursors. *Journal of Immunology* 171:2326-2330.
31. Allman, D., and J. P. Miller. 2005. The aging of early B-cell precursors. *Immunol Rev* 205:18-29.
32. Min, H., E. Montecino-Rodriguez, and K. Dorshkind. 2005. Effects of aging on early B- and T-cell development. *Immunol Rev* 205:7-17.
33. Min, H., E. Montecino-Rodriguez, and K. Dorshkind. 2004. Reduction in the developmental potential of intrathymic T cell progenitors with age. *Journal of Immunology* 173:245-250.
34. Rossi, D. J., D. Bryder, J. M. Zahn, H. Ahlenius, R. Sonu, A. J. Wagers, and I. L. Weissman. 2005. Cell intrinsic alterations underlie hematopoietic stem cell aging. *Proceedings of the National Academy of Science USA* 102:9194-9199.

35. Sudo, K., H. Ema, Y. Morita, and H. Nakauchi. 2000. Age-associated characteristics of murine hematopoietic stem cells. *Journal of Experimental Medicine* 9:1273-1280.
36. Medina, K. L., K. P. Garrett, L. F. Thompson, M. I. D. Rossi, K. J. Payne, and P. W. Kincade. 2001. Identification of very early lymphoid precursors in bone marrow and their regulation by estrogen. *Nat Immunol* 2:718-724.
37. Olsen, N. J., G. Olson, S. M. Viselli, X. Gu, and W. J. Kovacs. 2001. Androgen receptors in thymic epithelium modulate thymus size and thymocyte development. *Endocrinology* 142:1278-1283.
38. Olsen, N. J., X. Gu, and W. J. Kovacs. 2001. Bone marrow stromal cells mediate androgenic suppression of B lymphocyte development. *J Clin Invest* 108:1697-1704.
39. Igarashi, H., T. Kouro, T. Yokota, P. C. Comp, and P. W. Kincade. 2001. Age and stage dependency of estrogen receptor expression by lymphocyte precursors. *Proc Natl Acad Sci U S A* 98:15131-15136.
40. Kincade, P. W., H. Igarashi, K. L. Medina, T. Kouro, T. Yokota, M. I. Rossi, J. J. Owen, K. P. Garrett, X. H. Sun, and N. Sakaguchi. 2002. Lymphoid lineage cells in adult murine bone marrow diverge from those of other blood cells at an early, hormone-sensitive stage. *Seminars in Immunology* 14:385-394.
41. Heng, T. S. P., G. L. Goldberg, D. H. D. Gray, J. S. Sutherland, A. P. Chidgey, and R. L. Boyd. 2005. Effects of castration on thymocyte development in two different models of thymic involution. *J Immunol* 175:2982-2993.
42. Sutherland, J. S., G. L. Goldberg, M. V. Hammett, A. P. Uldrich, S. P. Berzins, T. S. Heng, B. B.R., J. L. Millar, M. A. Malin, A. P. Chidgey, and R. L. Boyd. 2005. Activation of thymic regeneration in mice and humans following androgen blockade. *The Journal of Immunology* 175:2741 - 2753.
43. Schmitt, T. M., and J. C. Zúñiga-Pflücker. 2006. T-cell development, doing it in a dish. *Immunol Rev* 209:95-102.
44. Porritt, H. E., L. L. Rumfelt, S. Tabrizifard, T. M. Schmitt, J. C. Zuniga-Pflucker, and H. T. Petrie. 2004. Heterogeneity among DN1 prothymocytes reveals multiple progenitors with different capacities to generate T cell and non-T cell lineages. *Immunity* 20:735-745.
45. Min, H., E. Montecino-Rodriguez, and K. Dorshkind. 2004. Reduction in the developmental potential of intrathymic T cell progenitors with age. *J Immunol* 173:245-250.
46. Heng, T. S., G. L. Goldberg, D. H. Gray, J. S. Sutherland, A. P. Chidgey, and R. L. Boyd. 2005. Effects of castration on thymocyte development in two different models of thymic involution. *J Immunol* 175:2982-2993.
47. Perry, S. S., R. S. Welner, T. Kouro, P. W. Kincade, and X. H. Sun. 2006. Primitive lymphoid progenitors in bone marrow with T lineage reconstituting potential. *J Immunol* 177:2880-2887.
48. Wu, L., P. W. Kincade, and K. Shortman. 1993. The CD44 expressed on the earliest intrathymic precursor population functions as a thymus homing molecule but does not bind to hyaluronate. *Immunology letters* 38:69-75.
49. Schwarz, B. A., A. Sambandam, I. Maillard, B. C. Harman, P. E. Love, and A. Bhandoola. 2007. Selective thymus settling regulated by cytokine and chemokine receptors. *J Immunol* 178:2008-2017.
50. Benz, C., and C. C. Bleul. 2005. A multipotent precursor in the thymus maps to the branching point of the T versus B lineage decision. *The Journal of experimental medicine* 202:21-31.
51. Rossi, F. M., S. Y. Corbel, J. S. Merzaban, D. A. Carlow, K. Gossens, J. Duenas, L. So, L. Yi, and H. J. Ziltener. 2005. Recruitment of adult thymic progenitors is regulated by P-selectin and its ligand PSGL-1. *Nature immunology* 6:626-634.

52. Gray, D. H., N. Seach, T. Ueno, M. K. Milton, A. Liston, A. M. Lew, C. C. Goodnow, and R. L. Boyd. 2006. Developmental kinetics, turnover, and stimulatory capacity of thymic epithelial cells. *Blood* 108:3777-3785.
53. Peschon, J., P. Morrissey, K. Grabstein, F. Ramsdell, E. Maraskovsky, B. Gliniak, L. Park, S. Ziegler, D. Williams, C. Ware, J. Meyer, and B. Davison. Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice. *J Exp Med* 180:1955-1960.
54. Mansson, R., A. Hultquist, S. Luc, L. Yang, K. Anderson, S. Kharazi, S. Al-Hashmi, K. Liuba, L. Thoren, J. Adolfsson, N. Buza-Vidas, H. Qian, S. Soneji, T. Enver, M. Sigvardsson, and S. E. Jacobsen. 2007. Molecular evidence for hierarchical transcriptional lineage priming in fetal and adult stem cells and multipotent progenitors. *Immunity* 26:407-419.
55. Perry, S. S., L. J. Pierce, W. B. Slayton, and G. J. Spangrude. 2003. Characterization of thymic progenitors in adult mouse bone marrow. *J Immunol* 170:1877-1886.
56. Umland, O., W. N. Mwangi, B. M. Anderson, J. C. Walker, and H. T. Petrie. 2007. The blood contains multiple distinct progenitor populations with clonogenic B and T lineage potential. *J Immunol* 178:4147-4152.
57. Wada, H., K. Masuda, R. Satoh, K. Kakugawa, T. Ikawa, Y. Katsura, and H. Kawamoto. 2008. Adult T-cell progenitors retain myeloid potential. *Nature* 452:768-772.
58. Martin, C. H., I. Aifantis, M. L. Scimone, U. H. von Andrian, B. Reizis, H. von Boehmer, and F. Gounari. 2003. Efficient thymic immigration of B220+ lymphoid-restricted bone marrow cells with T precursor potential. *Nat Immunol* 4:866-873.
59. Krueger, A., and H. von Boehmer. 2007. Identification of a T lineage-committed progenitor in adult blood. *Immunity* 26:105-116.
60. Sutherland, J. S., G. L. Goldberg, M. V. Hammett, A. P. Uldrich, S. P. Berzins, T. S. Heng, B. R. Blazar, J. L. Millar, M. A. Malin, A. P. Chidgey, and R. L. Boyd. 2005. Activation of thymic regeneration in mice and humans following androgen blockade. *J Immunol* 175:2741-2753.
61. Rossi, D. J., D. Bryder, J. M. Zahn, H. Ahlenius, R. Sonu, A. J. Wagers, and I. L. Weissman. 2005. Cell intrinsic alterations underlie hematopoietic stem cell aging. *Proceedings of the National Academy of Sciences of the United States of America* 102:9194-9199.
62. Offner, F., T. Kerre, M. De Smedt, and J. Plum. 1999. Bone marrow CD34 cells generate fewer T cells in vitro with increasing age and following chemotherapy. *British journal of haematology* 104:801-808.

Chapter 5

General Discussion

General Discussion

One of the hallmarks of the aging immune system is the deterioration of adaptive immunity. While this immunosenescence is often undetected in many older people because of their generally healthy state, it poses a severe threat to those who have undergone immuno-depleting circumstances, such as cytoreductive radiation- or chemo- therapies as well as chronic infectious diseases such as HIV. The underlying cause is a severe decline in T and B cell lymphopoiesis within the thymus and BM resepectively, with age. These age-related changes begin primarily from puberty and hence are temporally linked to increasing concentrations of sex steroids. The thymus in particular, is profoundly impacted by age and shrinks considerably in size and suffers progressive loss of functional tissue, replaced by the accumulation of adipoctyes (1). This results in a decline in the production of naive T cells and their receptor repertoire, ultimately leading to compromised peripheral T cell function. Similarly, albeit to a lesser extent, there is also accumulation of adipose tissue within the BM and a decrease in B cell generation output and function (2-4). The resulting ability of these organs to replenish lymphopoieisis in settings of lymphopenia is thus severely hindered leading to a prolonged period of immune depletion and hence increased susceptibility to opportunistic infections, malignant relapse yet ironically autoimmune diseases. The development of novel strategies for enhancing hematopoiesis, and in particular lymphopoiesis, is therefore of great clinical significance.

The correlation between sex steroid exposure and immune-deterioration has revealed the immuno-regenerative potential of sex steroid ablation (SSA) (1, 5-8). Following SSA, there is a restoration of the thymic internal architecture leading to enhanced T cell output and function (9-11). Since androgen receptors are expressed within both thymocyte and stromal compartments, it remains unclear what initiates the regeneration of the immune

system upon abrogation of sex steroid production (8, 12, 13). Possible hypotheses include 1) SSA rejuvenates hematopoieitic stem and progenitor function within the BM leading to an increase in both supply and quality of thymus-bound progenitors; 2) SSA regenerates the stromal compartment within the BM and thymus leading to improved HSPC development within the BM, and T cell development within the thymus; and 3) abrogation of sex steroids results in intrinsic changes within both HSPC and stromal compartments leading to an overall increase in to generate lymphocytes (8, 12, 14) . These findings presented here suggest that SSA is able to simultaneously enhance HSPC function intrinsically (Chapters 2 and 4) as well as aiding in the ability of the hematopoietic BM niche to support HSPC differentiation and function (Chapter 3) and the thymic microenvironment to generate T cells (Chapter 4).

With age, while there is a well-described accumulation of functionally inferior HSCs within the BM, they are primarily geared towards myelopoiesis (15-18). SSA was able to not only further expand these HSCs, but also enhance their function and reverse the skewing towards myelopoiesis. While these effects were evident at the population level, the improved functional propertions of HSCs were also demonstrated by limiting dilution competitive transplantation assays. These HSCs had a greater capacity for self-renewal in serial transplantation; increased ability to reconstitute hematopoiesis; and enhanced lymphoid differentiation capacity. This enhanced lymphoid function was linked to an increase in the number of downstream progenitors (such as LMPPs), although these cells were not functionally superior on a per cell basis. Previous studies have suggested that sex steroids can directly regulate lymphoid precursors within the BM (3).

Molecular analysis of primitive LT-HSCs has revealed deficiencies in DNA damage repair processes with age. Although these were not reversed early after SSA, this can be explained by the lack of enhanced HSC function observed with d2SSA LT-HSC (17). Interestingly however, d2SSA LT-HSC exhibited strengthened cell:cell and cell:extracellular matrix adhesion as seen with our pathway analysis and concordant with previous studies suggesting reduced cell adhesion of HSCs to the niche with age (19) . The d2SSA BM stroma also showed early signs of increased hematopoietic regulation (osteopontin, angiopoietin, Jagged-1 and stem cell factor); perhaps functional changes manifest within the HSC compartment with SSA are both cell autonomous but also very likely mediated through changes within the niche stromal components (14).

Several groups have revealed changes in the aging of BM stromal components, particularly osteoblasts (OBLs), but primarily only their osteogenic capacity has been studied, with little known about their hematopoiesis-supporting capacity (20, 21). The findings presented here demonstrate that there is an age-associated accumulation of high *Runx2* and osteopontin producing OBLs (or pre-OBLs) in the central marrow, that correlates with the numerical increase in HSCs (15). However, despite SSA further enhancing the number of HSCs, there was no equivalent numerical change in these or endosteal OBLs after SSA. Whilst this indicates that the two cell types (OBLs and HSCs) are not unequivocally linked numerically, the molecular changes that occurred in these OBLs suggest functional changes. Furthermore, this population of osteopontin-producing, SSA-responding high-*Runx2*-expressing OBLs in the central marrow have not been previousl described and warrant further investigation on their more precise role in hematopoiesis (22, 23).

Collectively, the data generated in this study clearly showed functional abnormalities in the BM HSPC compartment; there was improved reconstitution observed with the d7SSA HSCs compared to d7ShSSA controls. The logical implication being that these defects had at their source, associated abnormalities in the BM stromal niches that nurture and maintain them. Although numerically, there were only minimal changes to the supporting BM microenvironment after SSA, our data show the aged stroma to be functionally deficient through HSC dose dependent transplantation experiments, with improved reconstitution observed when the recipient stroma were SSA compared to the shSSA controls. Our microarray analysis of the BM stroma also indicated considerable early molecular changes that provide a tantalizing glimpse into how hematopoiesis is changing after SSA. However, similarly there were also considerable changes intrinsic to the HSCs themselves at the early timepoint after SSA. Consequently more research is required into the potential age-related functional abnormalities in the stroma and precise dissection of the sequential changes within this compartment relative to that occurring in the HSCs during the early stages of regeneration. This should help elucidate the degree of specific crosstalk interactions between HSCs and their supporting stromal niche that are vital for regulating quiescence, self-renewal and downstream differentiation of HSCs. The outcome of these studies are particularly relevant for designing more taional, strategic approaches to improving immune reconstition following HSCT or other cytoreductive therapies.

With the HSCs regenerating with SSA, and their mobilization unhindered with age, the next stage of lymphopoiesis, thymic entry and subsequently intrathymic development of HSPCs were next examined (19). Despite the vast increase in knowledge of BM hematopoiesis, the precise identity of the progenitor that egresses from the BM and homes into thymus remains unclear. Indeed many studies suggest the potential of more than one cell with the experimental capacity for thymus migration and T cell development, however their

physiological significance is poorly understood (24-28). Progenitors with both multi-lineage potential MPPs) in addition to committed lymphoid progenitors (CLPs and LMPPs) express the molecules deemed critical for thymic entry, CCR7, CCR9 and P-selectin glycoprotein ligand (PSGL-1). Interestingly, despite the considerable decline in thymus import, expression of these molecules remained unaltered with age (29-32). While SSA enhanced the overall thymic importation of both ETPs and CLP-2s, presumably as a consequence of the generalised increase in niche availability, short term homing assays indicated that SSA directly impacts on the ability of the CLP-2s but not ETPs to enter the thymus. Interestingly, this regenerative impact of SSA on CLP-2 was only observed in aged recipient mice implicating these cells in injury or stress responses and perhaps suggesting an thymic entry occurs via an alternate pathway to that mediated by the established CCR7, CCR9 and PSGL-1 (10).

The specific response with d7SSA in CLP-2s and not ETPs also suggest that under homeostatic conditions, ETPs represent a more canonical route to thymopoiesis with a superior T cell generating ability while CLPs, which are primarily B cell precursors, may serve as a non-canonical progenitor for T cell development with CLP-2s having a strong thymic homing capacity (27).

The thymic stromal cells remain vital in facilitating the development of intrathymic precursors into T cells and the re-establishment of their structure and function from an aged phenotype will be key in improving thymopoiesis and hence the clinical outcome of HSCT (1, 33, 34). While the degree of SSA-induced numerical regeneration of thymic epithelial cells remains controversial (35, 36), other findings from our laboratory have suggested a role for the non-epithelial supporting stroma in qualitatively rejuvenating

thymic epithelial cells (Chidgey, *manuscript in preparation*). It will be important to further research how SSA alters the signalling between the intrathymic T cell precursors and its supporting stroma allowing for its manipulation and perhaps more accelerated lymphoid regeneration.

Although the studies described herein have utilised surgical castration as a model for SSA, more clinically acceptable techniques are necessary. The most readily available is chemical SSA using the clinically approved and reversible luteinizing hormone releasing hormone (LHRH)/gonadotrophin releasing hormone (GnRH) analogues. Several options are available including LHRH-antagonists (which compete for LHRH receptor occupancy), LHRH-agonists (which work through a negative feedback loop) and direct sex steroid blockers (1, 37). All of these approaches achieve castrate levels of androgens and estrogens but have their advantages and disadvantages. For instance, while LHRH antagonists do not trigger the surge of androgens that LHRH agonists do, their use can be limited because pharmacologically it is more difficult to saturate a receptor than block the production of the hormone itself (37). Having taken advantage of the more precise timing in removing sex steroids with surgical SSA, the next iteration of this project will be to use the chemical SSA approach making the data more clinically relevant.

A major drawback of the current HSCT regime lies in the supply of HSCs for transplantation. Since SSA enhances the HSPC population and their capacity for restoring hematopoiesis within the BM and thymus *in vivo*, further clinical studies could be conducted to investigate the possibility of enhancing the expansion of HSCs *in vitro* and therefore addressing the issue of HSC supply.

It remains however, a major concern that the administration of cytoreductive agents such as cyclophosphamide (Cy) damage both the HSCs as well as the hematopoietic BM and thymic niche thus affecting the engraftment and differentiation efficiencies of the HSPCs. Although there is general consensus that the BM stroma is functionally altered with chemotherapy, the very nature of this damage is still unclear (38-40). More precise investigation into the effects of cyto-ablative therapies on both the HSPC and stromal niches at the cellular and molecular levels will be important for devising more strategic protocols. With previous studies indicating enhancement of immune recovery following HSCT with SSA, understanding the mechanism of chemotherapeutic damage to the hematopoietic system will enable more focused rejuvenation of key hematopoietic and stromal populations (5, 41, 42).

In conclusion, SSA allows for the rejuvenation of lymphopoiesis; simultaneously acting directly on the intrinsic capacity for HSCs to develop down the lymphoid lineage in addtion to the stromal microenvironment and its ability to support HSC function and lymphoid differentiation. While it is paradoxical that puberty and increasing age collectively depress the production and function of lymphoid cells, predisposing the individual to a wide range of both opportunistic and tratment-induced diseases, SSA provides a clinilcally safe and effective means of regenerating immunity. Not only does this address the multiple facets of immunodeficiency, but the improved BM and thymus function provides a platform for strategically manipulating the function of the immune system. Clearly SSA provides a clinically achievable means of immune restoration but as important as this is, it is also the catalyst to a new thrust for research focussed on its underlying molecular mechanisms and hence in the future enhancing more tailored therapies.

REFERENCES

1. Sutherland, J. S., G. L. Goldberg, M. V. Hammett, A. P. Uldrich, S. P. Berzins, T. S. Heng, B. R. Blazar, J. L. Millar, M. A. Malin, A. P. Chidgey, and R. L. Boyd. 2005. Activation of thymic regeneration in mice and humans following androgen blockade. *J Immunol* 175:2741-2753.

2. Medina, K., A. Strasser, and P. Kincade. 2000. Estrogen influences the differentiation, proliferation, and survival of early B-lineage precursors. *Blood* 95:2059-2067.

3. Medina, K. L., K. P. Garrett, L. F. Thompson, M. I. D. Rossi, K. J. Payne, and P. W. Kincade. 2001. Identification of very early lymphoid precursors in bone marrow and their regulation by estrogen. *Nat Immunol* 2:718-724.

4. Igarashi, H., T. Kouro, T. Yokota, P. C. Comp, and P. W. Kincade. 2001. Age and stage dependency of estrogen receptor expression by lymphocyte precursors. *Proc Natl Acad Sci U S A* 98:15131-15136.

5. Goldberg, G. L., J. S. Sutherland, M. V. Hammett, M. K. Milton, T. S. Heng, A. P. Chidgey, and R. L. Boyd. 2005. Sex steroid ablation enhances lymphoid recovery following autologous hematopoietic stem cell transplantation. *Transplantation* 80:1604-1613.

6. Olsen, N. J., S. M. Viselli, J. Fan, and W. J. Kovacs. 1998. Androgens accelerate thymocyte apoptosis. *Endocrinology* 139:748-752.

7. Olsen, N. J., P. Zhou, H. Ong, and W. J. Kovacs. 1993. Testosterone induces expression of transforming growth factor-beta1 in the murine thymus. *J Steroid Biochem Mol Biol* 45:327-332.

8. Viselli, S. M., N. J. Olsen, K. Shults, G. Steizer, and W. J. Kovacs. 1995. Immunochemical and flow cytometric analysis of androgen receptor expression in thymocytes. *Mol Cell Endocrinol* 109:19-26.

9. Goldberg, G. L., C. G. King, R. A. Nejat, D. Y. Suh, O. M. Smith, J. C. Bretz, R. M. Samstein, J. A. Dudakov, A. P. Chidgey, S. Chen-Kiang, R. L. Boyd, and M. R. M. van den Brink. 2009. Luteinizing hormone-releasing hormone enhances T cell recovery following allogeneic bone marrow transplantation. *J Immunol* 182:5846-5854.

10. Heng, T. S. P., G. L. Goldberg, D. H. D. Gray, J. S. Sutherland, A. P. Chidgey, and R. L. Boyd. 2005. Effects of castration on thymocyte development in two different models of thymic involution. *J Immunol* 175:2982-2993.

11. Goldberg, G. L., J. A. Dudakov, J. J. Reiseger, N. Seach, T. Ueno, K. Vlahos, M. V. Hammett, L. F. Young, T. S. Heng, R. L. Boyd, and A. P. Chidgey. 2010. Sex Steroid Ablation Enhances Immune Reconstitution Following Cytotoxic Antineoplastic Therapy in Young Mice. *The Journal of Immunology* 184:6014-6024.

12. Olsen, N. J., G. Olson, S. M. Viselli, X. Gu, and W. J. Kovacs. 2001. Androgen receptors in thymic epithelium modulate thymus size and thymocyte development. *Endocrinology* 142:1278-1283.

13. Olsen, N. N. J., S. S. M. Viselli, J. J. Fan, and W. W. J. Kovacs. 1998. Androgens accelerate thymocyte apoptosis. *Endocrinology* 139:748-752.

14. Olsen, N. J., X. Gu, and W. J. Kovacs. 2001. Bone marrow stromal cells mediate androgenic suppression of B lymphocyte development. *J Clin Invest* 108:1697-1704.

15. Rossi, D. J., D. Bryder, J. M. Zahn, H. Ahlenius, R. Sonu, A. J. Wagers, and I. L. Weissman. 2005. Cell intrinsic alterations underlie hematopoietic stem cell aging. *Proc Natl Acad Sci U S A* 102:9194-9199.

16. Yilmaz, O., M. Kiel, and S. Morrison. 2006. SLAM family markers are conserved among hematopoietic stem cells from old and reconstituted mice and markedly increase their purity. *Blood* 107:924-930.

17. Rossi, D., D. Bryder, J. Seita, A. Nussenzweig, J. Hoeijmakers, and I. Weissman. 2007. Deficiencies in DNA damage repair limit the function of haematopoietic stem cells with age. *Nature* 447:725-729.

18. Sudo, K., H. Ema, Y. Morita, and H. Nakauchi. 2000. Age-associated characteristics of murine hematopoietic stem cells. *J Exp Med* 192:1273-1280.

19. Xing, Z., M. A. Ryan, D. Daria, K. J. Nattamai, G. van Zant, L. Wang, Y. Zheng, and H. Geiger. 2006. Increased hematopoietic stem cell mobilization in aged mice. *Blood* 108:2190-2197.

20. Kassem, M., L. Ankersen, E. F. Eriksen, B. F. Clark, and S. I. Rattan. 1997. Demonstration of cellular aging and senescence in serially passaged long-term cultures of human trabecular osteoblasts. *Osteoporosis International* 7:514-524.

21. Kassem, M., and P. J. Marie. 2011. Senescence-associated intrinsic mechanisms of osteoblast dysfunctions. *Aging Cell* 10:191-197.

22. Stier, S., Y. Ko, R. Forkert, C. Lutz, T. Neuhaus, E. Grunewald, T. Cheng, D. Dombkowski, L. M. Calvi, S. R. Rittling, and D. T. Scadden. 2005. Osteopontin is a hematopoietic stem cell niche component that negatively regulates stem cell pool size. *The Journal of Experimental Medicine* 201:1781-1791.

23. Nilsson, S. K., H. M. Johnston, G. A. Whitty, B. Williams, R. J. Webb, D. T. Denhardt, I. Bertoncello, L. J. Bendall, P. J. Simmons, and D. N. Haylock. 2005. Osteopontin, a key component of the hematopoietic stem cell niche and regulator of primitive hematopoietic progenitor cells. *Blood* 106:1232-1239.

24. Bhandoola, A., and A. Sambandam. 2006. From stem cell to T cell: one route or many? *Nature Reviews* 6:117 - 127.

25. Bhandoola, A., H. von Boehmer, H. T. Petrie, and J. C. Zúñiga-Pflücker. 2007. Commitment and developmental potential of extrathymic and intrathymic T cell precursors: plenty to choose from. *Immunity* 26:678-689.

26. Porritt, H. E., L. L. Rumfelt, S. Tabrizifard, T. M. Schmitt, J. C. Zúñiga-Pflücker, and H. T. Petrie. 2004. Heterogeneity among DN1 prothymocytes reveals multiple progenitors with different capacities to generate T cell and non-T cell lineages. *Immunity* 20:735-745.

27. Martin, C. H., I. Aifantis, M. L. Scimone, U. H. von Andrian, B. Reizis, H. von Boehmer, and F. Gounari. 2003. Efficient thymic immigration of B220+ lymphoid-restricted bone marrow cells with T precursor potential. *Nat Immunol* 4:866-873.

28. Krueger, A., and H. von Boehmer. 2007. Identification of a T lineage-committed progenitor in adult blood. *Immunity* 26:105-116.

29. Rossi, F. M. V., S. Y. Corbel, J. S. Merzaban, D. A. Carlow, K. Gossens, J. Duenas, L. So, L. Yi, and H. J. Ziltener. 2005. Recruitment of adult thymic progenitors is regulated by P-selectin and its ligand PSGL-1. *Nature Immunology* 6:626-634.

30. Zlotoff, D. A., A. Sambandam, T. D. Logan, J. J. Bell, B. A. Schwarz, and A. Bhandoola. 2010. CCR7 and CCR9 together recruit hematopoietic progenitors to the adult thymus. *Blood* 115:1897-1905.

31. Krueger, A., S. Willenzon, M. Lyszkiewicz, E. Kremmer, and R. Forster. 2010. CC chemokine receptor 7 and 9 double-deficient hematopoietic progenitors are severely impaired in seeding the adult thymus. *Blood* 115:1906-1912.

32. Schwarz, B. A., A. Sambandam, I. Maillard, B. C. Harman, P. E. Love, and A. Bhandoola. 2007. Selective thymus settling regulated by cytokine and chemokine receptors. *The Journal of Immunology* 178:2008-2017.

33. Gray, D. H. D., T. Ueno, A. P. Chidgey, M. Malin, G. L. Goldberg, Y. Takahama, and R. L. Boyd. 2005. Controlling the thymic microenvironment. *Current Opinion in Immunology* 17:137 - 143.

34. Takahama, Y. 2006. Journey through the thymus: stromal guides for T-cell development and selection. *Nature Reviews* 6:127 - 135.

35. Gray, D. H. D., N. Seach, T. Ueno, M. K. Milton, A. Liston, A. M. Lew, C. C. Goodnow, and R. L. Boyd. 2006. Developmental kinetics, turnover, and stimulatory capacity of thymic epithelial cells. *Blood* 108:3777-3785.

36. Griffith, A. V., M. Fallahi, T. Venables, and H. T. Petrie. 2012. Persistent degenerative changes in thymic organ function revealed by an inducible model of organ regrowth. *Aging Cell* 11:169-177.

37. Hince, M., S. Sakkal, K. Vlahos, J. Dudakov, R. Boyd, and A. Chidgey. 2008. The role of sex steroids and gonadectomy in the control of thymic involution. *Cell Immunol* 252:122-138.

38. Domenech, J., E. Gihana, A. Dayan, D. Truglio, C. Linassier, I. Desbois, J. P. Lamagnere, P. Colombat, and C. Binet. 1994. Haemopoiesis of transplanted patients with autologous marrows assessed by long-term marrow culture. *British journal of haematology* 88:488-496.

39. Banfi, A., M. Podestà, L. Fazzuoli, M. R. Sertoli, M. Venturini, G. Santini, R. Cancedda, and R. Quarto. 2001. High-dose chemotherapy shows a dose-dependent toxicity to bone marrow osteoprogenitors: a mechanism for post-bone marrow transplantation osteopenia. *Cancer* 92:2419-2428.

40. Banfi, A., G. Bianchi, M. Galotto, R. Cancedda, and R. Quarto. 2001. Bone marrow stromal damage after chemo/radiotherapy: occurrence, consequences and possibilities of treatment. *Leukemia & lymphoma* 42:863-870.

41. Dudakov, J. A., G. L. Goldberg, J. J. Reiseger, A. P. Chidgey, and R. L. Boyd. 2009. Withdrawal of Sex Steroids Reverses Age- and Chemotherapy-Related Defects in Bone Marrow Lymphopoiesis. *The Journal of Immunology* 182:6247-6260.

42. Dudakov, J. A., G. L. Goldberg, J. J. Reiseger, K. Vlahos, A. P. Chidgey, and R. L. Boyd. 2009. Sex Steroid Ablation Enhances Hematopoietic Recovery following Cytotoxic Antineoplastic Therapy in Aged Mice. *The Journal of Immunology* 183:7084-7094.

Appendix 1

Stem Cells – Meet Immunity

J Mol Med (2009) 87:1061–1069
DOI 10.1007/s00109-009-0539-z

REVIEW

Stem cells—meet immunity

Tracy S. P. Heng · Jarrod A. Dudakov · Danika M. P. Khong · Ann P. Chidgey · Richard L. Boyd

Received: 12 July 2009 / Revised: 31 August 2009 / Accepted: 15 September 2009 / Published online: 16 October 2009

Abstract The ability of stem cells to differentiate into various different cell types holds great promise for the treatment of irreversible tissue damage that occurs in many debilitating conditions. With stem cell research advancing at a tremendous pace, it is becoming clear that one of the greatest hurdles to successful stem cell-derived therapies is overcoming immune rejection of the transplant. Although the use of immunosuppressive drugs can decrease the incidence of acute graft rejection, the burden of problems associated with prolonged immunosuppression must be reduced. Strategies inducing specific immunological tolerance complemented by enhanced immune function will bring stem cell therapies closer to reality.

Keywords Stem cells · Immune tolerance · Haematopoietic stem cell transplantation · Thymus regeneration

Introduction

The use of stem cells in medicine holds tremendous promise in many therapeutic areas and has generated enormous interest among researchers and unprecedented expectations from patients and their advocates worldwide. This unbridled enthusiasm, however, has to be tempered with realistic timelines to clinical implementation and many ethical, safety and practical hurdles.

Stem cells, by definition, have the ability to self-renew and differentiate into multiple specialised cell lineages. Several types of stem cells have been defined, namely embryonic stem (ES) cells, adult stem cells and induced pluripotent stem (iPS) cells. Presently, the difficulty in controlling the differentiation of these cells into specific cell populations appropriate for clinical use creates a challenging scenario. Although ES cells are the most versatile source of pluripotent cells, ethical issues associated with their derivation have limited their clinical use. Indeed, the applications for stem cells in medicine primarily have involved only adult stem cells until very recently with FDA approval being granted to Geron for the use of ES-derived neural stem cells to treat early spinal cord injury.

One major area that has, for many years, incorporated stem cells as part of its treatment regime is bone marrow (BM) transplantation or haematopoietic stem cell (HSC) transplantation (HSCT) for primary immunodeficiency disorders, bone marrow failure syndromes, non-malignant haematological disorders and haematological malignancies. The transplanted HSCs rebuild the haematopoietic compartment of myeloablated patients after engraftment in the BM and differentiate into lymphoid progenitors. Upon migration to the thymus, lymphoid progenitors develop into non-self-reactive T cells capable of recognising and eliminating foreign antigens.

HSCT can be performed using either the patient's own HSCs harvested prior to conditioning treatment (autologous HSCT) or from a matched donor (allogeneic HSCT). However, in the latter approach, the transplant may mount an immunological attack against the "foreign" host cells resulting in a systemic disorder known as graft-versus-host disease (GvHD), which can result in destruction of the thymus and increased opportunistic infections [1]. Such patients are thus treated with immunosuppressive drugs for extended periods of time. Additionally, the administration of total body irradiation or high-dose chemotherapy to

T. S. P. Heng · J. A. Dudakov · D. M. P. Khong · A. P. Chidgey · R. L. Boyd (✉)
Monash Immunology and Stem Cell Laboratories,
Monash University,
Clayton VIC 3800, Australia
e-mail: richard.boyd@med.monash.edu.au

deplete the cancer cells while conditioning the BM to allow for HSC engraftment severely depletes the immune system and leaves patients in a protracted immunocompromised state. Consequently, patients receiving HSCT have a significantly higher risk of morbidity and mortality associated with these cytoablative and conditioning regimes. This is particularly problematic in adult and older patients whose immune systems are highly inefficient at recovering from immunoablation because of age-related thymic atrophy and reduced BM function [2].

Prolonged immunosuppression is also critical in solid organ transplantation to prevent donor tissue rejection by the recipient's immune system. Unfortunately, such immunosuppressive drugs can also cause extensive loss of an important tolerance-inducing subset of thymic epithelial cells essential for deleting autoreactive T cells specific to selected peripheral antigens [3]. Hence, the ultimate challenge for successful translation of stem cell-derived therapies to the clinic is to restore the ageing or damaged thymus while establishing host immune acceptance of the donor cells and tissues. This can be achieved by combining thymic rejuvenation with HSCT using donor-derived HSCs leading to re-education of the emerging T cell repertoire to be tolerant to both donor and host cells [4].

Much ado about stem cells?

Since the identification of the first putative HSC over 40 years ago [5], stem cells have been widely used in the clinic as a curative therapy in severe haematological malignancies (leukaemia, lymphoma), non-malignant haematological disorders (sickle cell anaemia, thalassaemia) and immunodeficiency states (severe combined immunodeficiency) [6].

Other tissue-specific stem cells capable of regenerating specific biological systems have since been identified including neural stem cells in the subventricular zone of the brain [7], skin epithelial stem cells in the hair follicular bulge [8], intestinal stem cells in crypt base columnar cells [9] and skeletal stem cells in muscle satellite cells [10]. While these tissue-specific stem cells have the ability to differentiate into multiple lineages, they are more restricted in their potential than ES cells. ES cells have the ability to differentiate into all three germ layers, thereby giving them the capacity to generate all tissue-specific stem cells and consequently, all organs and tissues. Thus, the promise of ES cells is the custom-made development of specific types of cells and tissues to be transplanted on demand. If not properly differentiated before infusion, however, ES cells pose a grave health risk because they can form into lethal cancers called teratomas [11].

Several significant barriers need to be overcome before the therapeutic potential of stem cells can be realised on a large scale. Advances in the generation of iPS cells by reprogramming somatic cells [12] have assuaged ethical concerns about the use of human embryos and may even overcome the issue of immunological rejection of allogeneic tissue. Recently, the ability to generate human iPS cells using recombinant proteins that can penetrate across the plasma membrane [13, 14] eliminates the potential risk of unexpected genetic modifications induced from the retroviral vectors used in the original protocols [15–17]. However, iPS derivatives may again cause teratomas if infused without prior, complete and stable differentiation [18]. Thus, there still exists a need to design more efficient protocols to obtain pure populations of differentiated cells in sufficient quantities for clinical use. Moreover, evidence is emerging that diverse tissue-specific stem cell reserves and niches decline with age, manifest in increasing functional deficiencies [19], which may compromise stem cell therapies [20]. Nevertheless, the main hurdle to stem cell therapies is immunological recognition and rejection of the transplant by the recipient immune system (Fig. 1) [4].

Are stem cells really immune-privileged?

The immune system targets foreign antigens normally associated with infections. However, cells that are genetically different, such as cancers and those expressing non-self major histocompatibility (MHC) antigens, are also attacked. Several studies have shown that undifferentiated ES cells may be transplanted across MHC barriers without eliciting an immune response [21, 22], at least in the short term, suggesting that ES cells are immune-privileged. This is best explained by the fact that an acute allogeneic response is induced upon recognition of MHC proteins, and undifferentiated human ES cells do not express MHC class II and only low levels of MHC class I. However, the latter is upregulated upon differentiation of ES cells into primitive embryoid bodies or teratomas and, in the presence of IFN-γ [23], a cytokine released in inflammatory settings. ES cells in these settings may therefore be targeted for acute rejection. Similarly, transplanted ES cell derivatives may be eliminated by alloreactive T cells or alloantibodies induced following uptake of graft-derived antigens by host antigen-presenting cells [24]. The capacity of human ES cells to induce robust cellular and humoral responses is clearly demonstrated in a recent study tracking the fate of transplanted cells over longer periods of time, which were ultimately rejected [25].

Overcoming immune recognition of stem cell-derived therapies

As MHC alloantigens are the primary target for immune recognition, it may be possible to create a bank of

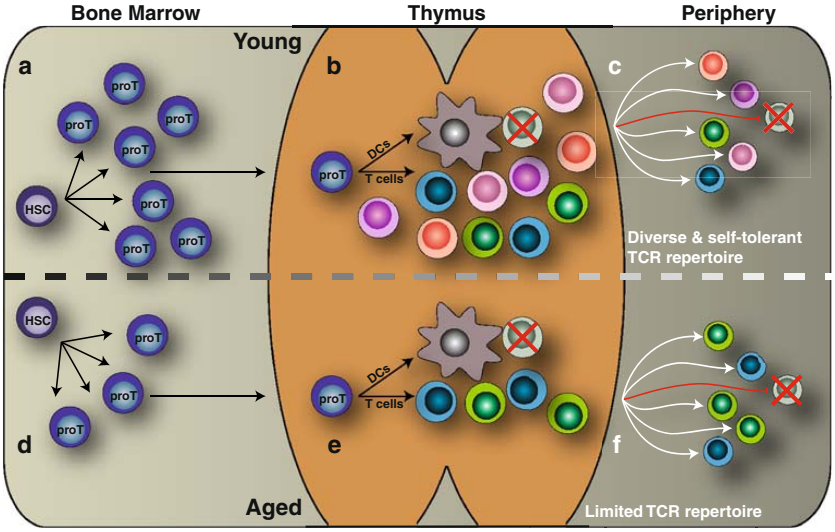


Fig. 1 Thymopoiesis is significantly impaired with age leading to a reduction in the peripheral immune repertoire. **a** HSCs in the BM differentiate into the lymphoid lineage and migrate to the thymus. **b** Once in the thymus, pro-T cells differentiate into the T-lineage pathway. Autoreactive thymocytes are deleted when they encounter their cognate self-antigens presented by thymic epithelium or BM-derived dendritic cells (*DCs*) in a process known as negative selection (central tolerance). **c** Populations of mature naïve T cells with a diverse T cell receptor (TCR) repertoire are released into the circulation to mediate adaptive immunity. **d** In the aged animal, BM and intrathymic pro-T cell numbers are reduced. **e** Decline in thymopoiesis decreases the number of T cells exported to the periphery. **f** While peripheral T cell numbers are maintained by homeostatic expansion of memory cells, the diversity of the peripheral TCR repertoire is significantly reduced in aged animals

immunotyped ES cells to enable patient–cell line matching [26]. However, mismatched minor histocompatibility antigens are sufficient to provoke graft rejection [27], thus the logistics of establishing a bank broad enough to represent diverse MHC polymorphisms are staggering. Furthermore, transplantation of grafts into "immune-privileged" sites such as the brain may not necessarily result in engraftment as MHC class I expression is induced in an inflammatory environment [28].

Deceiving the immune system

Although cells with low MHC class I expression normally elicit natural killer (NK) cell recognition [29], human ES cells appear to be protected from NK cell killing by their low or lack of expression of lysis receptors [23]. As such, abrogation of MHC molecules has been suggested as a strategy to generate less immunogenic cell lines. However, skin grafts from MHC-deficient mice are still rejected by thymic-derived $CD4^+$ T cells in allogeneic recipients possibly due to minor or non-classical MHC molecules [30, 31]. Knocking out MHC class I expression may also be dangerous since MHC class I molecules are essential for clearance of viral-infected or cancerous cells. Similarly, attempts to enforce ectopic expression of Fas ligand on transplanted cells to activate apoptosis of potentially alloreactive T cells have resulted in rejection when the transplanted cells themselves expressed Fas or were transplanted in Fas-expressing tissues [32].

Immunosuppression: drug administration versus the "natural" way

Immunosuppressive regimes have long been employed in the clinic to prevent transplant rejection. Immunosuppressants commonly used include anti-inflammatory corticosteroids, T cell-depleting or blocking antibodies, calcineurin inhibitors to block T cell proliferation and anti-proliferative drugs to interfere with lymphocyte activation [33]. While immunosuppressive drug therapy has enhanced initial graft acceptance rates, significant long-term graft survival is yet to be achieved. Chronic administration of non-specific immunosuppressive drugs is also associated with increased susceptibility to infections and malignancies as well as non-immunological complications such as liver toxicity and renal failure [33]. Moreover, thymic epithelial cell damage caused by the use of immunosuppressive drugs [3] might even lead to autoimmune disease by impairing central tolerance.

An alternative to drug-based therapies is to harness the inherent immunosuppressive properties of endogenous cell populations as a form of "natural" immunosuppression. One

such cell population is the Foxp3-expressing $CD4^+CD25^+$ regulatory T cell (Treg) subset essential for maintaining self-tolerance and preventing autoimmunity [34]. Although naturally arising Tregs are generated when developing T cells encounter self-antigens in the thymus, they may also be induced specifically to a chosen foreign antigen under subimmunogenic conditions [35] and confer transplantation tolerance [36].

Another cell population that has gained much interest over recent years for its striking immunosuppressive effects is the multipotent mesenchymal stromal cell (MSC). MSCs are adherent fibroblast-like cells with the ability to expand in culture and differentiate into bone, fat and cartilage [37]. Initially isolated from the bone marrow [38], MSCs have since been identified in various other foetal and adult tissues including umbilical cord blood [39], placenta [40] and fat [41]. MSCs can home to sites of injury and produce growth factors to alleviate inflammation and promote tissue repair [42]. Importantly, MSCs are highly immunosuppressive and evoke little immunity when transplanted, prompting their use in the clinic to facilitate hematopoietic engraftment and combat graft-versus-host disease [43]. The mechanisms underlying MSC-mediated immunoregulation remain largely unclear but likely involve interplay between IFN-γ, TGF-β, IL-1, nitric oxide and chemokines [44]. Although the significance of the immunomodulatory properties of MSCs under steady-state conditions is yet to be defined, co-transplantation of MSCs could well facilitate allograft acceptance by suppressing the activation of alloreactive T cells. Similarly, epithelial cells isolated from the amnion display multilineage differentiation potential and, unlike ES cells, are non-tumorigenic upon transplantation [45]. These cells may also possess a degree of immunosuppressive capacity [46], although their clinical application requires further in vivo studies.

Engineering pluripotent stem cells

In recent years, advances in nuclear cloning technology have brought us closer to patient-specific transplantation therapy. The conversion of differentiated cells to an embryonic state can be achieved by several techniques including transfer of a somatic nucleus into an enucleated oocyte (somatic cell nuclear transfer), cellular fusion of somatic cells with ES cells, selective expansion of pluripotent ES-like cells in culture and reprogramming of somatic cells by defined transcription factors (iPS) [47]. Such is the success of iPS technology that several groups have managed to engineer disease-relevant cell types by reprogramming somatic cells from patients [48, 49]. Although iPS cells are comparable in their developmental potential to ES cells, they are not genetically identical [50] and many questions remain with regards to their long-term stability (do they revert?), safety (will they become cancers?) and function (how comparable are iPS cell derivatives to their in vivo counterparts?) in humans.

Inducing tolerance

Although the use of patient-specific iPS cells may theoretically circumvent the need for inducing tolerance to donor grafts, there are many unknowns regarding cellular therapies based on iPS technology. On the other hand, the use of HSCT has been shown to be an effective means of preventing graft rejection by inducing central tolerance via the in vivo differentiation of donor HSCs into the dendritic cell lineage [51]. Intrathymic donor-derived DCs presenting donor antigens are capable of inducing tolerance by deleting the recipient's donor-reactive thymocytes [52], thereby ensuring permanent graft acceptance as happens for normal self-tolerance (Fig. 1).

In order to induce tolerance to donor grafts, however, HSCs must be derived from either the same donor or at least be MHC-matched to the transplant. Furthermore, for transplantation to be successful, the graft must also be tolerant to the recipient's cells or GvHD can occur. The induction of central tolerance of both donor and host lymphocytes can only be achieved with a functioning thymus. This is made difficult by the gradual atrophy of the thymus with age, which is further compounded by the myeloablative conditioning required to enhance HSC engraftment. One strategy to overcome this is to use low-dose myeloablation that is sufficient to create “space” for the HSCs to engraft but low enough to ensure there is no severe prolonged depletion of the immune system [53]. However, this strategy will still require immunosuppression of the pre-existing immune system to prevent graft rejection and immune regeneration through restoration of the thymus to enable the production of new cohorts of donor- and recipient-tolerant T cells.

Rebooting the immune system

Utilising the innate capacity of the thymus to induce central tolerance obviates the need to depend on long-term immunosuppression. Paradoxically, the thymus degenerates with age [2] (Fig. 1). This results in one of the fundamental problems with the use of HSCT to induce tolerance—the severe delay in immune recovery from immunoablation in adults and the elderly. Consequently, a critical challenge in transplantation biology is enhancing immune and, particularly, thymic regeneration.

Regenerating the immune system

Sex steroids have a profound impact on lymphoid development and immune function [54, 55], and consequently,

endocrine-based strategies can potentially restore the immune system. We and others have found that sex steroid ablation (SSA) enhances thymopoiesis and T cell function in adult or aged mice and humans [56, 57] as well as improve thymic recovery following chemotherapy treatment [58]. The effects of SSA are not restricted to the thymus as B-lymphopoiesis and lymphoid progenitor numbers are enhanced in the BM [59]. Furthermore, SSA enhances immune recovery following autologous [60] and allogeneic HSCT without exacerbation of GvHD [61, 62]. In this regard, we have recently demonstrated in a clinical study that treatment with a luteinising hormone releasing hormone (LHRH) agonist, which blocks sex steroids in a reversible manner, augments thymic and immune recovery following both autologous and allogeneic HSCT [63].

As an alternative to SSA, exogenously administered cytokines and hormones have also been investigated for their potential to reverse age-related or immunoablation-induced lymphopoiesis. Studies using keratinocyte growth factor (KGF) have found that thymic cellularity is significantly increased in aged animals and following radiation or chemotherapy [64, 65]. KGF acts directly on the proliferation of thymic epithelial cells [66] and also prevents GvHD-mediated thymic damage [67]. Furthermore, we have found that combination therapy with KGF and SSA has an additive effect on thymic reconstitution [68]. Interleukin-7 (IL-7), another candidate molecule, reverses age-associated increases in apoptosis of thymocytes [69] and enhances peripheral T cell function following allogeneic HSCT by promoting the proliferation of lymphocytes and lymphoid progenitors [70–72]. Likewise, Fms-like tyrosine kinase 3 ligand (Flt3L) predominantly promotes the expansion of BM progenitors [73] leading to an increase in both thymic-dependent and independent reconstitution [74, 75]. Another

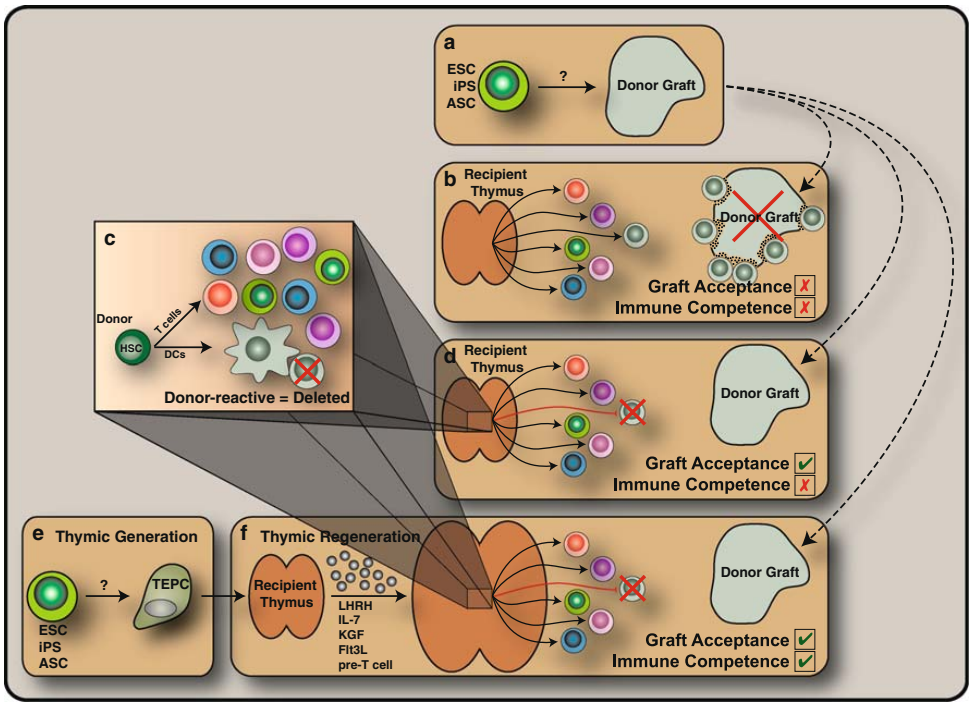


Fig. 2 Inducing allogeneic tolerance and enhancing immune reconstitution following HSCT. **a** Transplantable organs and tissues can be either donated or generated using embryonic stem cells (*ESCs*), induced pluripotent stem cells (*iPS*) or adult stem cells (*ASCs*). **b** Without tolerance induction or prolonged immunosuppression, donor grafts will be rejected by the patient's immune system. **c** One way to induce tolerance is transplantation of donor HSCs, which then differentiate to dendritic cells (*DCs*) capable of deleting alloreactive T cells in the thymus. **d** This process, however, is severely hampered by thymic involution which leads to significantly reduced thymic function. This can be a result of age or the immunoablative conditioning required for efficient engraftment of donor HSCs. Studies are focusing on many therapies that may facilitate thymic regeneration. **e** ESCs, iPS or ASCs may be directed to differentiate into thymic epithelial progenitor cells (*TEPCs*) which may either be transplanted into the existing thymus in vivo—thereby regenerating the thymic microenvironment—or a whole de novo thymus could be grown and transplanted. **f** Thymic regeneration can also be achieved using exogenously administered growth factors, such as KGF, IL-7, Flt3L and GH, transfer of in vitro generated T cell precursors or prior withdrawal of sex steroids. These therapies, shown to augment thymic regeneration, will not only result in graft acceptance but also allow for robust immune function

soluble factor with possible therapeutic potential is growth hormone (GH), which, despite concerns over its safety, has been shown to regenerate the aged [76] or immunodeficient thymus [77] and enhance BM HSC function [78]. Taken together, these studies indicate that these therapies may provide a novel clinical basis for enhancing immune reconstitution and, as a result, improving the induction of tolerance to transplanted allogeneic organs or tissues (Fig. 2).

Constructing a new immune system

Recently, the development of the OP9-DL1 culture system has enabled the generation of precursor T cells from HSCs [79] and ES cells [80]. Adoptive transfer of these in vitro-generated precursor T cells has been shown to enhance thymic reconstitution following allogeneic HSCT, thereby providing a clinical basis for their use [80]. A major concern with this approach, however, is the issue of safety since such cells have not been subject to normal selection processes in the thymus, particularly induction of self-tolerance.

It may be possible to enforce the de novo development of a tolerant cohort of cells by injecting patient-specific iPS cell-derived HSCs if their bone marrow has been severely damaged, but this needs to be coupled with a functional thymus. One could thus envisage employing a combination of emerging stem cell-based technologies to create a new thymus. We have previously isolated a population of thymic stromal cells enriched in stem cell or progenitor activity in the foetal mouse [81]. Identifying the equivalent in the adult human thymus and the important stem cell-niche interactions may lead to the ability to specifically activate resident thymic stem cells to repair or replace damaged tissue. Furthermore, it may be possible to use microRNAs and specific transcription factors to direct the differentiation of stem cells down the thymic epithelial pathway. Such thymic epithelial progenitor cells could then be seeded with donor, or same ES cell-derived, HSCs supporting mesenchyme and growth factors in sophisticated biomatrices to simulate the in situ thymus and promote the development of a new cohort of donor-tolerant cells [82] (Fig. 2).

Conclusions

Rapid advances in stem cell research have provided a tantalising glimpse into a future where “made-to-order” tissues and organs may be possible. However, there remains the fundamental complication of transplant rejection due to the immunological recognition of donor grafts by the recipient immune system. Current preventative measures mainly entail prolonged immunosuppression, which leaves the patient in a severely immunocompromised state. These problems need to be overcome in order for stem cell therapies to be a clinically viable option for the many millions of patients suffering from severe diseases or less life-threatening conditions such as neural degeneration, deafness, blindness, autoimmune disease, spinal disc repair and accident- or sporting-related injuries.

Although significant progress has been made in reverting autologous somatic cells into a stem cell-like state and thereby avoiding the problem of graft rejection, there are many questions that need to be addressed before iPS cell-derived therapies are deemed safe for clinical use. Co-transplantation with immunosuppressive MSCs may facilitate graft acceptance, but, ultimately, their use does not confer central tolerance to donor grafts. In this respect, the application of HSCT coupled to thymic regeneration for creating long-term immune tolerance has been an important step towards achieving acceptance of donor tissue grafts. However, severe and prolonged immune depletion due to conditioning regimes significantly increases the risk of patient morbidity or mortality. This is further exacerbated by the diminished capacity of the immune system to fully regenerate with age.

As LHRH agonists are already used routinely to treat prostate cancer, endometriosis and fibroids, their use is currently the most clinically feasible approach to address this problem. Furthermore, combination therapy with certain growth factors such as KGF or even thymic epithelial progenitor cells may provide an attractive platform to enhance immune recovery following HSCT while concomitantly inducing tolerance to allogeneic donor tissue. The ability to generate transplantable tissue, induce graft tolerance and enhance immune function will aid in achieving successful transplantation and herald an exciting future for regenerative medicine.

Acknowledgments Some of the studies reported in this review were supported by grants from the National Health and Medical Research Council of Australia, the Australian Stem Cell Centre and Norwood Immunology. J. A. D. was supported by a fellowship from the Cancer Council of Victoria.

Conflict of interest statement The authors declare that they have no conflict of interests.

References

1. Krenger W, Hollander GA (2008) The immunopathology of thymic GVHD. Semin Immunopathol 30:439–456. doi:10.1007/s00281-008-0131-6
2. Chidgey A, Dudakov J, Seach N, Boyd R (2007) Impact of niche aging on thymic regeneration and immune reconstitution. Semin Immunol 19:331–340. doi:S1044-5323(07)00080-2[pii]10.1016/j.smim.2007.10.006
3. Fletcher AL, Lowen TE, Sakkal S, Reiseger JJ, Hammett MV, Seach N, Scott HS, Boyd RL, Chidgey AP (2009) Ablation and regeneration of tolerance-inducing medullary thymic epithelial

cells after cyclosporine, cyclophosphamide, and dexamethasone treatment. J Immunol. doi:jimmunol.0900225[pii]10.4049/jimmunol.0900225
4. Chidgey AP, Layton D, Trounson A, Boyd RL (2008) Tolerance strategies for stem-cell-based therapies. Nature 453:330–337. doi:nature07041[pii]10.1038/nature07041
5. Till JE, Mc CE (1961) A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiat Res 14:213–222
6. Boyiadzis M, Pavletic S (2004) Haematopoietic stem cell transplantation: indications, clinical developments and future directions. Expert Opin Pharmacother 5:97–108. doi:10.1517/14656566.5.1.97
7. Doetsch F, Caille I, Lim DA, Garcia-Verdugo JM, Alvarez-Buylla A (1999) Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. Cell 97:703–716. doi:S0092-8674(00)80783-7[pii]
8. Taylor G, Lehrer MS, Jensen PJ, Sun TT, Lavker RM (2000) Involvement of follicular stem cells in forming not only the follicle but also the epidermis. Cell 102:451–461. doi:S0092-8674(00)00050-7[pii]
9. Barker N, van Es JH, Kuipers J, Kujala P, van den Born M, Cozijnsen M, Haegebarth A, Korving J, Begthel H, Peters PJ, Clevers H (2007) Identification of stem cells in small intestine and colon by marker gene *Lgr5*. Nature 449:1003–1007. doi:nature06196[pii]10.1038/nature06196
10. Sherwood RI, Christensen JL, Conboy IM, Conboy MJ, Rando TA, Weissman IL, Wagers AJ (2004) Isolation of adult mouse myogenic progenitors: functional heterogeneity of cells within and engrafting skeletal muscle. Cell 119:543–554. doi:S0092867404010360[pii]10.1016/j.cell.2004.10.021
11. Swijnenburg RJ, Tanaka M, Vogel H, Baker J, Kofidis T, Gunawan F, Lebl DR, Caffarelli AD, de Bruin JL, Fedoseyeva EV, Robbins RC (2005) Embryonic stem cell immunogenicity increases upon differentiation after transplantation into ischemic myocardium. Circulation 112:I166–I172. doi:112/9_suppl/I-166[pii]10.1161/CIRCULATIONAHA.104.525824
12. Amabile G, Meissner A (2009) Induced pluripotent stem cells: current progress and potential for regenerative medicine. Trends Mol Med 15:59–68. doi:S1471-4914(09)00016-1[pii]10.1016/j.molmed.2008.12.003
13. Zhou H, Wu S, Joo JY, Zhu S, Han DW, Lin T, Trauger S, Bien G, Yao S, Zhu Y, Siuzdak G, Scholer HR, Duan L, Ding S (2009) Generation of induced pluripotent stem cells using recombinant proteins. Cell Stem Cell 4:381–384. doi:S1934-5909(09)00159-3[pii]10.1016/j.stem.2009.04.005
14. Kim D, Kim CH, Moon JI, Chung YG, Chang MY, Han BS, Ko S, Yang E, Cha KY, Lanza R, Kim KS (2009) Generation of human induced pluripotent stem cells by direct delivery of reprogramming proteins. Cell Stem Cell 4:472–476. doi:S1934-5909(09)00214-8[pii]10.1016/j.stem.2009.05.005
15. Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126:663–676. doi:S0092-8674(06)00976-7[pii]10.1016/j.cell.2006.07.024
16. Wernig M, Meissner A, Foreman R, Brambrink T, Ku M, Hochedlinger K, Bernstein BE, Jaenisch R (2007) In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. Nature 448:318–324. doi:nature05944[pii]10.1038/nature05944
17. Park IH, Zhao R, West JA, Yabuuchi A, Huo H, Ince TA, Lerou PH, Lensch MW, Daley GQ (2008) Reprogramming of human somatic cells to pluripotency with defined factors. Nature 451:141–146. doi:nature06534[pii]10.1038/nature06534
18. Nishikawa S, Goldstein RA, Nierras CR (2008) The promise of human induced pluripotent stem cells for research and therapy. Nat Rev Mol Cell Biol 9:725–729. doi:nrm2466[pii]10.1038/nrm2466
19. Rossi DJ, Jamieson CH, Weissman IL (2008) Stems cells and the pathways to aging and cancer. Cell 132:681–696. doi:S0092-8674(08)00137-2[pii]10.1016/j.cell.2008.01.036
20. Carlson ME, Conboy IM (2007) Loss of stem cell regenerative capacity within aged niches. Aging Cell 6:371–382. doi:ACE286[pii]10.1111/j.1474-9726.2007.00286.x
21. Fandrich F, Lin X, Chai GX, Schulze M, Ganten D, Bader M, Holle J, Huang DS, Parwaresch R, Zavazava N, Binas B (2002) Preimplantation-stage stem cells induce long-term allogeneic graft acceptance without supplementary host conditioning. Nat Med 8:171–178. doi:10.1038/nm0202-171nm0202-171[pii]
22. Li L, Baroja ML, Majumdar A, Chadwick K, Rouleau A, Gallacher L, Ferber I, Lebkowski J, Martin T, Madrenas J, Bhatia M (2004) Human embryonic stem cells possess immune-privileged properties. Stem Cells 22:448–456. doi:22/4/448[pii]10.1634/stemcells.22-4-448
23. Drukker M, Katz G, Urbach A, Schuldiner M, Markel G, Itskovitz-Eldor J, Reubinoff B, Mandelboim O, Benvenisty N (2002) Characterization of the expression of MHC proteins in human embryonic stem cells. Proc Natl Acad Sci USA 99:9864–9869. doi:10.1073/pnas.142298299142298299[pii]
24. Drukker M, Benvenisty N (2004) The immunogenicity of human embryonic stem-derived cells. Trends Biotechnol 22:136–141. doi:10.1016/j.tibtech.2004.01.003S0167779904000198[pii]
25. Swijnenburg RJ, Schrepfer S, Govaert JA, Cao F, Ransohoff K, Sheikh AY, Haddad M, Connolly AJ, Davis MM, Robbins RC, Wu JC (2008) Immunosuppressive therapy mitigates immunological rejection of human embryonic stem cell xenografts. Proc Natl Acad Sci U S A 105:12991–12996. doi:0805802105[pii]10.1073/pnas.0805802105
26. Taylor CJ, Bolton EM, Pocock S, Sharples LD, Pedersen RA, Bradley JA (2005) Banking on human embryonic stem cells: estimating the number of donor cell lines needed for HLA matching. Lancet 366:2019–2025. doi:S0140-6736(05)67813-0[pii]10.1016/S0140-6736(05)67813-0
27. Robertson NJ, Brook FA, Gardner RL, Cobbold SP, Waldmann H, Fairchild PJ (2007) Embryonic stem cell-derived tissues are immunogenic but their inherent immune privilege promotes the induction of tolerance. Proc Natl Acad Sci U S A 104:20920–20925. doi:0710265105[pii]10.1073/pnas.0710265105
28. Mason DW, Charlton HM, Jones AJ, Lavy CB, Puklavec M, Simmonds SJ (1986) The fate of allogeneic and xenogeneic neuronal tissue transplanted into the third ventricle of rodents. Neuroscience 19:685–694
29. Liao NS, Bix M, Zijlstra M, Jaenisch R, Raulet D (1991) MHC class I deficiency: susceptibility to natural killer (NK) cells and impaired NK activity. Science 253:199–202
30. Grusby MJ, Auchincloss H Jr, Lee R, Johnson RS, Spencer JP, Zijlstra M, Jaenisch R, Papaioannou VE, Glimcher LH (1993) Mice lacking major histocompatibility complex class I and class II molecules. Proc Natl Acad Sci U S A 90:3913–3917
31. Dierich A, Chan SH, Benoist C, Mathis D (1993) Graft rejection by T cells not restricted by conventional major histocompatibility complex molecules. Eur J Immunol 23:2725–2728
32. Green DR, Ferguson TA (2001) The role of Fas ligand in immune privilege. Nat Rev Mol Cell Biol 2:917–924. doi:10.1038/3510310435103104[pii]
33. Golshayan D, Pascual M (2008) Tolerance-inducing immunosuppressive strategies in clinical transplantation: an overview. Drugs 68:2113–2130. doi:68154[pii]
34. von Boehmer H (2005) Mechanisms of suppression by suppressor T cells. Nat Immunol 6:338–344. doi:ni1180[pii]10.1038/ni1180
35. Kretschmer K, Heng TS, von Boehmer H (2006) De novo production of antigen-specific suppressor cells in vivo. Nat Protoc 1:653–661
36. Verginis P, McLaughlin KA, Wucherpfennig KW, von Boehmer H, Apostolou I (2008) Induction of antigen-specific regulatory T

cells in wild-type mice: visualization and targets of suppression. Proc Natl Acad Sci U S A 105:3479–3484. doi:0800149105[pii]10.1073/pnas.0800149105
37. Keating A (2006) Mesenchymal stromal cells. Curr Opin Hematol 13:419–425. doi:10.1097/01.moh.0000245697.54887.6f00062752-200611000-00004[pii]
38. Owen M, Friedenstein AJ (1988) Stromal stem cells: marrow-derived osteogenic precursors. Ciba Found Symp 136:42–60
39. Bieback K, Kern S, Kluter H, Eichler H (2004) Critical parameters for the isolation of mesenchymal stem cells from umbilical cord blood. Stem Cells 22:625–634. doi:22/4/625[pii]
40. In 't Anker PS, Scherjon SA, Kleijburg-van der Keur C, de Groot-Swings GM, Claas FH, Fibbe WE, Kanhai HH (2004) Isolation of mesenchymal stem cells of fetal or maternal origin from human placenta. Stem Cells 22:1338–1345. doi:22/7/1338[pii]
41. Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P, Hedrick MH (2002) Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell 13:4279–4295. doi:10.1091/mbc.E02-02-0105
42. Phinney DG, Prockop DJ (2007) Concise review: mesenchymal stem/multipotent stromal cells: the state of transdifferentiation and modes of tissue repair—current views. Stem Cells 25:2896–2902. doi:2007-0637[pii]10.1634/stemcells.2007-0637
43. Uccelli A, Moretta L, Pistoia V (2008) Mesenchymal stem cells in health and disease. Nat Rev Immunol 8:726–736
44. Ren G, Zhang L, Zhao X, Xu G, Zhang Y, Roberts AI, Zhao RC, Shi Y (2008) Mesenchymal stem cell-mediated immunosuppression occurs via concerted action of chemokines and nitric oxide. Cell Stem Cell 2:141–150. doi:S1934-5909(07)00314-1[pii]10.1016/j.stem.2007.11.014
45. Ilancheran S, Michalska A, Peh G, Wallace EM, Pera M, Manuelpillai U (2007) Stem cells derived from human fetal membranes display multilineage differentiation potential. Biol Reprod 77:577–588. doi:biolreprod.106.055244[pii]10.1095/biolreprod.106.055244
46. Wolbank S, Peterbauer A, Fahrner M, Hennerbichler S, van Griensven M, Stadler G, Redl H, Gabriel C (2007) Dose-dependent immunomodulatory effect of human stem cells from amniotic membrane: a comparison with human mesenchymal stem cells from adipose tissue. Tissue Eng 13:1173–1183. doi:10.1089/ten.2006.0313
47. Jaenisch R, Young R (2008) Stem cells, the molecular circuitry of pluripotency and nuclear reprogramming. Cell 132:567–582. doi:S0092-8674(08)00115-3[pii]10.1016/j.cell.2008.01.015
48. Raya A, Rodriguez-Piza I, Guenechea G, Vassena R, Navarro S, Barrero MJ, Consiglio A, Castella M, Rio P, Sleep E, Gonzalez F, Tiscornia G, Garreta E, Aasen T, Veiga A, Verma IM, Surralles J, Bueren J, Belmonte JC (2009) Disease-corrected haematopoietic progenitors from Fanconi anaemia induced pluripotent stem cells. Nature. doi:nature08129[pii]10.1038/nature08129
49. Dimos JT, Rodolfa KT, Niakan KK, Weisenthal LM, Mitsumoto H, Chung W, Croft GF, Saphier G, Leibel R, Goland R, Wichterle H, Henderson CE, Eggan K (2008) Induced pluripotent stem cells generated from patients with ALS can be differentiated into motor neurons. Science 321:1218–1221. doi:1158799[pii]10.1126/science.1158799
50. Chin MH, Mason MJ, Xie W, Volinia S, Singer M, Peterson C, Ambartsumyan G, Aimiuwu O, Richter L, Zhang J, Khvorostov I, Ott V, Grunstein M, Lavon N, Benvenisty N, Croce CM, Clark AT, Baxter T, Pyle AD, Teitell MA, Pelegrini M, Plath K, Lowry WE (2009) Induced pluripotent stem cells and embryonic stem cells are distinguished by gene expression signatures. Cell Stem Cell 5:111–123. doi:S1934-5909(09)00292-6[pii]10.1016/j.stem.2009.06.008
51. Weissman IL, Shizuru JA (2008) The origins of the identification and isolation of hematopoietic stem cells, and their capability to induce donor-specific transplantation tolerance and treat autoimmune diseases. Blood 112:3543–3553. doi:112/9/3543[pii]10.1182/blood-2008-08-078220
52. Gallegos AM, Bevan MJ (2004) Central tolerance to tissue-specific antigens mediated by direct and indirect antigen presentation. J Exp Med 200:1039–1049. doi:jem.20041457[pii]10.1084/jem.20041457
53. Sykes M (2007) Mechanisms of tolerance induced via mixed chimerism. Front Biosci 12:2922–2934. doi:2282[pii]
54. Olsen NJ, Kovacs WJ (2001) Effects of androgens on T and B lymphocyte development. Immunol Res 23:281–288. doi:IR:23:2-3:281[pii]10.1385/IR:23:2-3:281
55. Kincade PW, Medina KL, Payne KJ, Rossi MI, Tudor KS, Yamashita Y, Kouro T (2000) Early B-lymphocyte precursors and their regulation by sex steroids. Immunol Rev 175:128–137
56. Sutherland JS, Goldberg GL, Hammett MV, Uldrich AP, Berzins SP, Heng TS, Blazar BR, Millar JL, Malin MA, Chidgey AP, Boyd RL (2005) Activation of thymic regeneration in mice and humans following androgen blockade. J Immunol 175:2741–2753. doi:175/4/2741[pii]
57. Roden AC, Moser MT, Tri SD, Mercader M, Kuntz SM, Dong H, Hurwitz AA, McKean DJ, Celis E, Leibovich BC, Allison JP, Kwon ED (2004) Augmentation of T cell levels and responses induced by androgen deprivation. J Immunol 173:6098–6108. doi:173/10/6098[pii]
58. Heng TS, Goldberg GL, Gray DH, Sutherland JS, Chidgey AP, Boyd RL (2005) Effects of castration on thymocyte development in two different models of thymic involution. J Immunol 175:2982–2993. doi:175/5/2982[pii]
59. Dudakov JA, Goldberg GL, Reiseger JJ, Chidgey AP, Boyd RL (2009) Withdrawal of sex steroids reverses age- and chemotherapy-related defects in bone marrow lymphopoiesis. J Immunol 182:6247–6260. doi:182/10/6247[pii]10.4049/jimmunol. 0802446
60. Goldberg GL, Sutherland JS, Hammet MV, Milton MK, Heng TS, Chidgey AP, Boyd RL (2005) Sex steroid ablation enhances lymphoid recovery following autologous hematopoietic stem cell transplantation. Transplantation 80:1604–1613. doi:00007890-200512150-00015[pii]
61. Goldberg GL, Alpdogan O, Muriglan SJ, Hammett MV, Milton MK, Eng JM, Hubbard VM, Kochman A, Willis LM, Greenberg AS, Tjoe KH, Sutherland JS, Chidgey A, van den Brink MR, Boyd RL (2007) Enhanced immune reconstitution by sex steroid ablation following allogeneic hemopoietic stem cell transplantation. J Immunol 178:7473–7484. doi:178/11/7473[pii]
62. Goldberg GL, King CG, Nejat RA, Suh DY, Smith OM, Bretz JC, Samstein RM, Dudakov JA, Chidgey AP, Chen-Kiang S, Boyd RL, van den Brink MR (2009) Luteinizing hormone-releasing hormone enhances T cell recovery following allogeneic bone marrow transplantation. J Immunol 182:5846–5854. doi:182/9/5846[pii]10.4049/jimmunol.0801458
63. Sutherland JS, Spyroglou L, Muirhead JL, Heng TS, Prieto-Hinojosa A, Prince HM, Chidgey AP, Schwarer AP, Boyd RL (2008) Enhanced immune system regeneration in humans following allogeneic or autologous hemopoietic stem cell transplantation by temporary sex steroid blockade. Clin Cancer Res 14:1138–1149. doi:14/4/1138[pii]10.1158/1078-0432.CCR-07-1784
64. Alpdogan O, Hubbard VM, Smith OM, Patel N, Lu S, Goldberg GL, Gray DH, Feinman J, Kochman AA, Eng JM, Suh D, Muriglan SJ, Boyd RL, van den Brink MR (2006) Keratinocyte growth factor (KGF) is required for postnatal thymic regeneration. Blood 107:2453–2460. doi:2005-07-2831[pii]10.1182/blood-2005-07-2831
65. Min D, Panoskaltsis-Mortari A, Kuro OM, Hollander GA, Blazar BR, Weinberg KI (2007) Sustained thymopoiesis and improvement in functional immunity induced by exogenous KGF administration in

murine models of aging. Blood 109:2529–2537. doi:blood-2006-08-043794[pii]10.1182/blood-2006-08-043794
66. Rossi SW, Jeker LT, Ueno T, Kuse S, Keller MP, Zuklys S, Gudkov AV, Takahama Y, Krenger W, Blazar BR, Hollander GA (2007) Keratinocyte growth factor (KGF) enhances postnatal T-cell development via enhancements in proliferation and function of thymic epithelial cells. Blood 109:3803–3811. doi:blood-2006-10-049767[pii]10.1182/blood-2006-10-049767
67. Rossi S, Blazar BR, Farrell CL, Danilenko DM, Lacey DL, Weinberg KI, Krenger W, Hollander GA (2002) Keratinocyte growth factor preserves normal thymopoiesis and thymic microenvironment during experimental graft-versus-host disease. Blood 100:682–691
68. Kelly RM, Highfill SL, Panoskaltsis-Mortari A, Taylor PA, Boyd RL, Hollander GA, Blazar BR (2008) Keratinocyte growth factor and androgen blockade work in concert to protect against conditioning regimen-induced thymic epithelial damage and enhance T-cell reconstitution after murine bone marrow transplantation. Blood 111:5734–5744. doi:blood-2008-01-136531[pii]10.1182/blood-2008-01-136531
69. Andrew D, Aspinall R (2001) Il-7 and not stem cell factor reverses both the increase in apoptosis and the decline in thymopoiesis seen in aged mice. J Immunol 166:1524–1530
70. Alpdogan O, Muriglan SJ, Eng JM, Willis LM, Greenberg AS, Kappel BJ, van den Brink MR (2003) IL-7 enhances peripheral T cell reconstitution after allogeneic hematopoietic stem cell transplantation. J Clin Invest 112:1095–1107. doi:10.1172/JCI17865112/7/1095[pii]
71. Fry TJ, Moniuszko M, Creekmore S, Donohue SJ, Douek DC, Giardina S, Hecht TT, Hill BJ, Komschlies K, Tomaszewski J, Franchini G, Mackall CL (2003) IL-7 therapy dramatically alters peripheral T-cell homeostasis in normal and SIV-infected nonhuman primates. Blood 101:2294–2299. doi:10.1182/blood-2002-07-22972002-07-2297[pii]
72. Chu YW, Memon SA, Sharrow SO, Hakim FT, Eckhaus M, Lucas PJ, Gress RE (2004) Exogenous IL-7 increases recent thymic emigrants in peripheral lymphoid tissue without enhanced thymic function. Blood 104:1110–1119. doi:10.1182/blood-2003-10-36352003-10-3635[pii]
73. Wils EJ, Braakman E, Verjans GM, Rombouts EJ, Broers AE, Niesters HG, Wagemaker G, Staal FJ, Lowenberg B, Spits H, Cornelissen JJ (2007) Flt3 ligand expands lymphoid progenitors prior to recovery of thymopoiesis and accelerates T cell reconstitution after bone marrow transplantation. J Immunol 178:3551–3557. doi:178/6/3551[pii]
74. Fry TJ, Sinha M, Milliron M, Chu YW, Kapoor V, Gress RE, Thomas E, Mackall CL (2004) Flt3 ligand enhances thymic-dependent and thymic-independent immune reconstitution. Blood 104:2794–2800. doi:10.1182/blood-2003-11-37892003-11-3789[pii]
75. Kenins L, Gill JW, Boyd RL, Hollander GA, Wodnar-Filipowicz A (2008) Intrathymic expression of Flt3 ligand enhances thymic recovery after irradiation. J Exp Med 205:523–531. doi:jem.20072065[pii]10.1084/jem.20072065
76. Dixit VD, Yang H, Sun Y, Weeraratna AT, Youm YH, Smith RG, Taub DD (2007) Ghrelin promotes thymopoiesis during aging. J Clin Invest 117:2778–2790. doi:10.1172/JCI30248
77. Napolitano LA, Schmidt D, Gotway MB, Ameli N, Filbert EL, Ng MM, Clor JL, Epling L, Sinclair E, Baum PD, Li K, Killian ML, Bacchetti P, McCune JM (2008) Growth hormone enhances thymic function in HIV-1-infected adults. J Clin Invest 118:1085–1098. doi:10.1172/JCI32830
78. Carlo-Stella C, Di Nicola M, Milani R, Longoni P, Milanesi M, Bifulco C, Stucchi C, Guidetti A, Cleris L, Formelli F, Garotta G, Gianni AM (2004) Age- and irradiation-associated loss of bone marrow hematopoietic function in mice is reversed by recombinant human growth hormone. Exp Hematol 32:171–178. doi:10.1016/j.exphem.2003.11.007S0301472X03003941[pii]
79. La Motte-Mohs RN, Herer E, Zuniga-Pflucker JC (2005) Induction of T-cell development from human cord blood hematopoietic stem cells by Delta-like 1 in vitro. Blood 105:1431–1439. doi:2004-04-1293[pii]10.1182/blood-2004-04-1293
80. Schmitt TM, de Pooter RF, Gronski MA, Cho SK, Ohashi PS, Zuniga-Pflucker JC (2004) Induction of T cell development and establishment of T cell competence from embryonic stem cells differentiated in vitro. Nat Immunol 5:410–417. doi:10.1038/ni1055ni1055[pii]
81. Gill J, Malin M, Hollander GA, Boyd R (2002) Generation of a complete thymic microenvironment by MTS24(+) thymic epithelial cells. Nat Immunol 3:635–642. doi:10.1038/ni812ni812[pii]
82. Seach N, Layton D, Lim J, Chidgey A, Boyd R (2007) Thymic generation and regeneration: a new paradigm for establishing clinical tolerance of stem cell-based therapies. Curr Opin Biotechnol 18:441–447. doi:S0958-1669(07)00083-3[pii]10.1016/j.copbio.2007.07.001

Appendix 2

Feeding the fire: the role of defective bone marrow function in exacerbating thymic involution

Review

Feeding the fire: the role of defective bone marrow function in exacerbating thymic involution

Jarrod A. Dudakov[1], Danika M.P. Khong, Richard L. Boyd and Ann P. Chidgey

Immune Regeneration Laboratory, Monash Immunology and Stem Cell Laboratories (MISCL), Monash University, Wellington Rd, Clayton, Victoria, Australia 3800

Most of the steps of lymphopoiesis have been elucidated but contentious issues remain, particularly regarding the identity and function of the earliest lymphoid progenitors that leave the bone marrow and seed the thymus. Hematopoiesis is effectively continuous throughout life, but there is a profound decline in immune function with increasing age, driven by thymus involution and severely curtailed B cell development. A key question is whether defects in bone marrow progenitors, such as reduced differentiation and repopulation potential, are the common denominator. While thymic involution temporally precedes overt HSC functional decline, a logical supposition is that the latter exacerbates the former. This review explores this possible link, and concludes that improving bone marrow function is fundamental to sustained thymic regeneration.

Maintenance of hematopoietic stem cell function in the niche

Hematopoietic stem cells (HSCs) are crucial throughout life for their ability to differentiate and generate all hematopoietic lineages while maintaining the capacity for self-renewal. This requires populations of quiescent HSCs in the bone marrow (BM) that differentiate as needed, a process that is particularly important following hematopoietic stress, such as cytoreductive chemotherapy or radiation therapy for neoplastic malignancies. Maintenance of HSC quiescence, self-renewal and differentiation is a complicated process involving multiple interactions and signals derived from the surrounding microenvironment, or niche (Figure 1). HSC niches have been identified in the BM at the endosteal interface between the central marrow and bone and adjoining sinusoidal vascular endothelium; however, the individual roles of these niches and the extent of their involvement in hematopoiesis is currently in question [1]. Within these niches, several individual cell populations have been implicated in the maintenance of HSCs, including osteoblasts (OBL), CXCL12 abundant reticular cells (CAR), endothelium, mesenchymal stromal cells, adipocytes and osteoclasts [1,2]. Molecular signals from these cells including osteopontin (OPN), thrombopoietin (TPO), CXCL12, parathyroid hormone (PTH), angiopoietin-1 (Ang-1), stem cell factor (SCF), N-cadherin, and bone morphogenic protein (BMP)-4, as well as their downstream mediators, such as c-Myc, Bmi-1, Rac-1 and Rac-2, and the cyclin-dependent kinase inhibitors (CDKIs) $p16^{ink4A}$, $p21^{cip1/waf1}$, $p19^{Arf}$, and $p57^{kip2}$, initiate or repress programs that govern the localisation, quiescence, self-renewal or differentiation of HSCs (Figure 1) [2,3].

Lymphoid commitment of HSCs

Commitment and differentiation toward the lymphoid and T cell lineages within the BM is a complex process involving multiple paracrine signals and cell–cell interactions as well as their downstream transcriptional programs. Recent findings suggest that lymphopoiesis from HSCs involves passage through a lympho-myeloid progenitor that contains no erythroid potential (Figure 2) [4]. These BM-derived precursors, known as lymphoid-primed multipotent progenitors (LMPPs), are characterised by the upregulation of lymphoid associated genes such as PU.1 and Ikaros [5,6]. Ikaros is a member of the zinc finger transcription factor family implicated in upstream HSC function [7] and has a crucial role in HSC lymphoid lineage priming. Ikaros represses key myeloid genes and self-renewal while promoting the expression of lymphoid associated genes such as *Flt3, ckit* and *IL7R* [5,8]. However, while Ikaros is very important, the redundancy of hematopoiesis, which would be of evolutionary advantage, is evident through the presence of intrathymic early T-lineage progenitors (ETPs) in $Ikaros^{-/-}$ animals and T cell development still occurs in these mice, albeit at a reduced rate [9]. PU.1, which is a member of the Ets transcription factor family, is expressed in HSCs, downstream multipotent progenitors (MPPs) and common lymphoid progenitors (CLPs), but not in erythroid or thromboid cells, and is vital for balancing cell lineage decisions of HSCs between myeloid and lymphoid lineage choices [10]. A recent study revealed that along with promoting differentiation of HSCs down lympho-myeloid lineages, PU.1 might play a role in promoting self-renewal of HSCs [11] and so prove to be critical to asymmetric divisions of HSCs [12].

LMPPs are enriched within the $Flt3^{hi}CD34^{+}Thy1^{-}$ population of $Lin^{-}Sca1^{+}ckit^{+}$ (LSK) cells [4,13,14] and those expressing a recombination activating gene (RAG-1) were found to be surprisingly quiescent when labelled with bromodeoxyuridine (BrdU), suggesting that some lymphoid-committed precursors remain quiescent to sustain lymphopoiesis throughout life [15]. Furthermore, several

Corresponding author: Chidgey, A.P. (ann.chidgey@med.monash.edu.au)
[1] Present address: Department of Immunology, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10065, USA..

1471-4906/$ – see front matter doi:10.1016/j.it.2010.02.002 Available online 29 March 2010

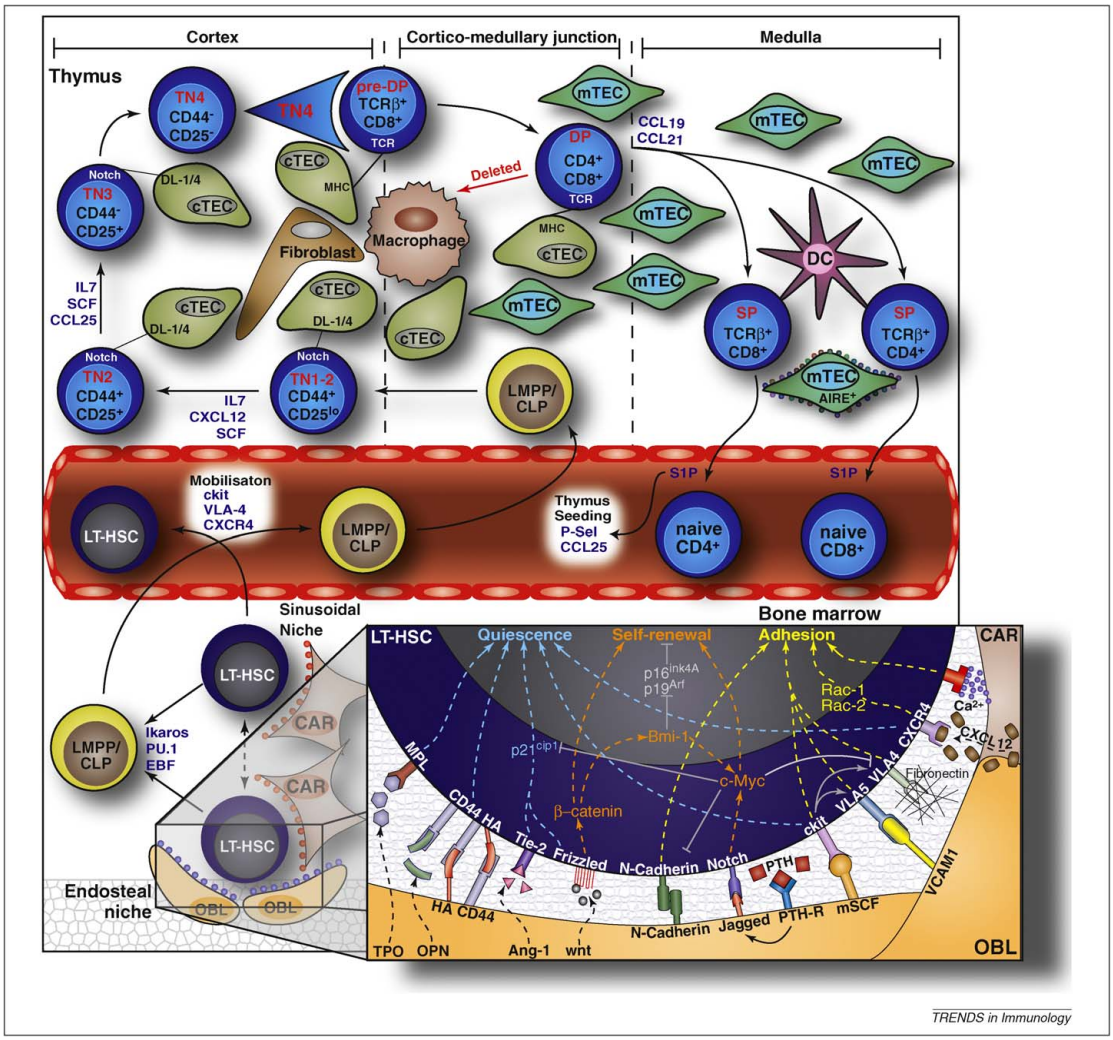


Figure 1. Maintenance of HSCs in the niche and their differentiation down lymphoid lineages in the BM and thymus. Long-term hematopoietic stem cells (LT-HSC) reside in specialised niches in the endosteum and adjacent to sinusoidal vascular epithelial cells [1]. CAR cells and a subset of hematopoiesis-supporting OBL are thought to be critical for HSC maintenance in the niche [1,2]. Adhesion of HSCs to these niches is mediated by signals through the calcium-sensing receptor, CXCR4, VLA-4 and 5, ckit and N-cadherin. The balance between self-renewal and quiescence is maintained through the Notch and β-catenin pathways as well as signalling through Tie-2, hyaluronic acid, CD44 and MPL by their respective ligands Ang-1, CD44, OPN and TPO [2,3]. Differentiation down the lymphoid lineage towards LMPP and CLP is associated with expression of PU.1 and Ikaros [10]. For T cell development to occur, hematopoietic progenitors periodically egress from the BM, associated with interactions through ckit, VLA-4, Flt3 and CXCR4, and enter the circulation [37]. Expression on the surface of circulating progenitor cells of CCR9 and PSGL-1 are crucial for the entry of hematopoietic progenitors into the thymus [28,32,33]. Once in the thymus, commitment to the T-cell lineage is secured by interaction with the Notch ligands delta-like 1 and 4 [10,30]. With appropriate interactions from the supporting stromal microenvironment, comprised primarily of cortical and medullary thymic epithelial cells (cTEC and mTEC respectively) and fibroblasts, T cell development progresses through distinct stages and positive and negative selection, to form self-tolerant single positive T cells [57]. Naïve T cells are exported from the thymus based on an S1P gradient that can influence the expression of P-selectin and CCL25 by thymic stromal cells and thereby influence thymic receptivity to circulating progenitors [39].

other BM-resident lymphoid progenitors with T-lineage potential have been identified, including CLP-1 and CLP-2 [16]. In fact, in the case of both CLP-1 and CLP-2 there is evidence to suggest their capacity for thymus-seeding [17,18], although their limited T cell differentiation potential compared to that of intrathymic ETPs [17,19] suggests a role in non-canonical T cell development or as a response to injury [20,21].

Journey from the BM to thymus

The thymus continues to function throughout life, albeit at a progressively reduced rate with age but, importantly, it contains no self-renewing lymphoid stem cells. Thymopoiesis is instead maintained by the periodic egress of progenitors from the BM into the circulation. It is this dependency that raises the question of a causal link between alterations in BM function and subsequent loss of

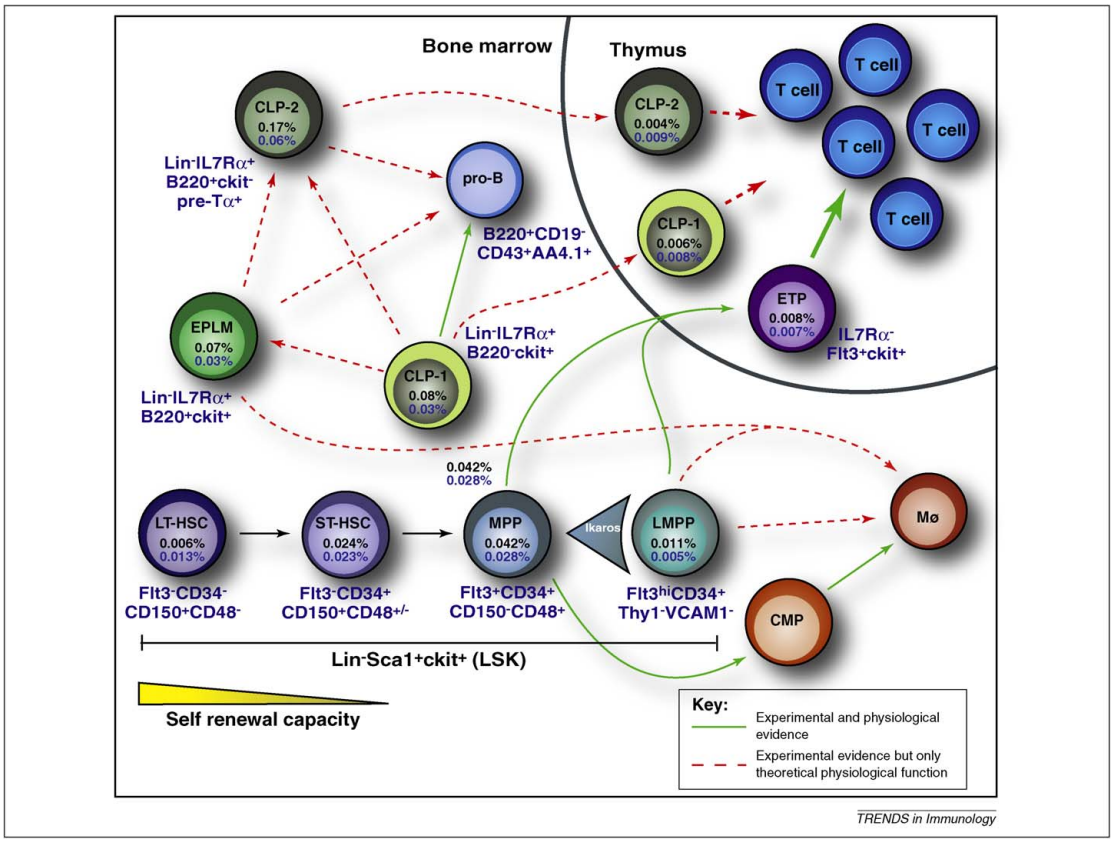


Figure 2. Identification and interrelationship between hematopoietic progenitor cells of BM and thymus. All blood cells derive from HSCs that lack expression of mature hematopoietic markers (such as CD3, CD4, CD19, CD45R, CD11b, CD11c, Mac-1 and TER-119) and express LSK. Using expression of Flt3, CD34, CD150, and CD48, LSK cells can be further subdivided according to their repopulation and self-renewal potential [105]. Within the MPP population, LMPPs can be isolated based on differential expression of Thy-1, Flt3, VCAM-1, CD27 and CD62L [4,14,22]. Numerous Lin-IL7Rα$^+$ bipotent lymphoid precursors have been identified and can be distinguished by expression of B220 and c-kit, including the ''classic'' CLP-1 and CLP-2 and early progenitor with lymphoid and myeloid potential (EPLM), all of which can form the earliest B cell precursors. Experimentally, many lymphoid progenitors can give rise to myeloid cells but the physiological relevance of this is not known [106]. Several potential routes of egress from the BM to the thymus have been proposed. The ''canonical'' route involves the egress of LMPP cells from the BM and their homing and settling in the thymus [9,25,26,28]. Despite only limited T cell potential, CLP-1 and CLP-2 cells have been shown to home to and seed the thymus, suggesting an alternate ''non-canonical'' route of T cell development [17,18] that might be involved in the regeneration following insult [20,21]. Indicated for each cell type are their percentages relative to total organ cellularity in 2-mo (black) and 9-mo (blue) C57Bl/6 mice.

thymopoiesis. LMPPs, a subset of which are phenotypically and functionally analogous to the earliest intrathymic ETP population [9,13,22], can be found in the circulation [22], making them a prime candidate as a thymus-seeding cell. However, while the identity of the thymus-seeding progenitor has yet to be elucidated unequivocally, it does not necessarily need to be a unique entity; indeed, numerous candidates with thymus-seeding ability and T lineage potential have been proposed, albeit with only limited data to imply their steady-state physiological functions (Figure 2) [23,24]. Moreover, the extent of T-lineage commitment before thymic entry is unclear [23]. Recent studies have found the earliest intrathymic T cell precursors retain myeloid potential [25,26]; however, there is still debate over their propensity, or lack thereof, for B cell potential [27,28]. Furthermore, populations of cells restricted to either the lymphoid [17,18] or specifically T-lineage [29] have been identified in the circulation and might contribute towards T cell development. These alternative lineage programs are soon repressed by engaging the Notch ligands delta-like 1 and 4, expressed by thymic stromal cells, ensuring commitment and progression down the T lineage [30,31].

The mechanisms underlying the entry of T-lineage progenitors into the thymus are only just being resolved. It is now clear that expression of chemokine receptors 7 and 9 (CCR7 and CCR9) and p-selectin glycoprotein ligand (PSGL)-1 by circulating progenitors and their respective ligands/receptors on thymic stromal cells is crucial for entry of progenitors into the thymus [32–35]. Indeed, it was shown recently that only CCR9-expressing MPPs or CLPs could seed the thymus and that expression of CCR9 was dependent on Flt3 signalling in the BM [33], which is also associated with lymphoid priming [4].

Through the use of elegant adoptive transfer experiments, it was demonstrated that free intrathymic microenvironmental niches control the importation of thymic progenitors [36] via the opening or closing of so-called intrathymic microvascular gates. This process is thought to occur in coordination with the periodic release of progenitors from the BM, suggesting a feedback loop exists between the BM and thymus [37]. While elucidation of this periodic link between the BM and thymus has remained difficult, a recent study found that both niche availability, possibly under the control of the early response gene 1 transcription factor [38], and the levels of circulating S1P, which controls the egress of naïve T cells from the thymus, can control expression of P-selectin and the chemokine CCL25, thereby directing thymic receptivity to circulating progenitors [39].

The aging thymus

The profound involution of the thymus is one of the most widely studied aspects of age on the immune system. It is a gradual process that begins at the onset of puberty, leading to the generally accepted role of sex steroids in inducing thymic involution (Box 1) [40] While the proportion of thymocyte subsets remains remarkably unaffected with age, emphasising the homeostatically regulated T cell development pathways, there is a considerable decline in total thymopoiesis and significant disruption to the thymic architecture (Figure 3) [40]. This results directly in a decline in the emigration of naïve T cells into the periphery [41], the numbers of which are then maintained by homeostatic expansion of pre-existing peripheral memory T cells. The subsequent decline in diversity of the T cell receptor repertoire leads to reduced responsiveness to new antigens [42].

Mechanistically, the production of IL-7, which is crucial for proliferation and differentiation from the triple negative 2 (TN2) stage of thymocyte development, is reduced significantly with age and,. thus, implicated in thymic involution [43]. However, exogenous administration of IL-7 does not reverse thymic atrophy significantly [43,44], suggesting the decline in IL-7 production might only partially explain thymic involution. Moreover, the impact of exogenous keratinocyte growth factor (KGF) or growth hormone, as well as the removal of sex steroids implicates these processes in thymic involution [40,39].

Box 1. Strategies for immune regeneration

Several strategies have been developed to reverse thymic degeneration as a consequence of age or following immunodepletion caused by cytoreductive treatments such as chemotherapy and radiation therapy. These include: administration of exogenous growth factors and cytokines, such as KGF, growth hormone, ghrelin, IL-7, IL-15, insulin-like growth factor-1, IL-12, or Flt3L; adoptive transfer of T cell precursors generated *in vitro*; the chemical or surgical ablation of sex steroids (Figure 3) [45,56,61,97,98] While most of these treatments primarily promote thymopoiesis directly, KGF and sex steroid ablation enhance thymic regeneration by acting on thymic stromal cells [40,45,61,99]. However, the success of growth factors such as IL-7, growth hormone and Flt3L is equivocal [40,44]. Furthermore, one of the main drawbacks to these therapies is that their efficacy is limited to the duration of administration due to the short half-life of these growth factors. Adoptive transfer of *in vitro* generated precursor T cells aids in thymic regeneration and even has long-lasting impacts on the thymus well after the transient progenitors have passed through the thymus; however, the mechanism behind this prolonged regeneration is poorly understood [100]. Among these therapies, sex steroid ablation has beneficial impacts on both the BM and thymus, reversing age-related thymic involution [56] and enhancing BM recovery following chemotherapy [98,101] and bone marrow transplantation [97]. These novel approaches to restore immune capacity through the translation of pre-clinical research are likely to result in the development of strategies to improve the outcome for a variety of patients who incur considerable morbidity and mortality from infections and relapse after high-dose chemotherapy and HSC transplant or following chemotherapy. Furthermore, these strategies could be used in a variety of other clinical settings to overcome lymphocytopenia, to enhance the effectiveness of adult vaccinations or to stimulate lymphoid regeneration following prolonged viral infection such as HIV. Finally, rejuvenation of the thymus might be used to re-establish self-tolerance in autoimmune disease or to prevent rejection of donor transplants.

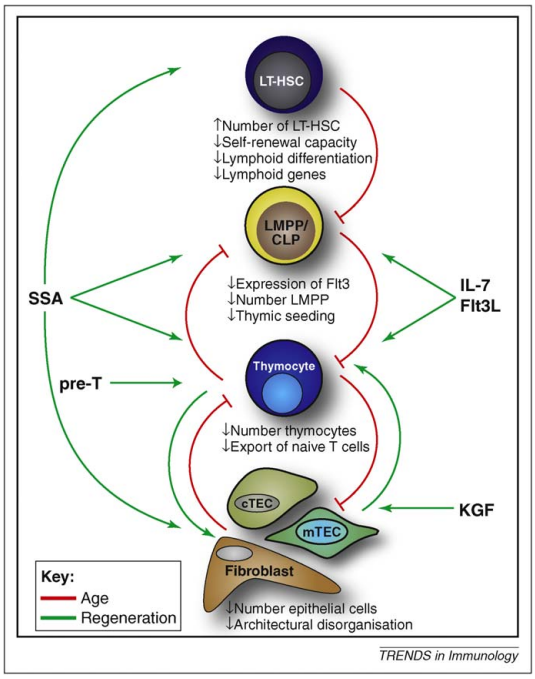


Figure 3. Age-related changes and regeneration of lymphopoiesis. There is a significant increase with age in the number of cells expressing the phenotype of long-term hematopoietic stem cells (LT-HSCs) [52,65]. Functionally, however, these cells are reduced in their self-renewal capacity [67]; ability to generate lymphocytes [52,68,72]; and repopulation potential [52]. These functional changes lead to a reduction in number and function of lymphoid progenitors [48]. In the thymus, there is a profound involution with age, with reductions in the number of developing thymocytes leading to reduced export of naïve T cells and a limited peripheral receptor repertoire [41,42]. In the thymus, impacts are observed also on the supporting stromal cells, such as epithelium and fibroblasts, which are radically reduced in number with an associated architectural disorganisation [40]. Several strategies have been proposed to assist thymic regeneration, all impacting on different elements of this hierarchy of degeneration. Directly impacting on thymocytes and early T-lineage progenitors are the cytokines IL-7 and Flt3, although their clinical effectiveness is equivocal [40,44]. KGF produced by fibroblasts impacts directly on the expansion of epithelial cells, thereby enhancing thymopoiesis indirectly [99]. Also, adoptive transfer of *in vitro* generated precursor T cells (pre-T) has been shown to enhance thymopoiesis and regenerate thymic stromal cells [100]. SSA has wide-ranging impacts on this hierarchy and regenerates both at the level of improved microenvironments and thymic and BM lymphopoiesis [40,56,97,98,101].

At the cellular level, while the aged thymus does not appear to be significantly impacted in its ability to import hematopoietic progenitors from the circulation [46,47], intrathymic T cell precursors, along with their upstream BM-resident lymphoid progenitors, deteriorate with age in frequency, number and their ability to produce T cells [48–50], although this functional defect has been challenged [51]; differences in the experimental techniques used might underlie the discrepancy. Expression of Flt3 on LSK cells is also reduced significantly with age [52] and might be associated with a decline in the absolute number of BM-resident lymphoid progenitors through the link to CCR9 [33].

It has been suggested recently that plasma levels of S1P, which is involved with regulating the export of naïve single positive thymocytes, also regulates the expression of P-selectin, CCL25 and subsequently thymic receptivity [39]. However, in the aged, where there is a decline in thymic export, curiously there is no apparent corresponding change in the expression of P-selectin or CCL25 and subsequently receptivity of the thymus to the entry of circulating progenitors [47]. The direct relationship between age, circulating S1P levels and thymic expression of P-selectin and CCL25 is still unresolved (Box 2). Considering the role of S1P in HSC migration and homing [53,54], however, a greater role for S1P might be on the egress of these progenitors from the BM, limiting thymus function indirectly. Interestingly, following thymic regeneration mediated by sex steroid ablation there is both an increase in thymic export [40] and enhanced thymic CCL25 expression and receptivity [55].

While supply of blood-borne hematopoietic or thymic progenitors is crucial for maintaining thymopoiesis throughout life, and there is a reduced BM capacity for these cells with age, a reduced ability for their importation does not appear to underlie the initiation, of thymic involution [47]. Just as there is significantly reduced developmental potential of T lineage progenitors and HSCs with age, there is a profound deterioration of the thymic microenvironment that initiates and controls the complex pathways of T cell differentiation and maturation [40,56,57]. Deciphering the initial events of thymic involution is difficult, particularly due to the crosstalk between developing thymocytes and the supporting stromal cells: reduced thymopoiesis can alter the thymic microenvironment significantly, just as microenvironmental defects can alter thymopoiesis [58–60].

While numerous treatments have been developed to combat thymic involution (Box 1), including growth hormone, KGF, IL-7, the use of *in vitro* generated precursor T cells, and sex steroid ablation (SSA) [40,45,61], it is probable that regeneration of both the thymic microenvironment and the HSC compartment are essential for rapid, long-term stable regeneration.

Linking HSC aging with the functional decline in thymopoiesis

It is becoming increasingly clear that there are significant impacts of aging on the intrinsic function of HSCs. While different across strains [62–64], in the most widely used C57Bl/6 there is a numerical accumulation of HSCs, in particular long-term (LT)-HSCs [52,65], but they are functionally far less effective in their homing and engraftment capacity [66]. HSCs from aged animals exhibit decreased repopulation and lymphoid differentiation potential [49,52] as well as reduced self-renewal capacity upon serial transplantation [67]. In repopulation experiments, defects in HSC engraftment were restricted to the lymphoid lineage and acquired HSC defects have been linked directly to a reduced antibody repertoire in B cells with age [68], further implicating an intrinsic skewing away from the lymphoid lineage. In this regard, HSCs from young animals transformed with the Bcr-Abl oncogene produce both myeloid and lymphoid leukemias, whereas HSCs from aged mice exhibit exclusively a myeloproliferative disorder [69]. These studies are consistent with an intrinsic bias away from lymphopoiesis with age and have led to a clinical hypothesis that myeloid-biased HSCs may be the transforming site in myelogenous leukemias, which are more prevalent with age [70,71]. Moreover, despite declining self-renewal capacity amongst multipotent HSCs, clonally there is an accumulation of phenotypically identical self-renewing myeloid-biased progenitors [72,73].

The mechanisms underlying these intrinsic age-related changes include upregulation of myeloid- and downregulation of lymphoid-associated genes [52], accumulation of mitochondrial and genomic DNA mutations [74,75], alterations in epigenetic regulation [76–78], deficiency in DNA repair [79,80] and altered p53 expression [81], all of which contribute to altered HSC function. Bmi-1, which has an

Table 1. Age-related changes to the expression of molecules associated with HSC differentiation towards the T-lineage .

Factor	Role	Changes with age	Ref.
HSC maintenance			
Bmi-1-p16^{Ink4A}/p19^{Arf}	Self-renewal and quiescence	Increase in p16 ^{ink4A} and p19 ^{Arf}	[79,82–84,86]
Tie2-Ang-1	HSC quiescence	Reduced Tie-2 expression in skeletal muscle	[88]
Lymphoid commitment and differentiation			
Ikaros	Lymphoid commitment	Higher turnover and lower expression	[89,90]
E2A, E47, Pax5	B-lineage commitment	Decreased	[90,102]
Notch	T-lineage commitment	Intrathymic Notch and Delta-like 1 increased	[103]
RAG-1	Lymphoid differentiation	Decreased	[104]
Flt3-Flt3L	Lymphoid differentiation	Reduction in Flt3 expression on LSK cells in BM	[52]
IL-7	Lymphoid development	Reduced IL-7 expression in both BM and thymus	[43,52]
Thymic seeding			
CCL25-CCR9	Thymic seeding	CCR9 potentially downregulated as expression is dependent on Flt3 signalling, which is decreased	[33]
L-, E-, P-selectin-PSGL-1	Thymic seeding	P-selectin might change if there is a change in S1P gradients	[39]

Box 2. Outstanding questions

- What is the role of niche aging in initiating functional changes to HSCs?
- Does the reduced lymphoid differentiation potential of HSCs lead to reduced thymic seeding by circulating progenitors?
- What are the relative contributions of reduced thymic seeding and an altered thymic microenvironment with age in causing thymic involution?
- What is the impact of age on circulating S1P and receptor expression?
- Is there a correlation between P-selectin or CCL25 expression in the thymus and LMPP or CLP egress from the BM and is there a role for S1P expression and naïve T cell export in this correlation?
- Is there a bi-directional communication between the BM and thymus with respect to progenitor migration and immigration?

integral role in self-renewal [82], has been implicated in the senescence and aging of HSCs [79,82], predominantly through its downstream targets, $p16^{ink4A}$ and $p19^{Arf}$, which are increased significantly with age (Table 1) [83,84], although it is possible that these are an adaptation towards cancer resistance [84,85]. Collectively, these changes result in a block in self-renewal and the promotion of cellular senescence [86]. The interaction between Ang-1 (expressed by the BM niche) and Tie-2 (expressed by HSCs) has been implicated in the maintenance of HSC quiescence within the niche [87]. In skeletal muscle, while there was no change in Ang-1 expression with age, there was a significant decrease in expression of its receptor Tie-2 [88], which, if translated to HSCs, could explain age-related declines in HSC quiescence [87].

Interestingly there is a significant age-related decline in the expression of the B-lineage transcription factors Pax-5 and E2A as well as the general lymphoid factor Ikaros [89,90]. While reduction in Ikaros expression is likely to impact on lymphoid lineage differentiation, other T lineage factors such as GATA-3, similar to Pax-5 and E2A, might underlie the reduced generation of T cells with age. Moreover, as Ikaros controls IL7Rα expression [5], the minimal impact of IL-7 administration on thymopoiesis could be explained by reduced signalling potential through the receptor (Box 2).

Defects in HSC function might arise through age-related changes to the niche. Microenvironmental defects in the BM have been linked to degeneration of HSC function with age [91,92] and the age of the BM microenvironment was found to be crucial for effective thymopoiesis, regardless of the degree of residual thymus tissue present [93]. Moreover, progressive shortening of telomeres by BM stromal cells leads to niche dysfunction with age, contributing to the myeloid-lineage bias with age [94]. Importantly a young microenvironment, and presumably more functional niches, is enough to, at least partially, restore age-related stem cell function [92,93,95,96].

As profound as these alterations are in the provision and function of BM progenitors, their role as the trigger for thymic involution remains speculative and, if anything, unlikely because the latter is more overt earlier in life. Furthermore, transferring young HSCs or ETPs alone does not induce regeneration of the aged atrophic thymus [46,51]. It is likely that chronologically, the decline in the structure and function of the individual thymic stromal microenvironments or niches related to the earliest TN stages are the primary cause. However, the exacerbation and prolongation of this thymic atrophy would appear to be markedly dependent on the subsequent loss in provision of adequately functional BM-derived HSCs and T-lineage progenitors.

Concluding remarks

The clinical impacts of age-related thymus involution are profound and represent a major therapeutic challenge. Understandably, much research has been levelled at thymus structure, development, maintenance and degeneration as a basis to developing strategies for its functional rejuvenation. However, since thymic function is intimately linked to a continual supply of relevant blood-borne progenitors, it would be logical to predict that age-related defects in the BM might have a major aetiological role in thymic degeneration, if not in its initiation, certainly in its severity and prolongation. As a corollary, improvement of BM function could provide an important catalyst for thymic rejuvenation. Indeed, it is now clear that thymus atrophy is a multifaceted process involving a confluence of events, including alterations in the BM niches, limited HSC function with respect to propensity for lymphoid lineages, decreased thymic importation of circulating progenitors and a dysregulated thymic microenvironment as a direct consequence of reduced T cell development. Ultimately, these collectively lead to reduced thymic export of naïve T cells, potentially even leading to the consequential reduction in the re-importation of circulating progenitors.

Clearly, a more rational approach to thymic regeneration requires more detailed investigations into the role of the BM niches in the induction of age-associated HSC defects. It will be important to reveal the feedback loops between the thymus and BM that allows for the periodic egress of progenitors from the BM and their uptake by the thymus and the dysregulation of these pathways leading towards age-related immune degeneration. Considering that at least some of these processes are reversed following surgical or chemical sex steroid ablation, it is important to understand the mechanism of action of sex steroids on the immune and hematopoietic systems as a first approach to delineating the complex molecular and cellular networks dictating lymphopoiesis.

Understanding the processes involved in thymic involution, in particular the crosstalk between developing thymocytes and their supporting stromal niches, is beginning to yield novel therapeutic strategies for thymic rejuvenation. It is now clear that consideration must be given to the cellular and molecular dialogue with the BM to provide a more strategic and efficient approach to thymus-based reconstitution of the immune system. The clinical applications for this include reducing morbidity and mortality in HSC transplant recipients following high-dose chemotherapy, which, in turn, allows for earlier re-immunisation against common pathogens and even potentially enhances the graft versus tumour phenomenon.

Acknowledgements
This work was supported by grants from the Australian National Health and Medical Research Council, the Australian Stem Cell Centre and Norwood Immunology. J.A.D. received support from a Cancer Council of Victoria Sydney Parker Smith postdoctoral fellowship.

References

1 Kiel, M.J. and Morrison, S.J. (2008) Uncertainty in the niches that maintain haematopoietic stem cells. *Nat. Rev. Immunol.* 8, 290–301
2 Wilson, A. and Trumpp, A. (2006) Bone-marrow haematopoietic-stem-cell niches. *Nat. Rev. Immunol.* 6, 93–106
3 Orford, K.W. and Scadden, D.T. (2008) Deconstructing stem cell self-renewal: genetic insights into cell-cycle regulation. *Nat. Rev. Genet.* 9, 115–128
4 Adolfsson, J. *et al.* (2005) Identification of Flt3+ lympho-myeloid stem cells lacking erythro-megakaryocytic potential a revised road map for adult blood lineage commitment. *Cell* 121, 295–306
5 Yoshida, T. *et al.* (2006) Early hematopoietic lineage restrictions directed by Ikaros. *Nat. Immunol.* 7, 382–391
6 Luc, S. *et al.* (2008) Down-regulation of Mpl marks the transition to lymphoid-primed multipotent progenitors with gradual loss of granulocyte-monocyte potential. *Blood* 111, 3424–3434
7 Papathanasiou, P. *et al.* (2009) Self-renewal of the long-term reconstituting subset of hematopoietic stem cells is regulated by Ikaros. *Stem Cells* 27, 3082–3092
8 Ng, SY-M. *et al.* (2009) Genome-wide lineage-specific transcriptional networks underscore Ikaros-dependent lymphoid priming in hematopoietic stem cells. *Immunity* 30, 493–507
9 Allman, D. *et al.* (2003) Thymopoiesis independent of common lymphoid progenitors. *Nat. Immunol.* 4, 168–174
10 Rothenberg, E.V. *et al.* (2008) Launching the T-cell-lineage developmental programme. *Nat. Rev. Immunol.* 8, 9–21
11 Iwasaki, H. *et al.* (2005) Distinctive and indispensable roles of PU.1 in maintenance of hematopoietic stem cells and their differentiation. *Blood* 106, 1590–1600
12 Takano, H. *et al.* (2004) Asymmetric division and lineage commitment at the level of hematopoietic stem cells: inference from differentiation in daughter cell and granddaughter cell pairs. *J. Exp. Med.* 199, 295–302
13 Lai, A.Y. and Kondo, M. (2007) Identification of a bone marrow precursor of the earliest thymocytes in adult mouse. *Proc. Natl. Acad. Sci. U. S. A.* 104, 6311–6316
14 Mansson, R. *et al.* (2007) Molecular evidence for hierarchical transcriptional lineage priming in fetal and adult stem cells and multipotent progenitors. *Immunity* 26 (4), 407–419
15 Pelayo, R. *et al.* (2006) Cell cycle quiescence of early lymphoid progenitors in adult bone marrow. *Stem Cells* 24, 2703–2713
16 Bhandoola, A. and Sambandam, A. (2006) From stem cell to T cell: one route or many? *Nat. Rev. Immunol.* 6, 117–126
17 Martin, C.H. *et al.* (2003) Efficient thymic immigration of B220+ lymphoid-restricted bone marrow cells with T precursor potential. *Nat. Immunol.* 4, 866–873
18 Serwold, T. *et al.* (2009) Reductive isolation from bone marrow and blood implicates common lymphoid progenitors as the major source of thymopoiesis. *Blood* 113, 807–815
19 Benz, C. *et al.* (2008) The stream of precursors that colonizes the thymus proceeds selectively through the early T lineage precursor stage of T cell development. *J. Exp. Med.* 205, 1187–1199
20 Maillard, I. *et al.* (2006) Notch-dependent T-lineage commitment occurs at extrathymic sites following bone marrow transplantation. *Blood* 107, 3511–3519
21 Krueger, A. *et al.* (2006) Phenotypic plasticity of T cell progenitors upon exposure to Notch ligands. *J. Exp. Med.* 203, 1977–1984
22 Perry, S.S. *et al.* (2006) Primitive lymphoid progenitors in bone marrow with T lineage reconstituting potential. *J. Immunol.* 177, 2880–2887
23 Bhandoola, A. *et al.* (2007) Commitment and developmental potential of extrathymic and intrathymic T cell precursors: plenty to choose from. *Immunity* 26, 678–689
24 Saran, N. *et al.* (2010) Multiple extrathymic precursors contribute to T-cell development with different kinetics. *Blood* 115, 1137–1144
25 Bell, J.J. and Bhandoola, A. (2008) The earliest thymic progenitors for T cells possess myeloid lineage potential. *Nature* 452, 764–767
26 Wada, H. *et al.* (2008) Adult T-cell progenitors retain myeloid potential. *Nature* 452, 768–772
27 Balciunaite, G. *et al.* (2005) The earliest subpopulation of mouse thymocytes contains potent T, significant macrophage, and natural killer cell but no B-lymphocyte potential. *Blood* 105, 1930–1936
28 Benz, C. and Bleul, C.C. (2005) A multipotent precursor in the thymus maps to the branching point of the T versus B lineage decision. *J. Exp. Med.* 202, 21–31
29 Krueger, A. and von Boehmer, H. (2007) Identification of a T lineage-committed progenitor in adult blood. *Immunity* 26, 105–116
30 Maillard, I. *et al.* (2005) Regulation of lymphoid development, differentiation, and function by the Notch pathway. *Annu. Rev. Immunol.* 23, 945–974
31 Rothenberg, E.V. and Scripture-Adams, D.D. (2008) Competition and collaboration: GATA-3, PU. 1, and Notch signaling in early T-cell fate determination. *Semin. Immunol.* 20, 236–246
32 Rossi, F.M. *et al.* (2005) Recruitment of adult thymic progenitors is regulated by P-selectin and its ligand PSGL-1. *Nat. Immunol.* 6, 626–634
33 Schwarz, B.A. *et al.* (2007) Selective thymus settling regulated by cytokine and chemokine receptors. *J. Immunol.* 178, 2008–2017
34 Zlotoff *et al.* (2010) CCR7 and CCR9 together recruit hematopoietic progenitors to the adult thymus. *Blood* 115, 1897–1905
35 Krueger *et al.* (2010) CC chemokine receptor 7 and 9 double-deficient hematopoietic progenitors are severely impaired in seeding the adult thymus. *Blood* 115, 1906–1912
36 Prockop, S.E. and Petrie, H.T. (2004) Regulation of thymus size by competition for stromal niches among early T cell progenitors. *J. Immunol.* 173, 1604–1611
37 Goldschneider, I. (2006) Cyclical mobilization and gated importation of thymocyte progenitors in the adult mouse: evidence for a thymus-bone marrow feedback loop. *Immunol. Rev.* 209, 58–75
38 Schnell, F.J. *et al.* (2006) Early growth response gene 1 provides negative feedback to inhibit entry of progenitor cells into the thymus. *J. Immunol.* 176, 4740–4747
39 Gossens, K. *et al.* (2009) Thymic progenitor homing and lymphocyte homeostasis are linked via S1P-controlled expression of thymic P-selectin/CCL25. *J. Exp. Med.* 206, 761–778
40 Chidgey, A. *et al.* (2007) Impact of niche aging on thymic regeneration and immune reconstitution. *Semin. Immunol.* 19, 331–340
41 Hale, J.S. *et al.* (2006) Thymic output in aged mice. *Proc. Natl. Acad. Sci. U. S. A.* 103, 8447–8452
42 Nikolich-Zugich, J. (2008) Ageing and life-long maintenance of T-cell subsets in the face of latent persistent infections. *Nat. Rev. Immunol.* 8, 512–522
43 Aspinall, R. (2006) T cell development, ageing and interleukin-7. *Mech. Ageing Dev.* 127, 572–578
44 Chu, Y.W. *et al.* (2004) Exogenous IL-7 increases recent thymic emigrants in peripheral lymphoid tissue without enhanced thymic function. *Blood* 104, 1110–1119
45 Holland, A.M. and van den Brink, M.R.M. (2009) Rejuvenation of the aging T cell compartment. *Curr. Opin. Immunol.* 21, 454–459
46 Mackall, C.L. and Gress, R.E. (1997) Thymic aging and T-cell regeneration. *Immunol. Rev.* 160, 91–102
47 Gui, J. *et al.* (2007) The aged thymus shows normal recruitment of lymphohematopoietic progenitors but has defects in thymic epithelial cells. *Int. Immunol.* 19, 1201–1211
48 Min, H. *et al.* (2004) Reduction in the developmental potential of intrathymic T cell progenitors with age. *J. Immunol.* 173, 245–250
49 Zediak, V.P. *et al.* (2007) Multiple prethymic defects underlie age-related loss of T progenitor competence. *Blood* 110, 1161–1167
50 Yu, S. *et al.* (1997) Thymocyte progenitors and T cell development in aging. *Mech. Ageing Dev.* 94, 103–111
51 Zhu, X. *et al.* (2007) Lymphohematopoietic progenitors do not have a synchronized defect with age-related thymic involution. *Aging Cell* 6, 663–672
52 Rossi, D.J. *et al.* (2005) Cell intrinsic alterations underlie hematopoietic stem cell aging. *Proc. Natl. Acad. Sci. U. S. A.* 102, 9194–9199
53 Kimura, T. *et al.* (2004) The sphingosine 1-phosphate receptor agonist FTY720 supports CXCR4-dependent migration and bone marrow homing of human CD34+ progenitor cells. *Blood* 103, 4478–4486

54 Massberg, S. *et al.* (2007) Immunosurveillance by hematopoietic progenitor cells trafficking through blood, lymph, and peripheral tissues. *Cell* 131, 994–1008
55 Williams, K.M. *et al.* (2008) CCL25 increases thymopoiesis after androgen withdrawal. *Blood* 112, 3255–3263
56 Sutherland, J.S. *et al.* (2005) Activation of thymic regeneration in mice and humans following androgen blockade. *J. Immunol.* 175, 2741–2753
57 Petrie, H.T. and Zuniga-Pflucker, J.C. (2007) Zoned out: functional mapping of stromal signaling microenvironments in the thymus. *Annu. Rev. Immunol.* 25, 649–679
58 Chen, L. *et al.* (2009) Foxn1 is required to maintain the postnatal thymic microenvironment in a dosage-sensitive manner. *Blood* 113, 567–574
59 Min, D. *et al.* (2007) Sustained thymopoiesis and improvement in functional immunity induced by exogenous KGF administration in murine models of aging. *Blood* 109, 2529–2537
60 Rossi, S.W. *et al.* (2007) Keratinocyte growth factor (KGF) enhances postnatal T-cell development via enhancements in proliferation and function of thymic epithelial cells. *Blood* 109, 3803–3811
61 Goldberg, G.L. *et al.* (2007) Clinical strategies to enhance T cell reconstitution. *Semin. Immunol.* 19, 289–296
62 de Haan, G. *et al.* (1997) Mouse strain-dependent changes in frequency and proliferation of hematopoietic stem cells during aging: correlation between lifespan and cycling activity. *Blood* 89, 1543–1550
63 Geiger, H. and Rudolph, K.L. (2009) Aging in the lympho-hematopoietic stem cell compartment. *Trends Immunol.* 30, 360–365
64 Geiger, H. *et al.* (2001) Age- and stage-specific regulation patterns in the hematopoietic stem cell hierarchy. *Blood* 98, 2966–2972
65 Pearce, D.J. *et al.* (2007) Age-dependent increase in side population distribution within hematopoiesis: implications for our understanding of the mechanism of aging. *Stem Cells* 25, 828–835
66 Liang, Y. *et al.* (2005) Effects of aging on the homing and engraftment of murine hematopoietic stem and progenitor cells. *Blood* 106, 1479–1487
67 Kamminga, L.M. *et al.* (2005) Impaired hematopoietic stem cell functioning after serial transplantation and during normal aging. *Stem Cells* 23, 82–92
68 Guerrettaz, L.M. *et al.* (2008) Acquired hematopoietic stem cell defects determine B-cell repertoire changes associated with aging. *Proc. Natl. Acad. Sci. U. S. A.* 105, 11898–11902
69 Signer, R.A.J. *et al.* (2007) Age-related defects in B lymphopoiesis underlie the myeloid dominance of adult leukemia. *Blood* 110, 1831–1839
70 Bell, D.R. and Van Zant, G. (2004) Stem cells, aging, and cancer: inevitabilities and outcomes. *Oncogene* 23, 7290–7296
71 Satoh, C. and Ogata, K. (2006) Hypothesis: myeloid-restricted hematopoietic stem cells with self-renewal capacity may be the transformation site in acute myeloid leukemia. *Leuk. Res.* 30, 491–495
72 Cho, R.H. *et al.* (2008) A new mechanism for the aging of hematopoietic stem cells: aging changes the clonal composition of the stem cell compartment but not individual stem cells. *Blood* 111, 5553–5561
73 Muller-Sieburg, C.E. *et al.* (2004) Myeloid-biased hematopoietic stem cells have extensive self-renewal capacity but generate diminished lymphoid progeny with impaired IL-7 responsiveness. *Blood* 103, 4111–4118
74 Yao, Y.G. *et al.* (2007) Age-dependent accumulation of mtDNA mutations in murine hematopoietic stem cells is modulated by the nuclear genetic background. *Hum. Mol. Genet.* 16, 286–294
75 Geiger, H. *et al.* (2005) Regulation of hematopoietic stem cell aging in vivo by a distinct genetic element. *Proc. Natl. Acad. Sci. U. S. A.* 102, 5102–5107
76 Dorshkind, K. *et al.* (2009) The ageing immune system: is it ever too old to become young again? *Nat. Rev. Immunol.* 9, 57–62
77 Fraga, M.F. (2009) Genetic and epigenetic regulation of aging. *Curr. Opin. Immunol.* 21, 446–453
78 Montecino-Rodriguez, E. and Dorshkind, K. (2006) Evolving patterns of lymphopoiesis from embryogenesis through senescence. *Immunity* 24, 659–662
79 Rossi, D.J. *et al.* (2007) Deficiencies in DNA damage repair limit the function of haematopoietic stem cells with age. *Nature* 447, 725–729
80 Nijnik, A. *et al.* (2007) DNA repair is limiting for haematopoietic stem cells during ageing. *Nature* 447, 686–690
81 Dumble, M. *et al.* (2007) The impact of altered p53 dosage on hematopoietic stem cell dynamics during aging. *Blood* 109, 1736–1742
82 Park, I.K. *et al.* (2003) Bmi-1 is required for maintenance of adult self-renewing haematopoietic stem cells. *Nature* 423, 302–305
83 Krishnamurthy, J. *et al.* (2004) Ink4a/Arf expression is a biomarker of aging. *J. Clin. Invest.* 114, 1299–1307
84 Signer, R.A. *et al.* (2008) Aging and cancer resistance in lymphoid progenitors are linked processes conferred by p16Ink4a and Arf. *Genes Dev.* 22, 3115–3120
85 Shanley, D.P. *et al.* (2009) An evolutionary perspective on the mechanisms of immunosenescence. *Trends Immunol.* 30, 374–381
86 Janzen, V. *et al.* (2006) Stem-cell ageing modified by the cyclin-dependent kinase inhibitor p16INK4a. *Nature* 443, 421–426
87 Arai, F. *et al.* (2004) Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. *Cell* 118, 149–161
88 Wagatsuma, A. (2006) Effect of aging on expression of angiogenesis-related factors in mouse skeletal muscle. *Exp. Gerontol.* 41, 49–54
89 Van der Put, E. *et al.* (2004) Decreased E47 in senescent B cell precursors is stage specific and regulated posttranslationally by protein turnover. *J. Immunol.* 173, 818–827
90 Riley, R.L. *et al.* (2005) Deficient B lymphopoiesis in murine senescence: potential roles for dysregulation of E2A, Pax-5, and STAT5. *Semin. Immunol.* 17, 330–336
91 Wagner, W. *et al.* (2008) Aging of hematopoietic stem cells is regulated by the stem cell niche. *Exp. Gerontol.* 43, 974–980
92 Mayack, S.R. *et al.* (2010) Systemic signals regulate ageing and rejuvenation of blood stem cell niches. *Nature* 463, 495–501
93 Donnini, A. *et al.* (2007) Intrinsic and microenvironmental defects are involved in the age-related changes of Lin–c-kit+ hematopoietic progenitor cells. *Rejuvenation Res.* 10, 459–472
94 Ju, Z. *et al.* (2007) Telomere dysfunction induces environmental alterations limiting hematopoietic stem cell function and engraftment. *Nat. Med.* 13, 742–747
95 Conboy, I.M. *et al.* (2005) Rejuvenation of aged progenitor cells by exposure to a young systemic environment. *Nature* 433, 760–764
96 Song, Z. *et al.* (2009) Cell intrinsic and extrinsic mechanisms of stem cell aging depend on telomere status. *Exp. Gerontol.* 44, 75–82
97 Goldberg, G.L. *et al.* (2009) Luteinizing hormone-releasing hormone enhances T cell recovery following allogeneic bone marrow transplantation. *J. Immunol.* 182, 5846–5854
98 Dudakov, J.A. *et al.* (2009) Sex steroid ablation enhances hematopoietic recovery following cytotoxic antineoplastic therapy in aged mice. *J. Immunol.* 183, 7084–7094
99 Alpdogan, O. *et al.* (2006) Keratinocyte growth factor (KGF) is required for postnatal thymic regeneration. *Blood* 107, 2453–2460
100 Zakrzewski, J.L. *et al.* (2006) Adoptive transfer of T-cell precursors enhances T-cell reconstitution after allogeneic hematopoietic stem cell transplantation. *Nat. Med.* 12, 1039–1047
101 Dudakov, J.A. *et al.* (2009) Withdrawal of sex steroids reverses age- and chemotherapy-related defects in bone marrow lymphopoiesis. *J. Immunol.* 182, 6247–6260
102 King, A.M. *et al.* (2007) Accelerated Notch-dependent degradation of E47 proteins in aged B cell precursors is associated with increased ERK MAPK activation. *J. Immunol.* 178, 3521–3529
103 Aw, D. *et al.* (2009) Phenotypical and morphological changes in the thymic microenvironment from ageing mice. *Biogerontology* 10, 311–322
104 Labrie, J.E., 3rd *et al.* (2005) Bone marrow microenvironmental changes in aged mice compromise V(D)J recombinase activity and B cell generation. *Semin. Immunol.* 17, 347–355
105 Wilson, A. *et al.* (2008) Hematopoietic stem cells reversibly switch from dormancy to self-renewal during homeostasis and repair. *Cell* 135, 1118–1129
106 Zuniga-Pflucker, J.C. and Schmitt, T.M. (2005) Unraveling the origin of lymphocyte progenitors. *Eur. J. Immunol.* 35, 2016–2018