

**The effect of Poly I:C induced inflammation on hematopoietic
stem and progenitor cell behaviour in the zebrafish
hematopoietic transplant model**

Marie McBrien

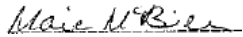
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Declaration of originality

I declare that all work presented in this thesis is my own, and that all material, including ideas, information, data, results and figures from another sources have been appropriately referenced or acknowledged.



Marie McBrien

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Abstract

Hematopoietic stem cells are a small but significant population of cells fundamental for generating and maintaining the hematopoietic system. These cells are used in the treatment of cancer and auto-immune patients. Studies in mammals suggest that inflammation and infection can modulate the biology of these cells, affecting their location, self-renewal capacity and directing differentiation. The aim of this work was to study the effect of repeated stimulation on the hematopoietic stem and progenitor (HSPCs) population in zebrafish (*Danio rerio*) to benefit from the live imaging potential of this model organism. It was hypothesised that early post-transplant HSPC behaviour (e.g. lodgement in the niche, self-renewal, mobilisation and differentiation) could be observed and would be indicative of the success or failure of HCT. Double transgenic Tg (*cd41:GFP*; *lysc:dsRed*) donors, in which HSPCs express green fluorescent protein (GFP+) and myeloid cells express red fluorescent protein (dsRed+) were used. HSPCs were sorted from donor whole kidney marrow (WKM) and transplanted into irradiated optically-transparent recipients, which were then imaged using wide-field microscopy, individually tracked for survival and hematopoietic reconstitution was assessed after 28 days by flow cytometry. Indeed, initial experiments showed that early observations of cells in the WKM correlated with hematopoietic recovery and survival of recipients, although the strength of the correlation was not sufficiently powerful for predicting recipient outcome.

This refinement of the HCT protocol lead to the potentiality of studying the behaviour of HSPCs in the context of inflammation. Inflammation was initiated with repeated intra-peritoneal injections of the viral mimic Polyinosinic:polycytidylic acid (poly I:C) in either the donor or recipient prior to transplant. Poly I:C injection of donors prior to transplant causes HSPCs to colonise the recipient WKM at a greater rate than HSPCs from sham (PBS-injected) donors. However, this did not appear to affect recipient survival or WKM reconstitution at 28 days. Poly I:C injection of recipients prior to transplant did not affect early post-transplant repopulation of the WKM, myelopoiesis, recipient survival, or WKM reconstitution at 28 days. Future work will use competitive transplants to confirm these findings and will explore alternative inflammation models. Furthermore, the confounding factor of irradiation-caused inflammation will be mitigated by transplanting HSPCs into optically-transparent *bloodless* recipients. Overall, this thesis has demonstrated that the zebrafish can provide valuable *in vivo* data for studying HSPC behaviour in the recipient post-transplant.

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List of abbreviations

5-FU	Fluorouracil
ABCG2	ABC transporter G family member 2
AGM	Aorta Gonad Mesonephros
Akt	Protein kinase B
Ang1	Angiopoietin
ANOVA	ANalysis Of VAriance
AP-1	Activator Protein 1
APC	Antigen Presenting Cells
BM	Bone Marrow
BMI-1	B cell-specific Moloney murine leukemia virus Integration site 1
BMP	Bone Morphogenic Protein
BMPR1A	Bone Morphogenic Protein Receptor type 1a mice
bp	base pair
BrdU	BromodeoxyUridine
c-CBL	Casitas B-lineage Lymphoma
CAFC	Cobblestone Area-Forming Cell
CAM	Cell Adhesion Molecule
CAR	CXCL12-Abundant Reticular
CaR	Calcium Receptor
CBFa1	Core Binding Factor alpha, also known as runx2
CBF β	Core Binding Factor beta
CD	Cluster of Differentiation
cDNA	copy DeoxyriboNucleic Acid
CDR	Complementarity Determining Regions
CFC	Colony Forming Cell
CFSE	CarboxyFluorescein Succinimidyl Ester
CFU-S	Colony Forming Unit-Spleen
CHT	Caudal Hematopoietic Tissue
cKit	stem cell factor receptor, also known as CD117
CLP	Common Lymphoid Progenitors
CML	Chronic Myelogenous Leukemia
CMP	Common Myeloid Progenitors
CSF1Ra	Colony Stimulating Factor 1 Receptor a; also known as CD115 or fms, macrophage colony stimulating factor receptor
CT	Cycle threshold
CXCL12	CXC motif chemokine ligand 12; also known as stromal cell derived factor-1
CXCR4	CXC receptor 4, also known as CD184
DAMPs	Damage-Associated Molecular Patterns
DAPI	4',6-diamidino-2-phenylindole
DiD	Dialkylcarbocyanine dye
DiI	Dialkylcarbocyanine dye, octadecyl (C18) indocarbocyanines
DNA	DeoxyriboNucleic Acid
dNTPs	deoxy-Nucleotide TriPhosphate
dpf	days post fertilization

dpIR	days post Ionising Radiation
dpt	days post transplant
dsRed	<i>Discosoma</i> red fluorescent protein
dsRNA	double stranded RiboNucleic Acid
E2	Embryo growing medium
Egr1	Early growth response protein 1
EHT	Endothelial-Hematopoietic Transition
EMP	Erythroid-Myeloid Progenitor
ETS	E26 transformation specific
FACS	Flourescence Acitvated Cell Sorting
FADD	FAS-associated Death Domain-containing protein
FDCP	Factor dependent Paterson mix, progenitor cell line
FSC	Forward scatter
G-CSF	Granulocyte Colony-Stimulating Factor
Gata1	GATA-binding factor 1
Gata2	GATA-binding protein 2
GFP	Green Fluorescent Protein
GM-CSF	Granulocyte Macrophage Colony-Stimulating Factor
GMP	Granulocyte-Monocyte restricted Progenitor
GvH	Graft versus Host
Gy	Grays
H2B	Histone 2B
HA	Hyaluronic Acid
HCT	Hematopoietic Cell Transplantation
HLA-DR	Human Leukocyte Antigen - D Related
hpt	hours post transplant
HSC	Hematopoietic Stem Cell
HSPC	Hematopoietic stem and progenitor cell
ICM	intermediate cell mass
Id1	DNA-binding Inhibitor protein 1
IFN	Interferon
IFNAR	Interferon alpha receptor
IFNGR	Interferon gamma receptor
Ig	Immunoglobulin
ikbaa	NfkB-Inhibitor alpha
IKK	Inhibitor of kappa-B Kinase
IL	Interleukin
IPS-1	IFN-beta Promoter Stimulator 1
IR	Ionising Radiation
IRF	Interferon Regulatory Factor
Jag	Jagged
JAK	Janus kinase
Kdrl	Vascular endothelial growth factor receptor kdr-like
L-plastin	Leukocyte specific actin binding protein
Lck	Lymphocyte-specific-protein-tyrosine-Kinase
LCMV	Lymphocytic ChorioMeningitis Virus

Lepr	Leptin Receptor
Lin	Lineage
Lmo2	Lim domain only
LMPP	Lymphoid-primed multi-potent progenitor
LPS	LipoPolySaccharide
LSK	Lin- Sca1+ cKit+; lineage negative, stem cell antigen positive, stem cell factor receptor positive
LT-HSC	Long Term Hematopoietic Stem Cell
LTC-IC	Long-Term Culture-Initiating Cell
LTR	Long Term Repopulating
lyz	lyzosome C
M1	classically activated pro-inflammatory macrophage
M2	alternatively activated anti-inflammatory macrophage
MAFIA	MAcrophage FAS-Induced Apoptosis
MDA5	Melanoma Differentiation-Associated gene 5
MEP	Megakaryocyte-Erythrocyte Progenitor
mFAP4	microFibrillar-Associated Protein 4
MHC	Major Histocompatibility Complexes
miR	microRNA
MMP	Matrix Metallo-Proteinase
MPEG1	Macrophage expressed 1
mpf	months post fertilisation
MPO	Myeloperoxidase
MPP	Multi-Potent Progenitor
mRNA	messenger Ribonucleic Acid
MSC	Mesenchymal Stem Cells
mTOR	mammalian Target Of Rapamycin
MyD88	Myeloid Differentiation primary response gene 88
N-cad	N-cadherin
NCID	Notch Intracellular Domain
Nes	Nestin
NfKB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NICD1a	Notch1a Intracellular Domain 1a
NK	Natural killer
NLS	Nuclear Localisation Signal
NOD/SCID	Non-Obese Diabetic/Severe Combined Immuno-Deficient
OPN	Osteopontin
PAMP	Pathogen-Associated Molecular Patterns
PB	Peripheral Blood
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PIGF	Placental Growth Factor
Poly I:C	Polyinosinic:polycytidylic acid
PPR	Parathyroid Hormone Receptor
PRR	Pattern Recognition Receptors
PTH	Parathyroid Hormone

Pu.1	PU box, also known as Spi1
qRT-PCR	Quantitative Real-time Polymerase Chain Reaction
rag	recombination activating gene
Rb	Retinoblastoma
RBI	Rostral Blood Island
Rho	Rhodimine 123
RLHs	Retinoic acid-Inducible Gene 1 (RIG-I)-Like Helicases
ROS	Reactive Oxygen Species
runx1	Runt-related transcription factor 1
S1P	Sphingosine-1-phosphate
S1PR	Sphingosine-1-phosphate receptor
Sca1	Stem cell antigen 1
SCF	Stem Cell Factor
Scl	Stem Cell Leukemia
SD	Standard Deviation
SEC	Sinusoidal Endothelial Cell
SLAM	Signalling Lymphocytic Activation Molecule
SNO	Spindle-shaped N-cadherin Osteoblast
SNP	Single Nucleotide Polymorphism
SP	Side Population
SSC	Side Scatter
ST-HSC	Short-Term Hematopoietic Stem Cell
STAT	Signal Transducer and Activator of Transcription
TAK1	TGF-beta Activated Kinase
TBK1	TANK-Binding Kinase 1
TCR	T-Cell Receptor
TEP	Thrombocyte-Erythrocyte Progenitor
Tg	Transgenic
TGF	Transforming Growth Factor
TLR	Toll Like Receptor
TNF	Tumour Necrosis Factor
TPO	Thrombopoietin
TRAF3	TNF-Receptor Associated Factor 3
TRIF	TIR-domain-containing Adapter-Inducing Interferon-beta
TRITC	Tetramethylrhodamine
Ubi	Ubiquitous
VCAM	Vascular Cell Adhesion Protein
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelium Growth Factor Receptor
VLA4	Very Late Antigen 4, also known as beta integrin
WKM	Whole Kidney Marrow
wpf	weeks post fertilization
WT	Wild type

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1 Introduction

1.1 Hematopoietic stem cells

Hematopoietic cell transplantation (HCT) has been used clinically since 1959 in the treatment of patients with cancers (leukemias, lymphomas), autoimmune diseases, or bone-marrow syndromes (such as aplastic anemia and thalassemia sickle cell anemia), with about 2,000 people in the UK in need of one every year (1). Hematopoietic stem cells (HSCs) are the precursor cells of all mature blood cells throughout vertebrate life and are an essential component of the transplanted population as they ensure long term reconstitution of the hematopoietic system. HSCs are rare, multi-potent, and self-renewing cells that maintain homeostasis by differentiating to replace hematopoietic cells. They were first discovered by Till and McCulloch in 1961 and a comprehensive stem cell theory was published with Becker in 1963 (2). HSCs are often studied as part of a larger population of hematopoietic stem and progenitor cells (HSPCs).

1.2 HSC phenotype

Functionally, HSCs are identified by their ability to repopulate irradiated recipients. Their cell-surface phenotype is determined by antibody binding and pre-transplant isolation by fluorescence-activated cell sorting (FACS). Some markers are used to positively select for stem cell associated proteins (see Table 1.1). Other markers are used to select against differentiated cells, referred to as lineage negative (lin⁻), using a complex combination of markers, thereby by excluding mature populations (lymphoid, myeloid and erythroid). Importantly, the markers used to isolate HSCs will depend on the organism (human, mouse, zebrafish) and the stage of development (foetal, adult).

1.2.1 Purification of HSCs from mammals

The most frequently used marker used for isolating HSCs from human peripheral blood in clinic is mucosialin (hereafter referred to as cluster of differentiation (CD) 34), a transmembrane protein whose function is still unclear (3). More primitive HSCs can be isolated by the lack of expression of ADP-ribosyl-cyclase 1 (hereafter CD38) and the major histocompatibility class II antigen Human Leukocyte Antigen D Related (HLA-DR) expression (4, 5). Hénon *et al.* reported better post-transplant engraftment and hematopoietic recovery with CD34⁺ CD38⁻ cells than total CD34⁺ cells (6). Later Drake *et al.* found even better engraftment and hematopoietic reconstitution with CD34⁺ CD38⁻ HLA-DR⁻ cells compared with CD34⁺ CD38⁻ cells (7). About 70% of human CD34⁺ CD38⁻ HSCs express the thrombopoietin receptor (also known as c-mpl, hereafter referred to as CD110) and these cells (CD34⁺CD38⁻ CD110⁺) show better engraftment than CD34⁺CD38⁻ CD110⁻ (8).

Marker	Mouse	Human	Zebrafish
CD34/Mucosialin	<i>Negative</i> LT-HSCs, <i>Positive</i> ST-HSCs (9)	<i>Positive</i> (10)	
CD38/ADP-ribosyl-cyclase 1	<i>Positive</i> mid-fetal to adult (11)	<i>Positive</i> (4)	
CD105/Endoglin	<i>Positive</i> (12)	<i>Positive</i> (13)	
c-Kit/CD117	<i>Positive</i> (14, 15)		
Sca-1	<i>Positive</i> (16)		
CD150/SLAM	<i>Positive</i> (17)		
Tie2/CD202	<i>Positive</i> (18)		
Mpl/CD110	<i>Positive</i> (8)	<i>Positive</i> mid-fetal to adult (19)	
CD90/Thy1		<i>Positive</i> (20, 21)	
Hoescht-SP	<i>Present</i> (22)	<i>Present</i> (23)	<i>Present</i> (24)
CD133/Prominin-1		<i>Positive</i> (25)	
VEGFR2/FLK1/CD309		<i>Positive</i> (26)	
Flt3/Flk2	<i>Negative</i> (27)		
CD41/alpha integrin	<i>Positive</i> emerging HSCs, myeloid biased adult HSCs (28)		<i>Positive</i> Nascent and adult HSCs and thrombocytes (29)
Runx1	<i>Positive</i> emerging HSCs (30)		<i>Positive</i> emerging HSCs (31)
C-myb			<i>Positive</i> emerging HSCs (32)

Table 1.1: A list of HSC markers (proteins/genes/dyes) and their expression in mouse, human and zebrafish. Long term HSCs (LT-HSCs) refers to a quiescent population of stem cells and Short term HSCs (ST-HSCs) refers to a population of stem cells which undergoes cell cycling more frequently (1.2.4).

Other proteins expressed by stem cells and immature progenitor cells include thymocyte antigen 1 (thy1, hereafter CD90), a glycoposphatidylinositol cell surface protein with an immune-globulin like structure (20, 21); prominin-1 (CD133), a transmembrane glycoprotein which may be involved in organization of the cell membrane (25); and vascular endothelial growth factor receptor 2 (VEGFR2; also known as KDR, Flk1 or CD309), involved in vascular endothelial growth factor (VEGF) signalling (26, 33). The family of *signal-regulatory proteins* have also been reported as candidate stem cell proteins expressed by CD34⁺ CD90⁺ CD133⁺ cells (34).

In contrast to human HSCs, mouse HSCs are CD34^{-/low} (Table 1.1). In the mouse, HSCs are typically isolated from the lineage negative (Lin⁻), stem cell antigen 1 (Sca1) (16) positive and stem cell factor receptor (hereafter c-kit; also known as CD117) positive subset of the bone marrow (BM): referred to as Lin⁻ Sca1⁺ Kit⁺ and hereafter abbreviated to LSK. Although consistently used in isolating HSCs, a study suggested that some pluripotent stem cells have low c-kit expression and are therefore omitted when using these selection parameters (15). In addition, the expression of Sca1 (Ly-6 A/E) varies between mouse strains (35). The LSK subset makes up between 0.1 and 0.01 % of total BM cell population and just 10 % of LSK cells confer long-term reconstitution. In C57BL/6 mice, the CD34⁻ LSK subset makes up about 0.005 % of total BM cells(36).

Subsequently, the expression of foetal liver kinase 2 (also known as fms-like tyrosine kinase 3; hereafter CD135), a tyrosine kinase receptor, was found to distinguish lymphoid bias progenitors from multi-potent HSCs (27, 37, 38). Therefore, the signature LSK CD34⁻ CD135^{low/-} is also used to define HSCs. Just like in the human HSC population (CD34⁺ CD38⁻), the thrombopoietin receptor CD110 is expressed on 70 % of mouse LSK HSCs and used to segregate mouse HSCs. These cells show better engraftment than LSK CD34⁻ CD110⁻ (8).

The most recent method to purify mice HSCs employ differential expression of signalling lymphocytic activation molecule (SLAM) receptors 1 and 2, hereafter referred to as CD150 and CD48 respectively (17). HSCs highly express CD150, but not CD48, and are thus enriched in the LSK CD150⁺ CD48⁻ population. This subset of LSK cells makes up less than 0.01 % of total BM cells but 47 % of purified single-cells reconstitution experiments had long-term reconstitution. Most LSK CD34⁻ cells are CD150⁺ and CD135⁻, therefore these populations are largely overlapping (39). One in 10⁵ BM cells are LSK CD34⁻ CD150⁺ CD135⁻, and a third (1 in 3) of these cells are functionally able to reconstitute the immune system long-term.

1.2.2 Studying HSCs in the zebrafish

Unfortunately, while antibodies are used for the isolation of mammalian cells, as of yet there are no antibodies available to isolate HSCs from zebrafish samples (40). However, this has not prevented the use of the zebrafish model to study the regulation of hematopoiesis over the last 20 years. Zebrafish, *Danio rerio*, have several advantageous features, including optical transparency which allows *in vivo* visualisation of real time events, and the possibility for genetic manipulation which allows control of gene expression and the creation of transgenic lines. Most notably, zebrafish have been instrumental in mapping the origins of HSCs with fate mapping (41), real-time tracking (42) as well as for elucidating signal pathways important in HSC generation (43, 44).

1.2.2.1 Isolating HSCs from teleost kidney

The zebrafish kidney (referred to as the whole kidney marrow, WKM) is the primary site of hematopoietic stem cell maintenance and thus the functional equivalent of the mammalian bone marrow. Based on limiting dilution assays, it is estimated the WKM contains between 1 in 65, 500 and 1 in 38, 140 cells with long-term reconstitution capacity (45, 46). Traver *et al.* first described HCT in zebrafish and showed that the kidney marrow contains repopulating hematopoietic cells. By separating the kidney marrow population by size and granularity using flow cytometry forward and side scatter, Traver *et al.* showed that cells with reconstitution ability are among a population of lymphoid cells with low forward and side scatter (47, 48). However, less than 2 % (2 of 110) of recipients transplanted with cells of the lymphoid fraction had long-term reconstitution, as assessed by production of donor derived erythrocytes 6 months after transplantation (48).

Subsequently, transgenic zebrafish lines were used to isolate HSC enriched populations based on fluorescent protein expression, driven by HSC related genes (Table 1.1) (49, 50). One of these transgenic lines is the Tg (cd41:GFP) line, in which the green fluorescent protein (GFP) expressing population has been shown to contain a pluripotent population capable of reconstituting all cells of the immune system in irradiated fish (51). In these fish, GFP expression is driven by the thrombocyte specific α_{IIb} integrin (hereafter referred to as CD41) promoter and labels thrombocytes (GFP^{high}) and HSPCs (GFP^{low}). CD41 is expressed by definitive HSCs in zebrafish from larvae to adulthood (51). CD41 only transiently marks foetal HSCs in mice (28, 52). Images of embryonic mouse slices indicate that CD41 is an important modulator of aortic generated HSCs and CD41 localises at the point of contact between cells in intra-aortic hematopoietic clusters (53, 54). Ma *et al.* showed that the reconstituting potential of kidney cells was primarily in cd41^{low} population. These cells make up 0.8% of WKM cells, with between 5 000 and 10 000 in a 6 months post fertilisation (mpf) adult. Limiting dilution assays suggest HSC frequency is 1 in 100 cd41^{low} cells (51). Recently, the *Zebrabrow* system was used to induce a unique heritable colour to each nascent cd41:GFP+ cells. Transplantation of these labelled clones confirmed that the majority of cells in this population are progenitors with a balanced myeloid/lymphoid output and capable of contributing up to 95 % of the recipient marrow, suggesting this population is multi-potent (55). In this thesis a number of fish lines were exploited (see Table 2.1). Importantly, the Tg (cd41:GFP) zebrafish line was crossed with Tg (lyz:dsRed) fish in which myeloid cells express dsRed fluorescent protein (56). This meant that donor derived myeloid cells could be distinguished from endogenous host myeloid cells.

More recently, Tg (runx1+23:GFP) and Tg (runx1+23:NLSmCherry) lines were created by Tamplin *et al.* for the study of HSC migration and seeding of HSCs in the endogenous embryonic kidney (42). Tamplin *et al.* created a transgenic in which fluorescent protein expression was under the control of a regulatory element from the first intron of the mouse runt-related transcription factor 1 (hereafter referred to as runx1; also known as acute myeloid leukemia 1 protein or core-binding factor subunit α -2) locus (42). The enhancer is an important cis-regulatory element which involves runx1 and important regulators of hematopoiesis: GATA-binding protein 2 (Gata2), E26 transformation specific (ETS) and stem cell leukemia (SCL) to drive runx1 expression and HSC emergence (57). Runx1 is a transcription factor required for definitive hematopoiesis and formation of intra-

aortic hematopoietic clusters (30). The insertion of the mouse regulatory element created a transgenic line in which the fluorescent protein was expressed in adult zebrafish HSPCs. This regulatory element is a 531 bp sequence in the 1st intron of *Runx1*, 23 kb downstream from the P1 promoter. The enhancer regulates early expression of *Runx1* in definitive hematopoietic cells in the mouse embryos (57). The nuclear localisation sequence (NLS) in the Tg (runx1+23:NLSmCherry) line results in a larger labelled population than in the Tg (runx1+23:GFP) line. In double transgenic fish Tg (runx1+23:NLSmCherry; cmyb:GFP), the population of runx1+23:NLSmCherry+ cells appears to overlap significantly with cmyb:GFP+ cells (58). Similarly, in double transgenic Tg (runx1+23:NLSmCherry; cd41:GFP) fish, the population of runx1+23:NLSmCherry+ cells appears to overlap significantly with cd41:GFP+ cells. However, it is not known how the double positive cells (runx1+ cd41+) differ from single positive cells (runx1+ cd41- and runx1- cd41+) in their gene expression, their engraftment potential or their reconstitution potential. Perhaps the expression of these genes reflect their degree of pluripotency or differentiation biases of these cells. Runx1:GFP+ cells make up 0.4 % of WKM cells, with between 1,000 and 3,000 in a 6 mpf adult. The HSC frequency is estimated at 1 in 35 runx1:GFP+ cells (42).

Lineage tracing of HSCs using the Cre/ loxP system has also been developed in the zebrafish. Bertrand *et al.* created the Tg (kdrl:Cre; β -actin2:loxP-STOP-loxP:dsRed) line in which the vascular cells (kdrl+) express Cre. Cre recombinase excises the LoxP sites which restrict dsRed expression from the β -actin promoter. Therefore, there is a permanent switch from non-fluorescent to dsRed+ those cells and in the progeny of those cells. With this, they were able to show that almost all blood cells were derived from hemogenic endothelium (41). Kobayashi *et al.* (59) and Butko *et al.* (60) found similar results with Tg (jam1a:CreERT2; β -actin2:loxP-STOP-loxP:dsRed) and Tg (gata2b:Gal4; UAS:Cre; β -actin:LoxP-STOP-LoxP-dsRed) lines respectively. In the former, (jam1a:CreERT2; β -actin2:loxP-STOP-loxP:dsRed), HSPC precursors express jam1a which drives expression of Cre to excise the LoxP sites preventing ubiquitous (β -actin driven) expression of dsRed. Thus enabling as before the lineage tracing of jam1a+ cells. Jam1 and Jam3 are reliable markers of mouse HSCs (61, 62) and play a role in cell adhesion to the BM niche. In the latter, Tg (gata2b:Gal4; UAS:Cre; β -actin:LoxP-STOP-LoxP-dsRed), gata2b expression by forming HSPCs, drives expression of Gal4, which in turn drives UAS promoters to express Cre, which excise the LoxP cassette inhibiting dsRed expression. Thus, they could show that gata2b expressing cells contribute to adult hematopoiesis. Gata2 is expressed by all mouse HSCs and plays an important role in the emergence of these cells in the embryo (63).

1.2.3 Other selection parameters

Less commonly used methods of HSC enrichment include isolation of a side-population (SP), into which HSCs fall because of their unique ability to efflux the DNA-binding dye Hoechst 33342 (22). The efflux of Hoechst is dependent on ABC transporter G family member 2 (ABCG2). Transplantation of a single cell with strong dye efflux activity could engraft long term in irradiated mice recipients (64). However, Morita *et al.* have shown long-term repopulating activity in about 50 % of non-SP cells (65). In zebrafish, the SP fraction of the kidney (24, 66, 67) was found to highly express HSC-related genes including *Scl*, LIM domain only 2 (*lmo2*), and transcription factor *c-myb* (24). Comparative microarray analysis of zebrafish lymphoid SP fraction with mouse and human HSCs

found they have a similar molecular signature, including expression of *gata2*, DNA-binding inhibitor protein 1 (*Id1*) and early growth response protein 1 (*Egr1*) transcription factors (67). In addition, 22 % of the SP population overlapped with zebrafish HSPCs (*cd41^{low}*). This suggests HSCs are found in both SP and non-SP populations.

Other characteristics of mouse HSCs used for isolation include low staining with the mitochondrial dye Rhodamine 123 (Rho), expression of angiopoietin receptor (also known as receptor tyrosine kinase 2, hereafter referred to as CD202b), and expression of Transforming growth factor beta (TGF β) receptor, endoglin/CD105 (68-70). The Rho efflux activity of HSCs is attributed to another ABC transporter, P-glyco- protein (71). Although the Rho-SP has been identified in ginbuna carp, it has not been studied in zebrafish and the population has not been tested in transplantation assays.

1.2.4 Long and short term HSCs

The mouse LSK population is functionally a heterogeneous mix of HSPCs. In the classic paradigm (Figure 1.1), the differentiation potential of a cell is restricted as it transitions from long term (LT-HSC), to short term (ST-HSC), to multi-potent progenitor (MPP). The LT-HSC and ST-HSC populations are both self-renewing multipotent populations but they differ in cell cycle dynamics. The LT-HSC population has a low replication cycle which reduces the risk of incorporating harmful genetic mutations during DNA replication. A slow replicative cycle is also important for retaining self-renewing potential as rapidly cycling ST-HSCs have lower reconstitution ability (72, 73). Similarly, LT-HSCs can become functionally exhausted by increased cell cycling activity or by serial transplantation (74). In mice, CD34 is not expressed by LT-HSCs but is expressed on ST-HSCs (Table 1.1). MPPs have high cycling kinetics and express CD34, CD135(39) and CD224, a member of the SLAM family (17) (Figure 1.1).

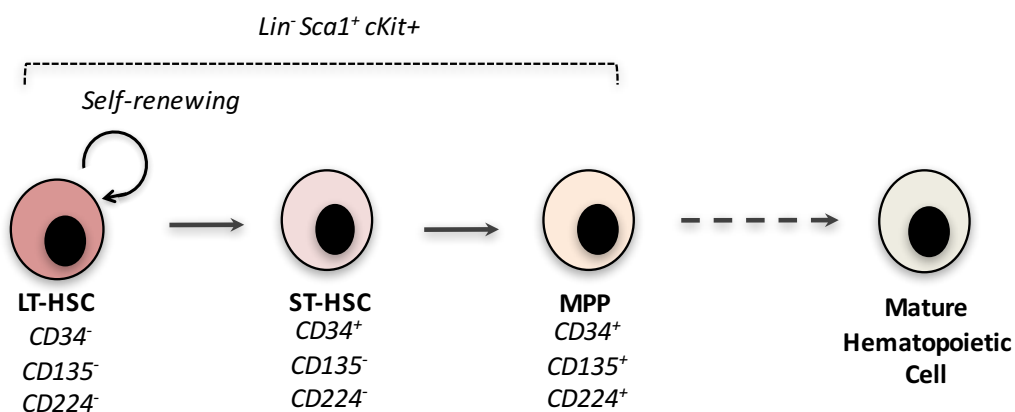


Figure 1.1: Subsets of the mouse hematopoietic stem and progenitor cell (HSPC) population. The immature HSPC population can be separated based on protein expression and cell cycle dynamics, which correlate with post transplantation reconstitution ability.

Label-retaining assays with bromodeoxyuridine (BrdU) or Histone 2B-GFP (H2B-GFP) dilution have been used to study the different cell cycle kinetics of the LSK population (72, 73). BrdU is a thymidine analogue that incorporates into the DNA while present, and then is lost when cells undergo mitosis in the absence of BrdU. However, there are doubts about the accuracy of the BrdU assay. For instance, Kiel *et al.* found that very few of BrdU labelled cells in neonatal mice samples expressed HSC markers (75). H2B-GFP labelling is done by doxycycline-induced expression of the histone protein 2B gene fused with GFP in all cells. When doxycycline is removed, the transcription of the H2B-GFP fusion gene is no longer induced and the production of GFP is stopped. As histone 2B is required for DNA hyperstructure, the H2B-GFP protein is gradually lost in replicating cells which produce H2B on demand. Foudi *et al.* found that transplanted HSCs (LSK CD150⁺ CD48⁻) with higher H2B-GFP expression had higher long-term reconstitution potential (72).

The ST-HSC subset (LSK CD34⁺ CD150⁺ CD48⁻ CD135⁻) is larger than the LT-HSC subset with about ~85% of LSK CD150⁺ CD48⁻ cells dividing within 4 weeks (as assessed by loss of BrdU or GFP; (72, 73). Cells in this population that have divided more than 5 times show significantly lower repopulating ability (72, 73). The smaller subset of LT-HSC (LSK CD34⁻ CD150⁺ CD48⁻ CD135⁻) contains infrequently dividing cells, also known as dormant or quiescent HSCs, and these cells divide about every 145 days (~5% retain H2B label after 72 weeks chase(72)). LT-HSCs do not respond to irradiation but can be activated by cytokines such as interferon- α (IFN- α) and granulocyte colony-stimulating factor (G-CSF; (73, 76). Dormant HSCs are also activated to proliferate following treatment with chemotherapeutic drug 5-Fluorouracil (5-FU), a drug used to kill proliferative cells.

Although quiescence seems to be a stem cell requirement, embryonic stem cells are highly proliferative and yet retain their *stemness*. It is not yet known if the cell cycling dynamics of an individual cell are consistent throughout its life; does a ST-HSC, characterised by its rapid loss of label (BrdU or GFP) continue rapid cell cycling, or can it switch to a quiescent state, characteristic of LT-HSCs. If so, the cell cycle behaviour could simple distinguish replicating clones from non-replicating clones, rather than determine the stem cell potential of that clone. Some evidence suggest that quiescent HSCs (Lin⁻ Sca1⁺ Thy^{low}) are better at engrafting when transplanted (77).

Importantly, similar cell cycling dynamics have not yet been demonstrated in the HSPC populations of the zebrafish. Perhaps identifying infrequently dividing cells in the HSPC population would permit the identification of other genes for isolating HSCs in the zebrafish and progress the use of zebrafish in the field. Currently, the use of zebrafish is limited by the lack of antibodies and imperfect transgenic lines (40).

1.2.5 HSC output bias

In the classic model of hematopoietic differentiation, mature blood cells are derived in a step-wise progression from a pluripotent stem cell through a tree-like hierarchy to a definitive progenitor cell (Figure 1.2).

In the current mammalian model of hematopoiesis, HSCs differentiate into MPPs and subsequently into oligo-potent progenitors, common myeloid progenitors (CMPs) and lymphoid-primed multi-potent progenitors (LMPPs). Recent mathematical models combining flow cytometry, transcriptomics and lineage fate data have challenged this simplistic *binary model*. For instance, Moignard *et al.* have used single cell expression profiling and powerful bioinformatics to identify the network of transcription factors controlling stem cell fate choices (78). As a result, the hematopoietic tree model with distinct branches has been reinterpreted as a *developmental continuum* with gradual separations that are a lot less distinct (79, 80). This model suggests a greater heterogeneity in the stem population than the former model. In addition, single cell sequencing evidence suggests HSCs can commit directly to more definitive stages and cells bypass conventional checkpoints (81). This model suggests a process by which a rapid response to demand is possible.

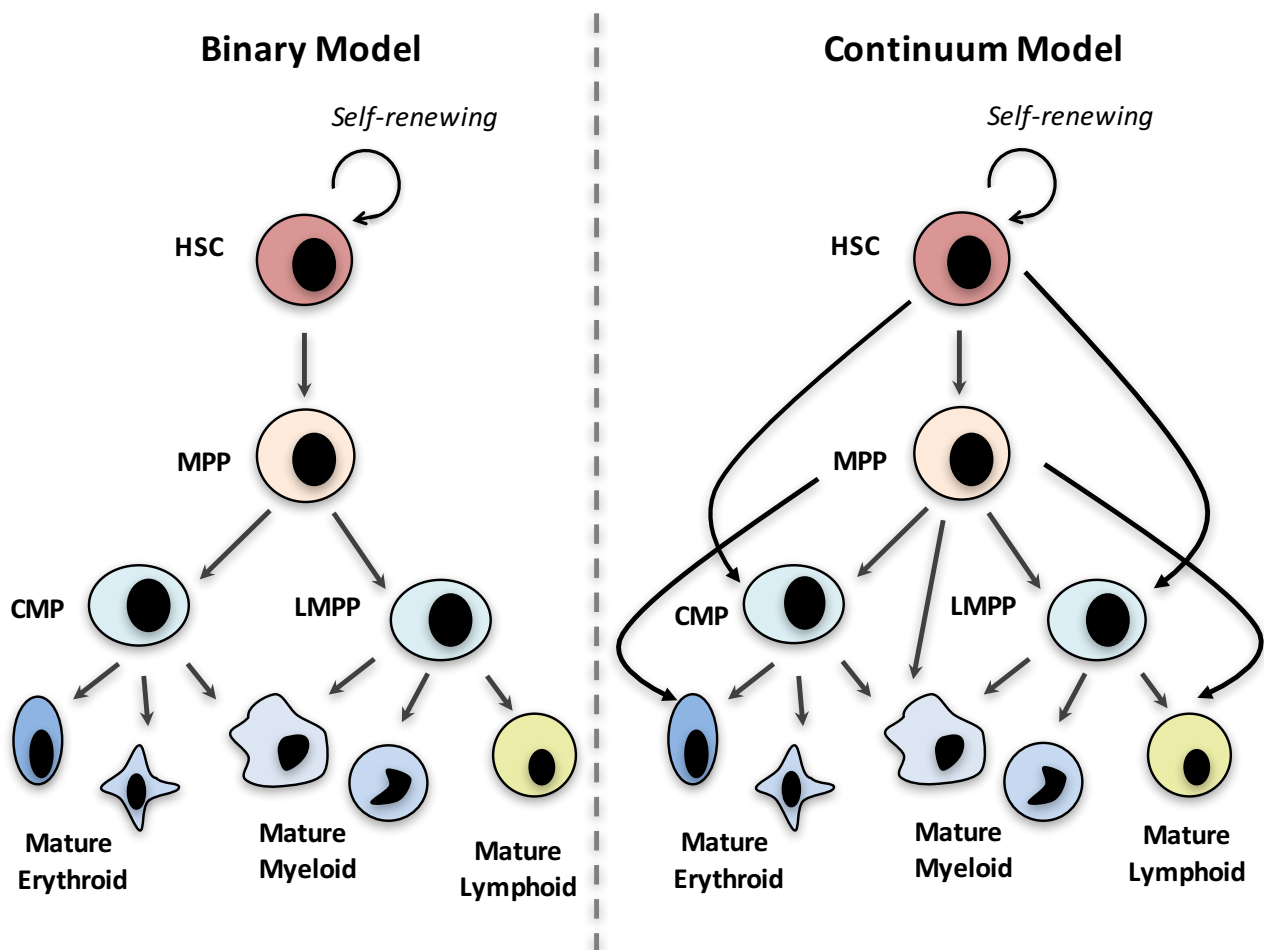


Figure 1.2: Hematopoietic differentiation progression. Past and present models of hematopoietic cell differentiation lineage hierarchy based on the human lineage hierarchy proposed by Görgens *et al.* (82). In the binary model, there is a linear sequence of events from HSC to MPP, to common myeloid progenitors (CMP) and lymphoid-primed multi-potent progenitors (LMPP), to mature hematopoietic cell. In the continuum model, the differentiation from HSC to mature hematopoietic cell does not require an intermediate cell type.

Conceptually, there are 3 possible outcomes when a stem cell divides (83): the mother cell produces 2 daughter cells of the same non-stem cell identity, having differentiated (Figure 1.3A); the mother cell asymmetrically divides into a daughter with the same stem cell identity as the mother cell and a daughter cell which has

differentiated (Figure 1.3B); or the 2 daughters retain the same identity as the mother cell, also known as self-renewal (Figure 1.3C). Studies are underway to decipher if, when and why these outcomes happen. Understanding the division of these cells may hold the key to understanding the lifespan of a HSC and how the drive to undergo cell division, in conditions such as inflammation, can affect the division of these cells.

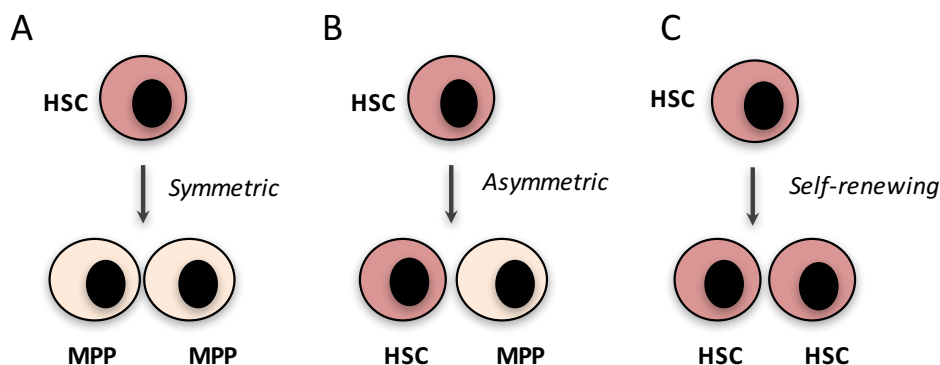


Figure 1.3: Symmetrical and asymmetrical division of hematopoietic stem cells. There are 3 conceptual outcomes when stem cells undergo mitosis. (A) The mother produces 2 non-stem cell daughters, (B) the mother cell produces 1 non-stem cell daughter and 1 daughter which retains stem cell qualities, or (C) two stem-cell clones are produced, often referred to as self-renewal.

Infection and immunological stress place a demand on innate and immune cells which have a limited lifespan and must be replenished. Hematopoietic requirements in response to infection and inflammation depend asymmetrically on either myeloid or lymphoid cells and both myeloid biased and lymphoid biased subsets appear to exist within the MPP population (distinguished by CD150 and CD229 expression in the mouse). There is not much information about the biases in the HSPC population of the zebrafish.

1.3 Immune homeostasis

Both zebrafish and mammals share all major blood cell types (48), making findings in the zebrafish translatable to mammalian systems. Just as in mammals, the erythroid lineage oxygenates the tissues while lymphoid and myeloid lineages give rise to adaptive and innate immune cells respectively (Figure 1.4). Recently, the mammalian hematopoietic tree has been revised by Görgens *et al.* (78, 82). They have challenged the model of lineage restriction of MPPs to common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs) by identifying a lymphoid-primed progenitor with partial myeloid potential (LMPP). This hierarchy has not yet been demonstrated in the zebrafish, but in my opinion is likely to be conserved and therefore is used in the schematic of hematopoiesis (Figure 1.4).

1.3.1 Erythroid lineage

In zebrafish, red blood cells/erythrocytes and thrombocytes/platelets are derived from a thrombocyte-erythrocyte progenitor (TEPs) functionally equivalent to mammalian megakaryocyte-erythrocyte progenitor

(MEPs). These cells are supplied from an erythroid-myeloid progenitor (EMP) population which also differentiates into a eosinophil and basophil progenitor (EoBP) population (Figure 1.4). Erythrocyte production is regulated by erythropoietin, and thrombopoiesis is regulated by thrombopoietin (TPO)(84).

Mature erythrocytes bind oxygen and transport it to tissues around the body. Both mammalian and zebrafish erythrocytes are defined by the transcription factor GATA-binding factor 1 (Gata1). Traver *et al.* showed that populations of the kidney marrow could be distinguished by flow cytometry (48). Zebrafish erythrocytes have an average size of 6.2 μm and can be separated from the rest of the kidney marrow cells by selecting, or gating, cells with low forward scatter and high side scatter (FSC^{low} SSC^{high}) (48). Single cell transcriptional analysis found erythroid cells of the WKM expressed erythroid-specific genes, including β -A1-globin, solute-carrier-family-4, and spectrin- β -erythrocytic (85). Teleost erythrocytes resemble primitive mammalian erythrocytes as they are nucleated. Zebrafish also form thrombocytes and these have been studied using the transgenic CD41 line (29). They are smaller than erythrocytes and have low forward and side scatter (FSC^{low} SSC^{low/intermediate}) when analysed by flow cytometry.

1.3.2 Myeloid lineage

In the revised hematopoietic tree, granulocytes (neutrophils, eosinophils, and basophils) and monocytes (including macrophages and dendritic cells) are derived from granulocyte-monocyte restricted progenitors (GMPs), and EMPs differentiated from CMPs and LMPPs (Figure 1.4). Myeloid cells are part of the innate immune system. Both zebrafish and mammals also have a complement system that assists the cells of the innate immune system. Traver *et al.* found that the myeloid fraction of the WKM has high forward and side scatter (FSC^{high} SSC^{high}) in a flow cytometry plot and these cells have an average size of 15.2 μm (48).

Macrophages, neutrophils and dendritic cells all have varying phagocytic capacity. They engulf pathogens and the debris of dead cells and digest them with enzymes to recycle their contents. Macrophage phenotypes are characterized as ‘classically activated pro-inflammatory (M1)’ or ‘alternatively activated anti-inflammatory (M2)’ cells. M1 macrophages promote inflammation, extracellular matrix destruction, and apoptosis and tend to lead to chronic inflammation. M2 macrophages promote extracellular matrix construction, cell proliferation and angiogenesis which tend to resolve inflammation and facilitate wound healing.

Both macrophages and neutrophils attack pathogens by activating a “respiratory burst” which produces free radicals. These cells also produce chemokines to recruit more cells to the site. Zebrafish neutrophils resemble human neutrophils, possessing segmented nuclei and myeloperoxidase (MPO)-positive cytoplasmic granules (86). Transgenic zebrafish lines include Tg (pu.1:GFP) (87) and Tg (lyz:dsRed) (56) in which the myeloid lineage is marked; Tg (mpx:GFP)(88) labels neutrophils; Tg (fms:mCherry) and Tg (mpeg:mCherry) (89) mark macrophages (Table 2.1). As in mammals (Figure 1.4), *leukocyte specific actin binding protein (l-plastin)* is expressed by monocytes, macrophages and also B-cells, T-cells, granulocytes and NK cells (86).

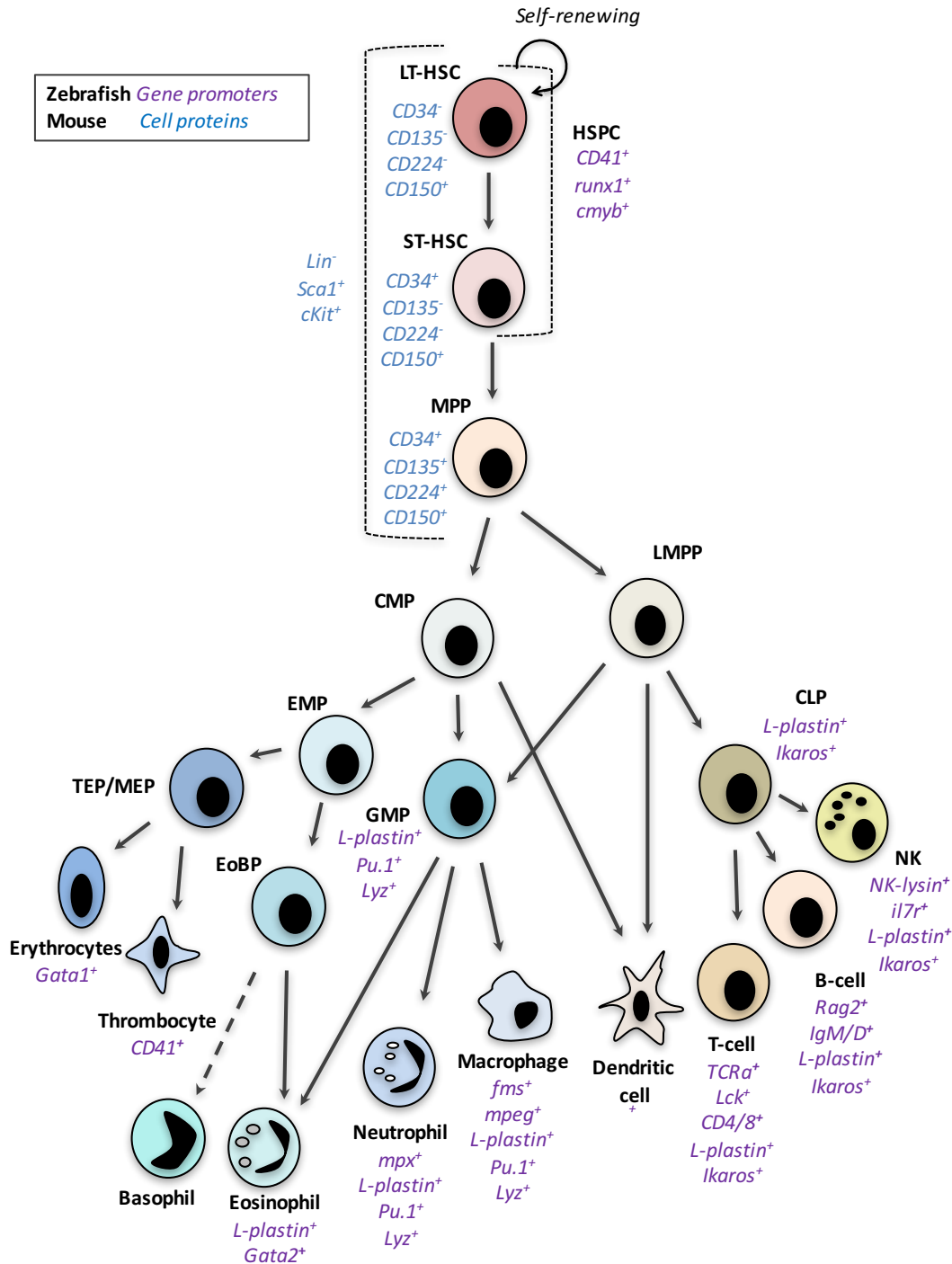


Figure 1.4: A schematic comparison of the mouse and zebrafish hematopoietic tree. The hematopoietic cell differentiation lineage hierarchy is adapted from Bryder et al. (90) combined with the current human hematopoietic hierarchy proposed by Görgens *et al.* (82). Long-term (LT-) and short-term (ST-) HSCs have only been described in mice (blue). In zebrafish (purple), a more general HSPC population is describe which may contain LT- and ST- populations. This HSPC population supply a population of MPPs which respond to demand and regulate myeloid and lymphoid production via LMPPs and CMPs. LMPPs supply a population of granulocyte-monocyte restricted progenitors (GMPs), producers of monocytes/macrophages and granulocytes and a population of common lymphoid progenitors (CLPs) producers of B-, T- and natural killer (NK) cells. LMPPs can also differentiate into dendritic cells and monocytes. CMPs also supply GMPs as well as an erythroid-myeloid progenitor population (EMPs). EMPs produce erythrocytes and, in the mouse megakaryocytes, via the megakaryocyte-erythrocyte progenitor population (MEP), or in the zebrafish, thrombocytes via the thrombocyte-erythrocyte progenitor population (TEP). EMPs can also differentiate into eosinophils and basophils (putative in the zebrafish) via the eosinophil-basophil progenitor (EoBP). Blue labels indicate mouse specific proteins used to identify corresponding cell population. Purple labels indicate zebrafish specific gene promoters/proteins used to identify corresponding cell populations.

Eosinophils and basophils are also granulocytes, derived from EoBPs, a subset of EMPs. They are implicated in allergic responses. In mammals, these cells degranulate in response to activation, releasing enzymes and cytokines. Eosinophils were isolated in a Tg (gata2:EGFP) zebrafish line and shown to degranulate in response to helminth infection (91). It is currently not known whether a basophil population exists in the zebrafish (86).

1.3.3 Lymphoid lineage

With the exception of natural killer (NK) cells, lymphoid cells (B- and T-cells) form the adaptive component of the immune system. Teleost fish are among the most ancient vertebrates possessing an adaptive immune system. These cells have low forward and side scatter (FSC^{low} SSC^{low}) in a flow cytometry plot of the WKM and have an average size of 8.3 μ m (48). In the current model of the hematopoietic tree, myeloid and lymphoid branches are much less distinct than in the classical model with CMPs and CLPs. Indeed, the monocytic/macrophage potential is retained by both CMPs and LMPPs, leading some to propose that the current variety of immune cell-types came from the evolved specialisations of a macrophage-like prototype (82).

NK cells are derived from CLPs and have attributes of both the innate and adaptive immune branches (92). Although zebrafish NK cells are not easily distinguished using flow cytometry forward and side scatter, recent single cell transcription analysis has found a population of NK-lysin expressing cells in the WKM suggesting a NK type cell does exist (85). In mammals, both cytotoxic (CD8 expressing) T lymphocytes and NK cells produce NK-lysins. Based on the gene expression analysis of NK-lysin expressing cells found in the zebrafish (no CD4 or CD8, but lymphocyte-specific-protein-tyrosine-kinase (Lck), T-cell Receptor (TCR) α , TCR- β and interleukin-7 receptor (IL7R, hereafter referred to as CD127) are expressed), these cells are a putative NK population (85).

CLPs also differentiate into B cells and T cells, components of the adaptive immune system. As in mammals, both of these populations are produced in the HSC niche (BM of mammals or kidney marrow fish), but most T cell progenitors must migrate to mature in the thymus (93). The cells of the adaptive immune system retain memory of specific pathogens encountered and provide a higher affinity response when the pathogen reoccurs. The identification of antigens on pathogens relies on the expression of immunoglobulin (Ig) domain-based antigen receptors that are diversified by DNA rearrangement, somatic mutation, and gene conversion. Like mammals, fish B cells express Ig proteins and T cells express TCR complexes. Both these receptor types are assembled by recombination activating gene 1 and 2 (rag1 and rag2) proteins (94). In the zebrafish, B- and T-cells can be distinguished in the lymphoid fraction with the use of transgenic markers of Rag2 and Lck respectively. Furthermore, unsupervised clustering of single cell transcriptional analysis of WKM lymphoid cells was able to separate B- and T-cells based on gene expression (85).

Ig receptors of B-cells consist of 2 heavy and 2 light chains. In both mammals and zebrafish, the variable antigen binding portion of the heavy chain is produced by DNA rearrangement of exons. Also in both, the constant portion of the heavy chain is specified by μ and δ exons (co-expressed by alternative splicing) to produce IgM and IgD on naïve B-cells. In mammals, μ and δ exons get switched to other isotypes (γ , ϵ or α). However, these other isotypes

do not exist in zebrafish (or teleost fish) and switching is done to another exon (ζ) to produce IgZ (95, 96). The binding of an antigen to the surface Ig causes transmission of a signal via CD79 to induce B-cell activation. Page *et al.* studied different stages of B cell development in adult zebrafish using a dual transgenic line Tg (rag2:dsRed;IgM:eGFP) line (96). Single cell transcriptional analysis of cells of the WKM found that many mammalian orthologues were expressed in zebrafish B-cells such as *paired-box 5* (Pax5), *tetraspanin membrane protein* (CD37), CD79a, IgM, IgD and rag2 (85).

In mammals, T lymphocytes express cell surface TCRs which are constructed from variable chains (α/β or γ/δ) and constant chains (CD3- γ , δ , ϵ)(97). The variable domains on α -, β -, γ - and δ -chains have complementarity determining regions (CDRs) that determine antigen specificity. The variability of the chains is due to recombination (by rag1 and 2), gene conversion (by cytidine deaminases, AID), and random nucleotide additions. CDRs can be edited by recombinases in a process termed “TCR revision”. Mammalian T-cells co-express glycoproteins CD4 and CD8 along-side the TCR. These enhance the specific recognition of class I and class II major histocompatibility complexes (MHCs) constitutively. Class I MHCs are expressed by most cells, while class II are only present on antigen presenting cells (APC) including dendritic cells, macrophages and B-cells.

While antibodies for discrete T-cell populations (CD4 and CD8) have not yet been studied in zebrafish, single cell transcriptional profiling of cells of the WKM found both CD4 and CD8 to be expressed within the lymphoid fraction (85). These cells also expressed *tcra*, *lck* and *CD127* (85). Furthermore, genetic analysis found that zebrafish have MHC genes on chromosomes 1, 3, 8 and 19 (47). Chromosome 19 contains class I genes (98, 99). Class II α and β genes are located on chromosome 8. Chromosome 1 contains an additional type of class I genes (termed “ze” genes) that are most similar to mammalian non-classical Class I genes (100). Finally, the “L” genes, class I genes unique to teleost fish, are located on chromosomes 3 and 8. Antibody labelling cannot be used to determine cell-surface expression and therefore the precise function of the class I and II genes cannot yet be determined in zebrafish (101). Perhaps reporter lines (in which fluorescent protein expression is driven by the promoter producing the MHC transcript) would help in determining the function of these genes.

1.4 Techniques for studying hematopoietic stem cells

1.4.1 Ex vivo: Culture

Ex vivo approaches offer the possibility performing experiments in a cell-autonomous manner to study actions within or by the single cell, independent of cells in the environment. This complements *in vivo* experiments to study actions of the cell in its environment. Mouse BM cells can be kept short-term in RPMI media supplemented with cytokines but despite three decades of efforts, it has not yet been possible expand or maintain for long periods *bona fide* HSCs *ex vivo* for regenerative medicine purposes. However, HSCs have been cultured to determine their long-term repopulating activity. *In vitro* assays include colony forming cell (CFC) assays and the pseudo-emperipolesis (migration) of HSCs between stromal cells and the dish surface, also known as the ability to form cobblestones (cobblestone area-forming cell assays). For CFC assays, cells are plated at a low density in

a semi-solid medium in the presence of cytokines and cell numbers and types of colonies formed are used to quantify progenitor number and differentiation bias. The long-term culture system allows quantification of precursor cells capable of initiating and sustaining hematopoietic cells. These cells, known as long-term culture-initiating cells (LTC-IC) share functional properties with cells capable of repopulating hematopoiesis *in vivo* (102).

Some long term hematopoietic progenitor lines exist, including the non-leukemic Factor dependent Paterson (FDCP)-mix (103). These mouse cells were cloned and isolated from long term bone marrow cultures infected with src-MoMuLV (a molecular recombinant virus where Rous sarcoma virus is under the influence of murine amphotrophic leukaemia virus). Although their stem-like properties only last up to 15 weeks in culture, early isolates can form spleen colonies (CFU-S) in irradiated mice and restore long term hematopoiesis on irradiated marrow stroma *in vitro*. FDCP-mix is used instead of a primary culture of BM cells in experiments where a homogenous population or a large number of cells is required. Another cell line, recently developed, is the HoxB8-FL cells which have lost self-renewal capacity and the MEP lineage, but can differentiate into myeloid and lymphoid lineages (104).

The Schroeder lab have been working recently using microfluidic devices to fluctuate dosing of cytokines to BM derived cells (105). To complement this work, they have developed powerful software for high through-put analysis of pictures (106). With this, they can continuously track individual cells over generations, recording position, cell divisions, cell interactions and the protein activity in live cells. This technology was recently used to quantify the response of HSCs (LSK CD135⁻ CD150⁺ CD48⁻ isolated from mice) to IL-1 β (25 ng μ L⁻¹) over 6 days in liquid culture (107). The analysis revealed that HSCs cultured with IL-1 β divided at a significantly higher rate because the delay between cell divisions was significantly shorter than in HSCs cultured without IL-1 β . Notably, the effect was specific to HSCs as IL-1 β did not affect survival or proliferation of GMPs and MPPs. In addition, they found that transcription factor PU box (Pu.1) activity is significantly increased in HSCs treated with IL-1 β , before the first cell division event. This type of data cannot be obtained by any other current method.

Until recently, culturing of zebrafish hematopoietic cells was not possible because of the divergence between mammalian growth factors and cytokines. Stachura *et al.* first developed a method for culturing hematopoietic cells in suspension over isolated *zebrafish kidney stromal* cells (108). Subsequently, Lund *et al.* isolated mesenchymal cells from the caudal tail tissue which they called *zebrafish stromal cells* and which can maintain kidney marrow cells for up to 2 weeks (109). These cells are capable of migrating to the kidney in adoptive transfer experiments (109). Most recently, Campbell *et al.* have cultured a *zebrafish embryonic stromal trunk* cell from the dorsal aorta which supports the expansion and differentiation of zebrafish HSPCs (cd41:GFP⁺) (110). These culture techniques can be used to study terminal differentiation of cells but do not provide data on differentiation events or self-renewal events at the single cell level. Svoboda *et al.* have subsequently developed a methylcellulose assay to examine clonal expansion and differentiation of HSPCs and TEPs in real time *ex vivo* (84, 111). Zebrafish hematopoietic cells grow best at 32 °C in a humidified 5% carbon dioxide environment (112).

1.4.2 In vivo: Transplants

The gold standard for assaying HSCs is to test their capacity to provide lifelong reconstitution of all blood-cell lineages after transplantation into lethally irradiated recipients. The most rigorous assay for assessing *stemness* of hematopoietic cells is serial transplantation. It requires that HSCs be serially re-transplanted to show that they retain self-renewal and multi-potential differentiation capacity. Limiting dilution assays are used to measure the stem cell frequency within a population by transplanting increasingly diluted cell preparations.

Although the order of events is not clear, it is thought that in both mammals and zebrafish, transplanted HSCs localise to hematopoietic sites, engraft in the tissue, self-renew and differentiate. “Homing” refers to the movement of HSCs through blood circulation from the site of transplant into the supportive microenvironment (either BM or kidney marrow) and is essential for “engraftment”. Engraftment is the process in which the HSC leaves the circulation, transitions through the vascular wall and enters the hematopoietic tissue (113). This latter behaviour has not yet been observed in transplant recipients (in any animal model) but is thought to resemble the endogenous process that occurs during development when HSCs seed the hematopoietic tissue (42). Engraftment and retention of HSCs in the correct microenvironment is termed “lodgement”. HSC proliferation can be symmetrical or asymmetrical. Although the spleen and circulating blood contain low a low frequency of HSCs, ultimately the mouse BM is the only niche which protects self-renewal and quiescence of mice HSCs (114). It is not yet known whether the kidney marrow of zebrafish is the only site which protects the stem properties of HSCs, or whether other sites, such as the caudal hematopoietic tissue (CHT), spleen or thymus, could also be involved.

Our understanding of early post-transplant events is limited because of technical obstacles. For instance, it is unclear whether transplanted HSCs begin to differentiate in circulation or whether this occurs after engraftment in the hematopoietic niche. It is also unclear whether early post-transplant proliferation of HSPCs is towards self-renewal or maturation (Figure 1.3).

1.4.2.1 Support/carrier cells

Although theoretically a single stem cell should be sufficient to initiate long-term host repopulation, there is some evidence that a minimal threshold of cells is required for engraftment (115-117). For instance, human HSCs (CD34⁺ Lin⁻) cells contributed more to the NOD/SCID mouse recipient hematopoietic system when transplanted as an unfractionated sample, even though both recipient groups received equivalent numbers of (CD34⁺ Lin⁻) cells (117). Therefore, it appears to be beneficial to add support cells when transplanting low cell numbers, although the reason for this is not understood. Bonnet *et al.* have hypothesised that cytokines and proteolytic enzymes, such as matrix metallo-proteinase 9 (MMP 9), could play a role (116). Janowsha-Wieczorek *et al.* have suggested a mechanism involving platelet derived micro-particles (118).

1.4.2.2 Hematopoietic cell transplantation in mice

Mouse hematopoietic transplantation experiments have been instrumental in our understanding the regulatory pathways and niche elements involved in the regulation of hematopoiesis and the HSC. However, the mouse model relies on monitoring physiological factors such as body weight, condition of hair and posture to assess the initial success of transplantation. Later, blood tests are used to assay the capacity for self-renewal and engraftment of transplanted cells. In a few cases, survival is monitored to assess successful engraftment, although most studies transplant sufficient support cells and do not have this as an end point.

The first attempt to study the post-transplant spatial distribution of progenitor cells (Lin⁻ population; Lin⁻ WGA^{dim-med} Rh⁺ population; and Lin⁻ WGA^{dim-med} Rh⁻ population) *in situ* was by Nilsson *et al.* in 2001 when they examined longitudinal femur sections to determine the proximity of transplanted carboxyfluorescein succinimidyl ester (CFSE) labelled cells to the cells of the recipient BM (119). The first *in vivo* imaging of the BM niche was in 2003 by Askenasy and Farkas. They used an imaging window over the distal femoral epiphysis to monitor transplanted cells, although they did not analyse the distribution of cells within the BM (120). Ultimately, the most effective method to image post-transplant cell behaviour has been developed by the Lo Celso group (121). They use intra-vital microscopy, scanning a 4 mm × 6 mm window on top of the skull, to form a 3D image of the central sinus and the surrounding BM. They combine transgenic mice and dialkylcarbocyanine dyes (DiD or DiI) for cell tracing to obtain a dynamic model of the environment structure and the behavior of the cells in it over time. They have documented cell mobilization and engraftment in irradiated transplant recipients and even a cell dividing. Their method was used to show that HSCs from *Trichinella spiralis* infected donors interact with a more diverse set of niche components than HSCs from non-infected donors (122).

Genetically altered organisms have been developed to study human HSCs in model organism. Humanised mice (123), sheep (124) and zebrafish (50) make it possible to study human HSC function and malignancies such as leukaemia. One of the most commonly used mice strains has a mutation in the DNA-dependent protein kinase gene and is known as *severe combined immuno-deficient* (NOD/SCID) mice. This mutation results in a lack of T-cells, B-cells and an altered cytokine environment. Another useful model is the Kit mutant (125-127). These mice lack functional HSCs and therefore do not require radiation treatment prior to transplant. Furthermore, Antonelli *et al.* have generated mice with a human BM scaffold to study the behaviour of human leukemic cells from patients (128).

1.4.2.3 Hematopoietic cell transplantation in zebrafish

Due to technical obstacles, observations of hematopoietic cell behaviour in mammals is limited and the animal burden is high as testing multiple conditions and time points requires many animals. In addition, data can be more informative if it is obtained from the same subject over time. This makes the zebrafish an interesting alternative model. Zebrafish have been instrumental in our understanding of developmental hematopoiesis (129-131). Naturally, they also lend themselves to studying HSCT and have been used for this purpose since the early 2000s (132). Original studies used unselected WKM cells, but gradually HSPC enriched populations have

been purified for transplant (Table 1.2). Over time the method of cell delivery has progressed from intra-peritoneal delivery to RO delivery (49). Transgenic lines developed for the dissection of the origins of hematopoietic cells have been co-opted as an alternative to immunohistochemistry for identifying HSCs.

Donor	Recipient	Carrier cells	Route	Readout	Reference
Tg(<i>gata1</i> :eGFP) 10 ² - 10 ³ adult WKM cells	Bloodless <i>gata1</i> ^{-/-} mutant 48 hpf embryos	None	Sinus venous	Imaging over 7 days and tracking recipient survival up to 6 months	Traver et al. (2003)
Tg(<i>βactin</i> :eGFP) 10 ⁵ adult WKM cells	Irradiated (25 Gy, day -2) adult Casper	2x 10 ⁵ PB	Cardiac puncture	Imaged 4 hpt, sacrificed for flow cytometry at 4 wpt	White et al. (2008)
Tg(<i>globin</i> :GFP) 1.5 - 2 x 10 ⁵ adult PB cells	Adult Casper	None	Retro-orbital	Visualise circulating blood cells	Pugach et al. (2009)
Tg(<i>βactin</i> :eGFP) 100 – 10 ⁵ adult WKM cells	Irradiated (20- 40 Gy, day -2) adult WT	10 ⁵ PB	Cardiac puncture, then retro-orbital	Tracking recipient survival up to 13-16 weeks, engraftment at 90 days, sacrificed for flow cytometry at 14-16 wpt	De Jong et al. (2011)
Tg(<i>cd41</i> :eGFP) 100 – 16000 <i>cd41</i> ^{lo} adult WKM cells	Irradiated (25-35 Gy, day -2 and -1) adult WT	5x 10 ⁵ to 8x 10 ⁵ WT WKM cells	Cardiac puncture	Imaged at 30 dpt for circulating GFP+ cells	Ma et al. (2011)
WT or Tg(<i>ikaros</i> :GFP) 2 – 40 x 10 ⁴ adult WKM cells	Bloodless <i>cmyb</i> ^{Δ25127} mutant	None	Retro-orbital	Imaging 7 dpt, sacrificed for flow cytometry at 9 wpt	Hess et al. (2013)
Tg(<i>ubi</i> :eGFP) 3 x 10 ⁵ or 5 x 10 ⁴ adult WKM cells	Immunocompromised, irradiated (10 Gy, day -2) adult <i>rag2</i> ^{E450R} mutant	None	Intra-peritoneal	Imaged at 30 and 45 dpt for circulating GFP+ cells or sacrificed for flow cytometry	Tang et al. (2014)
Tg(<i>runx1</i> +23: mCherry; <i>ubi</i> :GFP) 1 - 50 double positive cells from adult WKM cells	Irradiated adult Casper	2x 10 ⁴ WKM	Retro-orbital	Sacrificed for flow cytometry 3 mpt	Tamplin et al. (2015)
1, 2 or 4 double positive cells from embryo	2 dpf WT	2x 10 ⁴ WKM	Injected into circulation	Survival to 5 months; engraftment assessed by flow cytometry	

Table 1.2: List of zebrafish transplant papers. PB indicates peripheral blood, WKM indicates kidney marrow.

The first zebrafish transplant experiments were done with embryo recipients (in which the thymus has not fully developed and does not produce mature T-lymphocytes) which meant there was no problem of histocompatibility between recipient and donor. This approach continues to be used by many researchers but limits our understanding of HSC behaviour in the context of a fully developed hematopoietic system. Subsequently, adult recipients were used but required preconditioning to deplete the endogenous immune system. As in the mouse model, recipients can be preconditioned with radiation, 5-FU or antibodies (133). Therapeutic doses for humans, used in the treatment of people with cancer, are typically between 2 and 4 Gy, experimental mice are treated with 9 Gy (134), goldfish are treated to 30 Gy and zebrafish with as much as 40 Gy (135). More recently, in order to reduce rejection, studies use MHC- matched recipients (45) or immune deficient recipients (46).

Both goldfish (136) and zebrafish (50) have been used for xenogeneic transplantation studies. In the latter, human CD34⁺ umbilical cord cells were labelled with fluorescent dye PKH26 and detected either by fluorescent imaging of the transplanted zebrafish embryo or by flow cytometry. In both goldfish and zebrafish, human cells

survived in the kidney marrow for up to 6 days, suggesting that the environmental cues of the teleost niche are sufficiently conserved to sustain cells of diverse origins, albeit for a limited period.

1.5 Embryonic origin of HSCs

Vertebrates, including zebrafish, are known to undergo waves of hematopoietic activity during which separate foetal and adult hematopoietic populations form (Figure 1.5; (137, 138). The first “primitive” wave produces the hemangioblast, a progenitor of both hematopoietic and endothelial cells. Primitive erythroid and myeloid cells are produced at this time. Subsequently, there is a transition wave during which erythroid-myeloid progenitor (EMP) cells emerge and produce definitive erythroid and myeloid cells. Both primitive and definitive erythroid and myeloid cells are generated independently of definitive HSCs. In parallel, there is a “definitive” wave during which long term, self-renewing pluripotent hematopoietic stem cells emerge from the dorsal aorta, migrate to intermediate sites of hematopoiesis to proliferate, and then ultimately settle in definitive hematopoietic niches. They produce mature erythroid, myeloid and lymphoid cells.

1.5.1 Primitive wave: Hemangioblast derived hematopoiesis

Hematopoiesis in the embryo begins with the emergence of committed progenitors followed by true HSCs (Figure 1.5A). In both mice and zebrafish, the embryonic primitive wave transiently produces hematopoietic cells from the hemangioblast (139, 140), generating primitive erythrocytes (141, 142) and primitive macrophages (143). These primitive erythrocytes and macrophages are thought to be important for embryonic development and for immunity in the embryo.

The hemangioblast emerges from activation of VEGFR2 during gastrulation of the mouse blastula (144). In mammals and birds, these cells form in the yolk sac. In the zebrafish, vascular endothelial and proerythroblasts are formed from the hemangioblast in the intermediate cell mass (ICM) derived from posterior lateral mesoderm, while endothelial and primitive myeloid cells are formed both in the ICM and in the rostral blood island (RBI) derived from anterior lateral mesoderm in the developing embryo (145-147). Davidson *et al.* used *cdx4* mutant fish (mutation in the homeobox *kugelig* gene) to show that patterning during gastrulation is important for hematopoiesis to occur in the ICM (148).

Very recently, somite derived HSPCs have been discovered in the zebrafish that do not originate from the posterior lateral mesoderm (149). These cells are shown to persist to adulthood, and when transplanted in embryos, contribute to multiple hematopoietic lineages suggesting pluripotency (149).

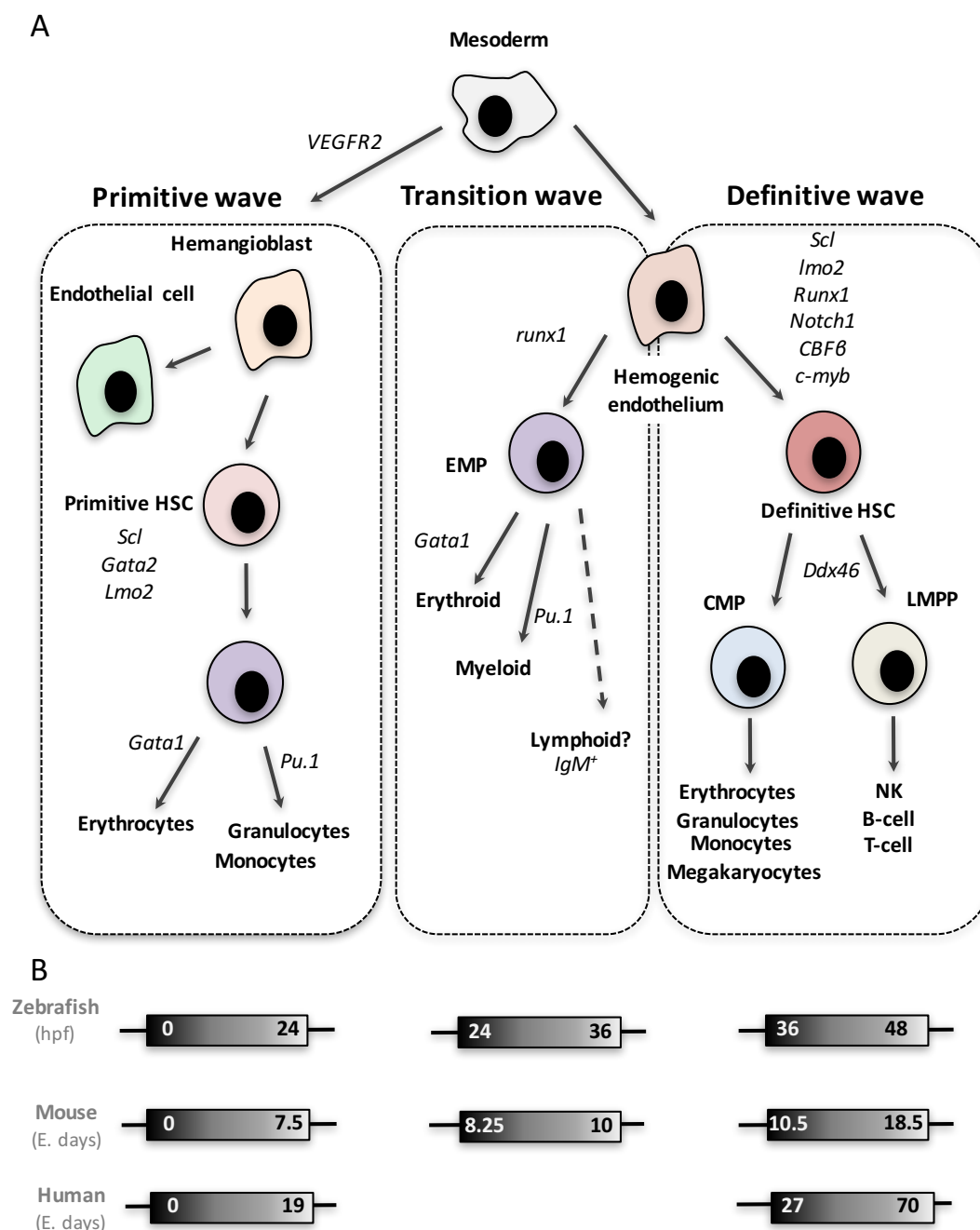


Figure 1.5: A schematic of the waves of hematopoiesis, adapted from Jing *et al.* (150) and Mikkola *et al.* (151). Mammals and teleost fish undergo waves of hematopoiesis. (A) Hematopoietic cells originate from mesoderm. The first (primitive) wave is derived from the hemangioblast, an endothelial and hematopoietic precursor. The hematopoietic cell has limited erythroid-myeloid potential. Subsequently, a transitional wave of erythroid-myeloid precursors (EMPs) is derived from hemogenic endothelium in the aorta-gonad-mesonephros. Lymphoid cells may be produced by this precursor, or another yet unidentified precursor population. Definitive hematopoiesis is produced from definitive HSCs, also derived from the hemogenic endothelium. These pluripotent cells supply a population of MPP which produce CMPs and CLPs. The CMPs give rise to adult erythroid and granulocyte cells. The LMPPs give rise to myeloid and lymphoid cells. Important genes are listed along the pathway. (B) The timings at which each wave occurs in the zebrafish (hours post fertilization), mouse (embryonic days) and human (embryonic days), ending when HSCs reach the kidney/bone marrow.

Around 6 hours post fertilisation (hpf) in the zebrafish embryo (Figure 1.5B), the primitive hematopoietic and endothelial progenitor known as the hemangioblast begins to differentiate in the ICM. These cells have been

imaged *in vivo* in zebrafish embryos using single cell laser activation of caged fluorescein dextran in individual cells of the ventral mesoderm (139). These progenitor cells express *Scl*, *Gata2* and *Lmo2* (60, 140, 152). The zebrafish *cloche* mutant does not have a primitive hematopoietic wave because of a lack of *Gata1* and *Gata2* which have a direct effect on the differentiation of endothelial and hematopoietic lineages (153). In addition, *Gata2* expression is regulated by microRNA MiR-451 (154). *Lmo2* is important for the timing of *c-myb* and *runx1* expression (152). Transcription factors *Scl*, *Lmo2* and *Gata2* are all required upstream regulators of erythrocyte transcription factor *Gata1* and myeloid transcription factor *Pu.1* in both zebrafish and mammals (147, 155). The balance of erythroid and myeloid cells is governed by the expression of *scl* and *lmo2* transcription factors (147).

At about 12 hpf of the zebrafish embryo, the erythrocyte specific gene *gata1* is observed in cells migrating to form the ICM in transgenic *gata1* reporter fish (156). These primitive erythrocytes enter circulation from 24 hpf and are the only erythrocytes for the first 4 dpf (157). It is not clear how long these cells survive, but ultimately they are replaced by mature erythrocytes cells that develop from the definitive hematopoietic wave (157).

The myeloid transcription factor *Pu.1* (*Spi1* in humans) is expressed in both the ICM and RBI by 16 hpf and subsequently only in the RBI from 20 hpf (86, 87, 143). The leukocyte specific actin binding protein (*l-plastin*) is expressed in the RBI from 18 hpf and detected in the ICM from 28 hpf (86). *Mpo*, an enzyme produced in granulocytes, is expressed by *pu.1* expressing cells but distinct from *l-plastin* expressing cells. This pattern suggests primitive myeloid cells originate from both the ICM and RBI sites. Embryonic myeloid cells distribute around the zebrafish yolk sac forming resident macrophages (158) and by 48 hpf form neutrophils (159). Although early macrophages have phagocytic activity (145), early neutrophils do not (159). It is not yet clear whether these cells persist or get replaced by cells differentiating in the definitive hematopoietic wave.

Erythroid and myeloid cells are also detected in the mouse yolk sac at E7.5 (embryonic day 7.5), prior to the emergence of definitive HSCs (160, 161) (Figure 1.5B). Primitive erythrocytes emerge from E7.5 (162) and macrophages progenitors appear in the yolk sac at E8.0. These cells subsequently migrate into the embryo. As in the zebrafish, the erythroid and myeloid cells only exist transiently, and lack lymphoid and self-renewal potential. In the human embryo, the yolk sac generates early hematopoietic cells from day 19 (Figure 1.5B) with limited myeloid potential.

1.5.2 Transient definitive wave: EMP derived hematopoiesis

Definitive erythrocytes and myeloid cells initially differentiate from committed EMPs (Figure 1.5) (129). In mice, these cells emerge at E8.25 from hemogenic endothelium in the yolk sac and colonise the foetal liver by E10.5 (Figure 1.5B). The term “definitive” has been applied to both yolk sac-derived EMPs that give rise to adult-type cells and embryo-derived HSCs. However, here we make a distinction as, although they engraft and transiently produce erythrocytes, EMPs do not have the capacity for long term reconstitution of hematopoiesis of myeloablated mice (163). In addition, the phenotype and proliferation capacity of EMP derived erythrocytes is significantly different from primitive erythrocytes derived from the hemangioblast during the primitive wave and definitive HSC-derived erythrocytes (141), making this wave distinct from the other two. Both EMP and

definitive HSCs are *runx1* dependent (30). However, Notch activity is required for definitive HSC specification (43, 59, 164), but not for the specification of EMPs (165). Downstream transcriptional regulators *gata2* (60) and *c-myb* (166, 167) are required.

EMPs also produce megakaryocytes and macrophages, but these are hard to distinguish from hemangioblast-derived and HSC-derived myeloid cells. This makes studying them difficult. It is hypothesised that their presence is to support erythrocytes and the mobilisation of definitive HSCs arising from the dorsal aorta (168).

Interestingly, embryos with persistent EMP emergence, but no definitive hematopoietic wave, produce a population of lymphoid cells. Furthermore, lymphoid cells have been detected in the mouse yolk sac at E9.5, before definitive HSC activity and prior to *rag1* expression in the embryo at E10.5 (169-173). Current evidence suggests that the lymphoid lineage is derived from a progenitor population, which temporally coincides with, but is independent of, EMPs (174).

In zebrafish, the adaptive immune system develops over 3 weeks and it is not clear whether these cells are derived from EMPs, specific lymphoid progenitors or definitive wave HSCs. The earliest IgM expressing cells (B-cells) appear between the dorsal aorta and posterior cardinal vein and in the kidney about 20 hpf, prior to the emergence of definitive HSCs at 2 dpf (96). Although some expression of *Rag1* and IgM was reported in the pancreas 4 to 11 dpf (175), this has not been subsequently confirmed (96). At about 3 dpf, *ikaros*, which in mammals is expressed in lymphoid progenitors, is expressed in the zebrafish thymus (176). *TCR α* is expressed from 4 dpf in the thymus, with expression increasing over 3 weeks (177).

1.5.3 Definitive wave: HSC derived hematopoiesis

The “definitive” wave, which produces the long term, self-renewing pluripotent hematopoietic stem cells that are able to generate into all mature lineages for the lifetime of the organism, begins at 2 dpf in the zebrafish (130) and E10.5 in the mouse (178, 179) (Figure 1.5B). Definitive HSCs emerge after vascular remodeling from an endothelial-hematopoietic precursor along the dorsal aorta of the zebrafish embryo, equivalent to the aorta-gonad-mesonephros (AGM) of mice. They populate the liver at E14.5 and the BM is colonised by E18.5. In humans, definitive HSCs appear in hematopoietic clusters along the aorta from day 27 (180, 181). HSCs proliferate in the liver and the BM is colonised by week 10 (180).

In the zebrafish, nascent HSCs are identified as *kdr*⁺ *runx1*⁺ in Tg (*kdr*:NLS:mCherry;*runx1*:23:GFP) embryos. These cells migrate to both the CHT (at the posterior end of the zebrafish, close to the cloaca) and to the pronephric tissue (at the anterior end of the zebrafish, the area above the heart and gut that will develop into the adult kidney marrow; (32, 182). Similarly, in the mouse, nascent HSCs migrate from the AGM to the foetal liver, to the spleen, and ultimately to the BM at the end of gestation (183). HSCs in the zebrafish CHT or mammalian foetal liver proliferate to expand the population. Time lapse imaging of the CHT in zebrafish has shown that

runx1⁺ HSCs are “cuddled” by 5 to 6 endothelial cells which control their orientation and division (42). *Ex vivo* imaging of mouse samples showed similar interactions between foetal liver cells and Sca1⁺ cells (Ly6a:GFP) (42).

Hematopoietic stem cells emerge from the aorta floor through a process termed endothelial-hematopoietic transition (EHT) (41, 184). This process requires *runx1* expression in endothelial cells but not hematopoietic cells (30, 42, 185). Knocking out *runx1* in mice is lethal to the embryo (186) while knock down in zebrafish, inhibits EHT (185). Furthermore, the *runx1*^{w84x} zebrafish mutant embryo lacks a definitive wave (185). Core-binding factor-beta (CBFβ) forms a heterodimer with Runx1, and is required for the release of definitive HSCs into circulation (187, 188). However, unlike Runx1, CBFβ is not required in transient hematopoiesis and EMP production (187, 188).

Another marker of definitive HSCs is the proto-oncogene *c-myb*. Like CBFβ it is not required in primitive hematopoiesis (189) but is required for migration of definitive HSCs to the CHT at 3 dpf (166). In the absence of *c-myb*, HSCs numbers decrease as a result of apoptotic death. The zebrafish *c-myb*^{t25127} mutant lacks definitive hematopoiesis. This mutant fish line has been exploited as a transplant recipient that does not require pre-conditioning (46, 167).

Many more genes involved in definitive hematopoiesis have been identified by the generation of mutant zebrafish using chemical mutagenesis. For instance, in *grechetto* mutants (mutation in the cleavage and polyadenylation specificity factor 1 gene, *cpsf1*), like in *c-myb* mutant, HSCs fail to migrate from the aorta to the CHT (190). Two other mutants created this way, *rumba* (mutated nuclear C2H2 zinc-finger factor) and *samba* (mutated human augmin complex subunit 3 gene, *haus3*) produce HSCs in the aorta which migrate to the CHT, but expansion and maintenance are defective and the result is a significantly reduced HSC population (191).

Scl and its partner *Lmo2*, are both required in primitive and definitive hematopoiesis. Morpholino-mediated knock down of *scl* expression in embryonic zebrafish resulted in a loss of both primitive and definitive hematopoiesis (192). Similarly, knock down of *lmo2* also resulted in a lack of both primitive and definitive hematopoiesis (152).

Finally, in the zebrafish, DEAD-box RNA helicase is expressed by HSCs in the CHT and required for the multi-lineage differentiation of definitive HSCs, possibly regulating *pu.1* and *gata1* expression (193).

1.5.4 Inflammatory signals regulate HSC emergence

Definitive HSC formation and maintenance is regulated by endogenous signalling molecules associated with inflammation signalling (44, 194-197). Genetic analysis of hematopoietic cell clusters in mouse AGM (195) and of *runx1*⁺ HSPCs in the zebrafish CHT (198) show that emerging definitive HSCs have an immune profile (with high expression of Toll-like receptors (TLRs) and MHCs) and inflammatory profile (with expression of IFN-regulated genes). As described earlier, erythroid and myeloid cells are produced during the primitive

hematopoietic wave, before the arrival of definitive HSCs and can therefore influence the emergence of these cells. It is hypothesised that these primitive cells are present both for immune protection and to provide the signalling molecules required for activating HSC specification. Indeed, there is significant evidence that cytokines such as type I and II IFNs, tumour necrosis factor (TNF) and ILs secreted by neutrophils, and to some lesser extent macrophages, have a direct impact on the size of the emerging HSPC population (44, 195, 198). In both the mouse (199) and the zebrafish (41), Notch1 and its ligand Jagged-1 (Jag1) are essential for the formation of the hemogenic endothelium and successive EHT (59, 164, 200). The activation of cytokine receptors often leads to activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NfκB) pathway. NfκB is highly expressed in the dorsal aorta of Tg (NfκB:EGFP;kdrl:mCherry) zebrafish between 24 and 30 hpf, prior to EHT (44). These cells also have high levels of *il-1β* and *NfκB-inhibitor alpha (ikbaa)* mRNA. Loss of NfκB in endothelial cells of Tg (fli1a:Gal4;UAS:dn-ikbaa) resulted in a significant reduction in the number of cmyb⁺ HSPCs (44).

Neutrophil secreted TNFα appears to drive endogenous HSC emergence. Espin-Palazon *et al.* used titrated doses of morpholinos to decrease mRNA levels of TNFα and its receptors TNFr1 and TNFr2. They showed the number of nascent HSCs (kdrl⁺; runx1⁺ and cmyb⁺) at the dorsal aorta was decreased in the absence of TNFα signalling, without effecting changes to the vasculature (44). This is supported by He *et al.* who also showed a reduction in of HSPCs (runx1⁺; cmyb⁺ kdrl⁺) in *tnfr2* morphants (198). Furthermore, conditional overexpression of TNFr2 specifically in endothelial cells of the vasculature in Tg (fli1a:Gal4; UAS:RFP; cmyb:GFP; UAS:tnfr2) resulted in an increase in HSC (cmyb:GFP⁺) numbers (44). Altogether these results indicate that neutrophils produce TNFα which binds to TNFr2 on endothelial cells and promotes EHT of the endothelial cells. Moreover, TNFα may also act through the Notch and NfκB pathway (44). Using Tg (tp1:eGFP) in which Notch-activated cells express GFP, Espin-Palazon *et al.* showed that TNFα signalling activates Notch1 in the dorsal aorta. Endothelial expression of Notch1a intracellular domain (NICD1a), the transcriptional factor released upon Notch1 activation, in Tg (kdrl:Gal4; UAS:NICD1a-myc) restored runx1⁺ cells in TNFr2 depleted zebrafish. Treatment of the anaemic zebrafish mutant *diamond-blackfan*, caused by a mutation in ribosomal protein S19 which alters the 40S ribosomal subunit, with the TNFα inhibitor Etanercept, rescues erythropoiesis and indicates a link between TNFα the erythroid transcription factor Gata1 (201).

Recently the TLR4 and Myeloid differentiation primary response gene 88 (MyD88) pathway was also associated with EHT in both mice and zebrafish. He *et al.* found that reducing TLR4 (in *tlr4bb* and *myD88* zebrafish morphants and *tlr4* knock out mice embryos) reduced the number of definitive HSCs (runx1⁺; cd41⁺; cmyb+kdrl⁺) (198). Notch target genes were decreased in these morphants, and the number of HSCs could be rescued by driving Notch intracellular domain (NCID) expression. Furthermore, vasculature expression of the dominant negative IkBaa, which prevents translocation of NfκB to the nucleus, also reduced the number of HSCs, demonstrating that the effect is mediated by NfκB. The effect could be rescued by the overexpression of IL-1β (198).

Another cytokine involved in early hematopoiesis in zebrafish is the IFN γ homolog IFN γ 1.2 (196). Although it does not appear to drive EHT, it positively regulates HSC proliferation and maintenance during development. In heat induced Tg (hsp:ifng1-2; cd41:EGFP; kdrlHRAS:mCherry), the number of HSCs (cd41:GFP+) along the dorsal aorta increased by 30 % (196). Furthermore, fish with a mutated IFN γ 1.2 receptor (IFNGR), had fewer HSCs (runx1:GFP+). Similarly, morpholino-mediated knockdown of IFN γ 1.2 and IFNGR resulted in fewer HSCs. They showed that IFN γ acts downstream of Notch as HSC numbers decreased in response to Notch-signalling inhibitor N-[N- (3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester (DAPT) but could be rescued by over expression of IFN γ . The application of DAPT was limited a period of 24 h beginning 24 hpf so as to not interfere with development of the vasculature 10-12 hpf. These findings were supported by Li *et al.* who also showed morpholino-depletion of type I interferon (IFN ϕ) also reduced HSC (cd41:GFP) numbers in the AGM of zebrafish (195). Furthermore, they showed that IFN α and IFN γ knock-out mouse embryos had fewer functional HSPCs (CD45⁺ Ly6a⁺) in the AGM at E11.5, although their maintenance was not affected.

Furthermore, Lu *et al.* have shown that microRNA (miR)-142-3p is specifically expressed in both zebrafish and mouse HSCs and regulates the expression of interferon regulatory factor (IRF) 7 (*irf7*) and G-CSF receptor (*Gcsfr*) (154). In miR-142-3p morphants, where *Irif7* expression is increased and *Gcsfr* is decreased, there was a decrease in the number of HSPCs (cmyb⁺; runx1⁺) in the AGM and CHT. Similarly, in transplants of mouse hematopoietic cells from the AGM at E11 (CD34⁺ ckit⁺), the addition of an inhibitor of miR-142-3p reduced the number of CFU-S in recipient mice. Thus, HSC expansion and differentiation is regulated by inflammation signalling between IRF-7 and G-CSF receptor.

IL-1 is a pro-inflammatory cytokine which has also been implicated in the early differentiation of HSC. Orelia *et al.* found that the ventral side of the dorsal aorta of embryonic mice express IL-1, its receptor (IL1rI) and the signalling mediators at E11 (202). Although there was only a minor increase of HSCs (ckit⁺ CD34⁺; 1.2 fold) in the AGM of IL1rI knock out mice, there was a significant increase (1.7-2 fold) in myeloid progenitors (202) suggesting that IL-1 signalling shifts the differentiation bias towards the lymphoid lineage.

Runx1 is a previously mentioned transcription factor involved in regulating HSC numbers. This transcription factor also drives IL-3 expression. Robin *et al.* showed that IL-3 could increase the number of HSCs (c-kit⁺ CD34⁺) in haplo-insufficient runx1 (+/-) AGM explants at E11. Moreover, the AGM of *il-3* knock out embryos had significantly fewer HSCs (ckit⁺ CD34⁺). Thus, IL-3 signalling increases proliferation and survival of HSCs during EHT (203).

Notably, recent *in vitro* experiments have not been able to use these cytokines (TNF α , IL-1 β or IFN γ) to drive human pluripotent progenitor cells to form hemogenic endothelial progenitors (CD31⁺CD34⁺CD45⁻ or CD34⁺CD43⁺CD73⁻) or hematopoietic cells (CD43⁺CD45⁺), irrespective of the developmental window (204). Furthermore, knockdown of endogenous Nf κ B signalling by small molecule BAY65-5811 inhibitor did not impact hematopoietic differentiation (204). Despite these contrary findings, there is significant evidence from both

mouse and zebrafish models that inflammatory signalling regulates HSC emergence. These *in vitro* experiment results are likely to reflect the limitations of current protocols and the physiological differences between cells *in vivo* and cells in culture.

Finally, macrophages have been observed in association with emerging human HSPCs (CD34⁺ CD45⁺) and emerging zebrafish HSPCs in Tg (cd41:GFP; mpeg1:mCherry) (168). Inhibition of MMPs results in accumulation of HSPCs at the AGM and reduced colonisation of subsequent hematopoietic sites (CHT or foetal liver). This suggests that their role may be to secrete proteolytic enzymes to degrade the extracellular matrix and facilitate HSPC mobilisation (168).

1.6 HSCs in the adult

Definitive HSCs are derived from the aorta, expanded in the liver, and finally maintained in the BM of the mouse. An analogous process has been observed in the zebrafish, where HSCs are derived from the dorsal aorta, expanded in the CHT, and maintained in the kidney marrow. Wright *et al.* used genetically marked para-biotic mice with shared circulation to show that circulating HSPCs continuously enter and exit circulation as part of normal hematopoiesis (205). Levels of progenitors in circulation are low in hematopoietic homeostasis as HSCs are maintained in quiescence by specialised environments known as the niche (206). Entry and exit from the BM is regulated by intrinsic and extrinsic factors.

The HSC niche was first put forward by Schofield in 1978 where he proposed that stem cell behaviour could only exist in association with supportive cells in the microenvironment (114). Essentially, stromal cells would support a stable HSC pool by inhibiting the cell's ability to proliferate, differentiate and exit the niche. There is some debate over which of the sub-regions of the BM (osteoblast and perivascular) are most important for the support of mammalian HSCs (207-210). Indeed, perhaps a variety of cells is necessary to fulfil all the niche requirements: maintenance, quiescence, self-renewal and inhibition of differentiation. For instance, a quiescent cell may be maintained away from self-renewing cells in an area that could potentially signal for differentiation of the daughter cell (83). If osteoblastic and vascular surfaces serve different support functions, this could explain why studies have not been able to pin down a specific cellular niche.

The BM is the major mammalian hematopoietic niche, although HSCs are also found in the spleen and circulating blood. Mouse bones are smaller and more compact than humans which have larger, less compact BM with more adipocytes and larger blood volume. On the other hand, zebrafish hematopoiesis occurs in the very distinct kidney which retains little structural similarity to the BM. Both of these regions are discussed below.

1.6.1 The bone marrow is the HSC niche in mammals

The BM niche is typically split into the anatomically distinct regions of endosteal and perivascular compartments, but in actual fact, *ex vivo* imaging of femur slices found that the two compartments are not distinct (211).

Furthermore, live imaging of the mouse calvarium shows that the majority of osteoblasts (90%) are within 20 μm of vascular surfaces (121).

1.6.1.1 The osteoblast niche

It was first reported that functional pluripotent hematopoietic cells (capable of colony formation) resided close to the endosteal surface of bones, and not the central marrow region (212). Indeed, osteoblasts and osteoclasts, responsible for depositing and reabsorbing the lining of the bone surface respectively, provide important factors necessary to maintain HSCs such as G-CSF (213), granulocyte-macrophage colony-stimulating factor (GM-CSF) (213), stem cell factor (SCF) (214), and IL-6 (215) which support the maintenance of immature hematopoietic phenotypes *in vitro* (216). Furthermore, long term cultures of BM cells contain osteoblasts.

Osteoblasts and osteoclasts (Figure 1.6) have been implicated in the form and function of the BM niche of mice due to knock out and conditional mutants. For instance, mice lacking core-binding factor alpha1 (CBF α 1, also known as runx2) have defective osteoblast formation resulting in reduced BM hematopoiesis and increased extramedullary hematopoiesis (217). In addition, Visnjic *et al.* reported that conditional osteoblast ablation in Col2.3delta-tk mice (targeting thymidine kinase expression with ganciclovir) resulted in the destruction of the endosteal surface and a significant loss in hematopoiesis (initially differentiated cells, and over time HSPCs) (218, 219). It also appears that osteoclasts play an important role by secreting proteolytic enzymes (such as MMP9 and proteinase 3) which degrade adhesion molecules in the BM, thus releasing HSCs from their tether (220).

The first functional evidence for the osteoblast niche came from 2 groups publishing the same year. In 2003, both Zhang *et al.* and Calvi *et al.* experimentally increased osteoblasts to show a direct role for osteoblasts in HSC regulation (221, 222). Zhang *et al.* examined the role of bone morphogenic protein (BMP) on HSCs using polyinosinic:polycytidylic acid (hereafter poly I:C) inducible mutant BMP receptor type 1a mice (BMPRI1A) (221). Mice lacking BMPRI1A had increased long-term (LSK BrdU⁺) and short term HSPC (LSK BrdU⁻) frequency, and these HSPCs competitively reconstituted irradiated recipients. As this receptor is not expressed by HSPCs, the effect must be due to the microenvironment. Further, histological examination of the bone found that BMPRI1A mutant mice had increased numbers of spindle-shaped N-cadherin osteoblast (SNO) stromal cells expressing N-cadherin (N-cad⁺) and β -catenin and that these cells were adjacent to N-cad⁺ expressing HSPCs (223). Together these data suggest that BMP regulates the number of HSPCs and links the increase in SNO cells with increased HSPC (LSK) numbers. These findings are supported by Sugimura *et al.* who reported that genes *flamingo* and *frizzled* are expressed by N-cad⁺ HSCs and permit non-canonical Wnt signalling with SNO cells that helps maintain the quiescence of HSCs in homeostasis (224).

In parallel, Calvi *et al.* drove osteoblast expansion using parathyroid hormone (PTH) on transgenic mice with osteoblast specific constitutive expression of parathyroid hormone receptor (PPR) (222). These mice had an increased number of osteoblasts and increased HSPC (LSK) frequency which competitively reconstituted irradiated recipients. Also, treating recipients with PTH increased transplant survival from 27 to 100 % and

substantially altered the histology of the recipient BM. Osteoblasts from PPR transgenic mice produced higher levels of *CXC motif chemokine ligand 12* (*Cxcl12*, also known as *Sdf1*), *il-6*, *Scf* and *Jag1* than wild type (WT) controls.

Although the paper suggests a link between high *jag1* expression by osteoblasts and the increase in HSPC frequency, further analysis by Mancini *et al.* demonstrated that Notch1/Jag1 interactions were not required for HSC maintenance (225). Neither the reconstitution ability, nor the frequency of HSPCs (LSK) was affected in conditional knock out mutants of *Jag1* and *Notch1*.

Osteoblasts lining the bone surface provide some of the requisite factors to maintain HSCs (Figure 1.6). For instance, *Scf* is expressed by stromal cells and is a ligand for c-Kit (CD117), a receptor on hematopoietic cells (although expression of c-Kit changes in certain conditions and therefore not always used as a marker). Antibody blocking of the interaction between c-KIT and SCF allows HSCs to engraft without irradiation (226). A number of c-Kit mutant mice have been exploited as transplant recipients that do not require preconditioning as the endogenous HSCs do not engage with the niche (227, 228).

The role of osteoblasts in supporting HSCs, in particular LT-HSCs was further supported by the finding that the angiopoietin receptor CD202b and angiopoietin 1 (Ang1) interaction was shown to protect HSPCs from the myelo-ablative compound 5-FU. Arai *et al.* found that a subpopulation of HSPCs (LSK-SP CD202b⁺) express CD202b and bind to angiopoietin 1 (Ang1) expressing osteoblasts (69). The CD202b/Ang1 signalling increased N-cadherin levels and reduced HSC cell cycling post-transplant. Furthermore, CD202b knock out mice fail to maintain HSPCs in the BM (18).

Another osteoblast product, Osteopontin (OPN), is also implicated in conserving the HSC population (Figure 1.6). OPN is a phosphorylated glycoprotein expressed specifically by osteoblasts on the endosteal surface of the BM and binds with alpha4-integrin and CD44 expressed by HSPCs. Both Nilsson *et al.* and Stier *et al.* used OPN knock out mice to show that in the absence of OPN, the HSPCs (LSK CD34⁻) fraction is increased, and that therefore OPN is a negative regulator of HSPC numbers (229, 230). While Stier *et al.* reports only modest differences in cycling HSPCs at early time points (<10 days), Nilsson *et al.* found a significant increase in cycling HSPCs (LSK) at later time points (> 4 weeks) suggesting a delayed effect. Transplanting OPN^{-/-} HSPCs had no effect on reconstitution, indicating OPN is a non-cell autonomous factor. OPN^{-/-} transplant recipients were better reconstituted than WT counterparts, although surprisingly, the HSPCs were significantly further from endosteal surfaces in these recipients (229).

Yet another niche product, TPO was initially discovered as a regulator of platelet production, but subsequently was shown to preserve HSPC quiescence and self-renewal *in vivo* (231). For instance, the knock-out of TPO or its receptor CD110 significantly reduces functional HSPCs in transplantation. TPO was shown to enhance intracellular expression of VEGF-A in HSPCs (LSK) promoting cell survival and proliferation (232).

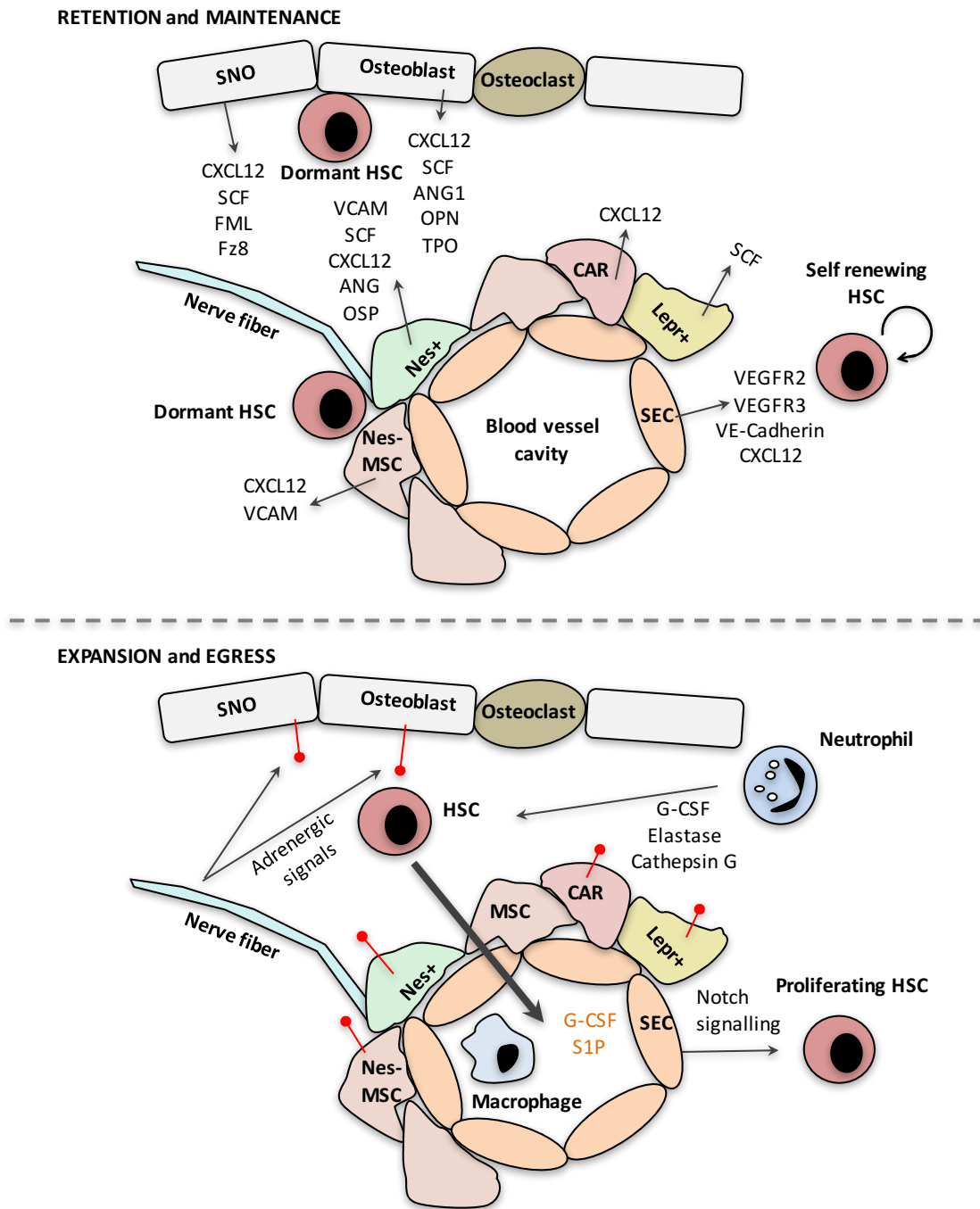


Figure 1.6: Retention and mobilisation factors of the bone marrow niche. Various cell types are involved in maintaining and trafficking of the HSC population from the bone marrow including: osteoblasts, including spindle-shaped N-cadherin osteoblasts (SNO), sinusoidal endothelial cells (SECs), mesenchymal stromal cells (MSCs), including CXCL12-abundant reticular (CAR) cells, Leptin receptor (Lepr+) stromal cells and Nestin (Nes+) expressing MSCs, macrophages and neutrophils. Retention factors (including CXCL12, SCF, VCAM, Vascular endothelial cadherin (VE-cadherin) Flamingo (FML) and Frizzled (Fz8) involved in non-canonical Wnt, Angiopoietin (ANG1), thrombopoietin (TPO), osteopontin (OPN)) expressed by niche cells are indicated. Mobilisation requires down regulation of these factors, indicated by red pin heads, as well as mobilisation signals (including G-CSF and sphingosine-1-phosphate (S1P)). Sympathetic nerves provide a circadian delivery of adrenergic signals. Macrophage egress signals for HSC proliferation and egress.

Finally, the osteoblast specific expression of *dickkopf1*, an inhibitor of canonical Wnt signalling, was shown to decrease Wnt in HSCs (LSK) which led to increased cell cycling (233). Furthermore, exposure to the Wnt inhibitor

reduced reconstitution ability of these cells, even when transplanted into an environment without the Wnt inhibitor. Together, this collection of data strongly supports the influence of the niche on the function of HSCs.

More recently, intra-vital imaging has shown that contact with osteoblasts is also important for transplanted hematopoietic cells. Lo Celso *et al.* transplanted HSCs (LSK CD48⁻ CD135⁻) dyed with DiD into osteoblast reporter mice (Col2.3:GFP) with a c-kit mutation (125). These recipient mice have GFP expression in osteoblast to allow distance measurements to be made and a c-Kit mutation that impairs endogenous HSPCs to engage with the niche, thus allowing transplanted HSCs to engraft without chemotherapy or radiation (228). Using 3-D live imaging they found that HSPCs were within 16 μ m of vasculature and within 20 μ m of endosteal surfaces. In contrast, in WT non-conditioned recipients, HSPCs were always > 30 μ m from endosteum surfaces, and none of these cells ever engrafted.

Furthermore, live imaging of HSPCs (LSK CD48⁻ CD135⁻) transplanted into transgenic PPR mice (increased osteoblasts due to constitutive expression of parathyroid hormone receptor) found that transplanted cells locate closer to the endosteum than in WT mice, even when adjustments were made to allow for the reduced marrow cavity size (121). This is possibly due to the high expression of the anchoring molecules expressed by osteoblasts. The proximity to osteoblasts suggests preservation of the stem phenotype and self-renewal rather than differentiation of transplanted cells.

However, some data has suggested that osteoblasts are not required for HSC maintenance. For instance, Kiel *et al.* found that neither (LSK CD150⁺ CD48⁻ CD41⁻) or (LSK CD135⁻) HSC populations expressed N-cadherin and that hematopoietic cells that did express N-cadherin possessed low reconstituting ability (3 in 30) (234). They argued that the majority of HSCs do not depend on the endosteal surface, although a small subset might (14 % of HSPCs were localised near endosteal surfaces).

Kiel *et al.* also studied the effect of osteoblasts on HSPCs in a biglycan deficient mouse model (234). These mice have fewer osteoblasts due to the depletion of the extracellular matrix proteoglycan. However, the frequency and reconstitution capacity of HSPCs (LSK CD150⁺ CD48⁻ CD41⁻) from these mice is not significantly different to WT HSPCs. These findings are contrary to the finding in the CBF α 1 mutant mouse model and the conditional osteoblast ablation in Col2.3delta-tk mice which both resulted in significant loss of hematopoiesis (217-219). These contrary findings may be due to the severity of the osteoblast ablation: in the CBF α 1 mutant and conditional Col2.3delta-tk mice, osteoblasts are not formed or severely impaired, while in the biglycan deficient mouse model the loss of osteoblasts is progressive over time. Together, these data suggest that the HSPC population can be maintained by low osteoblast numbers.

In addition to the results described above, Lo Celso *et al.* also found that, post-transplant, non-committed HSCs (LSK CD48⁻ CD135⁻) located closest to the osteoblasts, while more mature MPPs and committed progenitors were further away (121). This supports previous findings by Nilsson *et al.* which also found that the majority (60 %)

of non-committed progenitors (Lin⁻ Wga⁺ Rho^{low}) located closer to endosteum, while more mature cells were found in the central cavity (235).

There is evidence of bidirectional regulation of the endosteal niche. While the endosteal niche regulates HSC number and function, there is also evidence that HSPCs (LSK cells and LSK CD150⁺ CD48⁻ CD41⁻ cells) can direct the differentiation of mesenchymal cells into osteoid lineages *in vitro* and *in vivo* (236). Jung *et al.* injected HSCs (LSK CD150⁺ CD48⁻ CD41⁻) into pre-established "vossicles" (ectopic bone formations created from lumbar vertebrae) from CBF α 1 (bone transcription factor) reporter mice (236). They found that HSCs induced CBF α 1 activity in the vossicles, thus inducing differentiation of BM stromal cells into osteoid lineages. In addition, CBF α 1 activity was increased when vossicles were injected with HSCs from stressed (phlebotomised) mice, compared to sham injected vossicles and vossicles injected with HSCs from non-stressed mice. Furthermore, they found that stressed HSCs increased osteogenesis by producing higher levels of BMP2 and BMP6 (236).

1.6.1.2 The perivascular and endothelial niche

Given that hematopoietic cells arise from hemogenic endothelium precursor in the dorsal aorta, it is not entirely surprising that HSCs closely associate with vascular endothelial cells. While the endosteum surface is closely associated with the vascular surface, the central marrow cavity is made up of vascular and extravascular support cells (121, 211). These cells include CXCL12-abundant reticular cells (CAR) (237), sinusoidal endothelial cells (SECs) (238), neural glial 2 expressing pericytes (239) and mesenchymal stem cells (MSCs) (240, 241) including Leptin receptor (Lepr⁺) cells (242) and Nestin (Nes⁺) expressing cells (241) (Figure 1.6). The chemokines CXCL12 and SCF are important factors of the perivascular niche.

Although initially studies determined the endosteal niche was regulating HSPCs, Kiel *et al.* argued that the perivascular structures were also relevant to HSC support. They used the expression of SLAM proteins to distinguish HSCs in stained sections of BM and found that only 14 % of HSCs (LSK CD150⁺ CD48⁻ CD41⁻) localised to the endosteal surface. On the other hand, 60 % of HSCs were found by sinusoidal vascular endothelium (17). In support of this, both Tokoyoda *et al.* (243) and Sugiyama *et al.* (237) found HSCs (LSK CD150⁺ CD48⁻ CD41⁻) scattered throughout the central BM cavity.

SECs constitute the predominant surface area of the vascular niche (Figure 1.6). These cells express adhesion molecules that assist the migration and homing of HSPCs to the BM. SECs cells express VEGFR2, VEGFR3 and vascular endothelial cadherin and are essential for engraftment and reconstitution of hematopoiesis (238). Indeed, co-culturing of hematopoietic cells with SECs leads to incremental self-renewal of HSCs. SECs also play a role in regeneration after BM injury and assist in hematopoietic cell differentiation. However, the molecular pathways that modulate this function are not yet understood but may be through the production of cell autonomous factors. For instance, during recovery, protein kinase B (hereafter referred to as Akt) and mitogen-activated protein kinases regulate reconstitution (244).

Imaging by both Lo Celso *et al.* (121) and Xie *et al.* (211) found that proliferative HSPCs were mostly seen in the central marrow region. Xie *et al.* report that HSPCs in the same field of view had different behaviours; one stable and the other actively dividing (211). This may be due to cell autonomous features, or their position relative to vascular surfaces which would alter the vascular signals they are receiving.

Histological sections of CXCL12 reporter mice showed CXCL12 expression throughout the BM cavity (237), including perivascular stromal cells, endothelial cells, osteoblasts, and some hematopoietic cells (245, 246). Interestingly, the majority (97 %) of HSPCs (LSK CD150⁺ CD48⁻ CD41⁻) cells were found in contact with CAR cells (237). Conditional deletion of CXCL12 receptor CXCR4 (CXC receptor 4, CD184) reduced the fraction of HSPCs (LSK CD34⁻ and SP) in the BM, but also increased the MPP fraction (LSK CD34⁺). In support of this, Ding *et al.* targeted deletion of CXCL12 from specific support cells (246). HSPCs (LSK CD150⁺ CD48⁻) were depleted when CXCL12 was deleted from endothelial cells, and to a greater extent when deleted from perivascular cells (246). However, HSPCs were not depleted when CXCL12 was deleted from osteoblasts, although HSPCs became more migratory (240). This demonstrates a role for non-endosteal surfaces in the quiescence of HSCs. Transplantation of HSPCs, followed by induced deletion of CXCR4, showed that effects were cell autonomous and thus CXCR4 expression by HSPCs is important for HSPC quiescence.

In vivo imaging by Lo Celso *et al.* further noted that single cells were located closer to the endosteum while clusters (>3 cells) were further away (121). By combining DiD and DiI labelling of transplanted cells, they also revealed that the clusters of HSPCs observed in the marrow are due to the proliferation of single cells, and not the clustering of cells transplanted together.

Although distributed alongside perivascular cells (Figure 1.6), a distinct population of Nes⁺ cells have been investigated for their role in supporting HSPC quiescence because femoral sections from Nestin reporter mice showed a close association between these cells and HSPCs (LSK CD150⁺ CD48⁻). Nes⁺ cells express significant levels of ANG1, CXCL12, SCF, IL-7, vascular cell adhesion molecule (VCAM) 1, and OSP, genes implicated in HSPC retention in the BM (241). Furthermore, the highest level of these factors is found in a subset of Nes⁺ cells (CD45⁻ Ter199⁻ CD31⁻ PDGFRa⁺ CD51⁺) (247). Significantly, conditional knock out of Nes⁺ cells caused a 50 % decrease in HSPCs (LSK CD150⁺ CD48⁻) in the BM, and an increase in the spleen. This indicates a role for Nes⁺ MSCs in HSPC migration.

On the other hand, *ex vivo* imaging data by Xie *et al.* that found that HSPCs (LSK CD135⁻) transplanted into non-conditioned WT recipients were in direct contact with MSCs (both Nes⁺ and Nes⁻) indicating that HSPCs interact with a diverse set of niche components (211). Both Nes⁺ and Nes⁻ MSCs express important amounts of CXCL12 and both have been found to contribute to HSPC maintenance in the BM (240, 241).

1.6.1.3 Resident macrophages

Recently, BM resident macrophages have emerged as important regulators of HSPCs (Figure 1.6) (248). They influence whether HSPCs remain within the BM or enter the circulation (249, 250). They also regulate steady state erythropoiesis and granulopoiesis (251-254). Resident macrophages are located both in the central cavity and on the bone lining where they support osteoblasts (250, 253). The heterogeneity of the macrophage populations (5 markers currently being used) means that it is difficult to determine distinct population contributions.

In demand-driven hematopoiesis, sensors of cell loss feedback to HSPCs to increase cell proliferation and differentiation. Granulopoiesis induced by the loss of neutrophils during infection requires macrophage phagocytosis of dead cells. The exit of macrophages from the BM, needed to increase phagocytic capacity in the periphery, results in HSPC proliferation and granulopoiesis, mediated by G-CSF, IL-1 and TLR4 signalling (255). Recently, Boettcher *et al.* found that endothelial cells are robust producers of G-CSF in response to lipopolysaccharide (LPS) (256). Endothelial expression of this factor may be important for inducing macrophages egress from the BM.

Methods to deplete macrophages in mice (such as macrophage FAS-induced apoptosis (MAFIA), CD169 -DTR mice and clodronate-liposomes (249, 250)) leads to an increase in circulating HSPCs. In these models, the levels of retention factors including CXCL12, ANG1, Kit and VCAM1 are all reduced both at the mRNA (RT-qPCR of total BM cells (249) and endosteal cells (250)) and protein level (ELISA of cultured MS-5 cells (249)). The depletion of macrophages also results in an increase of both proliferative and dormant HSPCs in the BM suggesting that macrophages limit HSC proliferation and population size (257). Thus macrophages promote quiescence and retention while they are present, and hematopoietic cell production when they exit the BM.

Granulocyte production, granulopoiesis, is stimulated by G-CSF during infection (258). G-CSF is a common mobilisation agent used for harvesting donor progenitors today (259). It has been shown to reduce the amount of CXCL12 (260) and VCAM1 (261) in the BM, causing mobilisation of the HSPCs into the circulation (Figure 1.6). It also reduces the number of BM resident macrophages (250), which may also be the cause of HSPCs entering the circulation.

1.6.1.4 HSC mobilisation and retention factors

The niche factors ANG1/CD202b, SCF/c-Kit, N-Cadherin, TPO/CD110 and OPN/ alpha4-integrin/CD44 described earlier all play a role in HSC quiescence and retaining LT-HSCs in the BM (Figure 1.6). It is worth noting that HSC mobilisation and quiescence are linked. Thus, although listed here as distinct, mobilisation requires the downregulation of these niche factors as well as an upregulation of factors that lead to HSC migration out of the BM.

To begin, under normal physiological conditions, HSC trafficking to and from the BM is regulated by the circadian release of adrenergic signals from sympathetic nerves in contact with Nes⁺ mesenchymal cells (262). Signalling through the coupling β 3-adrenergic receptor *Adrb2* leads to down regulation of maintenance genes (CXCL12 and Specificity protein 1 *Sp1*) and egress of HSCs. The sympathetic nervous system also regulates HSCs through indirect influence on osteoblast function (263).

Migration and homing of hematopoietic cells, in response to injury or post-transplant, involves gradients of chemotactic factors and cell adhesion molecules (CAMs). The chemotactic activity of the previously mentioned CXCL12, as well as phosphosphingolipids, extracellular nucleotides and calcium ions also appear to play an important function (264-267). The lodgement of HSPCs is then orchestrated by CAMs which include selectins, integrins and CXCL12. Furthermore, the expression of hyaluronic acid (HA), a component of the extracellular matrix, appears to be a key molecule in regulating HSC distribution.

The chemokine CXCL12, is an important chemo-attractant of HSCs (240, 268, 269) and lymphocytes. This ligand is constitutively expressed by BM stromal cells, in particular by CXCL12-abundant reticular (CAR) cells surrounding sinusoidal endothelial cells or near the endosteum (237), and by endothelial cells. The main receptor for CXCL12 is CXCR4 which is expressed by immature hematopoietic cells. CXCL12 binding to CXCR4 signals, through Gi-protein messengers, to activate Inositol triphosphate *IP3*, Protein kinase C *PKC* and the Janus kinase (JAK) and Signal Transducer and Activator of Transcription (STAT) cascade. The JAK/STAT pathway is associated with HSC (LSK CD150⁺ CD48⁻) proliferation (270). Transplanted human HSCs (CD34⁺ CD38⁻) can engraft in mouse BM, and subsequently proliferate there, because of this signalling (271, 272). This pathway is also conserved in the zebrafish (see 1.6.2.2).

While CXCL12 is an important ligand assisting HSC retention in the BM, data suggests that this protein pair is less important for BM homing (273). In the absence of CXCL12 and CXCR4, homing and retention of HSPCs (CD34⁺) appears to rely on very late antigen-4 (VLA-4; part of the β integrin family) and the endothelial expression of its receptor VCAM1 (273). Indeed, a number of β integrins (including VLA-5 and lymphocyte function associated antigen-1) appear to be involved in trans-endothelial migration and engraftment. Using antibodies to block these proteins on human CD34⁺ cells prior to transplant, partially or completely prevented engraftment in NOD/SCID mice (274).

In support of this, Levesque *et al.* found that elastase and cathepsin-G are released by neutrophils during G-CSF induced mobilisation and these enzymes cleave VCAM1, preventing VLA-4 binding and thus contributing to cell mobilisation (261). VLA-4 expression on hematopoietic cells (CD34⁺) is regulated by cytokines including SCF (275), GM-CSF (276), IL-3 (276) and TGF β (277). Thus, quiescence and mobilisation is regulated by cytokines.

In addition to VCAM1, human BM endothelial cells also express E and P- selectins important for the homing of transplanted cells (278). Mazo *et al.* took direct observations of migrating progenitor cells (FDCP-mix) rolling

along microvessels. They showed reduced rolling of transplanted cells in the absence of E and P selectins (using neutralising antibodies and knock out mice) (279). In addition, Rood *et al.* found that blocking antibodies against β integrins did not inhibit adhesion in culture unless E-selectin mediated interaction was blocked (280).

VEGF and placental growth factor (PlGF) are chemokines important for HSPC proliferation and migration (33, 281). Hattori *et al.* showed that VEGF-A binds to VEGFR1 on human and mice HSPCs (CD34⁺ and LSK respectively) increasing mobilisation of these cells into circulation. Inhibiting VEGFR1 prevented hematopoietic recovery after myelo-ablation with 5-FU (33). PlGF signals through VEGFR1 to promote migration of BM HSPCs. This signalling is dependent on MMP9 as PlGF was not able to induce proliferation in MMP9 knock out mice (33). In corroboration with this, Heissig *et al.* have shown that myeloablation by 5-FU causes an increase in CXCL12 which increased MMP9, followed by the release of soluble Kit-ligand and ultimately causing progenitor cell recruitment and migration (282).

More recently, sphingosine-1-phosphate (S1P) and its receptor (S1PR) have been found to be strong chemotactic signals for the recruitment of HSCs when vascular injury occurs (Figure 1.6). S1PRs are expressed by hematopoietic progenitors (CD34⁺ CD38⁻) (283) which sense S1P released by various cell types including endothelial cells and activated platelets (284). Indeed, mice deficient in S1P or its receptor have reduced HSPC (LSK) mobilisation (285). In injury, plasma levels of S1P increase during mobilisation due to activation of the complement cascade and membrane attack complex (286). Indeed, it has been proposed that a combination of CXCR4 antagonist and S1PR agonist would increase HSPC mobilisation when harvesting cells from donors which would avoid the use of G-CSF (287).

HA is a major component of the extracellular matrix and cell movement increases with exposure to or expression of HA while cell movement is inhibited by HA degradation or blocking of receptors. HA is expressed by human (CD34⁺ CD38⁻) and mouse (LSK; Lin⁻ Rho^{low} and LSK CD150⁺ CD48⁻) primitive hematopoietic cells (288, 289). Expression of HA was higher on HSCs found at the endosteal surface than on HSCs or lineage committed cells found in the central marrow (288, 289). Furthermore, CT-imaging of the femur from *Has3* knockout mice (in which HA levels are reduced) showed transplanted HSPC took longer to trans-endothelially migrate into BM (290). These findings suggest that HA is important for regulating cell distribution post-transplant.

CD44 is expressed on primitive hematopoietic cells (Lin⁻ CD34⁺) and endothelial cells. It binds to numerous components of the extracellular matrix including HA (291), collagen (292), fibronectin (293), and transmembrane receptors such as E-selectin (294). CD44 regulates homing and retention of HSCs in the spleen, foetal liver and BM (295). CD44 knock out mice, have significantly larger HSC (LSK CD150⁺ CD48⁻) and progenitor (LSK CD150⁺ CD48⁺) populations, when normalised to their size (as CD44 knock out mice are significantly smaller). Interestingly, migration from the foetal liver to the bone marrow was delayed by 2 days, correlating with an increase in CXCL12 over this period, in CD44 knock out mice and may be the reason for increased proliferation.

Transplantation studies found that the absence of CD44 in the recipient micro-environment did not affect homing and distribution of WT HSCs. However, absence of CD44 on transplanted HSCs (both mouse LSK CD150⁺ CD48⁻ (289) and human CD34⁺ (295)) reduced homing in WT recipients. This effect was accentuated in CD44 knock out recipients. Altogether, the data indicates that CD44 expression on HSCs is critical for lodgement and that the effect is worsened if CD44 is absent in the microenvironment.

Finally, Adams *et al.* have argued that calcium sensing is important for HSC retention and protecting HSC function. Mouse HSPCs (LSK) express the calcium sensing receptor (CaR). The HSC population in knockout mice (CaR^{-/-}) are normal in number and function but do not retain close proximity to the endosteal surface of the BM (267). However, when competitively transplanted, CaR^{-/-} HSCs contribute only 2.7 % of the hematopoietic system after 16 weeks while WT HSCs contributed to 84.6 % of BM. Furthermore, HSPCs that do not reside close to the endosteal surface are not functionally competitive against WT HSCs. These results indicate a role for calcium sensing or endosteal contact in maintaining the HSPC population and in post-transplant reconstitution of the BM.

1.6.1.5 Cell autonomous factors for HSC quiescence

Recently, Vionnie *et al.* published the results of a set of cleverly designed experiments that strongly indicated that much of the behaviour of hematopoietic cells is cell autonomous. They created a mouse strain they called “Hue” in which Cre-induced recombination of 20 cassettes (encoding fluorescent genes) resulted in a range of coloured hematopoietic cells. Individual clones in transplant recipients behaved as they had in the endogenous hosts (proliferation and lineage commitment) and in response to stress (LPS or total body irradiation). They found that clones identified with the same marker set (LSK CD150⁺ CD48⁻) but which behaved differently (proliferation rate and lineage bias) had distinct epigenetic patterning (296).

A number of transcription factors and proteins have been shown to regulate HSC cell cycle which, if absent will cause unregulated cell cycling and stem cell exhaustion (297). These factors include *mammalian target of rapamycin*, a regulator of protein synthesis and metabolism, and its negative regulators *promyelocytic leukemia protein* and *F-box-and-WD-repeat-domain-containing-7*. Deletion of either of these proteins increased cell cycling and proliferation which led to exhaustion of the population (298, 299).

Similarly, the forkhead box protein (FoxO) family is regulated by Akt and usually inhibits cell cycle entry. Loss of FOXO3a in mice increased cell cycling and apoptosis of HSCs and this is mediated by reactive oxygen species (ROS) (300). In zebrafish, foxo3b is mediated by *ELL associated factor 1*. When the transcription of *ELL associated factor 1* was knocked down by morpholino, the number of HSPCs (runx1⁺ and cmyb⁺) were significantly reduced, although knock down of foxo3b did not have an effect on HSPC number (155). This suggests that related proteins might compensate for the role of foxo3b in the zebrafish. Together this data suggests the FOXO family are regulators of HSPCs.

Activator protein (AP-1) transcription factor JunB is a transcriptional regulator of myelopoiesis that is linked to chronic myelogenous leukemia (CML). Interestingly, Passegue *et al.* found that cell autonomous overexpression of JunB in the LT-HSC led to a decrease in the number of LT-HSCs (self-renewal), while inactivation led to an expansion of LT-HSCs (301) and CML.

The global developmental regulator pre-B-cell leukemia transcription factor *PBX1* has also been shown to regulate quiescence. In a conditional knockout mouse, the quiescent stem cell pool is reduced and their sensitivity to 5-FU as well as their lower reconstituting potential in transplantation suggest an increased cycling leads to exhaustion (302). Likewise, the conditional knockout of the all 3 retinoblastoma genes (*Rb*, *p107* and *p130*) causes increased cell cycling of HSPCs and exhaustion (303). *Egr1* is an early transcription factor, highly expressed in HSCs and significantly down regulated in differentiated cells. Loss of *Egr1* causes HSCs proliferation, mobilisation from the niche and ultimately shortens their lifespan (304). Reduced *Egr1* correlates with increase in polycomb group protein BMI-1 which increases HSC self-renewal and restricts ROS production (305). Bmi-1 targets are frequently mutated tumour suppressors.

Lastly, E3 ubiquitin ligase Casitas B-lineage lymphoma (c-CBL), expressed in HSCs, also restricts their cycling activity (306). The absence of this gene causes an increase in the HSPC population, although HSCs from these knockout donors have increased repopulating ability in transplant assays.

1.6.2 The kidney marrow is the HSC niche in zebrafish

In the zebrafish, the kidney (and the mesonephros) is the primary site of hematopoietic stem cell maintenance. There are some similarities between mammalian BM and zebrafish kidney marrow. Firstly, both are vascularised so hematopoietic cells can distribute throughout the body. Secondly, both are populated by resident macrophages. Thirdly, both niches express retention factors such as CXCL12 (307). On the other hand, a major component of the BM is the endosteal niche which retains quiescent cells through osteoblast expression of factors (ANG1, SCF, N-Cadherin, TPO and OPN) and corresponding receptor expression (CD202b, cKit, N-Cadherin, CD110, alpha4-integrin and CD44) by HSCs. Although zebrafish are osseous (308), the kidney is considered distinct from these cells and therefore the role of bone cells has not been investigated. This contributes to a lack of research into the role of these cells to provide retention factors. However, orthologues of *Ang1* (*Ang2* in the zebrafish) (309), TPO (29, 84) and OPN (*spp1* in the zebrafish) (310) have been found to regulate ossification in the zebrafish. Furthermore, *Ang2* was found to be expressed in glomeruli of the developing embryo (309).

There are a number of reasons for using the zebrafish to study HSCs. Firstly, the conserved features between teleost and mammalian hematopoietic systems (see 1.3). Secondly, the optical transparency of zebrafish allows visualization of the cells in the HSC niche. Despite the obvious differences between the BM and kidney, human CD34⁺ HSCs have successfully homed to the kidney marrow of irradiated zebrafish suggesting that the zebrafish kidney provides an adequate niche for the recruitment of human HSCs (50). Humanised fish are only just being developed but there is hope that this model can be used to study *in vivo* behaviour of subsets of the CD34⁺

population. An analysis of these subsets could identify the best cell population for human therapy. Furthermore, comparative studies in mice and zebrafish should help identify key components/molecules for regulating HSCs.

1.6.2.1 Structure of the kidney

The kidney of the zebrafish shares many features (cell types and developmental patterning) with other vertebrate kidneys and serves to remove waste from the blood and to maintain osmotic balance, as well as maintain hematopoietic cells (311). The functional unit of the kidney is the nephros, made up of the glomerus and tubular epithelium. Blood flows through the glomerus, a net-like structure of specialised epithelial cells connected by adheren junctions, to be filtered for ions and small molecules (312). This filtrate is transported down the lumen of proximal and distal kidney tubules and drained by collecting ducts.

The pronephros is the first kidney to form during embryogenesis and is the functional kidney of larval zebrafish (311). It consists of two nephros with glomeri fused at the midline on the ventral side of the dorsal aorta (Figure 1.7). The tubular epithelium extending from the glomeri to the cloaca is subdivided into 2 proximal: proximal convoluted tubule, PCT and proximal straight tubule, PST; And 2 distal tubules: distal early, DE and distal late, DL (Figure 1.7). Both the PCT and PST express receptors that indicate a role in the absorption of salts, sugars and protein that pass the glomeri filters. The DE and DL are the diluting segments which help retention of sodium chloride and regulation osmotic balance. About 2 weeks post fertilisation (wpf), progenitor mesonephritic nephrons migrate around and along the length of the pronephros, and fuse with the pronephros forming the final adult kidney. The kidney continually generates mesonephros throughout the life of the fish (313).

1.6.2.2 Hematopoietic function of the kidney

In teleost fish, all lineages of hematopoietic differentiation are observed in the kidney, and thus, in this respect at least, the teleost kidney is thought to be the primary hematopoietic niche, equivalent to the mammalian BM (314). The kidney is the primary site of hematopoiesis and termed the kidney marrow (48). It is a flat organ that adheres to the dorsal body wall via connective tissues, in close contact with the osseous spine (315). The kidney can conceptually be segmented into the head, mid and tail. The head kidney has the fewest renal tubes, is the most vascularised section of the kidney and the section with the greatest hematopoietic activity (314). The mid and tail kidney sections are less vascularised and have lower hematopoietic activity (314).

Vascularisation of the glomerulus occurs relatively late, at 36-40 hpf. Time-lapse imaging shows HSPCs (cd41⁺ and cmyb⁺) migrate from the dorsal aorta, along pronephritic tubules, to the anterior glomeruli from 48-96 hpf (32) and seed the kidney. When migrating cells reach the glomeruli, CD41 expression is down regulated and differentiation marker CD45 is upregulated, suggesting the pronephros signals for HSC maturation (32). Blood filtration begins around 48 hpf and is well established by 4 dpf (316).

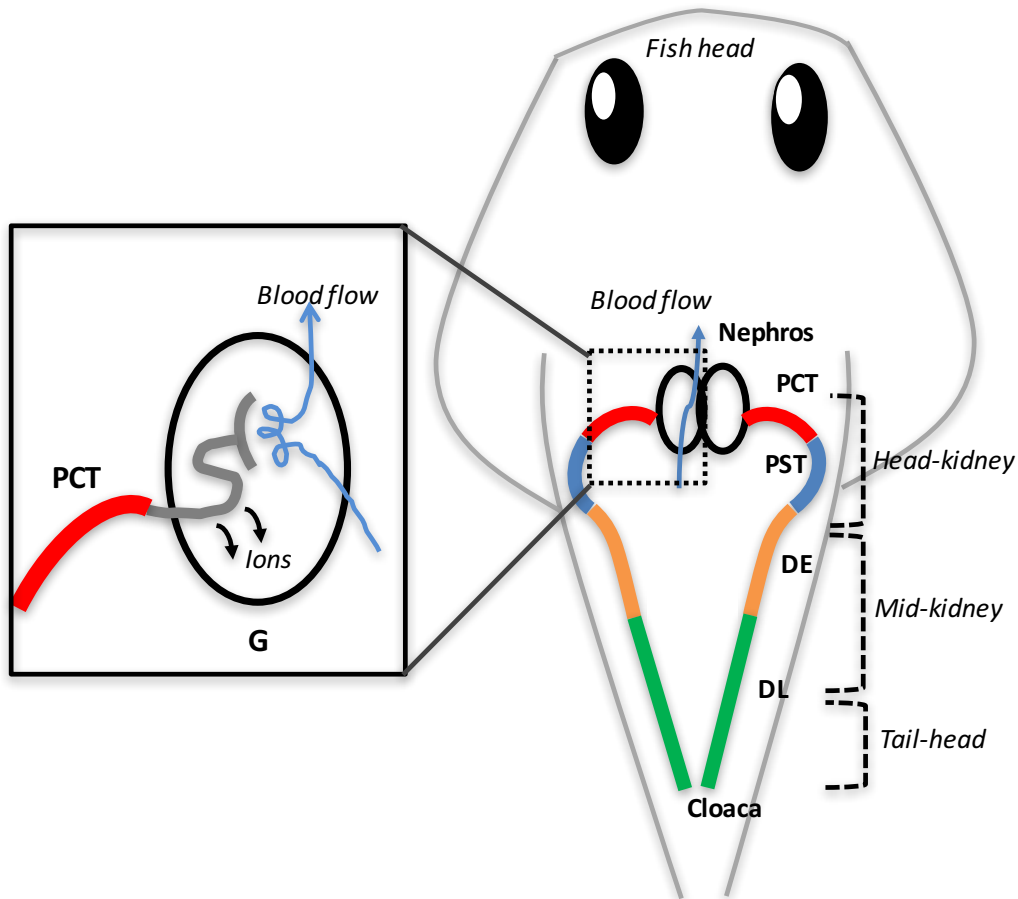


Figure 1.7: Structure of the embryonic zebrafish kidney. Dorsal view of the zebrafish. Blood flows through the glomeruli (inset, G) of the nephros where ions, sugar, and proteins are reabsorbed into the body. The waste solute is transported down the proximal convoluted tubule (PCT, red), into the proximal straight tubule (PST, blue), then through the distal early (DE, orange) and distal late (DL, green) structures to the cloaca. Hematopoietic stem cells migrate to and colonise the highly vascularized head kidney.

Not much is known about the signalling factors that retain HSCs in the kidney marrow and many of the factors found to regulate HSCs in the mouse BM has not yet been investigated. For instance, zebrafish have an angiopoietin orthologue (Ang2) which is expressed in the developing pronephric glomeruli (309) and thrombopoietin is expressed in the zebrafish but its expression pattern and function in the kidney marrow has not yet been studied (29, 84).

Zebrafish have 2 copies of the previously mentioned CXCL12 (see 1.6.1.4), with about 45 % nucleotide similarity with the human gene (317). Both *cxcl12a* and *cxcl12b* are expressed in kidney tubules, gills, and the skin (307). CXCL12a is the predominant form (317). As in mammals, it CXCL12a is a ligand for CXCR4, expressed by hematopoietic cells, and this coupling is important for regulating cell migration/retention in the BM. Although homing in *in vivo* transplant experiments was not affected when HSCs were pretreated with AMD3100 to block CXCR4, transplanting hematopoietic cells from *odysseus* mutants (which have a mutated form of *cxcr4b*) resulted in fewer homed cells (307). It would be interesting to know if CXCR4 could be used as a transgenic marker to distinguish thrombocytes and HSCs in the CD41⁺ population.

CXCL12 is also a ligand for the receptor CXCR7 (318). This alternative receptor is expressed on the surfaces of activated endothelial cells, on foetal liver cells and on some tumour cell lines (318). In zebrafish, this receptor was shown to regulate primordial germ cells migration as knockdown of CXCR7 alters the CXCL12a gradient and thus migration fails (319). This receptor has not yet been associated with hematopoietic migration but further research is required.

1.7 Adult HSC response to inflammation

The effect of inflammation signals on HSCs was previously addressed in relation to the role of cytokines (IFN γ , IFN ϕ , TNF α , IL-1 β , G-CSF) in regulating the size of the HSPC population during EHT and embryonic specification of HSCs (see 1.5.4). However, there is some indication that inflammatory cytokines can also profoundly affect the normal physiological population of HSCs in the adult. Systemic inflammatory insults such as cigarette smoke and diet have a significant impact on immunity and the immune system. A retrospective cohort study found that current smokers spent more days hospitalized post HSCT and had overall lower survival than previous smoker and non-smokers (320). *In vitro*, human HSPCs exposed to cigarette smoke extract had increased intra-and-extracellular expression of TLRs 2,3 and 4 and produced IL-8 and TGF β (321). Transplantation assays with mice found that recipients of BM cells from donor mice exposed to smoke for 14 days prior to transplant were normally reconstituted. However, when recipients were exposed to smoke post-transplant there was a six fold decrease in CFU ability of BM cells 12 days after transplant (322). High cholesterol diets have been associated with inflammatory diseases such as diabetes and cardio-vascular disease. Both obese mice and human patients have been found to have an increased number of circulating progenitors (CD34 $^{+}$ or LSK) (323, 324) and a pro-inflammatory (M1-like) macrophage profile. Both Singer *et al.* and Feng *et al.* have reported that the dietary priming of mouse HSCs (LSK CD150 $^{+}$) caused a myeloid bias output which was maintained in transplant recipients (324, 325). Together, this information advocates for an increased examination of how inflammation is affecting HSCs.

1.7.1 HSCs respond both directly and indirectly to infection

Mature immune cells are responsible for resolving infection and injury. Resident immune cells at the site of infection or injury use pattern recognition receptors (PRRs) to recognise pathogen-associated molecular patterns (PAMPs) and damage-associated molecular pattern (DAMPs) (326). Intracellular signalling cascades within these immune cells cause the production of type I IFNs, pro-inflammatory cytokines and chemokines. Type I IFNs stimulate hundreds of genes in infected cells and induce megakaryopoiesis and lymphopoiesis. Together IFNs and cytokines cause systemic inflammation. The secretion of chemokines recruits immune cells to the site of infection. After the wave of type I IFNs, stimulated T- and NK cells produce type II IFNs. These seem to bias progenitor differentiation towards myeloid production. Monocytes and neutrophils clear dead cells. Once the stimulus has been successfully cleared, the inflammatory response is then resolved, and the immune system returns to a homeostatic state. Dysregulation of the inflammatory response due to chronic inflammation has been linked to autoimmune diseases such as diabetes (327) and atherosclerosis (328).

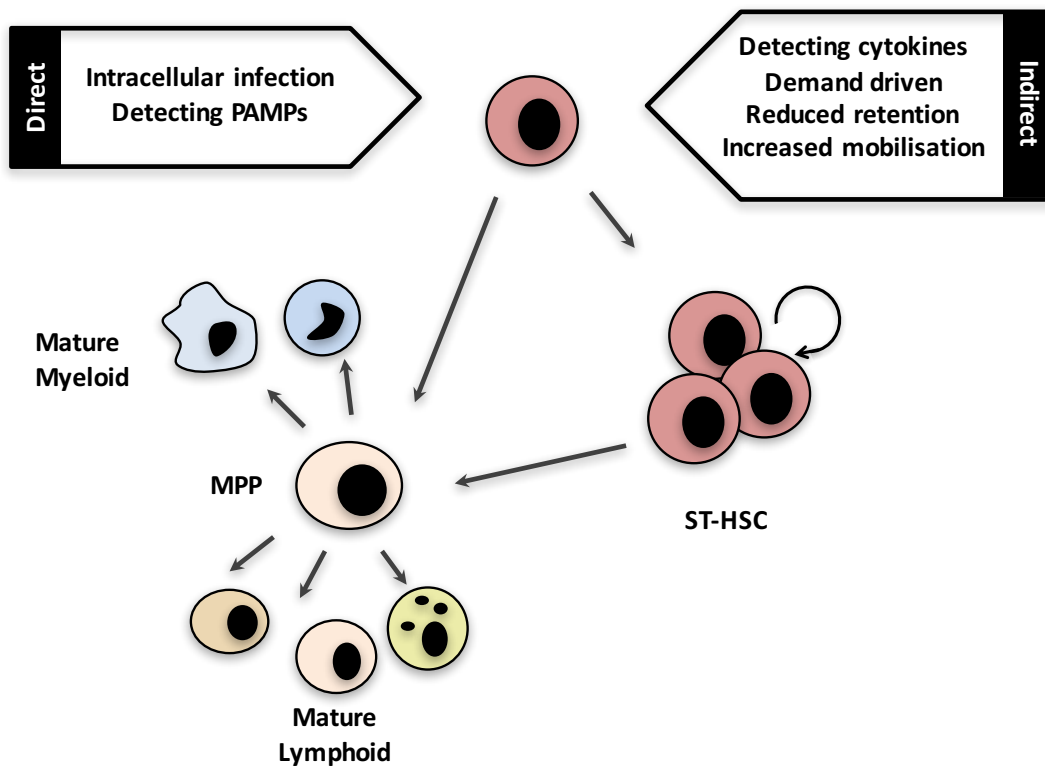


Figure 1.8: Direct and indirect HSC stimulation. HSCs respond to direct and indirect stimulation. Direct mechanisms include direct infection and direct recognition of a pathogen. Indirect mechanisms include cytokine stimulation, reduction in niche retention factors and increase in mobilization factors or responding to demand for mature cells.

Stimulatory effects on the mouse LSK compartment have been observed in viral, bacterial and candida models of infection (194, 329-331). In addition, zebrafish hindbrain infection with *Salmonella enterica* also caused emergency granulopoiesis and increased the HSPC population (cmyb⁺ and kdrl⁺ runx1⁺ cells) (332). There is growing evidence that HSCs can respond to infection either by direct or indirect mechanisms (Figure 1.8). Direct mechanisms include direct infection and direct recognition of a pathogen. Indirectly, HSCs can sense pathogens either by responding to cytokines, responding to niche signals or responding to demand for mature cells. Disruption to HSC quiescence through immune activation could have long term consequences for the hematopoietic system. Indeed, several studies indicate chronic inflammation can cause self-renewal and functional defects in the HSC population (76, 194, 333, 334).

1.7.1.1 Direct stimulation

There is some evidence that immature progenitors (CD34⁺) can be directly infected (Figure 1.8) (335, 336), although it is likely that the more primitive quiescent LT-HSCs are protected from direct infection (337). The consequence of a direct infection is that intracellular pathogens can severely alter cellular pathways which may lead to malignancies, either suppressing hematopoiesis or causing aberrant hematopoiesis.

The other mechanism by which HSCs respond directly to infection is through the recognition of PAMPs by PRRs (Figure 1.8). PRRs include TLRs and retinoic acid-inducible gene 1 (RIG-I)-like helicases (RLHs). Both human and mouse HSCs (CD34⁺ and LSK CD127⁻ respectively) express TLRs and the intracellular components necessary for transmitting the signals (338-341). Different TLRs have distinct effects on HSPC differentiation (342). For instance, TLR4 and TLR9 stimulated HSPCs differentiated into dendritic cells, while TLR3 stimulated HSPCs increased neutrophil production (343).

Of note, it has been shown that HSCs (Lin⁻ CD34⁺ CD38^{low}) express TLR3, 7 and 8 which are anchored in the endosomal membrane meaning they detect PAMPs in the cytosol (340, 344). It was shown that *in vitro* stimulation (TLR 1, 2, 7 and 8) prompted cell cycle entry and myeloid differentiation of both mouse and human HSCs (340, 342, 344). Furthermore, Megias *et al.* transplanted WT progenitors (LSK) into TLR knock out mice to show that proliferative response to TLR agonists *in vivo* was due to direct binding with progenitor cells (345). It should be mentioned however, that HSCs probably proliferate and differentiate quickly upon arrival into a transplant recipient. Therefore, it may be that the expansion of the HSC population was a secondary feedback response from the mature hematopoietic cells to TLR agonists rather than the transplanted progenitor cells. To test this, the proliferation of WT progenitors in TLR knock out recipients should be compared with the proliferation of TLR knock out progenitors (from conditional knock out mice in which only LSK cells lack TLRs) in TLR knock out recipients. Moreover, Takizawa *et al.* used prolonged LPS exposure to show that TLR4 activation of HSCs reduces HSC fitness in transplantations (346).

The expression of TLRs has not yet been shown on zebrafish HSPCs. However, a knock down of TLR4 and MyD88 in embryos decreased HSC production in the larvae, as determined by *runx* and *c-myb* expression (198). Furthermore, LPS stimulation of larval zebrafish causes emergency granulopoiesis (347) mediated by G-CSF, which expanded the HSPC compartment (increased *runx* and *c-myb* expression) (348). Thus, TLR binding does affect hematopoiesis in the zebrafish.

1.7.1.2 Indirect stimulation

HSCs also respond to infection and inflammation through indirect mechanisms (Figure 1.8). Both immune and non-immune cells secrete cytokines and chemokines in response to PRR ligation. For instance, LPS binding to TLR4 on peripheral hematopoietic cells causes signalling to the nucleus and transcription of NfκB, a transcription factor which regulates the production and release of numerous pro-inflammatory cytokines (IFNγ, IL-6, IL-1, TNF) (349-352). Cytokines such as type I and II IFNs, TNF and some interleukins (IL-1 and IL-6) appear to induce proliferation and differentiation of HSCs both during homeostasis and under hematopoietic stress (194, 353-355). HSPCs (CD34⁺) also express purigenic receptors and signalling components. Inflammation and infection can alter the production of adenosine triphosphate *ATP* and uridine triphosphate *UTP* which bind to purigenic receptors and influence HSC migration, proliferation and differentiation (264, 266, 356).

Human HSCs (CD34⁺) express a number of TNF receptors (357). In general, TNF has been shown to restrict HSCs *in vivo* in mice (358). However, transplants of HSCs from TNF-receptor knock out mice failed to engraft in recipients, suggesting that TNF and the 2 receptors may have a role in migration and homing (359).

It remains to be determined whether HSCs express the IL-1 receptor. However, there is evidence that the HSPC population responds to both acute and chronic exposure to IL-1 α and IL-1 β both *in vitro* and *in vivo* (107). While acute exposure triggers myeloid priming, chronic exposure (0.5 μ g daily for 70 days) did not appear to affect the size of the HSC (LSK CD135⁻ CD150⁺ CD48⁻) population but severely reduced self-renewal capacity in transplantation assays (107).

Type I IFNs bind to IFN- α receptor (IFNAR). Type II IFNs bind to the IFN- γ receptor (IFNGR). Both are expressed by hematopoietic progenitors (LSK) (76, 355). Indeed, quantitative Real-time Polymerase Chain Reaction (qRT-PCR) has found that HSCs (LSK CD135⁻ CD34⁻) express more IFNGR than T-, B- cells and granulocytes (355). IFN α has been shown to induce proliferation of dormant HSCs in mice and exhaustion of HSCs after 4-5 round of serial transplantation (76). Interestingly, HSCs (CD34⁻ LSK) produce the highest levels of IFN-2, a negative regulator of type I interferon signalling, indicating that preventing type I IFN signalling is important for maintaining this population (360).

Interferon gamma (IFN γ) is a Th1-type pro-inflammatory cytokine that directs innate and adaptive immune responses. Typically, IFN γ classically activates macrophages (cytokine production, phagocytosis, and antigen presentation to T cells) and drives the production of monocytes, monopoiesis. *In vitro* IFN γ drives monopoiesis bias by inhibiting G-CSF and thus lessening granulopoiesis. IFN γ can also impact the HSPC population by altering the macrophage population (334, 361). HSPC proliferation during *M. avium* infection is IFN γ dependent (355). McCabe *et al.* also found that HSPC differentiation was increased after IFN γ treatment, which did not occur in the absence of macrophages suggesting a role for these cells in influencing HSPC differentiation (257). They suggest that macrophages produce inflammatory factors in the niche that drive differentiation. Additionally, it has been suggested that the egress of macrophages recruited from the BM causes HSC proliferation and migration.

In short term methycellulose assays, cytokines SCF, GM-CSF and IL-3 all increased the adhesion of human CD34⁺ cells to HA in a CD44 dependent manner. This is an important indicator that cytokines could control proliferation and differentiation of hematopoietic progenitors, particularly in relation to cell engraftment (291).

1.7.2 HSC response to irradiation

The biological effects of radiation are complex and affect both cells directly exposed to the radiation, as well as cells not directly exposed to radiation (also called “non-targeted effects” or “bystander effect”) (362). In cells directly exposed to the radiation, the gamma rays energise the atoms of the cell *directly* causing cellular damage, and they produce ROS which *indirectly* cause damage to non-irradiated tissue, inducing a systemic response (Figure 1.9). Although there is a lot of evidence of damage in non-targeted cells, the mechanism of transmission

are complex and controversial (362). It is likely that multi-factorial pathways are involved and effects may depend on the dose and quality of exposure.

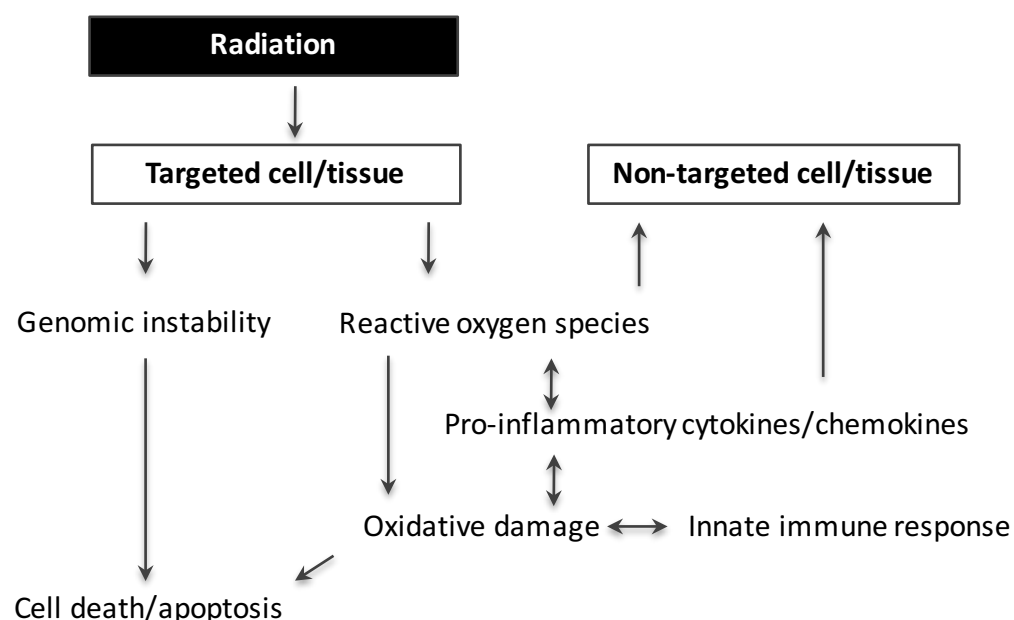


Figure 1.9: Ionising radiation causes systemic inflammation and cell death by multiple mechanisms. Schematic adapted from Kim *et al.* 2014. Arrows represent influence of one mechanism on another. Targeted cells have significant genomic damage and produce excessive reactive oxygen species. Together these cause cell death and apoptosis. Reactive oxygen species also induce pro-inflammatory cytokines and recruit immune cells. Together these have an effect on non-targeted cells, resulting in prolonged systemic inflammation.

Ionising radiation (IR) causes the most significant damage to DNA, causing single and double strand breaks in enough places as to make the repairs of the breaks impossible. The quantity of breaks (which induces apoptosis), as well as the high probability of mis-repair (which result in mutations), makes this damage the most important for cell death (363). There is evidence of genomic instability in both radiation targeted and non-targeted cells (364, 365). The damage done to DNA is most significant during mitosis, making rapidly dividing cells the most susceptible to radiation (363).

ROS are produced in excess following radiation and cause DNA damage as well as the oxidation of proteins and lipids (366). ROS have paradoxical roles in the cell. They are produced by mitochondria through the cytochrome C pathway and regulate genes that control various process of the cell (such as NFκB or hypoxia-inducible factor 1 alpha *HIF-1α*) as well as tumour suppressors (p53 and Phosphatase and tensin homolog, *PTEN*). ROS are derived through Nicotinamide-adenine dinucleotide phosphate (NADPH)-oxidase at the cellular membrane. They are also generated by the radio-lysis of water in the cell or produced by leukocytes.

Another indirect cause of tissue damage and cell death is the induction of inflammation through leukocyte production of pro-inflammatory cytokines and other chemokines (including IL-1, IL-6, TNFα and TGFβ (367-

369)) both immediately, and again later, post irradiation (366, 370). In addition to their pro-inflammatory effects, both TNF α and IL-1 also stimulate the secretion of MPPs. TNF α inhibitors have been used to mitigate radiation induced skin injury in mice (371). TGF β is considered to play a central role in mediating radiation-induced tissue fibrosis (372, 373).

Injured and dying cells are known to release pro-inflammatory DAMPs that can also stimulate inflammatory response. These molecular signals can affect non-irradiated cells, similar to a sterile inflammatory response such as wound healing (374). For instance, the clearance of radiation induced apoptotic cells induces the activation of macrophages and infiltration by neutrophils, contributing to an inflammatory cytokine storm (374, 375). Jaal *et al.* have shown increased activity of macrophages (CD105⁺) in irradiated skin tissue beginning shortly after irradiation and persisting for several weeks (376). This is supported by Jenrow *et al.* who have shown increased macrophage activity in the irradiated skin beginning 3 days after a single dose of 30 Gy in mice and persisting for more than 2 months thereafter even after the clearance of apoptotic bodies (377). In addition, macrophage recruitment from the BM is a factor that increases HSC migration and reduced homing and engraftment. Thus, it has been suggested that blocking phagocytic cell infiltration could mitigate irradiation injury and reduce systemic inflammation.

Typically, the endosteal region supports HSC quiescence. However, when recipients were irradiated before being transplanted with WT HSPCs, both Lo Celso *et al.* and Xie *et al.* saw proliferating HSPCs located nearer to endosteal regions. These results indicate that radiation damage of the microenvironment played a role in stimulating proliferation (121, 211). This may be because damage increased vascular signalling and attenuated inhibitory signalling from the endosteal compartment. It may also be that irradiation damage drove cell autonomous factors which can influence hematopoietic cell localisation. Indeed, Henninger *et al.* recently published that the clonal diversity of HSCs is significantly reduced by radiation (55). They used the *Zebrabrow* system to induce a unique heritable colour to each nascent cd41:GFP⁺ cell prior to transplantation into irradiated recipients. They found that clonal diversity was greater in the donor (at the time of transplant) than in the recipients 2.5-3 months post-transplant. This indicates clonal selection occurs in the irradiated environment.

1.8 Outstanding questions and the aims of this thesis

HSCs are a small but significant population of cells fundamental for hematopoiesis and the immune system. There are many outstanding questions that remain to be answered in order to improve cell therapies for cancer and autoimmune patients as well as to better understand the immune response to inflammation and infection.

Firstly, the definition of a HSC is straight forward, but identifying and selecting these cells from a heterogeneous mix of cells is difficult. How do cells within the sorted HSC (LSK CD150⁺ CD48⁻) population differ? Can this population be split into further subsets and how important are the differences between these subsets? In the zebrafish, the progenitor population is distinguished by *runx1*, *c-myb* and *cd41* expression. How does this population compare with mammalian HSPCs? Does the zebrafish HSPC population contain cells with distinct cell

cycle kinetics? Does a quiescent population exist and are these cells the long-term repopulating cells of the most pluripotent population? A primary objective of this thesis was to determine if this population exists using a label retaining assay. The hypothesis is that the cd41:GFP⁺ population can be split into label retaining cells with HSC qualities and non-label retaining cells which are HSPCs.

Secondly, there are questions about how the location of a HSC is influenced (the osteoblast versus vasculature debate), and how the location of HSCs can influence the environment. Examining this in homeostatic conditions has been difficult because HSCs are extremely rare, and so far, observations have been contradictory. If osteoblastic and vascular surfaces serve different support functions, this could explain why studies have not been able to pin down a specific cellular niche. This thesis will use observation of post-transplant migration and engraftment of HSCs to answer some questions such as: Where do transplanted HSCs migrate to following transplantation and is it important for HSCs to lodge into the niche (s) within a particular time frame for successful engraftment? Does subsequent expansion (proliferation of HSPCs and differentiation along myeloid and lymphoid lineages) depend on HSC biology prior to transplant? Do transplanted cells prioritise self-renewal or hematopoiesis? Does the recipient environment affect expansion of transplanted HSCs? Do HSCs differentiate in the primary niche or at secondary sites of hematopoiesis such as the thymus or spleen? The second objective of this thesis was to determine early post-transplant behaviour of transplanted cells using live optical imaging of transgenic labelled donor HSPCs in optically transparent recipients. In addition, could a method such as live optical imaging be a good predictor of engraftment and successful transplant, thus replacing survival as a readout (a severe protocol under UK Home Office Regulation) and significantly reduce animal suffering? The hypothesis is that if HSCs locate to the WKM niche soon after transplant will increase hematopoietic recovery. Furthermore, prioritising hematopoietic differentiation over self-renewal is a sign of hematopoietic stress, while prioritising self-renewal over differentiation is a sign of hematopoietic recovery. Therefore, HCT failure can be determined before recipient death.

Finally, there are many questions about how HSCs are affected by hematopoietic stressors (e.g. by infection or under inflammatory conditions). The current understanding is that, while HSC stimulation for hematopoiesis is considered beneficial during acute insults, prolonged and chronic activation can result in hematopoietic failure (in the mouse). It is not known whether this is true of zebrafish hematopoiesis but my hypothesis is that despite being highly regenerative, a similar failure in hematopoiesis will be observed. The final aim of this thesis is to determine if repeated stimulation can affect the ability of HSCs to reconstitute depleted recipient WKM. Recent research with the Zebrabrow system indicates the hematopoietic output (myeloid /lymphoid ratio) of cd41⁺ cells is conserved from donor marrow to transplant recipient marrow (55). Thus, transplant of cd41:GFP⁺ cells can be used to determine if HSC stimulation in the donor/recipient prior to transplant affects hematopoietic output in transplant recipients.

The hypothesis is that transplanted cd41:GFP+ HSCs will behave differently in the WKM, depending on whether they were stimulated prior to transplant or transplanted into an inflamed environment. In summary, the aims of this thesis were to:

1. Refine the zebrafish HCT protocol in order to observe transplanted cell behaviour *in vivo*.
2. Create an early post-transplant read-out that would predict HCT failure with the aim of reducing animal suffering with this protocol.
3. Examine whether repeated stimulation by hematopoietic stress /administration of inflammatory-inducers of donor hematopoiesis affects the post-transplant behaviour of transplanted HSPCs in the zebrafish
4. Examine whether a stimulated recipient environment affects the post-transplant behaviour of transplanted HSPCs in the zebrafish.

2 Materials and Methods

2.1 Zebrafish maintenance

Zebrafish were bred and maintained according to best practice and procedures conformed to UK Home Office Regulation (ASPA 1986). Details of the transgenic fish lines outline in Table 2.1.

Mutant/Transgenic	Advantage
Tra ⁻ Nac ⁻	Non-pigmented fish that are optically translucent. Ideal recipients as they allow single cells to be imaged in the live organism.
Tg(ubi:GFP)	All cells are express green fluorescent protein. Used as a marker of donor derived cells in recipient TraNac.
Tg(cd41:GFP)	Marks HSPCs (GFP ^{low}) and thrombocytes (GFP ^{high}). Used as a marker for sorting HSPCs for transplant and used as a marker of donor derived cells in recipients post-transplant.
Tg(runx1+23:GFP)	Marks HSPCs. Used as a marker for sorting HSCs for transplant and used as a marker of donor derived cells in recipients post-transplant.
Tg(lyz:dsRed)	Myeloid cells express dsRed. Used as a marker of donor derived cells in recipients TraNac.
cmyb ^{t12725}	Immuno-deficient fish. Ideal recipients as they do not require harmful pre-conditioning prior to transplant.

Table 2.1: Comprehensive list of the transgenic fish lines and their uses for the work throughout this thesis.

Embryos were initially reared in 50 μ L of methylene blue ($3 \times 10^{-5}\%$) (M9140; Sigma-Aldrich). At 5 dpf, larvae were transferred to 3 L tanks containing embryo growing medium (E2; 15 mM sodium chloride (S7653; Sigma-Aldrich), 0.5 mM potassium chloride (P9333 Sigma-Aldrich), 1 mM magnesium sulfate (M7506, Sigma-Aldrich), 150 μ M potassium phosphate monobasic (P5655; Sigma-Aldrich), 50 μ M sodium phosphate dibasic (S7907; Sigma-Aldrich), 1 mM calcium chloride (C5670; Sigma-Aldrich), 0.7 mM sodium bicarbonate (S6297; Sigma-Aldrich) and 10 U/mL penicillin streptomycin (15070-063; Life Technologies). At 12 dpf, larvae were moved onto the aquarium system, supplied by a combination of dechlorinated tap water and reverse osmosis water at a ratio of 1:5. Room conditions were kept at 28.5 °C with a cycle of 14 h light and 10 h dark. Fish were fed twice daily a combination of brine shrimp *Artemia*, larval dry food (ZM; Sytem) and adult dry food (Hikari Tropical Micro pellets; Hikari).

2.2 Genotyping fish lines

2.2.1 Obtaining genomic DNA from tail clip tissue

Tail clips were obtained from anaesthetised fish (founders were screened as adults >1 mpf; homozygous mutant recipients were confirmed with a tail clip after death) and DNA extracted from cells using “TaqMan sample to SNP” kit (Applied Biosystems). Genomic DNA was obtained according to manufacturer’s instructions, with the following adaptation: tissue was added to 10 µL of lysis buffer and boiled at 95 °C for 10 min. Samples were then cooled to room temperature and 10 µL of DNA stabiliser agent was added.

2.2.2 Identification of c-myb^{t25127} mutants

A mixture of fertilised eggs from c-myb heterozygote mutants was obtained from I. Hess (46). Fish were raised and heterozygote founders were selected by PCR. PCR reactions were set up using 1 µL of gDNA (as described in 2.2.1) with 10 µL 2X Taq PCR master mix (Qiagen), 1 µL of each primer (10mM; Fwd (TTTGAAGAAGCTTGAGGGTGA) and Rev (GTTTCAGGTGCCATTTCCACT)) and 7 µL of PCR grade water. Samples were run in a thermal cycler (Applied Biosystems) program: 94 °C for 4 min, 40 repeats of (94 °C for 30 s, 54 °C for 30 s, 72 °C for 1 min), finally 72 °C for 5 min. The PCR product was purified from the PCR reaction with the QIAquick (Qiagen) PCR clean-up kit according to manufacturer’s instructions. Samples were eluted in 30 µL elution buffer.

The t25127 mutation was detected by digesting the PCR product with restriction enzyme Hpy188iii (New England Biolabs). Purified samples (30 µL) were incubated 2 h with 4 µL of 10X buffer and 0.5 µL of Hpy188iii enzyme in a 37 °C water bath. Finally, samples were mixed with 4 µL orange loading dye (New England Biolabs) and loaded into a 2.5% agarose gel with ethidium bromide (1:1000) and run at 50 V for 30 min. This method could distinguish homozygote mutants (2 bands; 260 bp, 184 bp) from heterozygotes (4 bands; 260 bp, 184 bp, 146 bp, 114 bp) and homozygous WT (3 bands; 184 bp, 146 bp, 114 bp). For diagram see Figure 3.11.

2.2.3 Identification of transgenic runx1+23 fish

Fertilised eggs from Tg (runx1+23:GFP) and Tg (runx1+23:NLSmCherry) fish were obtained from O. Tamplin and L. Zon (42). Fish were raised and founders were selected by PCR. This method was also used to identify runx+ fish from crosses with Tg (lyz:GFP), Tg (lyz:dsRed) and Tg (ubi:GFP). PCR reactions were set up using 1 µL of gDNA (as described above) with 10 µL 2X Taq PCR master mix (Qiagen), 1 µL of each primer (10mM; Fwd (ACTGATAACGTGGGCAGCTT)) and Rev (GCTCTGCACTGCACTAAGGA) and 7 µL of PCR grade water. Samples were run in a thermal cycler (Applied Biosystems) program: 94 °C for 5 min, 35 repeats of (94 °C for 30 s, 60 °C for 1 min, 72 °C for 30 s), finally 72 °C for 7 min.

As the primers are specific for the enhancer sequence derived from the mouse genome, only Tg (runx1+23:GFP) and Tg (runx1+23:NLSmCherry) fish would produce a band. PCR reactions (20 µL) were mixed with 4 µL orange loading dye (New England Biolabs) and loaded into a 1% agarose gel with ethidium bromide (1:1000) and run at 50 V for 30 min. This method did not distinguish homozygotes from heterozygotes.

2.3 BrdU labelling

2.3.1 Labelling

BrdU labelling requires a “pulse” period of exposure and a “chase” period without exposure. The pulse was achieved either by intra-peritoneal injections or by dissolving BrdU in system water to make a solution to swim fish in. Fish received 8 intra-peritoneal injections of 5 µL every 12 h (BrdU; BD Bioscience; 10 mM solution in Phosphate Buffered Saline, PBS). Alternatively, BrdU (Sigma Aldrich) was dissolved in system water or embryo growing medium (E2) to make 100 mL volume of 0.5 mM, 1 mM, 5 mM, 10 mM, 20 mM and 40 mM to swim fish in.

2.3.2 Staining and flow cytometry

After the appropriate chase period, fish were humanely killed by anaesthetic overdose in 4 mg/mL tricaine (4 mg/mL; MS222; E10521; Sigma Aldrich) and brain concussion (secondary confirmation). The larvae body was dissociated in PBS containing 0.25% trypsin and 5 mg/mL collagenase P (Roche) at 37 °C for 30 min. Adults were dissected to analyse the WKM. BrdU was detected in WKM cells using the BD Biosciences APC BrdU Flow kit. In brief, cells were fixed in Cytofix/Cytoperm for 25 min on ice. Samples were washed with 1X Permeabilisation/Wash buffer and permeabilised in Permeabilisation Buffer Plus for 10 min on ice. Cells were washed and fixed again for 5 min at room temperature. Cells were washed again and incubated in 30 µg DNase (Invitrogen) at 37 °C for 1 h. Samples were washed and incubated with 5% goat serum in 1X Permeabilisation/Wash buffer for 20 min, washed with 1X Permeabilisation/Wash buffer and finally incubated with anti-BrdU antibody mouse monoclonal antibody (clone MoBU-1) Alexa Fluorophore 647 (1:50; Life technologies) and anti-GFP antibody conjugated with Alexa Fluorophore 555 (1:400; Life technologies). Stained cell samples were examined on a BD Biosciences LSR Fortessa cell analyser. Profiles were analysed using FlowJo v10.0.7.

2.4 Flow cytometry analysis of WKM populations

Flow cytometry analysis was done on single cell suspensions with BD LSR Fortessa (BD Biosciences). A minimum of 100 000 cells were acquired for WKM analysis. Data was analysed with FlowJo software (Treestar). Cell viability was assessed by resuspending cells in PBS -X with 1 µg/mL 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; D9542; Sigma) 2 min before flow cytometry analysis.

2.5 Transplants

2.5.1 Irradiation

TraNac, WT, Tg (runx1+23:GFP), Tg (runx1+23:NLSmCherry) and Tg (cd41:GFP) fish were pre-conditioned with γ -radiation with a ¹³⁷ Cesium source irradiator. Fish were contained in 30 mL system water and placed atop a 7.7cm spacer to place them centrally in the container and achieve a consistent dose rate of 3.65 Gy/min. Fish received two doses of 15 Gy (48 h and 24 h before transplant) or a single dose of 30 Gy (24 h before transplant).

2.5.2 Poly I:C treated recipients

Poly I:C treated recipients were anaesthetised with 0.17 mg/mL tricaine (MS222; E10521; Sigma Aldrich) and received 2 intra-peritoneal injections with a Hamilton syringe (30 Gauge) of 1 μ L containing 10 μ g poly I:C (P1530; Sigma Aldrich) 6 and 4 days prior to transplant. Control recipients were not injected. Both recipient groups were irradiated 48 h and 24 h prior to transplant.

2.5.3 Harvesting donors

Donors were selected for their particular requirements (Table 2.1), and in some cases donors were treated prior to transplant (described below). The whole kidney marrow was dissected from donors as described by Gerlach *et al.* (315). In brief, fish were killed by schedule 1 methods; anesthetic overdose in 4 mg/mL tricaine (MS222; Sigma Aldrich) and brain concussion (secondary confirmation). The thorax was opened ventrally using dissection scissors and the cavity organs removed. The kidney was removed from the dorsal wall using fine forceps and transferred to a 40 μ m nylon filter (Falcon). A single cell suspension was obtained by filtering through with ice cold PBS-X (0.9X PBS (Sigma-Aldrich), 2% fetal calf serum (Gibco)). Usually 4 donor cell samples were pooled.

2.5.4 Sorting donor WKM cells

The single cell suspension was sorted, at 2,000 events/s, using a BD FACSAria III operated by C. Simpson, J. Srivatava or J. Rowley (ICL, London) to obtain the population of interest. WKM cells were gated to exclude debris (using forward and side scatter, both use 488 nm laser, 480/10 filter). Cells were then split by GFP (488 nm laser, 490/20 filter) and dsRed (561 nm laser, 610/20 filter) or mCherry (561 nm laser, 480/10 filter) fluorescence and gates set to sort. Cells were sorted into PBS-X at 4 °C.

Carrier cells were obtained from WT fish. Fish were killed by anaesthetic overdose in 0.17 mg/mL tricaine (MS222; Sigma Aldrich). The heart was punctured with a 10 μ L pipette tip containing PBS-X with heparin (0.9X phosphate buffered saline (Sigma-Aldrich), 2% fetal calf serum (Gibco), 1000 U/mL heparin (H3393, Sigma-Aldrich)). The PBS-X with heparin was dispensed into the cavity and then the peripheral blood aspirated from the heart region while massaging the fish. The concentration of donor cells (sorted and carrier) was determined using a haemocytometer ($\times 10^4$ cells/mL).

2.5.5 Poly I:C donors

Tg (cd41:GFP;lyz:dsRed) donors were anaesthetised with 0.17 mg/mL tricaine (MS222; E10521; Sigma Aldrich) and received 8 intra-peritoneal injections of 5 μ L at 48 h intervals using a Hamilton syringe (30 Gauge). Controls were injected with 0.9% saline (9 g NaCl/L, Sigma-Aldrich) and poly I:C donors were injected with 10 μ g poly I:C (P1530, Sigma-Aldrich).

2.5.6 Retro-orbital hematopoietic cell transplantation

Transplants were carried out by retro-orbital (RO) injection as described by Pugach (49). In brief, the recipient is anaesthetised with 0.17 mg/mL tricaine (MS222; E10521; Sigma Aldrich) and placed laterally on a sponge. The needle is inserted at a 45 angle between the eye ball and socket and situated near the heart. Initially, adult fish were transplanted with 4-6 μ L of Tg (ubi:GFP) WKM suspension mixed with 20 μ g dextran conjugated to tetramethylrhodamine (TRITC; 120kDa), using a Hamilton syringe (30 Gauge, 5 μ L). Subsequent transplants were carried out with specific quantities of cells using an IM 300 micro-injector (Narishige, Japan). Micro-injector needles were pulled from glass capillaries and cut to size to obtain an injection volume of 1 to 1.2 μ L.

2.5.7 Survival

Transplants recipients were recovered from the transplant procedure in system water. Fish were separated into individual tanks, in system water but off the system, once imaging and tracking began at 4 days post-transplant (dpt). The water was changed every 2 days and individual fish were monitored for adverse signs until they were harvested at 28 dpt. In order to reduce suffering, fish showing adverse effects (lack of colour, oedema, gasping for air) were monitored more frequently and humanely killed with anaesthetic overdose (4 mg/mL tricaine; MS222; E10521; Sigma Aldrich) if the symptoms persisted > 2 h. Data from these fish was stored and included in the analysis.

2.6 Histology

Fish were killed by overdose of tricaine (4 mg/mL; E10521; Sigma-Aldrich) and fixed in 4 % paraformaldehyde (PFA; 18814; Polysciences Inc) at 4 °C overnight. Fish were positioned in a 3 % agar mount and transferred to 70 % ethanol. Samples were processed through increasing gradients of ethanol and paraffin and finally embedded in paraffin blocks. Sections (4 μ m) were cut with a Leica rotary microtome RM2235, mounted on slides, deparaffinised in xylol and stained with H&E on a Leica ST5020-CV5030 workstation by Lorraine Lawrence (Department of Medicine, ICL, London).

2.7 Cytospin

Live or fixed cells (with 4 % PFA; 18814; Polysciences Inc) were sorted with a Fortessa Aria and cytospun onto glass slides at 300 rpm for 3 min. Cells were stained with Wright Giemsa.

2.8 Imaging

In vivo imaging of the recipients was done using a wide field Zeiss Axiovert 200 inverted microscope. Optical depth was not calculated; only “in focus” fluorescent cells were counted. Fish were anaesthetised with 0.17 mg/mL tricaine (E10521; Sigma-Aldrich) and placed on a 4 cm petri dish. Data was collected using the 20X objective (LD Plan-Neofluar 20x 0.40 KORR). Fluorescence was observed using a filtered halogen lamp (Zeiss HAL 100) and Zeiss filter cubes: GFP (excitation 470/40, emission 525/50) and dsRed (excitation 562/40, emission 624/40). Pictures were taken by Hamamatsu ORCA-ER camera using Velocity software and processed with FIJI.

2.9 Gene expression by qRT-PCR

2.9.1 mRNA extraction

Gene expression was measured in the whole organism or in dissected organs such as the gill, spleen and WKM. Samples were collected and mRNA was extracted using the RNeasy mini kit (Qiagen) as per manufacturer’s instructions. In brief, the sample was homogenised in RLT buffer with a Pestle-Motar Mixer (Argos Technologies) and frozen overnight. Samples were thawed and spun down to remove non-macerated tissue. Samples were mixed with an equal volume of ethanol and then spun through a silica membrane. The membrane was washed with RW1 and RPE solutions. The filter was then spun to dry and the mRNA eluted with 30 μ L of elution buffer. Concentration was measured by spectrophotometry (Thermo Scientific Nanodrop 1000).

2.9.2 cDNA synthesis

mRNA was converted to cDNA using High Capacity cDNA Reverse Transcription kit (ThermoScientific) according to manufacturer’s instructions. In brief, 125 ng mRNA was mixed with dNTPs, random primers, and TaqMan master mix. Reactions were run in a thermal cycler (Applied Biosystems) program: 25 °C for 10 min, 37 °C for 120 min and then 95 °C for 10 min. cDNA was diluted 5 times (80 μ L added to 20 μ L reaction).

2.9.3 Relative gene expression

mRNA levels were measured using TaqMan® Probe-Based Gene Expression Assay (Applied Biosystems) or SYBR Green fast Assay (Applied Biosystems). Each Taqman Probe reaction takes 1 μ L of cDNA, 0.5 μ L of Taq fast primer/probe (Table 2.2), 5 μ L Master mix and 3.5 μ L qRT-PCR grade water. Each SYBR primer reaction takes 1 μ L of cDNA, 0.5 μ L of forward primer, 0.5 μ L of reverse primer (Table 2.2), 5 μ L Master mix and 3 μ L qRT-PCR grade water. Expression of *18S* was used as an internal control. All reactions were run in duplicate and the mean

value used (difference between duplicates <1.2 Ct). The qRT-PCR program ran 95 °C for 20 s, and then repeated [95 °C for 30 s, 60 °C for 30 s].

The $\Delta\Delta CT$ method used to measure relative differences in gene expression. In brief, cycle threshold (CT) values were obtained at a threshold of 0.2. The CT value of the gene-of-interest was subtracted from the 18S Ct value to normalise CT values between samples (ΔCT). Relative quantification was obtained by subtracting the median control sample ΔCT value from all the experiment's ΔCT values ($\Delta\Delta CT$). Finally, the fold change was calculated by $2^{-\Delta\Delta CT}$.

2.10 Statistical analysis

All statistical analysis was done using Graphpad 7.0 software (Graphpad software, CA, USA). Normal distribution was tested with the D'Agostino-Pearson omnibus test. The student t-test was used to compare 2 groups with Welch's correction if the standard deviation was significantly different. The Mann-Whitney test was used where data was non-parametric. One-way Analysis of Variance (ANOVA) with Tukey's multiple comparison test was used to compare more than 2 groups. Kruskal –Wallis test with Dunn's multiple comparison test was used if the data was non-parametric. Repeated Measures ANOVA was used to compare treatments over time. Significance is demonstrated by right-up-arrow with time (4, 6 and 8 dpt) on the x-axis and treatment groups (poly I:C and control) on the y-axis. Interaction in the angle. Pearson's correlation coefficient was used to test correlations between X and Y factors (usually number of cd41:GFP+ vs number of lyz:dsRed+). Mantel-cox test, also known as Log rank test, was used to compare survival.

P values less than 0.5 were deemed significant. Non significance was marked *ns*, p values as * $p < 0.5$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Error bars indicate standard deviation (SD).

Gene	Assay	Species	Identification code/ Sequence
18S	TaqMan	Eukaryote	4319413E
il1 β	TaqMan	Zebrafish	Dr03114368_m1
tnf α	TaqMan	Zebrafish	Dr03126850_m1
ifn ϕ 1	TaqMan	Zebrafish	Dr03100938_m1
lfny1.1	TaqMan	Zebrafish	Dr03109489_g1
lfny1.2	TaqMan	Zebrafish	Dr03081923_m1
cxcl-c1c	TaqMan	Zebrafish	Dr03436643_m1
cxcl12a	TaqMan	Zebrafish	Dr03119119_m1
l-plastin (lcp)	TaqMan	Zebrafish	Dr03099284_m1
mpx	TaqMan	Zebrafish	Dr03075659_m1
gata1	Taqman	Zebrafish	Dr03086722_m1
mpeg	Taqman	Zebrafish	Dr03439207_g1
runx1	Taqman	Zebrafish	Dr03074179_m1
c-myb	Taqman	Zebrafish	Dr03432766_m1
csf1ra	Taqman	Zebrafish	Dr03125163_m1
mFAP4	Taqman	Zebrafish	Dr03452550_g1
il8	SYBR	Zebrafish	F: 5' – TGTGTTATTGTTTTCTGGCATTTC – 3' R: 5' – CTGTAGATCCACGCTGTCTGC – 3'
il6	SYBR	Zebrafish	F: 5' – TCAACTTCTCCAGCGTGATG – 3' R: 5' – TCTTCCCTCTTTCTCCTG – 3'
lck	SYBR	Zebrafish	F: 5' – ACGCCGAAGAAGATCTC – 3' R: 5' – GCTTGGGGCAGTTACA – 3'

Table 2.2: Comprehensive list of the Taqman probe identification codes and SYBR primer sequences used for the work in this thesis.

3 Refinement of hematopoietic cell transplantation in zebrafish

3.1 Introduction

This chapter presents work establishing the zebrafish transplant protocol which allows visualisation of kidney marrow colonisation and engraftment by donor cells. This work builds on the decade of zebrafish transplant studies which have been important for the understanding of hematopoiesis but have not taken advantage of the *in vivo* imaging potential that this model provides. This chapter sets out to establish a transplant model that would allow early post-transplant cell mobilisation to be quantified and to determine if this can be used to predict recipient survival and immune reconstitution. Determining early markers of recipient recovery is an important goal as it will significantly reduce the severity of this protocol.

3.1.1 *In vivo* monitoring of transplanted hematopoietic cells

Mammalian models have used transplantation experiments to identify HSPC populations and mechanisms regulating hematopoiesis for over 50 years (2). HCT can be used to test the self-renewal and differentiation capacity of hematopoietic cells. Despite decades of research, the early post-transplant processes in the irradiated recipient are still poorly understood. It is still unclear whether and how post-transplant cell behaviour affects the success of transplants.

Although the order of events is not clear, it is thought that transplanted HSCs localise to hematopoietic sites, engraft in the tissue, self-renew and differentiate. Due to technical obstacles, most of these events have not been observed *in vivo* and therefore there is a limited understanding of the events after transplantation. For instance, it is unclear whether transplanted HSCs begin to differentiate in the circulation or whether this occurs after engraftment in the hematopoietic niche.

The first attempt to study the post-transplant spatial distribution *in situ* was by Nilsson *et al.* in 2001 (119). They labelled sorted cell populations with the cell fluorescent dye CFSE and transplanted them (0.8×10^5 to 3×10^5 cells, depending on population) into recipient mice. Longitudinal sections of the recipient mouse femur were analysed to determine labelled cell proximity to the endosteal (<12 cells from endosteum) or central cavity (>12 cells from endosteum). They found that both Lin⁺ and Lin⁻ population were evenly distributed throughout the femur at 1 hpt. However, significantly more (~72%) of HSCs (Lin⁻ Rho^{dull}) were found in the central marrow region at this time. Interestingly, over the following 5 h, over half of the HSC population was found nearer to the endosteal surface, while only 30% of the more mature Lin⁻ population (Lin⁻ Rho^{high}) were in this region. Together this study suggests that transplanted HSCs prefer the endosteal region while mature populations distribute around the central marrow region. These findings are supported by previous evidence that maturing hematopoietic cells consistently distribute in specific locations. However, it is important to note that the *in situ* results presented by Nilsson *et al.* were done using non-ablated recipients in order to keep an intact micro-

Refinement of hematopoietic cell transplantation in zebrafish environment. However, this meant that none of the transplanted cells would engraft. It is also important to note that these results could not provide information about how this distribution affected the success of transplants because mice were humanely killed to obtain the data.

In order to correlate post-transplant cell behaviour with transplant success, real-time *in vivo* imaging of the bone marrow is required. In 2003, Askenasy and Farkas described a method for tracking Lin⁺PKH-labelled cells *in vivo* using an imaging window over the distal femoral epiphysis (120). This was the first example of real-time monitoring of hematopoietic cells post-transplant, although, the distribution of transplanted cells was not described. More recently in 2009, both Xie *et al.* and Lo Celso *et al.* imaged post-transplant behaviour of hematopoietic cells in mice.

Xie *et al.* isolated and transplanted 2,000 HSCs (LSK CD135⁻) from β -actin promoter driven GFP transgenic mice and transplanted these into irradiated and non-irradiated recipient mice. They used 2 photon microscopy (to image cell behaviour *ex vivo*) in conjunction with longitudinal sectioning (to take measurements of cell distribution). They found that HSCs were more likely to associate with the endosteum (<16 μ m) in the irradiated recipient bone marrow than in non-irradiated recipients. By staining the longitudinal sections, they found GFP expressing HSCs were in direct contact with MSCs. Again, early post-transplant cell behaviour was imaged, this time in irradiated recipients. However, again these results could not provide information about how this distribution affected the success of transplants because mice were humanely killed to obtain the data.

The first live imaging was published by Lo Celso *et al.* in 2009. Recipient osteoblast reporter mice (Col2.3:GFP) were transplanted with HSCs (LSK CD48⁻ CD135⁻) labelled with DiD. The recipient mice were fitted with an imaging window over the calvarium (top of the skull) and imaged with a two-photon hybrid microscope which rendered a 3-D image of the bone and vasculature. They found that in non-conditioned WT recipients, HSPCs were always > 30 μ m from endosteum surfaces, and it was known that these cells would not engraft. However, in irradiated recipients, they noted that single cells were located closer to the endosteum, while clusters (>3 cells) were further away. By combining DiD and DiI labelling of transplanted cells, they also revealed that the clusters of HSPCs observed in the marrow are due to the proliferation of single cells, and not the clustering of cells transplanted together. They also used recipient osteoblast reporter mice (Col2.3:GFP) with a c-Kit mutation (125). The c-Kit mutation impairs endogenous HSPCs to engage with the niche, thus allowing transplanted HSCs to outcompete and engraft in the recipient without chemotherapy or radiation (228). They showed that, in this intact micro-environment, transplanted cells were within 16 μ m of vasculature and within 20 μ m of endosteal surfaces. These results provided significant data about the early post-transplant behaviour of transplanted HSCs in the recipient. However, the experimental setup meant that this behaviour could not be correlated with transplant outcome in the same recipient. Firstly, the invasiveness of the procedure means that the mice would not be recovered after imaging. Secondly, the experiments involved transplantation of a larger number of HSCs (5×10^3 to 15×10^3 cells) in order to consistently observe 3 to 15 cells in the field of view within 20 minutes of

Refinement of hematopoietic cell transplantation in zebrafish

transplant. This guaranteed the recipients were rescued and therefore there would be no behavioural data for unsuccessful transplants.

3.1.2 Early post-transplant behaviour of hematopoietic cells in zebrafish

As mentioned in the introduction chapter 1.2.2, the zebrafish model has made significant contributions to the hematopoietic field (40, 150, 378). One of the major advantages of the zebrafish is the non-invasive imaging potential, particularly in embryos and juveniles (see Table 1.2). For this reason, zebrafish have become an attractive tool to address the question of early post-transplant behaviour and its effect on recipient outcome.

Until now, the visualisation of donor cells in the recipient fish has been quite basic. In the first attempt, by White *et al.*, 2×10^5 WKM cells from Tg (β -actin:GFP) with ubiquitous GFP expression were transplanted into transparent (Casper) recipients and it was found that it took 2 weeks to visualise GFP+ cells in the WKM of adult recipients (379). Casper and TraNac zebrafish lines have been used as recipients (Table 1.2) as they are optically transparent (48, 379-381). However, as they grow into adult fish, the tissues and organs scatter light which interferes with image acquisition and therefore limits their use. Thus, in this thesis, larvae are used for image acquisition and adults are harvested for flow cytometry.

More recently, Li *et al.* showed they could measure *relative engraftment* as a ratio of fluorescence intensity from competing donor populations *in vivo* in adult recipients 4 wpt (381). They co-transplanted WKM from ubiquitous GFP and DsRed2 expressing donors (Tg (β -actin:GFP) and RedGlow respectively) and calculated the “relative engraftment capability” as the ratio of GFP/DsRed2. This method is useful for comparing the relative fitness of 2 populations of cells, but does not reveal when HSPCs arrive in the niche or the behaviour of single cells in the recipient.

Some of the best images of transplanted cells in recipients come from embryos at 48 hpf which were transplanted with Tg (gata1:dsRed; β -actin:EGFP) in which gata1:dsRed+ erythrocytes and non-erythroid β -actin:GFP+ cells could be seen circulating in the blood vessels 1 day after transplant (48). One of the reported advantages of using embryo recipients is that they have not yet developed their adaptive immune system and therefore are not pre-conditioned with irradiation. However, studies suggest that lymphoid progenitors colonise the thymus from 3 dpf, even if they are not mature until 3 wpt (96, 176, 177). In this thesis, adult recipients were used in order to study transplantation in a fully mature immune system with the requirement of preconditioning with *gamma*-radiation.

3.1.3 The effects of irradiation

Radiation is used as a hematopoietic-ablative therapy for the treatment of certain cancers and autoimmune diseases in order to kill unwanted malignant cells. For HCT, immuno-ablation seems to be important for allowing transplanted cells to be in contact with niche stroma and engraft (121). Indeed, CXCL12 increases in the

Refinement of hematopoietic cell transplantation in zebrafish

irradiated host (24 to 48 h), and this contributes to cell homing and engraftment (245). Furthermore, Weiss *et al.* found that recipient mouse survival improved if cells were transplanted 24 h or 48 h after irradiation instead of straight away (382). The authors propose that this delay allows the establishment of adequate niche space. However, radiation-induced toxicity is an important factor in the recovery of recipients. Gamma-rays of energy cause irreparable DNA breaks (363-365), initiate reactive oxygen species (366), and pro-inflammatory cytokines and chemokines (367-369) which result in progressive damage after exposure. The efficacy of gamma-radiation to kill cells and an increase in non-target effects has been associated with temperature (383, 384). This may explain why mammals with a body temperature of 37 °C are treated with 9 Gy, and zebrafish with a body temperature of 28 °C receive the much higher dose of 30 Gy. The higher radiation dose in zebrafish may have an influence on the non-target effects in the two models.

The effect of irradiation on zebrafish have been studied in regards to the depletion of immune cells in the WKM and the survival of irradiated fish (135). Traver *et al.* showed a significant decrease in lymphocytes and precursors 24 h post irradiation (hpIR) with 20 Gy, with populations beginning to recover 8 days post ionizing radiation (dpIR) and recovering to pre-irradiation levels by 14 dpIR. The inflammatory response to irradiation has not been investigated in zebrafish. Irradiation induced inflammation is potentially a confounding factor when studying cell transplantation and the role of the niche in the successful (engraftment and long term reconstitution) transplantation of cells and therefore warrants further examination.

3.2 Aims

The aim of this thesis is to assess the response of HSCs under conditions of inflammation. HSCs will be tested using transplantation experiments. The aim of this chapter is to report the foundation experiments that will establish a valuable transplant and imaging procedure for the rest of the thesis.

More specifically, the aims were to:

1. Prepare a protocol for the preconditioning of transplant recipients and examine the effect of the protocol on cell depletion and production of pro-inflammatory signals.
2. Optimise a technique for the visualisation of transplanted cells.
3. Determine if hematopoietic reconstitution and recipient survival can be predicted from early post-transplant cell behaviour.
4. Identify a LT-HSC population in the zebrafish by labelling slow cycling hematopoietic cells.

3.3 Results

3.3.1 The effect of radiation on zebrafish

HSC “stemness” and function is assessed by its ability to reconstitute the immune system of an hematopoietic-depleted host. For HCT, recipients are treated with sub-lethal doses of irradiation in order to deplete resident hematopoietic populations and allow donor cells to engraft. Previous work has suggested that the dose of irradiation should be assessed for specific zebrafish lines as the sensitivity of lines varies greatly (47).

The major blood populations of the WKM can be distinguished simply using forward (directly proportional to cell size) and side scatter (a measure of cell granularity) as measured in flow cytometry (48). This was used to determine the proportion of erythrocytes, lymphocytes, myelomonocytes, and precursor cells in the WKM following treatment. Cells most radio-sensitive would decrease in percentage and cells most radio-resistant would proportionally increase in percentage.

3.3.1.1 30 Gy depletes populations in the kidney marrow

Initially, WT fish were used to establish if a single dose of 30 Gy or a split dose (twice 15 Gy, 24 h apart) would be most effective in depleting the WKM. Fish were irradiated and the WKM was harvested 5 dpIR (would be 4 days after transplant) for flow cytometry (Figure 3.1A). The results showed that both protocols caused a significant decrease in populations of the WKM following exposure to 30 Gy irradiation (Figure 3.1B, C). The lymphocyte, myelomonocyte, and precursor populations decreased significantly (Figure 3.1B, C). and are therefore the most sensitive to irradiation. The erythrocyte population proportionally increased relative to these 3 populations (Figure 3.1C). In irradiated samples, there is a greater percentage of events that do not fall within the boundaries (gates) of the 4 main populations, indicating that these events are “debris” as they do not have the scatter properties of the cells typically in the WKM. It is unclear whether this debris is evidence of non-hematopoietic kidney marrow cells (ie. tubular, vascular, or epithelial cells) more prominent in the sample because the hematopoietic cells of the WKM are gone; or perhaps the debris is evidence of irradiation damaged WKM cells that have changed shape as they die. In addition, the erythrocyte gate can be contaminated with debris that scatters in the top left quadrant. To assess whether an increase in erythrocytes is real, or whether it reflects an increase in debris, WKM cells were cytopspun onto slides and stained with Wright-Giemsa (Supplementary Figure 7.1). All 4 populations were significantly under represented as seen in the flow cytometry profiles, and the majority of recovered events appeared to be enucleated and cytoplasm rich cell structures. The number of erythrocytes could not be quantified accurately, but qualitatively these cells were not as abundant in irradiated samples.

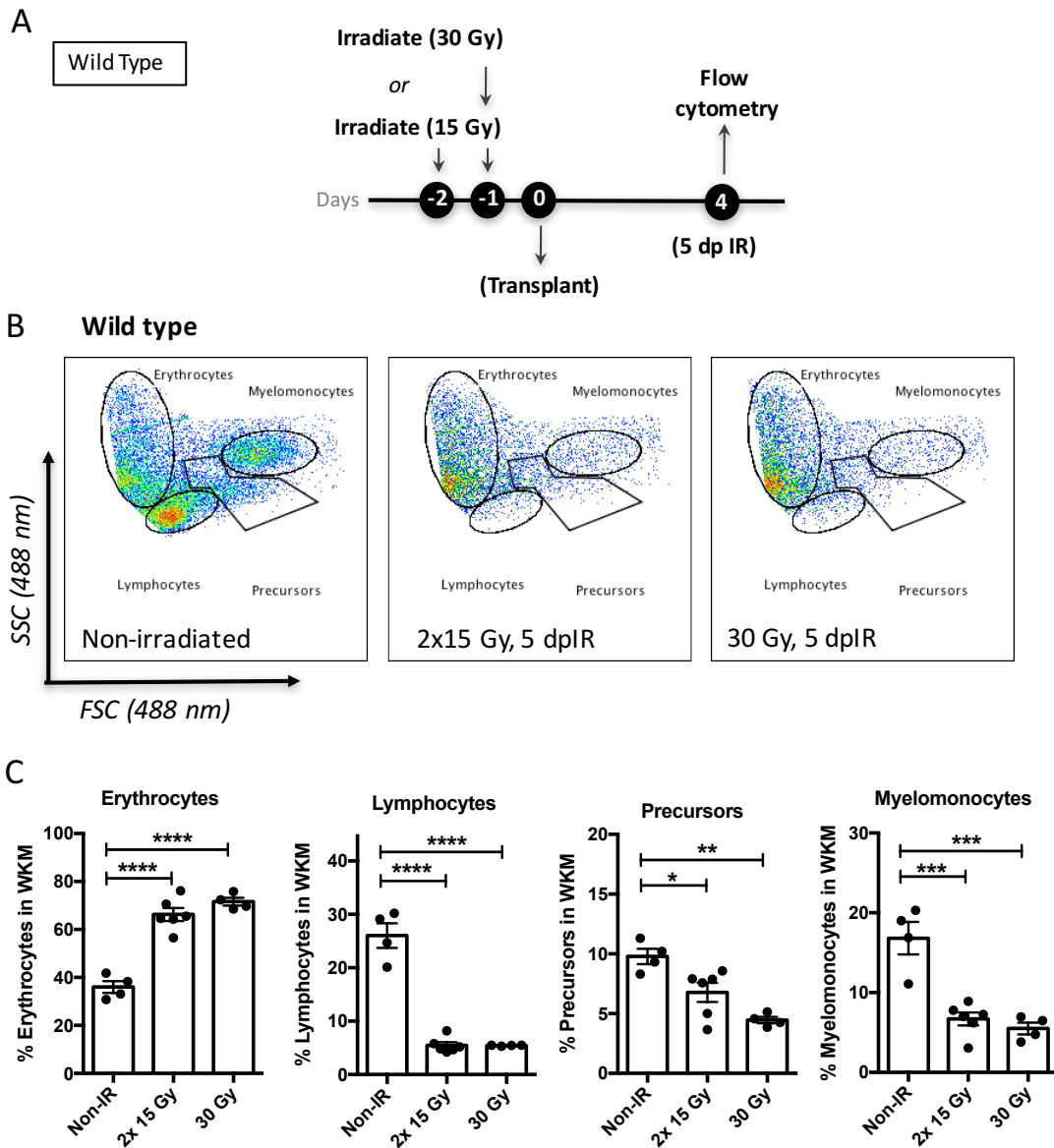


Figure 3.1: Depletion of cells in the wild type kidney marrow by irradiation. WT fish were treated with 2 doses of 15 Gy or 1 dose of 30 Gy and the WKM was harvested after 5 days. (A) Procedure overview. This protocol does not involve transplantation but (Transplant) on day 0 indicates when these irradiated fish would usually receive a transplant. (B) Representative WKM flow cytometry profile of non-irradiated and irradiated WT 5 days post irradiation. SSC indicates side scatter; FSC indicates forward scatter (both use 488 nm laser, 488/10 filter). Percentage of (C) erythrocytes, myelomonocytes, lymphocytes and precursors cells in the WKM. One experiment shown (n>4). Experiment repeated twice with WT, twice with TraNac. Each dot represents an individual fish. Statistical significance using one-way ANOVA and Tukey post-test. ****=p<0.0001, ***=p<0.001, **=p<0.01, *=p<0.05.

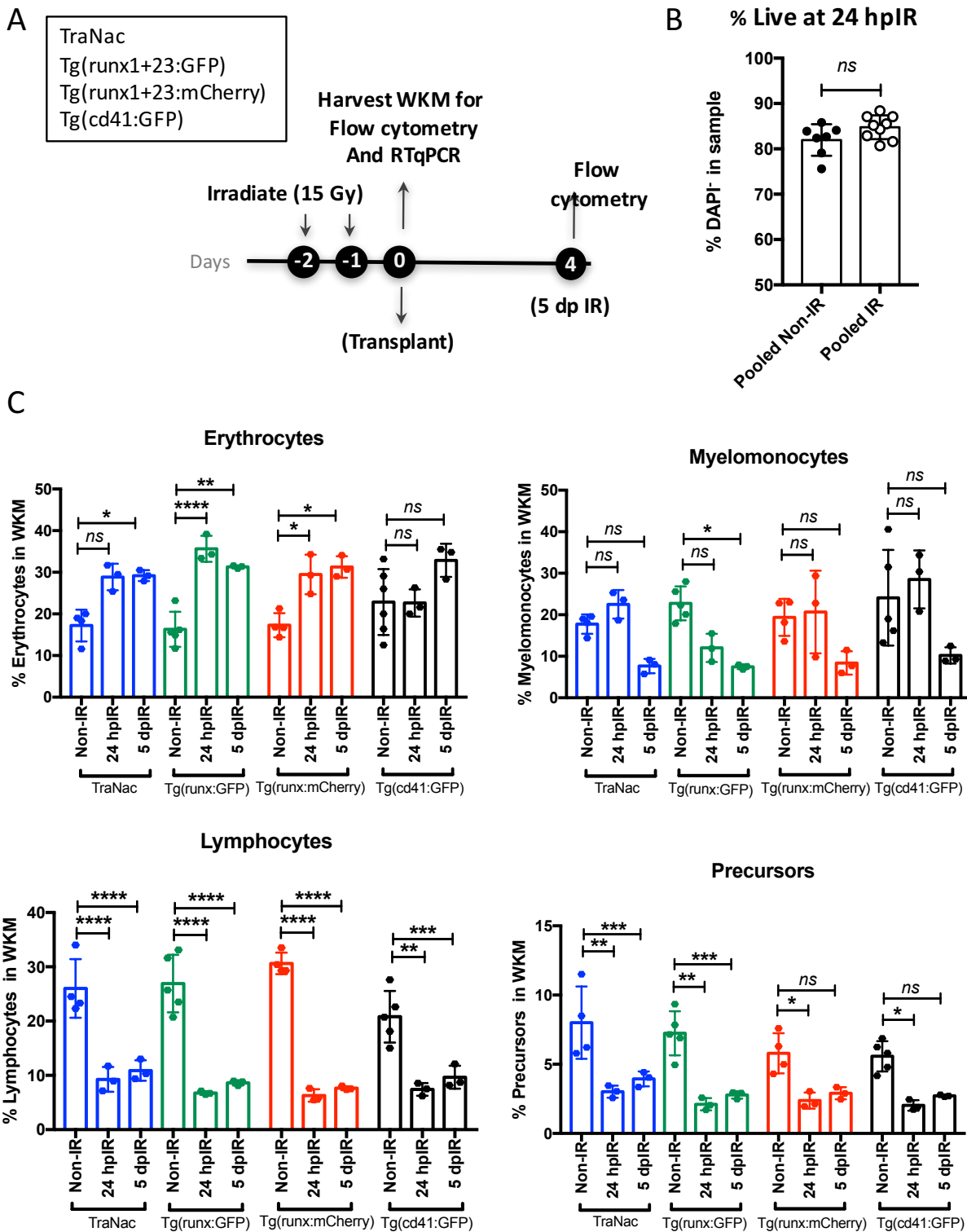


Figure 3.2: Depletion of kidney marrow cells in various lines following 2 doses of 15 Gy irradiation. TraNac, Tg (runx1+23:GFP), Tg (runx1+23:mCherry) and Tg (cd41:GFP) fish were treated with 2 doses of 15 Gy and the WKM was harvested 24 h (24 hpIR) and 5 days (5 dpIR) post irradiation. One experiment ($n>3$). Work in collaboration with D. Polos. Each dot represents an individual fish. (A) Procedure overview. (B) Percentage of live cells as determined by DAPI staining in samples harvested 24 h after 2nd irradiation dose. "Pooled" indicates both TraNac and Tg (cd41:GFP) fish are presented in the same column. Statistical significance using student t-test. (C) Percentage of erythrocytes, myelomonocytes, lymphocytes and precursors cells (as determined by flow cytometry forward/side scatter) in the WKM of non-irradiated (Non-IR), 24 hours after irradiation (24 hpIR) and 5 days after irradiation (5 dpIR) fish. One experiment shown ($n>4$). Experiment repeated twice with TraNac, once with the other lines. Statistical significance using one-way ANOVA and Tukey post-test. ****= $p<0.0001$, ***= $p<0.001$, **= $p<0.01$, *= $p<0.05$.

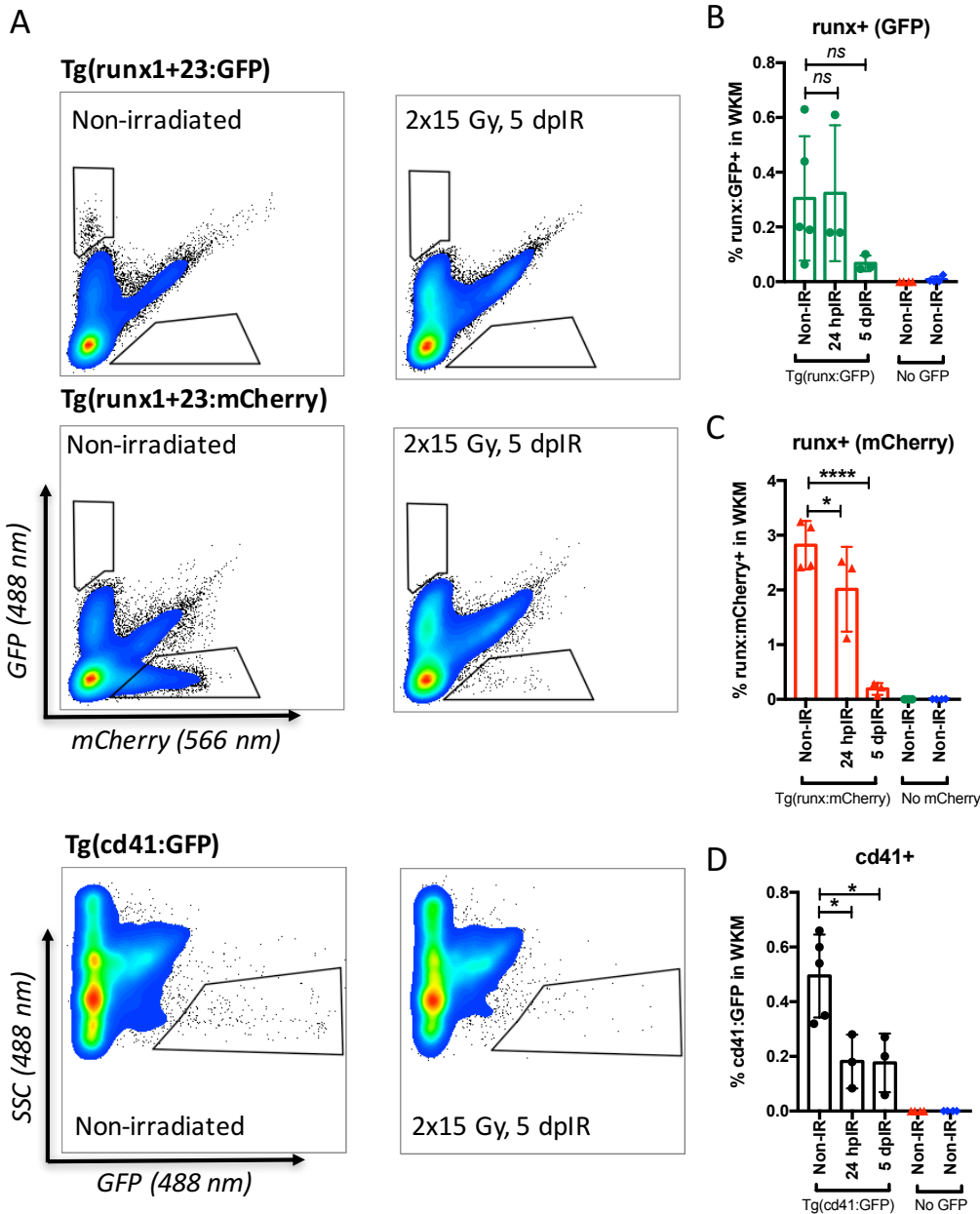


Figure 3.3: The percentage of HSPCs is significantly decreased post irradiation. TraNac, Tg (runx1+23:GFP), Tg (runx1+23:mCherry) and Tg (cd41:GFP) fish were treated with 2 doses of 15 Gy and the WKM was harvested 24 h (24 hpIR) and 5 days (5 dpIR) post irradiation. Same experiment as Figure 3.3. One experiment shown (n>3). Experiment repeated twice with TraNac, once with the other lines. Each dot represents an individual fish. Work in collaboration with D. Polos. (A) WKM flow cytometry profile of non-irradiated and irradiated (5 dpIR) Tg (runx1+23:GFP), Tg (runx1+23:mCherry) and Tg (cd41:GFP) fish. SSC indicates side scatter (488 nm laser, 488/10 filter); GFP (488 nm laser, 530/30 filter) mCherry (561 nm laser, 610/20 filter). (B) Percentage of GFP+ in Tg (runx1+23:GFP) with non-irradiated TraNac and Tg (runx1+23:mCherry) negative controls. (C) Percentage of mCherry+ in Tg (runx1+23:mCherry) with non-irradiated TraNac and Tg (runx1+23:GFP) negative controls. (D) Percentage of GFP+ in Tg (cd41:GFP) with non-irradiated TraNac and Tg (runx1+23:mCherry) negative controls. Statistical significance using one-way ANOVA and Tukey post-test. ****=p<0.0001, *=p<0.05

Radiation leads to wide spread cell loss, as seen by the reduction of lymphocytes, myelomonocytes and precursors. DAPI, a DNA binding molecule which is retained in live cells, was used to compare percentages of dead cells (without a viable cell membrane). There was no significant difference in the percentage of dead cells in pooled radiated and pooled non-irradiated samples, when harvested at 5 dpIR (Figure 3.2B).

Although the only recipients used for transplantation are the translucent TraNac mutants, the Tg (cd41:GFP), Tg (runx1+23:GFP) and Tg (runx1+23:mCherry) lines can be used to assess if the HSPC populations which are labelled are radio-sensitive. The 4 lines were treated with 2 doses of 15 Gy and harvested at 24 h (would be day of transplant) and 5 days (would be 4 days after transplant) after the 2nd irradiation dose (Figure 3.2A). The results show similar changes in the 4 populations that were seen in the WT, 5 days after the 2nd dose (Figure 3.2C).

Examination of the major populations at 24 h (would be day of transplant) shows that both the lymphocyte and precursor populations are severely decreased within 24 hpIR (Figure 3.2C). Interestingly, the myelomonocyte population, which decreased by 5 dpIR, is not significantly changed by 24 hpIR (Figure 3.2C). Again, the erythroid population appears to proportionally increase.

The results also show a significant decrease in the HSPC population based on GFP+ gating of Tg (cd41:GFP) and Tg (runx1:23:GFP) and mCherry+ gating of (runx1:23:mCherry) fish (Figure 3.3). At 24 h and 5 dpIR, the percentage of cd41:GFP+ cells is at half the untreated levels. The runx1:mCherry+ population is significantly decreased by a third after 24 h and by 80 % after 5 days. Interestingly, the runx1:GFP+ population was not affected after 24 h, and appeared to be dropping at 5 dpIR, although to a significantly lower percentage.

qRT-PCR was used to confirm the flow cytometry data by measuring transcript levels of cell specific markers in the whole organism. As before, TraNac fish were treated with 2 doses of 15 Gy, 24 h apart, and then the decapitated body was harvested 24 h later (at time of transplant) for RNA extraction and qRT-PCR analysis (Figure 3.4A). Interestingly, the erythroid specific gene *gata1* was significantly decreased (Figure 3.4B), supporting the possibility that the percentage increase determined in the flow cytometry data was due to an increase in the contaminating debris or a relative increase due to the decrease of the other cell populations (Supplementary Figure 7.1, Figure 3.2C). It is important to note that relative gene expression was measured from the whole body rather than the WKM and it is not clear how this would affect the results.

Equally, the T-cell specific gene *lymphocyte specific protein tyrosine kinase (lck)* was significantly decreased, correlating with the significant decrease in the lymphocyte designated population in the flow cytometry data (Figure 3.2C).

Macrophage specific genes *microfibrillar-associated protein 4 (mfap4)* and *macrophage expressed 1 (mpeg1)* remain unchanged, reflecting the flow cytometry data at 24 hpIR (Figure 3.2C). On the other hand, macrophage and monocyte gene *colony stimulating factor 1 receptor a (csf1ra)*; also known as CD115) increased, implying an increase in monocytes expressing this receptor. The pan leukocyte marker *l-plastin* expressed by immune cells (385), remained unchanged. Together, these data are consistent with the flow cytometry data (Figure 3.3).

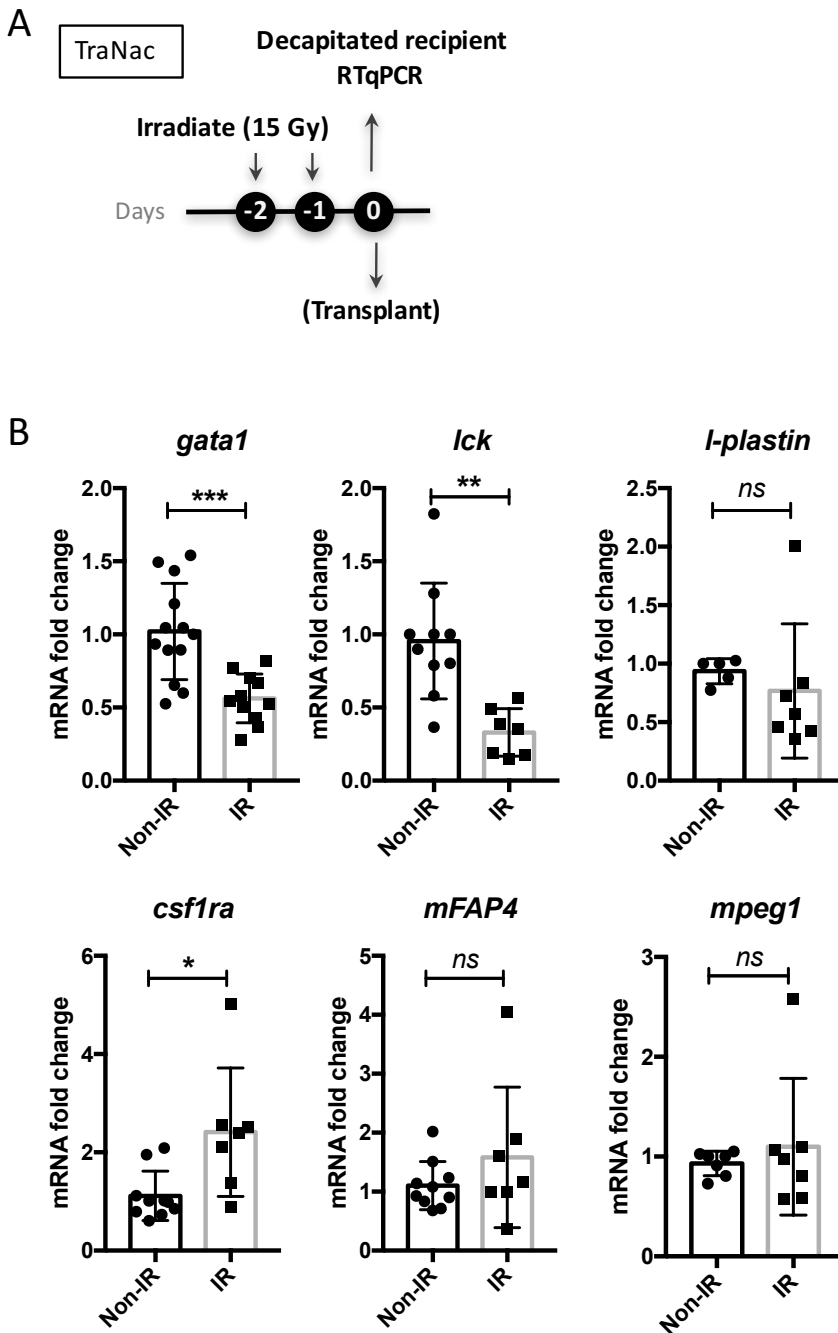


Figure 3.4: Irradiation changes the prevalence of blood population markers in the whole recipient, at the time of transplant (24 hpIR). (A) Procedure overview. Fish were left untreated (Non-IR) or irradiated (IR) with 2 doses of 15 Gy, 24 h apart, and were killed 24 h after the 2nd dose. RNA was extracted from the decapitated body and qRT-PCR was performed on samples. (B) Expression levels of *gata1*, *mFAP4*, *csf1ra*, *lyz*, *l-plastin* and *mpeg1* were normalised to 18S using the $\Delta\Delta CT$ method. Fold change relative to the median control value. 2 experiments pooled, $n > 7$. Each dot represents an individual fish. Statistical significance using Mann-Whitney (non-parametric t-test). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.3.1.2 Irradiation causes increased expression of cytokines in the whole recipient

Irradiation induces severe DNA damage in dividing cells throughout the organism, causing death in these cells and the production of pro-inflammatory cytokines. In order to assess the inflammation in the recipient environment, the levels of some previously mentioned cytokines (see chapter 1.7.2; *il-1 β* , *tnf α* , *il-6* and *tgfb β* (367-369)) were measured at 24 hpIR, the projected time of transplant (Figure 3.4A). Transcript levels were

determined using the $\Delta\Delta CT$ method (see chapter 2.9.3). Since transplanted cells are not delivered directly into the WKM (the RO injection technique is used, depositing cells near the heart), the inflammatory environment of the recipient could have a biological impact on the cells, perhaps influencing their migration or stimulating differentiation, before they reach the WKM.

The results show that transcripts of many of the classical cytokines and chemokines (*il-1 β* , *tnf α* , *il-6*, *ifn γ 1.1*, and *cxcl-c1c*) are increased in irradiated fish (Figure 3.5). Both *il-1 β* and *ifn γ 1.1* were increased ≈ 3 fold. *Tnf α* was increased ≈ 2.5 times. *Il-6* levels were approximately doubled. The chemokine *cxcl-c1c* was ≈ 4 fold higher and the most significantly upregulated transcript. Neither *ifn ϕ* nor *ifn γ 1.2* transcripts were induced, as judged by analysis at the whole body level, by the irradiation treatment.

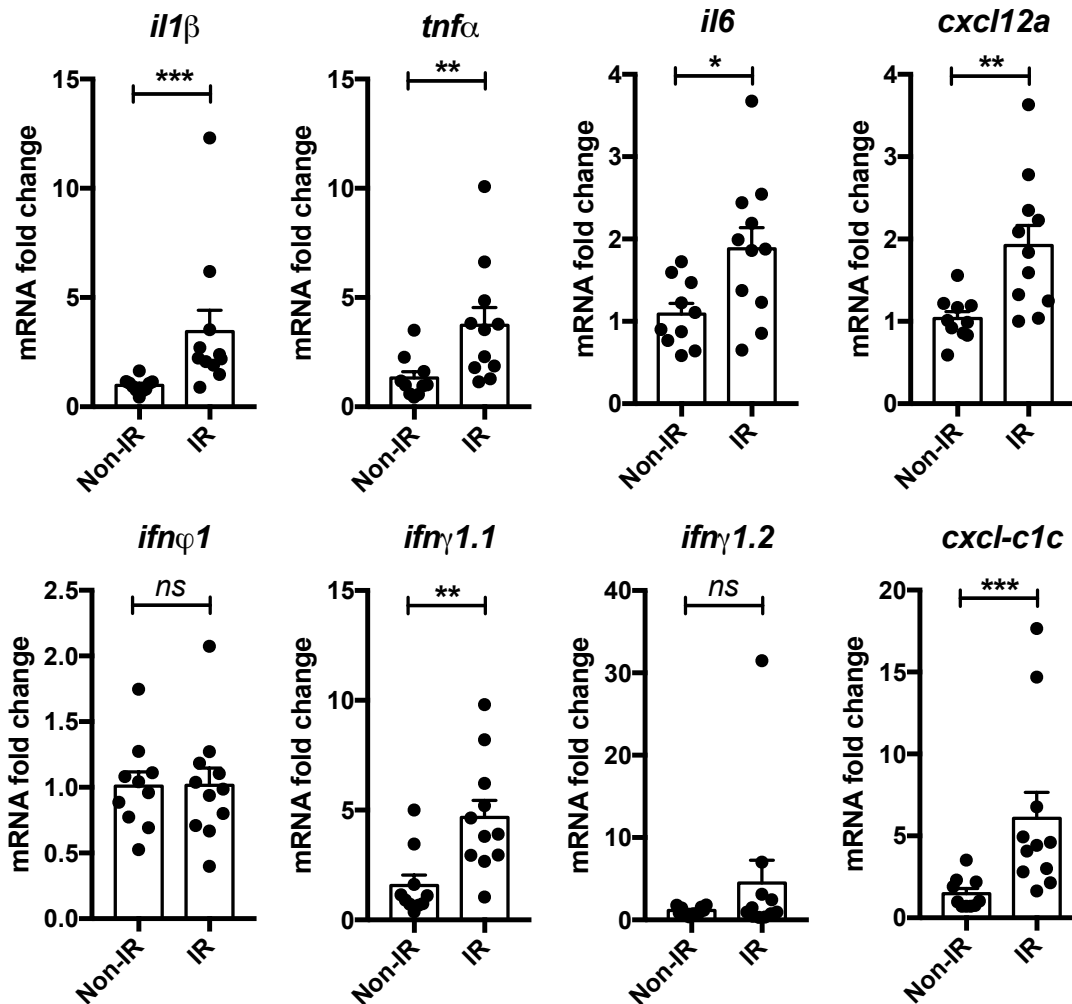


Figure 3.5: Irradiation causes an increase in inflammatory gene expression in the whole recipient, at the time of transplant (24 hpIR). Fish were left untreated (Non-IR) or irradiated (IR) with 2 doses of 15 Gy, 24 h apart, and were killed 24 h after the 2nd dose. RNA was extracted from the decapitated body and qRT-PCR was performed on samples. Expression levels of *il-1 β* , *tnf α* , *il-6*, *cxcl12a*, *ifn ϕ 1*, *ifn γ 1.1*, *ifn γ 1.2* and *cxcl-c1c* were normalised to 18S, using the $\Delta\Delta CT$ method. Fold change relative to the median control value. 3 experiments pooled, $n > 10$. Each dot represents an individual fish. Statistical significance using Mann-Whitney (non-parametric t-test). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.3.1.3 Irradiation causes inflammation in the WKM

In order to determine if the inflammatory environment assessed in the whole organism extended specifically to the kidney marrow, TraNac fish were treated to 2 doses of 15 Gy, 24 h apart and the WKM was harvested at 1, 3, 8 and 24 h after the 2nd irradiation treatment for RNA extraction and qRT-PCR analysis (Figure 3.6A). The time points were chosen to determine when the changes in expression occurred relative to the time of exposure and to relate with changes seen in at the whole body level at 3 hpIR.

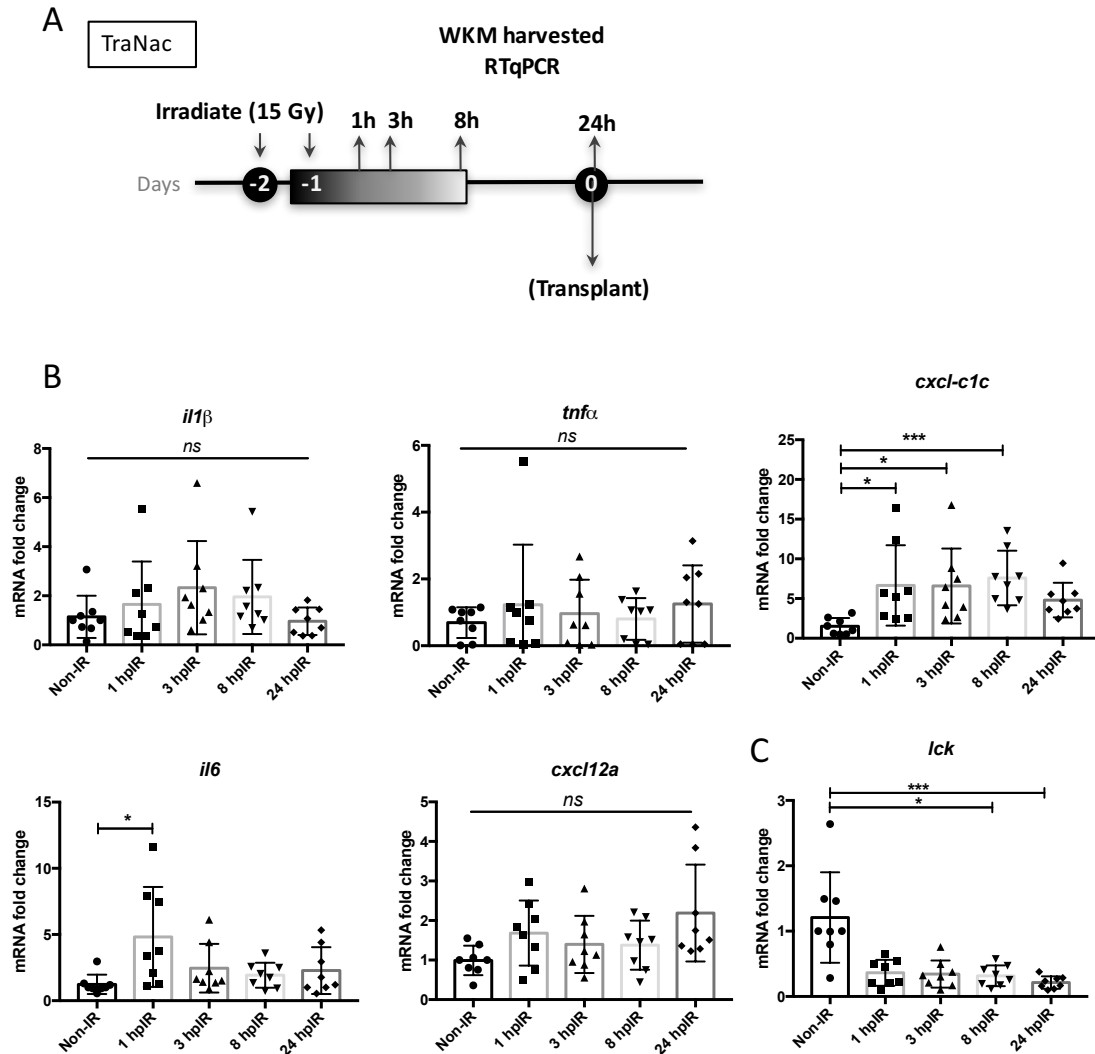


Figure 3.6: Irradiation causes an increase in inflammatory gene expression in the whole kidney marrow. TraNac fish were left untreated (Non-IR) or irradiated (IR) with 2 doses of 15 Gy, 24 h apart, and the WKM was harvested 1, 3, 8, and 24 h after the 2nd dose. RNA was extracted and qRT-PCR was performed on samples. 2 experiments pooled. Work done in collaboration with D. Polos. (A) Procedure overview. (B) Relative expression levels of *il-1β*, *tnfa*, *cxcl-c1c*, *il-6* and *cxcl12a*. (C) Relative expression of *lck*. Fold change calculated by normalising to 18S and relative to the median control value. 2 experiments, n=8. Each dot represents WKM from an individual fish. Statistical significance using one-way ANOVA.

Although transcript levels of chemokines *il-1β*, *tnfa* and *cxcl12a* were increased in the whole irradiated fish (Figure 3.5), the levels in the kidney marrow did not change significantly in the WKM over time (Figure 3.6B). Expression of *cxcl-c1c* significantly increased over the time course, being highest between 1 and 8 hpIR, and falling to ≈5 fold levels at 24 hpIR (Figure 3.6B). In the whole organism the levels of *cxcl-c1c* are also ≈5 fold

higher in irradiated samples at 24 hpIR (Figure 3.5). Levels of *il-6* peak at 1 hpIR, and decrease to ≈ 2 fold levels at 24 hpIR (Figure 3.6B). In the whole organism the levels of *il-6* are also ≈ 2 fold higher in irradiated samples at 24 hpIR (Figure 3.5). Altogether, the transcript levels of cytokines in the WKM somewhat reflect the conditions in the entire organism. Transcript levels of *ifn γ 1.2* and *ifn ϕ 1* were also measured but did not change (Supplementary Figure 7.2).

As the flow cytometry data shows the lymphocyte population is significantly decreased in the whole organism by 24 hpIR (Figure 3.2C), the mRNA from this time course was used to look at T-cell specific *lck* expression to determine how quickly this population is affected after exposure to *gamma*-radiation. The level of *lck* was ≈ 50 % lower at 1 hpIR and ≈ 80 % lower by 24 hpIR (Figure 3.6C) suggesting the death of lymphocytes is rapid.

3.3.1.4 Survival of irradiated fish without transplant

Of note, the dose of irradiation given to TraNac recipients is sub-lethal, with some fish surviving to 28 days (Figure 3.7). Half of non-transplanted fish died by 11 dpt. In order to reduce suffering of animals, fish showing adverse symptoms were humanely killed (see chapter 2.5). The transplant is done using the RO injection technique (49). Fish were injected this way with PBS to assess whether the technique affected the survival of fish. The method did not significantly reduce survival (Figure 3.7).

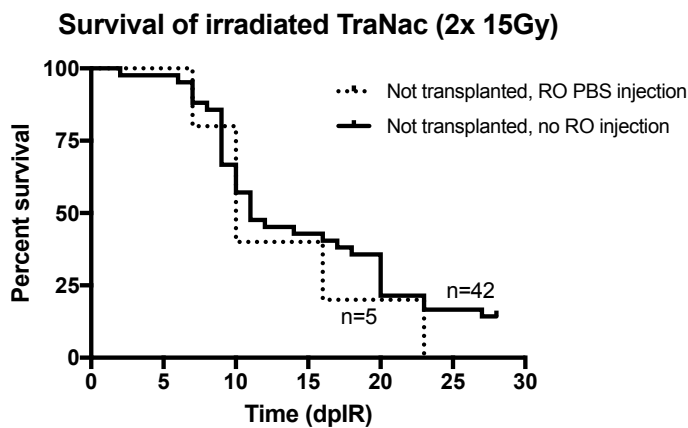


Figure 3.7: Survival of TraNac following 2 doses of 15 Gy irradiation, 24 h apart. TraNac fish were treated with 2 doses of 15 Gy or and tracked for 28 days. Survival chart of IR fish and IR fish retro-orbitally injected with 5 μ L PBS at day 0 (24 h after the 2nd dose of 15 Gy). (Pooled data from 2 experiments with data from “Not transplanted” fish from 8 experiments presented in Figures 3.9D, 3.15E, 3.19, 3.21; Total n = 42; Retro-orbital injection of PBS in 1 experiment, n=5). Mantel-cox test, ns.

3.3.2 Delivery of cells via retro-orbital injection

3.3.2.1 Dextran confirms correct delivery of cells in hematopoietic cell transplantation

In order to investigate the effect of inflammation on HSC function, a baseline reconstitution ability of WKM cells from healthy donors was established. Optically transparent TraNac recipients were irradiated (2 x 15 Gy) to deplete the resident hematopoietic populations. Cells from donor Tg (ubi:GFP) fish were harvested on the day of

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transplant and delivered using the RO injection technique (Figure 3.8A). The *ubiquitous (ubi)* gene promoter allows expression of green fluorescent protein in >90 % of WKM cells. These Tg (*ubi:GFP*) donors allowed us to determine the contribution of donor cells (*ubi:GFP*+) to the recipients' reconstituted immune system. Single recipients received the whole WKM, from 0.9 to 1.4 x 10⁶ WKM cells, from a single donor (hereafter referred to as "1:1") or recipients received 10⁵ or 5 x 10⁴ cells of unselected WKM from a pool of donors.

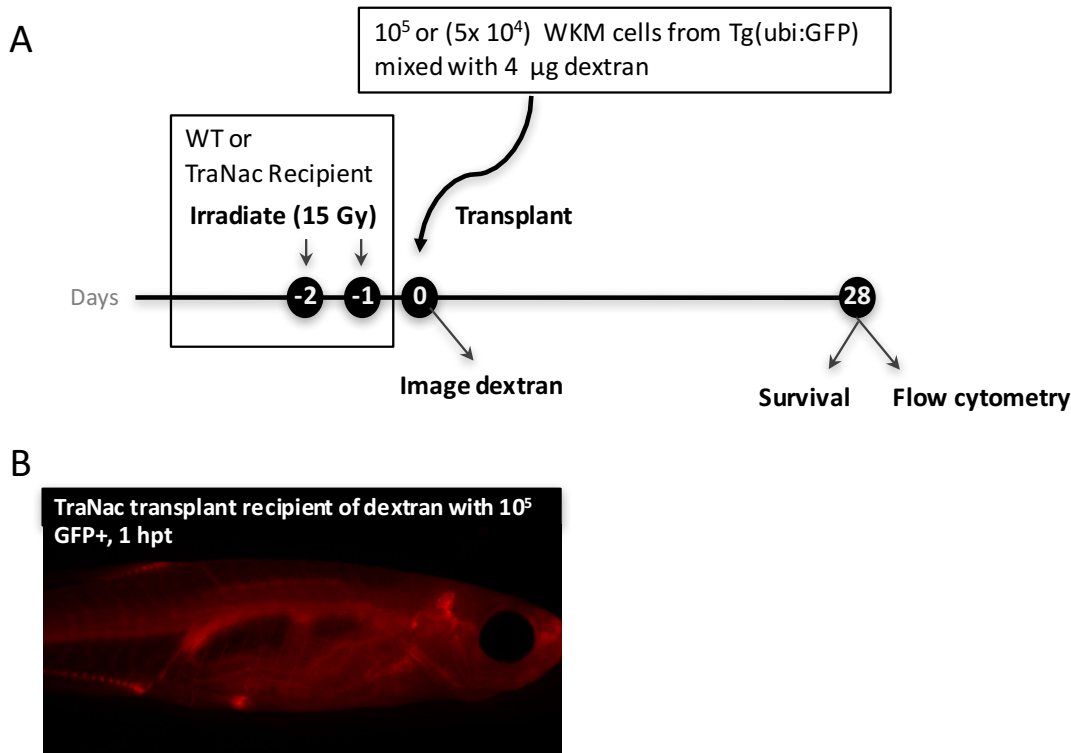


Figure 3.8: Retro-orbital injection successfully delivers into the vasculature. (A) Procedure overview. TraNac or WT fish were irradiated with 2 doses of 15 Gy and either not transplanted or transplanted with 10⁵ WKM cells from a Tg (*ubi:GFP*) donor, mixed with dextran. **(B) Fluorescent imaging of recipient TraNac injected with 4 μg TRITC-conjugated dextran, 1 hpt.**

In order to establish and validate the delivery technique, dextran was added to the cells. Dextran is a glucan (120kDa) used as a tracer because it marks the vasculature by binding to the endothelium and erythrocytes. In this case, it is conjugated to TRITC so it can be detected with fluorescent microscopy. Upon injection, the cell solution will enter the blood stream and the entire vasculature will be visible.

It was found that when these *ubi:GFP*+ WKM cells were injected together with dextran, the vasculature was visible but the *ubi:GFP*+ cells were not discernible at 4 or 8 dpt (Figure 3.8B). Three weeks after transplant, single cells were still not detectable but there was a region of fluorescence in the WKM area.

3.3.2.2 Transplantation of heterogeneous WKM cells successfully reconstitutes the immune system of TraNac recipients

Although single cells were not detectable under the fluorescence widefield microscope, a region of fluorescence in the WKM region could be seen, suggesting the transplants had been successful. Survival of transplant

recipients was significantly higher than in non-transplanted irradiated fish. Recipients of 1:1 transplants had a better survival than recipients of lower doses (Figure 3.9D). To confirm the transplanted cells had rescued these fish, and to assess donor contribution, surviving fish were harvested at 28 dpt and the reconstitution of the WKM was determined using flow cytometry (Figure 3.9A).

Donor contribution was assessed by gating ubi:GFP+ cells within the debris exclusion gate (gating of around the densest portion of the acquired data to exclude outliers), using donor WKM as a GFP+ positive control and WT WKM as a GFP negative control (Figure 3.9B). The WKM of recipients contained on average 55 % ubi:GFP+ cells (ranging from 30 to 65 %). As Tg (ubi:GFP) donor fish have 75 % ubi:GFP+ cells, this suggests ~20 % (ranging from 5 to 40 %) of the cells in the transplant recipients were not derived from Tg (ubi:GFP) donor cells (Figure 3.9C). This ~20 % could represent hematopoietic cells derived from the recipient, or these cells could be contaminating structural WKM cells derived from the recipient. It is not clear whether the ubi:GFP+ cells detected at 28 dpt are the cells that were originally delivered at the time of transplant (0 dpt), and/or cells derived from transplanted HSCs (a result of engraftment and hematopoiesis of transplanted HSCs). To fully assess engraftment of HSCs, fish would need to be assessed after at least 6 months.

All recipients had multi- lineage reconstitution. To confirm that titrated transplant doses were able to rescue all the populations of the recipient WKM, both the GFP positive and negative populations of the recipients' WKM were gated for the major blood populations (Supplementary Figure 7.3). In general, the recipients had similar population percentages in the ubi:GFP+ fraction as the GFP negative fraction of WT WKM or the GFP positive fraction of Tg (ubi:GFP) donor WKM.

In Tg (ubi:GFP) donors, the majority of the GFP negative cells scatter in the erythrocyte gate, suggesting that this gate is contaminated by debris that is not part of the WKM cellular composition (Figure 3.10A). The percentage of erythrocytes in the GFP negative fraction of the recipient WKM (70 %) was similar to donor Tg (ubi:GFP) WKM (60 % to 80 %), but higher than the percentage of erythrocytes in the GFP negative fraction of WT WKM (40 %). This is because all cells in the WT WKM are GFP negative and therefore the percentage is split between the 4 cell groups. On the other hand, the majority of cells in recipients of Tg (ubi:GFP) donor fish are GFP+ and therefore there are few GFP- cells of the 4 cell groups. This had the effect of disproportionally increasing the percentage of cells in the erythrocyte population.

A significant proportion of recipient myelomonocyte, lymphocyte and precursor cells were GFP positive. The GFP positive fraction was 25 % myelomonocytes, while the GFP negative fraction had <5 % scattering in the myelomonocyte gate. The percentage of ubi:GFP+ myelomonocytes in the recipient WKM was not significantly different to the percentage of myelomonocytes in the GFP- WT WKM (20 %) or the percentage of myelomonocytes in the ubi:GFP+ of Tg (ubi:GFP) WKM (20 %; Figure 3.10B). The same is seen for lymphocytes, as shown in (Figure 3.10C).

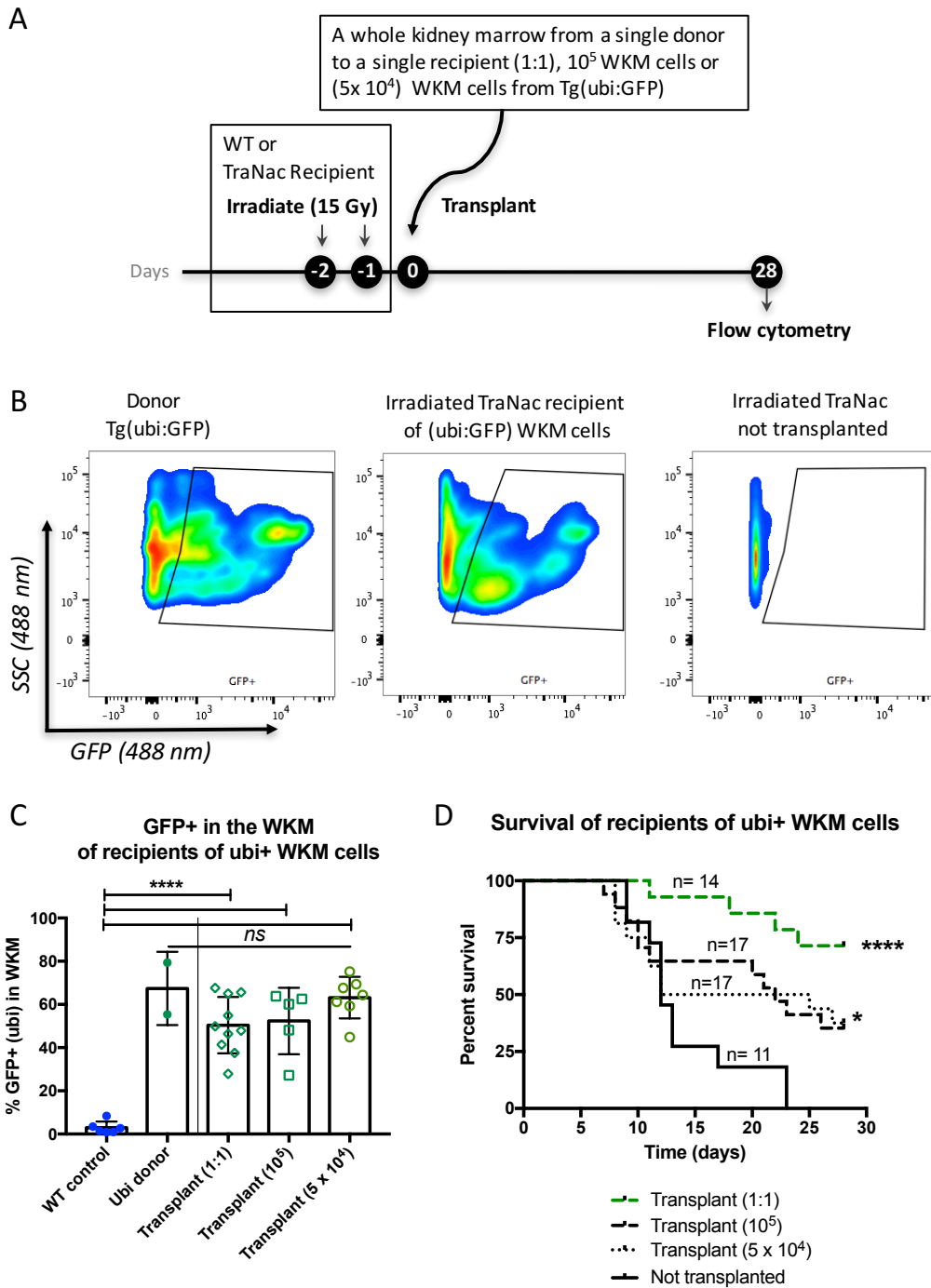


Figure 3.9: A transplant of heterogeneous WKM cells successfully populates the WKM and rescues irradiated TraNac recipients at least 28 dpt. TraNac or WT fish were irradiated with 2 doses of 15 Gy and retro-orbitally injected with a whole Tg (ubi:GFP) donor WKM (1:1), 10^5 WKM cells or 5×10^4 cells from a Tg (ubi:GFP) donor. 7 experiments pooled, not all groups were present in each repeat; 3 (1:1) transplants, 2 (10^5) and 2 (5×10^4), $n > 14$. (A) Procedure overview. (B) Flow cytometry scatter profile of Tg (ubi:GFP) donor, WT, transplant recipient TraNac 28 dpt and non-transplanted irradiated TraNac recipient. SSC indicates side scatter (488 laser, 488/10 filter); GFP indicates (488 laser, 530/30 filter). (C) Percentage of GFP+ in the WKM cells of recipients, WT and donor Tg (ubi:GFP). Each dot represents an individual fish. (D) Survival. Mantel-cox test between “not-transplanted” and each transplanted group. **** $p < 0.0001$, * $p < 0.05$. Non-transplanted recipients did not receive 10^5 WT PB cells.

Interestingly, the percentage of ubi:GFP+ precursors in the recipient WKM (22 %) was twice as high as the percentage of precursors in the non-transplant control (WT and Tg (ubi:GFP)) WKM (10 %; Figure 3.10D). This suggests an expansion of the precursor population following transplantation.

Taken together with the dextran data, these results show that the retro-orbital method is able to deliver cells into the recipient blood stream. They also show that the donor cells were successful at integrating into the recipient WKM and to outcompete any remaining host derived cells.

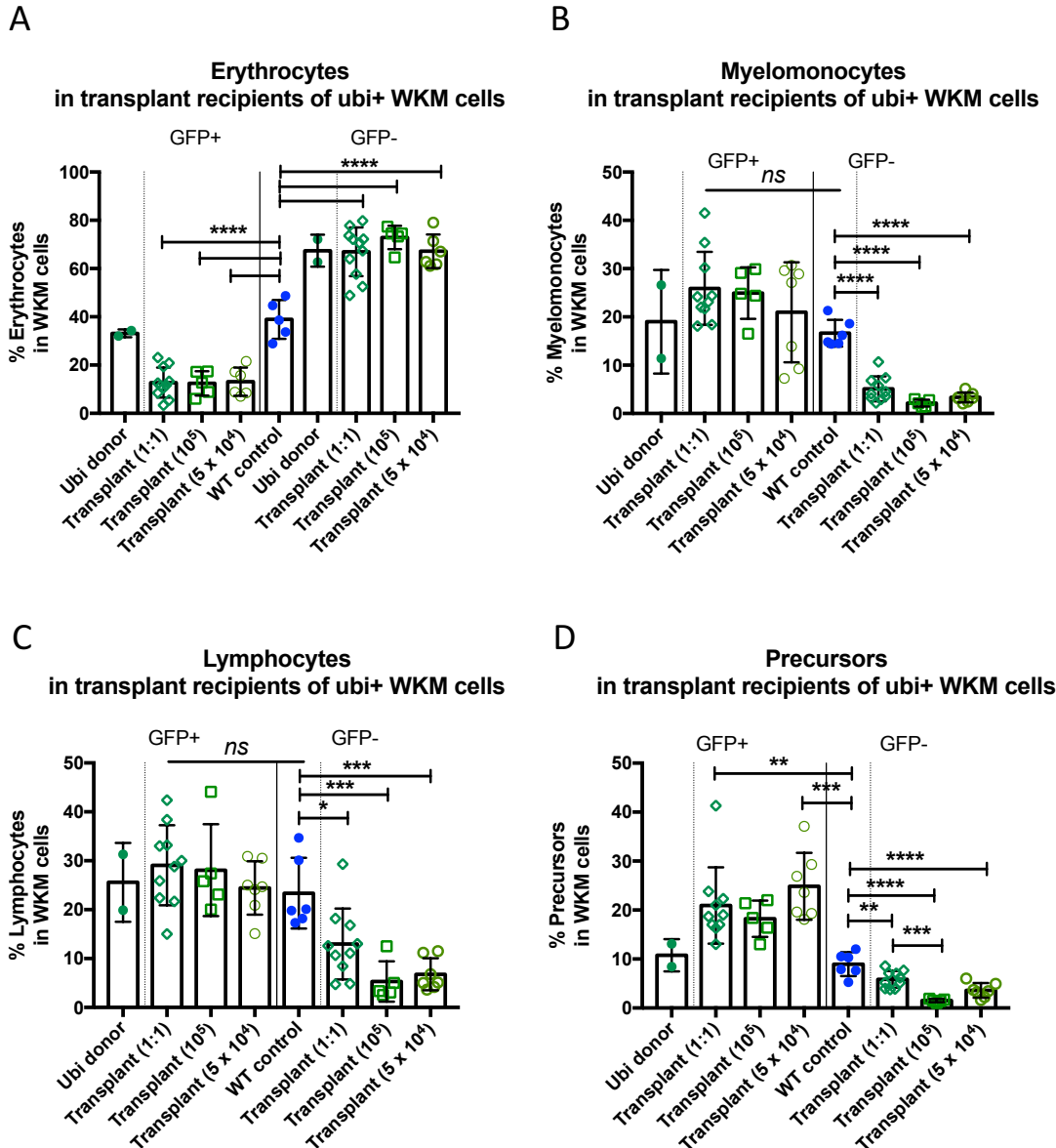


Figure 3.10: Transplant recipients have a larger precursor population at 28 dpt than non-irradiated, non-transplanted fish. TraNac or WT fish were irradiated with 2 doses of 15 Gy and retro-orbitally transplanted with a whole Tg (ubi:GFP) donor WKM (1:1), 10⁵ WKM cells or 5 x 10⁴ cells from a Tg (ubi:GFP) donor. 7 experiments pooled; 3 (1:1) transplants, 2 (10⁵) and 2 (5 x 10⁴), n>11; Each dot represents an individual fish. (A) Flow cytometry scatter profile of a standard Tg (ubi:GFP) donor, standard WT fish, irradiated and transplant recipient TraNac 28 dpt and irradiated non-transplanted TraNac recipient 28 dpIR. SSC indicates side scatter (488 laser, 488/10 filter); GFP indicates (488 laser, 530/30 filter). The percentage of (A) erythrocytes, (B) myelomonocytes, (C) lymphocytes, and (D) precursors in the ubi:GFP+ and ubi:GFP- gates of cells in the WKM, as determined by flow cytometry.

3.3.3 Establishing the rescue of bloodless $c\text{-myb}^{t25127}$ mutant recipients

Conventionally, transplant recipients are preconditioned with irradiation to deplete the recipient's immune system. This is often necessary, for instance in the treatment of leukaemia or auto-immune disease, as the purpose is to remove malignant cells and replace them with a healthy population. However, as shown by the cytokine data, this treatment causes general inflammation throughout the whole organism. A particular concern would be if the supportive cells of the HSC niche are damaged. In order to study hematopoietic engraftment in a non-inflamed environment (see chapter 5), we obtained a line of $c\text{-myb}^{t25127}$ mutants that do not undergo the definitive wave of hematopoiesis and are therefore “*bloodless*” after the cells from the primitive wave have been exhausted.

Homozygous $c\text{-myb}^{t25127}$ mutants do not live long enough to not reach sexual maturity. Because of this, homozygotes for the mutation can only be obtained by breeding heterozygotes. The homozygote mutants (1 in 4) are selected for transplant based on phenotype and then subsequently confirmed as such by genotyping DNA from a tail clip post-transplant (Figure 3.11A). In brief, genomic DNA is obtained from a tail clipped adult, and a polymerase chain reaction with gene targeting primers amplifies the region of interest. In the WT, the sequence amplified has 2 digestion sites for the Hpy188III enzyme, resulting in 3 products. However, in the $c\text{-myb}$ mutant, the mutation that causes the bloodless phenotype is a transversion mutation of thymine to adenine in the DNA binding domain. This point mutation removes the target sequence of restriction enzyme Hpy188III and therefore the amplified sequence just gets cut once, resulting in 2 products. This PCR and restriction digest method can therefore be used to distinguish WT from $c\text{-myb}$ mutants (Figure 3.11B). Unlike $c\text{-myb}$ homozygotes and heterozygotes, the WT will not have the larger 260 base pair (bp) sequence. Homozygote $c\text{-myb}$ mutants will present only 2 bands (260 bp, 184 bp); WT will present 3 bands (184 bp, 146 bp, 114 bp); Heterozygote $c\text{-myb}$ mutants will present 4 bands (260 bp, 184 bp, 146 bp, 114 bp) because they have both a copy of the gene with the mutation site and a copy without.

As described by Hess, when transplanted, the rescued $c\text{-myb}$ mutant is indistinguishable from its WT sibling (46). Therefore, the ability to maintain hematopoietic cells is not compromised. To better understand the phenotype and examine the physical structure of the kidney organ, histological sections of these fish were prepared. The kidney marrow was anatomically similar to WT but qualitative analysis suggests there are structural changes and fewer cells among the nephritic tubules (Figure 3.11C).

The $c\text{-myb}^{t25127}$ line is difficult to maintain because it lacks a competent immune system. Fish were raised in low antibiotic embryo growing medium (E2) but morbidity was high and juveniles did not live past 12 wpf and would not grow past 1 cm in length. Hess *et al.* suggest that size could be restricted by the requirement for oxygen exchange across the epithelium (46). The small size of the fish meant it was too difficult to inject with a Hamilton syringe, as it is typically done for zebrafish transplants. Therefore, the RO technique was refined using a micro-injector, typically used for injecting embryos. The ability to rescue these mutants at a smaller and younger stage meant a greater proportion of rescued fish.

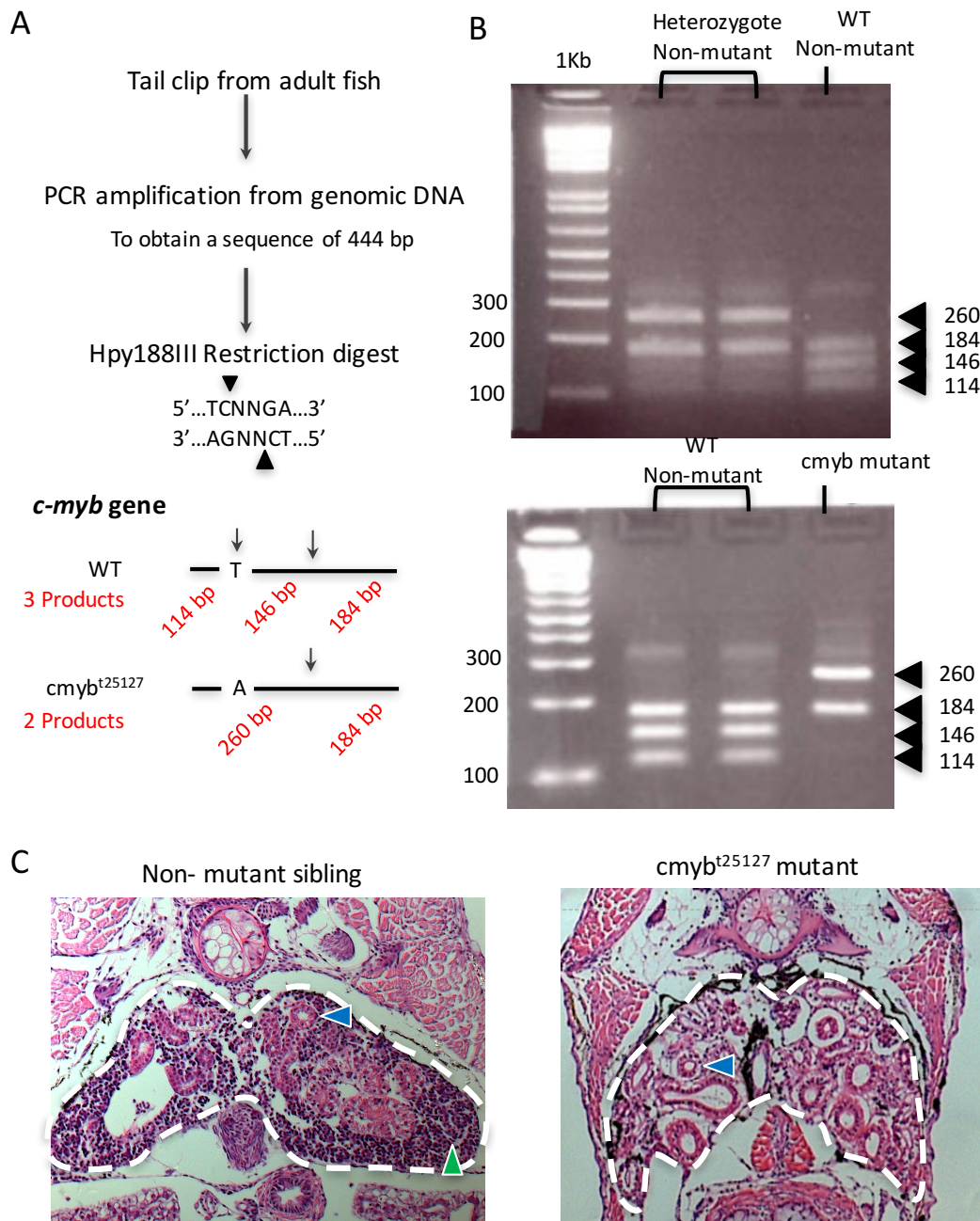


Figure 3.11: Characterisation of the c-myb^{t25127} mutant. Heterozygotes for the c-myb mutant gene were bred and embryo were reared with low-antibiotic-containing-embryo growing media (E2) on the bench. Homozygous mutants were identified phenotypically and receive a transplant at 5 wpf. (A) Genotyping was done on tail clip DNA by PCR amplifying a portion of the c-myb gene and using restriction enzyme Hpy188III to cleave sequence (indicated by arrows); WT sequence cleaved at 2 sites, mutant sequence cleaved at 1 site. Fragment sizes in base pair (bp). (B) The digested PCR product was run on 1.5% agarose gel. Restriction digest of WT produces 3 bands, c-myb heterozygotes produces 4 bands, c-myb homozygotes produces 2 bands. Ladder size on the left, fragment size on the right. (C) Representative sagittal sections of non-mutant (left) and c-myb^{t25127} mutant (right) at 4 wpf. WKM is outlined in dashed white line. Kidney tubule indicated with blue arrow, hematopoietic cells indicated with green arrow (Non-mutant only).

Transplantation of 7.5×10^4 WKM cells from Tg (lyz:dsRed) fish was used to assess the method of transplant in these fish (Figure 3.12A). Just like Tg (ubi:GFP), lyz:dsRed⁺ cells can be used to determine the contribution of donor cells in the recipient. The *lysozyme C* (lyz) gene promoter expresses red fluorescent protein in the myeloid population (56). These donors were also used because red fluorescent protein has a higher fluorescence intensity

which would help visualisation of cells in the recipient. WT and *c-myb* heterozygote siblings from the same batch were irradiated (15 Gy 48 h and 24 h prior to transplant) and transplanted alongside the homozygote mutants.

Despite the pigmentation of the *c-myb* mutant recipients (as they are not on a translucent background such as TraNac), donor cells could be visualised in the tail WKM of the recipient but could not be accurately quantified (Figure 3.12B).

Fish were humanely killed at 33 dpt for flow cytometry to assess multi-lineage reconstitution (Figure 3.13A). There were no differences in the reconstitution of the WKM erythrocyte, myelomonocyte, lymphocyte and precursor populations (Figure 3.13B). In addition, the percentage of *lyz:dsRed*⁺ cells in the WKM was not significantly different ($p=0.4396$) between *c-myb*^{t25127} mutants and non-mutant recipients (Figure 3.13C). Finally, the survival of *c-myb*^{t25127} mutants was slightly lower (although not significantly different) than irradiated non-mutant recipients. The genotype of the recipients was verified by taking a tail clipping at the time of harvest (33 dpt).

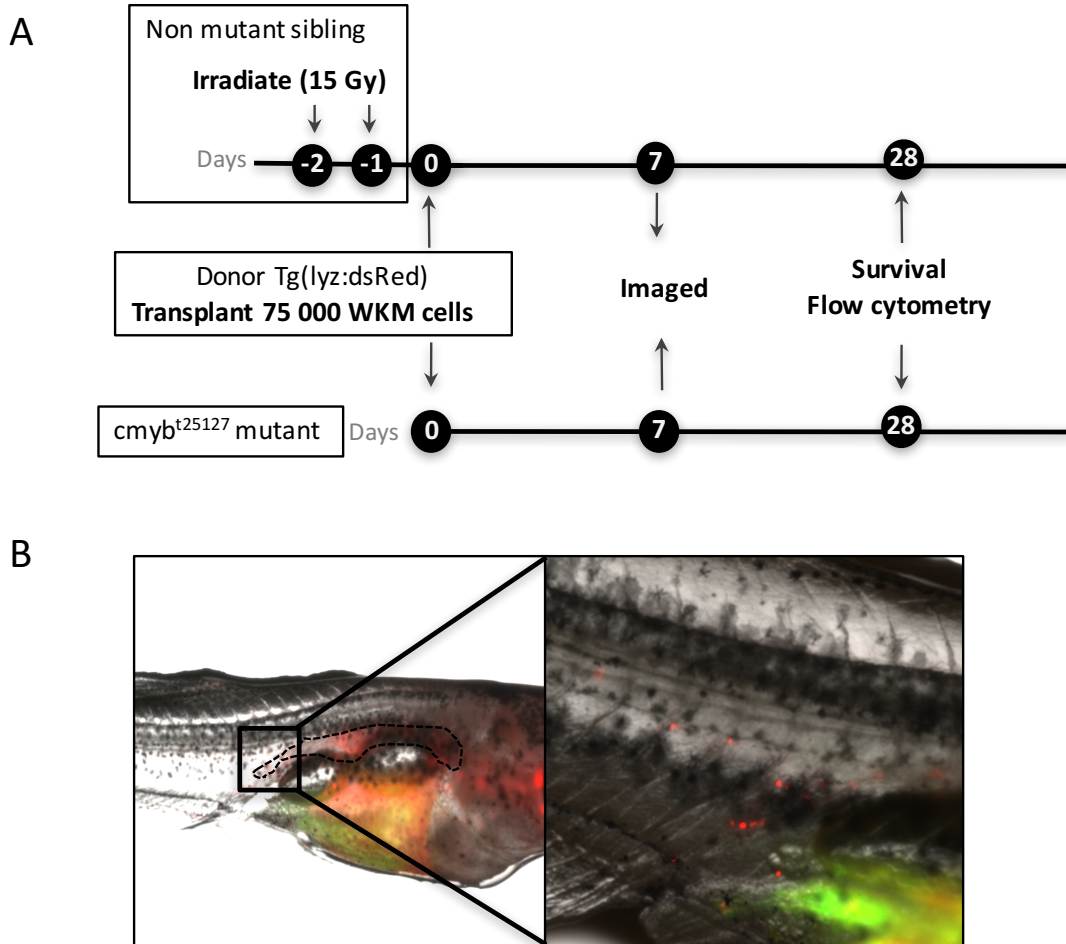


Figure 3.12: Transplanting *c-myb*^{t25127} mutant with 7.5×10^4 unsorted WKM cells from Tg (*lyz:dsRed*) donors. *C-myb*^{t25127} mutant fish were raised in E2 (embryo medium) to 7 wpf and then transplanted with 75 000 WKM cells from a pool of 3 Tg (*lyz:dsRed*) donors. Non-mutant siblings were irradiated twice with 15 Gy, 24 h apart and transplanted 24 h later, alongside the mutants (A) Procedure overview. (B) Representative widefield image (Olympus CKXC41 fluorescent microscope) of *c-myb*^{t25127} mutant recipient 7 dpt; the region of interest is enlarged.

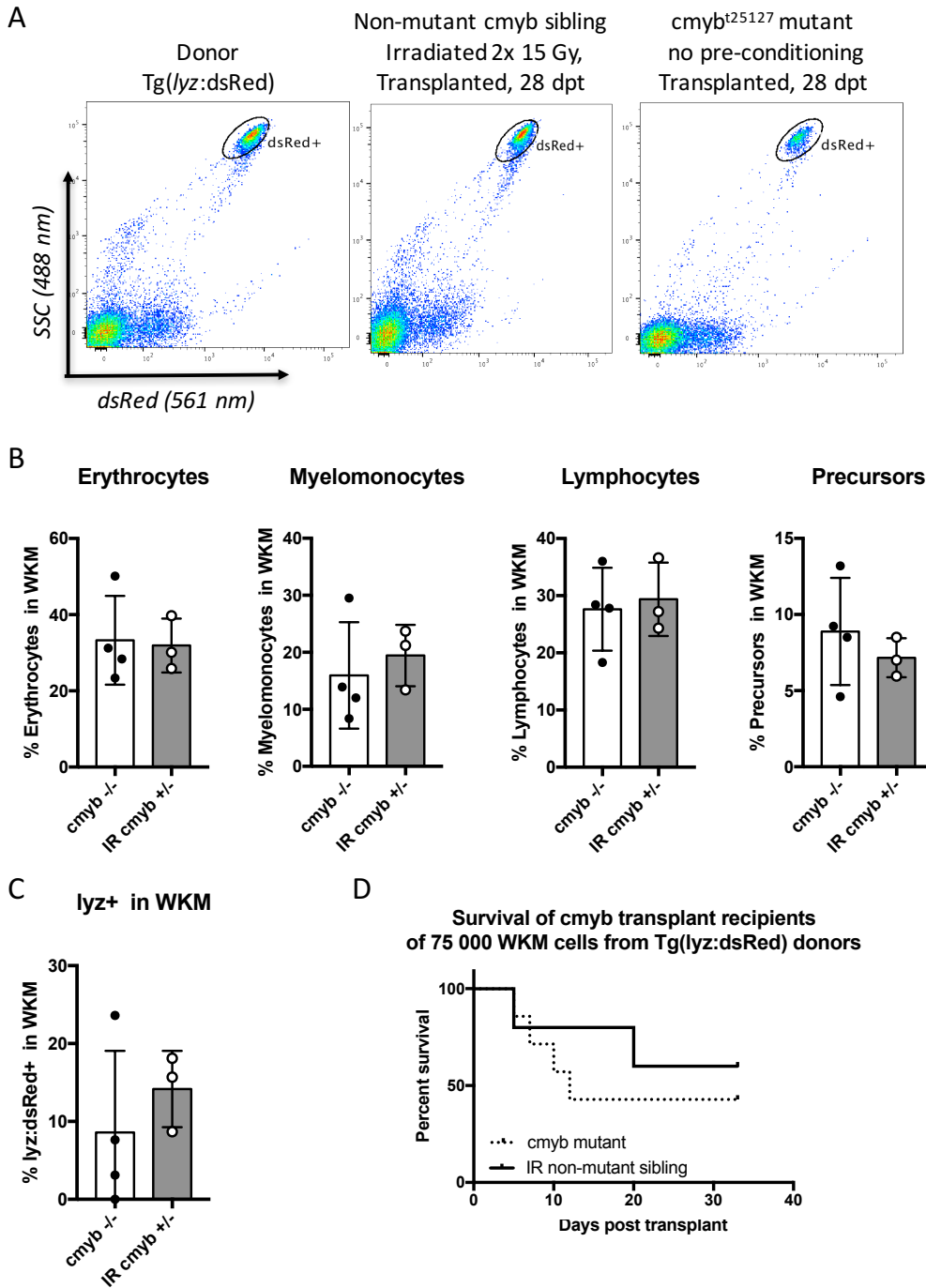


Figure 3.13: The WKM of *c-myb*^{t25127} mutants transplanted with a heterogeneous mix of WKM cells is populated with donor cells 33 dpt. *c-myb*^{t25127} mutant fish were raised in E2 (embryo medium) to 7 wpf and then transplanted with 75 000 WKM cells from a pool of 3 Tg (*lyz:dsRed*) donors. Non-mutant siblings were irradiated twice with 15 Gy, 24 h apart and transplanted 24 h later, alongside the mutants. Recipients were harvested at 33 dpt for flow cytometry. 1 experiment (mutant (*c-myb*^{-/-}) *n* = 7; Non-mutant (*c-myb*^{+/-}) *n* = 5). (A) Scatter profile of Tg (*lyz:dsRed*) donor, irradiated and transplanted non-mutant (*c-myb*^{+/-}) recipient and transplanted *c-myb* mutant (*c-myb*^{-/-}) recipient. SSC indicates side scatter (488 laser, 488/10 filter); dsRed (561 nm laser, 610/20 filter). (B) The percentage of erythrocytes, myelomonocytes, lymphocytes, and precursors in the WKM of recipients. (C) The percentage of *lyz:dsRed*⁺ cells in the WKM of recipients. (D) Post-transplant survival of recipients. Mantel-cox test.

Taken together, this experiment confirms that the *c-myb*^{t25127} mutant can be rescued by HCT. Surprisingly, the data indicates that immune reconstitution of *c-myb*^{t25127} mutants is not significantly different from their

Refinement of hematopoietic cell transplantation in zebrafish
irradiated non-mutant heterozygous or homozygous WT siblings. The ability to distinguish single cells in the recipient 7 dpt highlighted the possibility of using juvenile TraNac recipients to visualise the post-transplant establishment of donor cells in the kidney marrow.

3.3.4 Early post-transplant events

3.3.4.1 Transplanted HSPCs can be visualised migrating in smaller TraNac recipients

Auxiliary work with *c-myb bloodless* mutants revealed it was possible to image single cell behaviour in smaller fish. In order to visualise early post-transplant HSPC lodgment, mobilisation, proliferation and differentiation, the previously described micro-injection technique above was used to transplant irradiated juvenile (4-5 wpf) TraNac fish. This would minimise trauma to the recipient and optimize visualization of cells in the transplant recipient (a combination of a small optical depth with the fluorescence of cells from transgenic donors). A preliminary experiment in which 32 recipients were injected with WKM cells mixed with dextran showed that the fluorescence of the dextran interfered with visualisation of cells in the recipient. For this reason, dextran was omitted from subsequent experiments.

As our interest lies in the effects of inflammation on the behaviour of the HSPC population (and not the immune system as a whole), the experiment relied on obtaining a HSPC population for transplantation studies. As mentioned in the introduction, CD41, integrin alpha chain 2b, is an integral membrane protein expressed on early hematopoietic progenitors and thrombocytes in the zebrafish (28, 29). Ma *et al.* showed that only GFP^{low} cells transplanted into irradiated adult zebrafish could reconstitute the hematopoietic system up to 68 weeks after transplantation (51). Tg (cd41:GFP) fish were crossed with Tg (lyz:dsRed) fish, in which myeloid cells express dsRed fluorescent protein (56). The Tg (cd41:GFP;lyz:dsRed) donors allowed us to sort a HSPC enriched population for transplantation, and to determine the contribution of cd41:GFP⁺ donor derived myeloid cells to the recipient's reconstituted immune system.

Firstly, the reconstitution ability of a heterogeneous population of WKM cells without mature myeloid cells (cd41:GFP⁺, lyz:dsRed⁻) was assessed. This population is richer in the various cell types needed to reconstitute an ablated immune system and so recipient rescue should be higher than when cd41:GFP⁺ HSPCs are transplanted alone. As HSCs make up <1 % of the WKM, it was necessary to transplant a large population to improve the probability of survival and long term reconstitution.

Juvenile TraNac were irradiated (2 doses of 15 Gy, 24 h apart) and transplanted with 2×10^5 lyz:dsRed negative cells (gating in Figure 3.14C) and 10^5 WT carrier peripheral blood (PB) cells. Recipient fish were imaged daily from 3 to 6 dpt (Figure 3.14A). The sorting of cells can take up to 2.5 h, therefore DAPI was used to assess whether there was a change in cell viability over time. The percentage of live cells at the start of the sort (97.1 %) was comparable to the percentage at the end of the sort (96.6 %), indicating that the time taken for the sorting process did not cause significant cell death (Figure 3.14B).

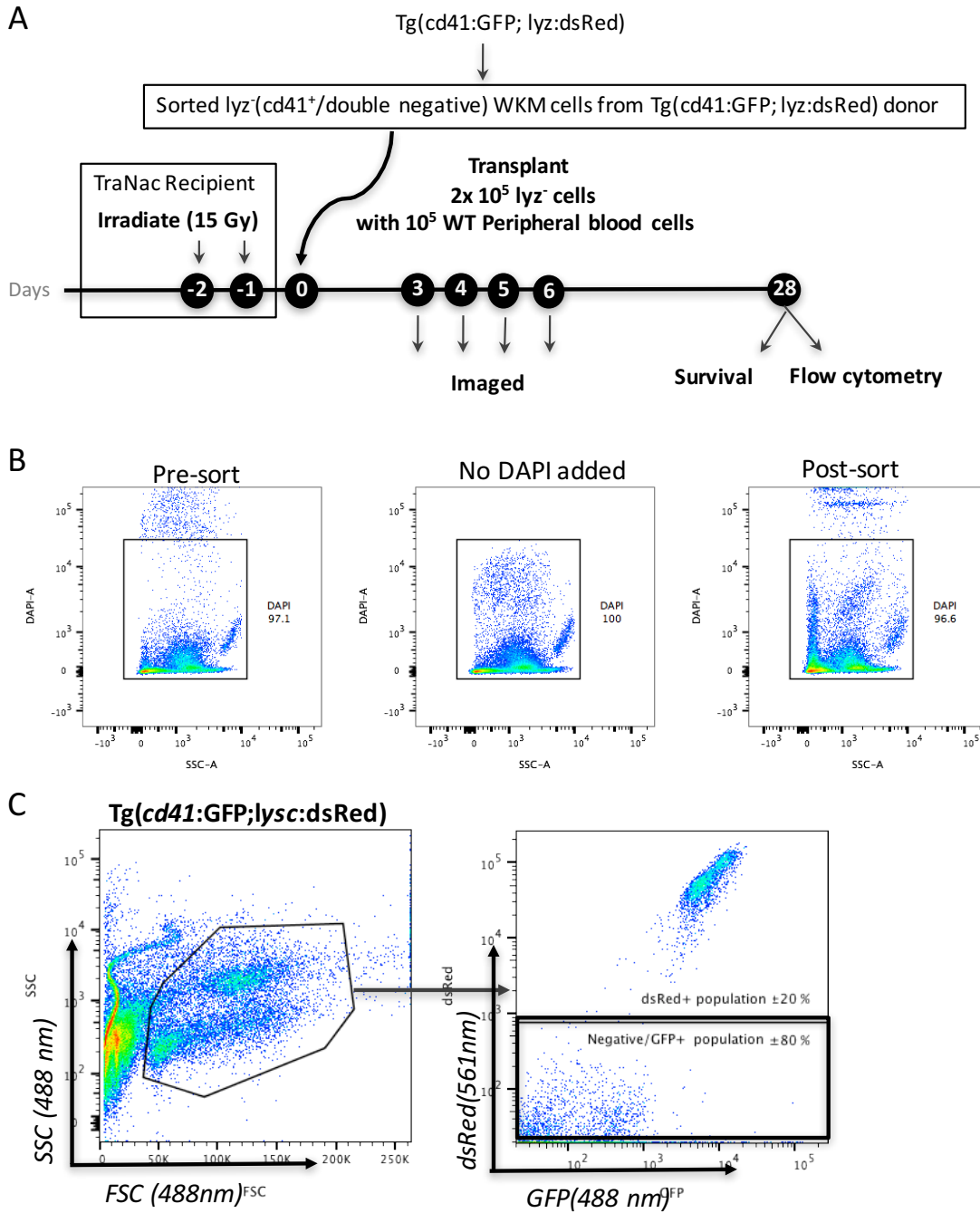


Figure 3.14: Transplantation of WKM cells depleted of mature myeloid cells (lyz negative) into irradiated TraNac recipients. WKM cells from Tg (cd41:GFP; lyz:dsRed) were sorted for lyz negative (dsRed⁻, GFP⁺/-) cells. (A) Protocol overview. (B) 100 μ L of pooled donor WKM was stained with DAPI pre- and post-sort. Flow cytometry scatter of donor WKM with DAPI gating. SSC indicates side scatter, DAPI (355 nm laser, 450/20 filter). (C) Flow cytometry scatter of donor WKM cells with gating used to obtain negative/GFP⁺ donor population. SSC indicates side scatter; FSC indicates forward scatter (both use 488 nm laser, 488/10 filter). The debris exclusion gated population is separated by dsRed (561 nm laser, 610/20 filter) and GFP (488 nm laser, 530/30 filter) scatter to obtain a lyz⁻ population.

Recipient fish were kept in E2 following the transplant, and then imaged from 3 dpt. The number of cd41:GFP⁺ and lyz:dsRed⁺ cells in the head, mid and tail regions of the WKM were counted.

The transplanted cd41:GFP+ population was visible in the kidney marrow of most fish by 5 dpt (Figure 3.15Di). Live imaging of the fish revealed the cd41:GFP+ cells behave in 2 different ways. One population is highly mobile, and the other is quite sedentary. It was not possible to distinguish high and low expression of GFP because the observed fluorescence intensity could be affected by the position of the cell in the fish.

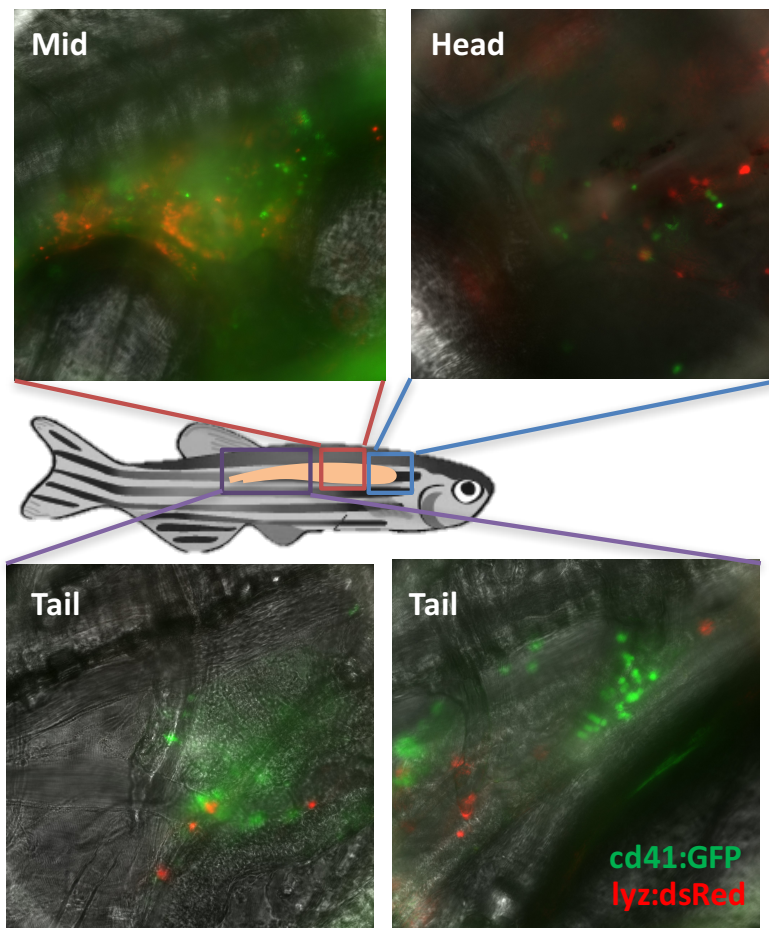
The lyz:dsRed+ population was observable in the fish WKM from 4 dpt (Figure 3.15Dii). However, some lyz:dsRed+ could be seen around the eye (the site affected by the transplant needle insertion) from 3 dpt. The number of cells in the WKM grew steadily over time. In order to determine if the lyz:dsRed+ cells have a location bias, the distribution of cells was analysed across 3 sections of the WKM: head, mid and tail (Figure 3.15B, C). This revealed that on day 4, the majority of cells are in the head kidney and very few are in the mid or tail kidney. However, by day 5, although the number of lyz:dsRed+ in the head remained the same, the cell numbers increased in the mid kidney and were highest in the tail kidney. This suggests that the head kidney gets colonised before the cells progress to the tail kidney. It is not clear whether the spread to the tail kidney is due to migrating mature cells or the migration and subsequent differentiation of precursors in these regions.

Taking the lyz:dsRed+ observations together with the cd41:GFP+ observations, the data suggests that precursors are present in the fish before they become observable by microscopy based on GFP expression and that there may be a limit to detection. To determine if cells could be detected in the WKM prior to when they are observed at day 4, recipients' head and their WKM were harvested at 3 dpt for flow cytometry analysis. No distinct population of fluorescent cells was detected in the scatter of either tissue at 3 dpt (data not shown). The cells were perhaps too few to detect, or had lost fluorescence as they temporarily stop expressing cd41 and GFP, or the transplanted cells have differentiated into a type of multi-potent progenitor which is not labelled by CD41 or Lyz proteins.

Recipients were monitored for survival (Figure 3.15E). Survival is shown for the first 28 days. The survival of irradiated but not transplanted fish was unusually high in this pool of 3 experiments, as usually it is around 15 % (Figure 3.7) and can be as low as 0 % (Figure 3.9D). This may reflect a problem with the irradiation step, or is due to variation in fish response to the irradiation treatment. The survival of transplanted fish was higher than non-transplanted irradiated siblings with 8 out of 17 fish receiving a transplant surviving past 28 dpt. The longest survival was 55 dpt for transplant recipients, and 41 days for irradiated but not transplanted fish.

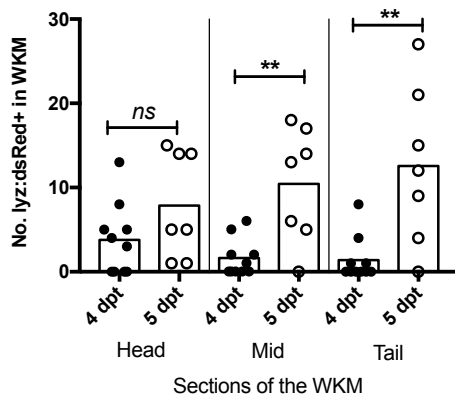
Together these experiments show that transplanting 2×10^5 lyz:dsRed negative cells can rescue irradiated TraNac recipients at least 4 wpt. They also show that single cells can be observed in small adult TraNac recipients.

A



B

WKM distribution and no. of lyz:dsRed+ in recipient of 2×10^5 lyz:dsRed- cells



C

Distribution of lyz:dsRed+ in WKM 4 and 5 days post transplant

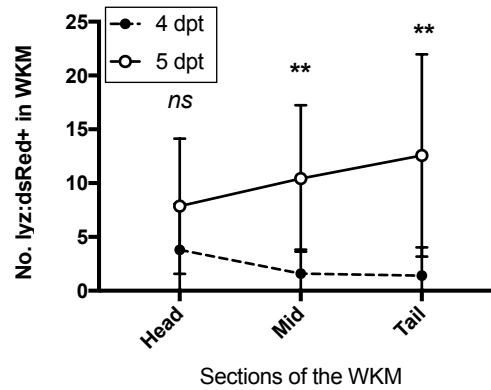


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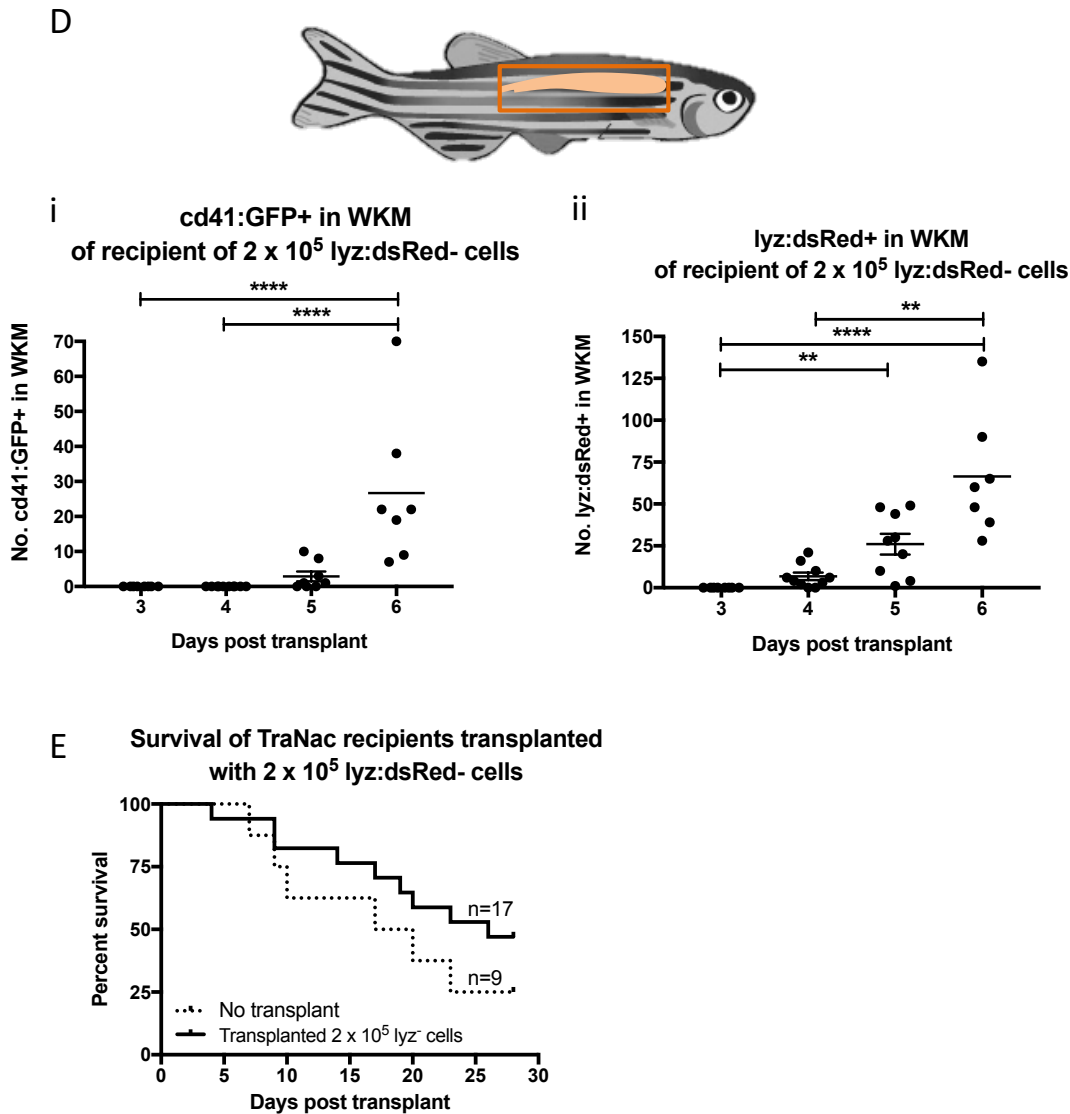
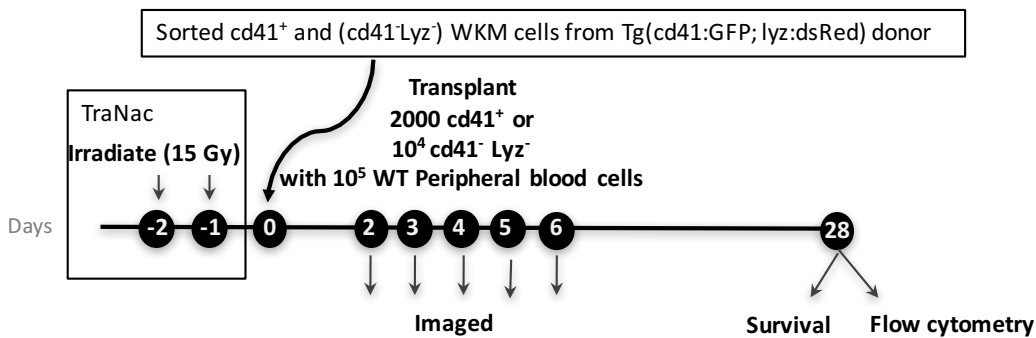


Figure 3.15: Irradiated TraNac recipients of WKM cells depleted of mature myeloid cells (lyz:dsRed-) have mature myeloid cells (lyz:dsRed+) in the WKM from 4 dpt. TraNac recipients were treated with 2 doses of 15 Gy and either did not receive a transplant, or were transplanted with 2×10^5 dsRed negative WKM cells from Tg (cd41:GFP; lyz:dsRed) donors and 10^5 WT PB cells. Non-transplanted recipients did not receive 10^5 WT PB cells. Fish drawing reproduced with permission from E. Griffith (A) Recipients were imaged 3 to 6 dpt under a Zeiss Axiovert 200 inverted confocal microscope with 488 and 506 nm lasers. The kidney marrow was divided into 3 sections and dsRed + cells were counted in the head, mid and tail segments. (B) Bar graph shows mean number of dsRed+ in sections of the kidney marrow (dots represent individual fish) at 4 and 5 dpt. Student's t-test for each section (4 v 5 dpt), ** $p < 0.01$. Data from a single experiment. (C) The line graph shows the mean number (dots) of dsRed+ (with lines connecting sections) at 4 and 5 dpt. (the same data as in B). (D) The sum total of dsRed+ and GFP+ in the 3 sections from 3 to 6 dpt was plotted; each dot represents a fish ($n > 9$). One-way ANOVA (Kruskal-Wallis) with Dunn's post test, ** $p < 0.01$, **** $p < 0.0001$ (E) Survival chart. Mantel-cox test non-significant. Experiment repeated 3 times and data pooled.

3.3.4.2 Both *cd41:GFP+ lyz:dsRed-* and *cd41:GFP- lyz:dsRed-* cells can give rise to *lyz:dsRed+* cells in the transplant recipient

Having shown that transplantation of *lyz:dsRed* negative cells rescues irradiated fish and allows differentiation of *lyz:dsRed+* progeny, the next step was to further partition this population. The *lyz:dsRed* negative population was split into a double negative (*cd41:GFP- lyz:dsRed-*) population and a *cd41:GFP+* population (*cd41:GFP+ lyz:dsRed-*), as shown in (Figure 3.16B). As *cd41:GFP+* cells are enriched for HSCs, it was posited that *cd41:GFP+* cells could produce *lyz:dsRed+* cells, unless these cells were lymphoid biased HSPCs. It was also posited that the double negative (*cd41:GFP- lyz:dsRed-*) would produce *lyz:dsRed+* cells because this heterogeneous mix of WKM cells would include myeloid precursors. However, this population may not rescue irradiated recipients long-term because it does not contain *cd41:GFP+* HSCs. It may also generate *cd41:GFP+* cells. These cells could be thrombocytes derived from TEPs, or precursors to a *cd41:GFP+* HSPC population.

A



B

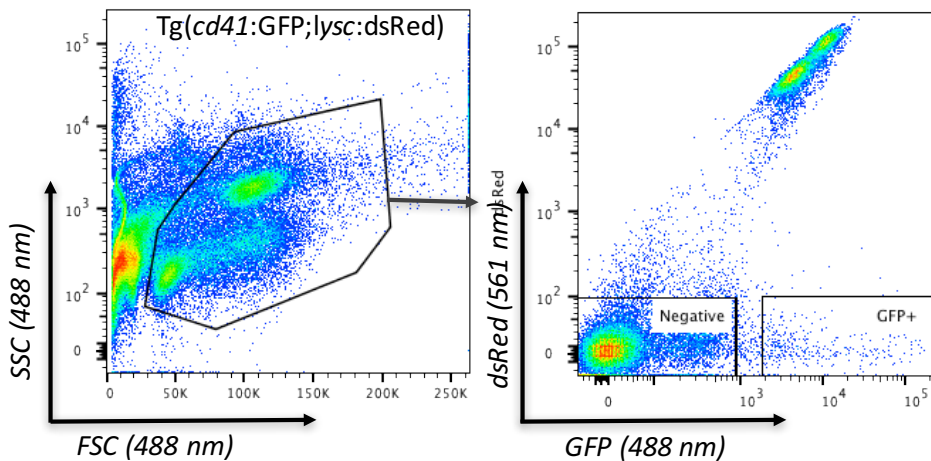


Figure 3.16: Transplantation of HSPCs (*cd41:GFP+*) or WKM cells depleted of mature myeloid cells and HSPCs (*lyz:dsRed* negative, *cd41:GFP* negative). WKM cells from Tg (*cd41:GFP*; *lyz:dsRed*) were sorted for double negative (*lyz:dsRed-* *cd41:GFP-*) and *cd41:GFP+* (*lyz:dsRed-* *cd41:GFP+*) cells. (A) Protocol overview. (B) Flow cytometry scatter of donor WKM cells with gating used to obtain populations from donors for transplant. SSC indicates side scatter; FSC indicates forward scatter (both use 488 nm laser, 488/10 filter). The debris exclusion gated population is separated to obtain a double negative (*lyz:dsRed-* *cd41:GFP-*) and *cd41:GFP+* (*lyz:dsRed-* *cd41:GFP+*) population using GFP (488 nm laser, 530/30 filter) and dsRed (561 nm laser, 610/20 filter).

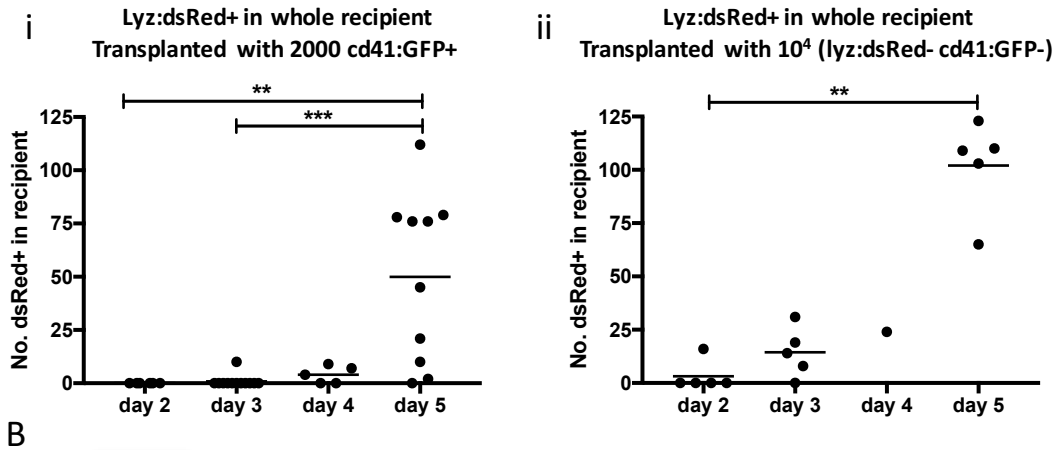
As before, recipient TraNac fish were irradiated (2 doses of 15 Gy, 24 h apart) prior to transplant of either 2,000 cd41:GFP+ lyz:dsRed-, or 10⁴ double negative WKM cells (cd41:GFP- lyz:dsRed-). The number of cd41:GFP+ cells to be transplanted was reached by estimating the number of cd41:GFP+ cells that would have been present in the previous experiments with ubi:GFP donors (Figure 3.9). As the typical WKM contains 0.5 % cd41:GFP+ cells, then 2 x 10⁵ ubi:GFP+ cells would contain approximately 1000 cd41:GFP+ cells. This number was doubled to increase the likelihood of a successful transplant. The number of double negative cells to be transplanted was reached by reducing the number of cells that would have been present in the previous experiments with ubi:GFP donors by an order of magnitude and halving. This number is approximately 1 % of the WKM, but this population is unlikely to contain HSCs.

Cells of interest, either 2,000 cd41:GFP+ lyz:dsRed-, or 10⁴ double negative WKM cells (cd41:GFP- lyz:dsRed-), were transplanted with support cells (Table 1.2) therefore all recipients also received 10⁵ support PB cells (45). Recipients were imaged from 2 to 6 dpt (Figure 3.16A). A transplant dose of 2,000 cd41:GFP+ cells was chosen because adult Tg (cd41:GFP) fish typically have about 5,000 cd41:GFP+ cells and also based on previous experiments (51).

The number of cd41:GFP+ and lyz:dsRed+ cells were counted at non-hematopoietic sites such as around the eye (the site of transplant), the organs, and down the body (Figure 3.17A), as well as in the WKM area (Figure 3.18A).

In recipients of 2,000 cd41:GFP+ cells, cd41:GFP+ cells could be seen in the WKM from 3 dpt, although numbers are constant initially, they increase significantly from 5 dpt (Figure 3.18Ai). Lyz:dsRed+ cells are observed both in the WKM (Figure 3.18Bi) and around the recipient's eye at the same time, 4 dpt (Figure 3.17Ai). As cd41:GFP+ cells were observed in the recipient WKM before lyz:dsRed cells were present in the WKM, it is likely that cd41:GFP+ cells populate the WKM before they differentiate to produce myeloid cells (either directly or through intermediary precursor cells). Importantly, taken with the previous experiment, when 2x 10⁵ lyz:dsRed- cells (a mix of cd41:GFP+ and double negative cells) were transplanted into recipients, lyz:dsRed+ cells also appeared from 4 dpt (Figure 3.15). Furthermore, cd41:GFP+ cells were only detected at 5 dpt, while in these experiments they are visible 2 days earlier, at 3 dpt. Although this seems inconsistent, it is important to note that the number of cd41:GFP+ cells does not vary much between 3 and 5 dpt in this set of results (Figure 3.17Ai). This might indicate that although the cells were not identified at 3 and 4 dpt in the previous transplants, they were possibly already there. In addition, my ability to distinguish the fluorescent cells possibly improved with experience. As the increase in cd41:GFP+ cells in the WKM happens between 5 and 6 dpt in both sets of experiments, and the increase in lyz:dsRed+ cells in the WKM happens between 4 and 5 dpt, together these observations suggest that cd41:GFP+ colonise the WKM at 3 dpt, prioritise production of myeloid cells at 4 dpt and then switch to proliferation at 5 dpt.

A



B

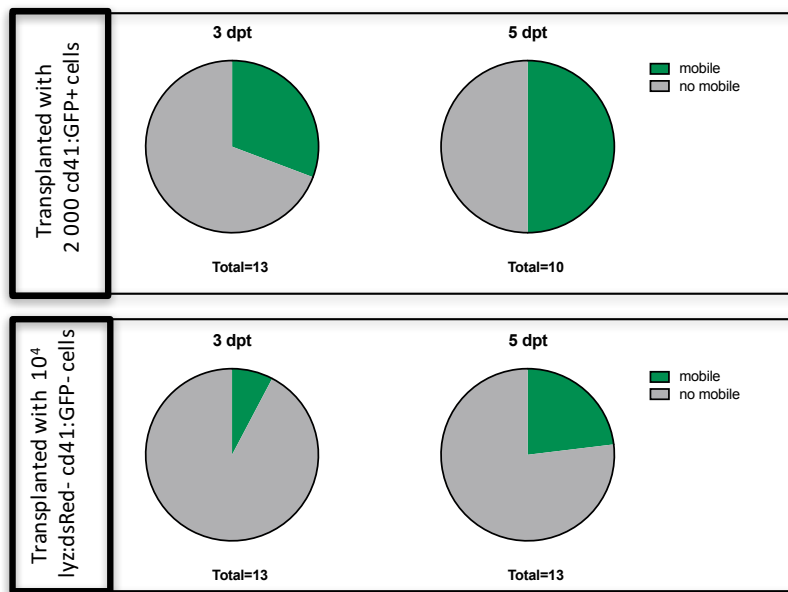


Figure 3.17: Both cd41:GFP+ cells and cd41:GFP- give rise to myeloid cells in the recipient, early post-transplant. WKM cells from Tg (cd41:GFP;lyz:dsRed) were sorted for double negative (lyz:dsRed- cd41:GFP-) and cd41:GFP+ (lyz:dsRed- cd41:GFP+) populations. Irradiated TraNac were transplanted with either 2,000 cd41:GFP+ (lyz:dsRed- cd41:GFP+) or 104 double negative (lyz:dsRed- cd41:GFP-) WKM cells and 10^5 WT PB cells. Non-transplanted recipients did not receive 10^5 WT PB cells. Individual fish were imaged in vivo (widefield Zeiss Axiovert 200 microscope) daily from 2 to 6 dpt with 488 and 506 nm lasers. Fish reproduced with permission from E. Griffith. (A) The number of lyz:dsRed+ cells throughout the recipient, excluding the WKM. One-way ANOVA (Kruskal-Wallis) with Dunn's post-test. Each dot represents an individual fish. (B) The proportion of recipients with mobile cd41:GFP+ throughout the recipient. 3 experiments pooled (n=12).

In recipients of 10^4 double negative cells, a maximum of 15 cd41:GFP+ cells were observed in the recipient for the first 6 dpt (Figure 3.18Aii) and only half as many had mobile cd41:GFP+ cells (23 % and 50 % respectively; Figure 3.17B). On the other hand, lyz:dsRed+ cells were seen in recipients of double negative cells from 3 dpt, a day earlier than fish transplanted with 2,000 cd41:GFP+ cells (Figure 3.17A). They appear in the recipient WKM at 5 dpt, similar to fish transplanted with 2,000 cd41:GFP+ cells (Figure 3.18B).

Finally, survival of transplant recipients suggest that the transplant of double negative cells does not rescue irradiated fish ($p=0.931$; Figure 3.19A). However, transplanting 2,000 cd41:GFP+ did improve survival ($p=0.0576$; Figure 3.19B). The same experiment was also carried out using *bloodless* c-myb^{t25127} mutant recipients (Figure 3.19C). Due to the pigmentation of these fish, cell number could not be accurately assessed using imaging. However, survival of these fish shows that a transplant of either double negative cells or cd41:GFP+ cells can rescue the mutant to 42 dpt (Figure 3.19D). Almost half (47 %) of recipients transplanted with 2,000 cd41:GFP+ cells survive past 28 dpt, significantly more than non-transplanted mutants (Figure 3.19E). The longest survival was 73 dpt; significantly more than non-transplanted siblings, but not as good as the reported 55 % survival to 12 wpt (84 dpt) following transplantation of 4×10^5 WT WKM cells (46).

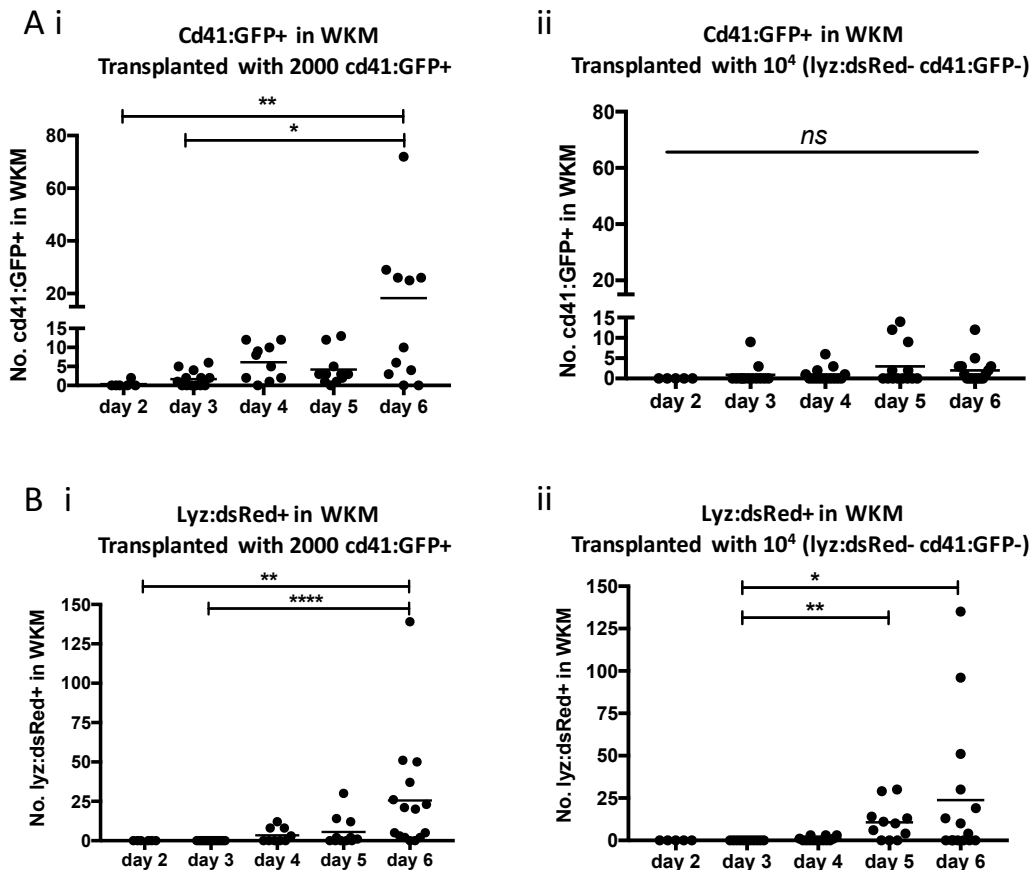
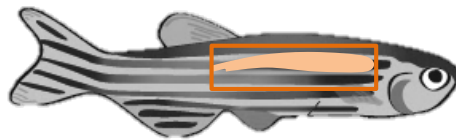


Figure 3.18: Both cd41:GFP+ cells and double negative cell transplantation gives rise to myeloid cells in the WKM of irradiated TraNac transplant recipients. WKM cells from Tg (cd41:GFP;lyz:dsRed) were sorted for double negative (lyz:dsRed- cd41:GFP-) and cd41:GFP+ (lyz:dsRed- cd41:GFP+) populations. Irradiated TraNac were transplanted with either 2,000 cd41:GFP+ or 10^4 double negative (lyz:dsRed- cd41:GFP-) WKM cells and 10^5 WT PB cells. Non-transplanted recipients did not receive 10^5 WT PB cells. Individual fish were repeatedly imaged in vivo under a Zeiss Axiovert 200 microscope daily from 2 to 6 dpt with 488 and 506 nm lasers. Fish reproduced with permission from E. Griffith. (A) The number of cd41:GFP+ cells in the WKM of the recipients. (B) The number of lyz:dsRed+ cells in the WKM of the recipients. Each dot represents an individual fish. One-way ANOVA (Kruskal-Wallis) with Dunn's post-test. 3 experiments pooled ($n=12$).

Together these data show that 2,000 cd41:GFP+ cells have the potential to rescue irradiated recipients and produce both cd41:GFP+ and lyz:dsRed+ cells (in all 12/12 fish). Fish injected with double negative cells produced very few cd41:GFP+ cells, but lyz:dsRed+ cells were visible in these recipients. The lower survival rate of these recipients suggests that cd41:GFP+ cells are required for HCT rescue.

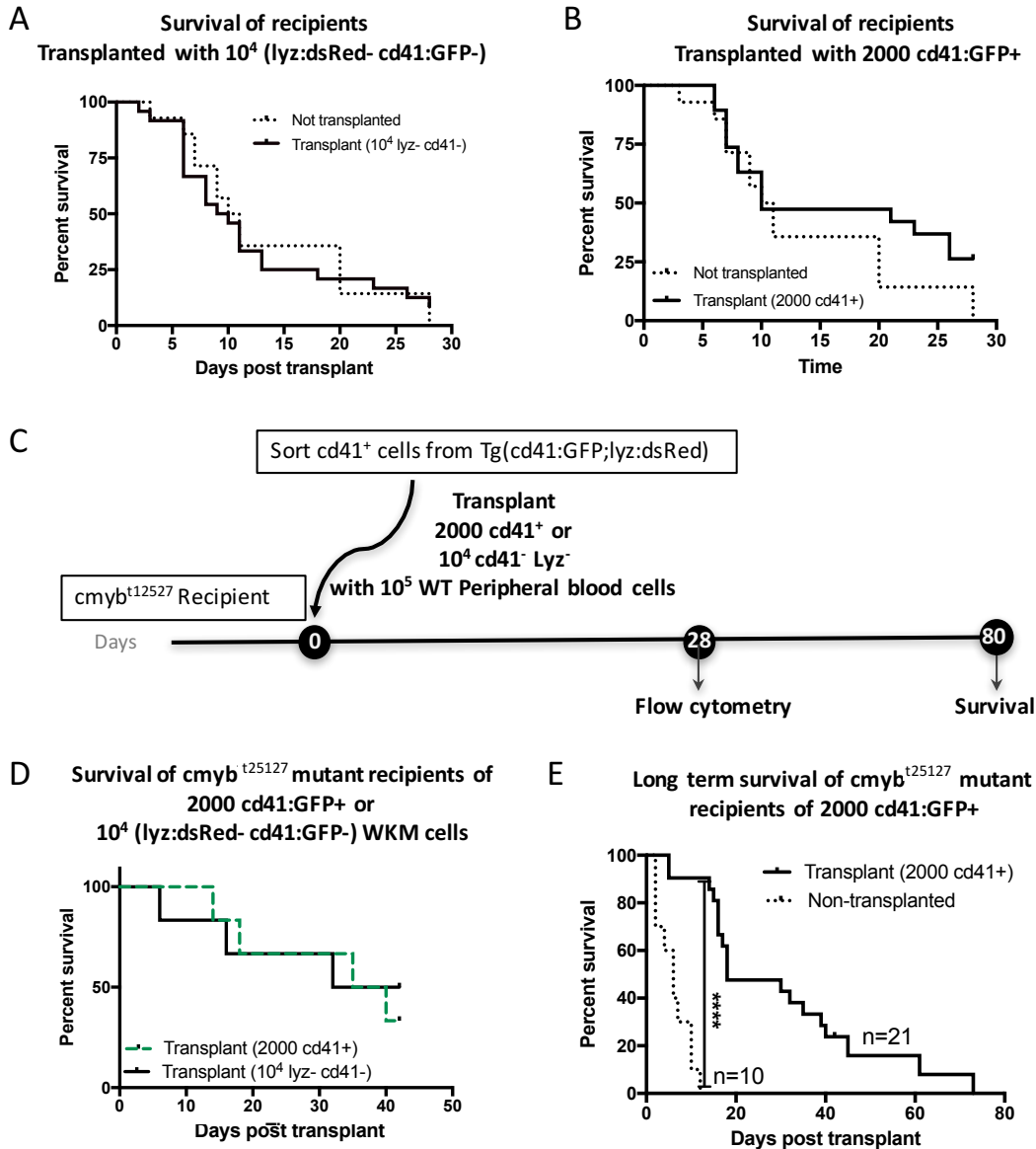


Figure 3.19: Survival of irradiated TraNac recipients of 2,000 cd41:GFP+ or 104 double negative WKM cells (lyz:dsRed-cd41:GFP-). WKM cells from Tg (cd41:GFP;lyz:dsRed) were sorted for double negative (lyz- cd41-) and GFP+ (cd41+) populations. Irradiated TraNac and c-myb bloodless mutants were transplanted with either 2,000 cd41:GFP+ or 10^4 double negative (lyz- cd41-) WKM cells and 10^5 WT PB cells. Non-transplanted recipients did not receive 10^5 WT PB cells. (A) Survival chart of TraNac recipients of 10^4 double negative (lyz- cd41-) WKM cells. 3 experiments pooled, n =24. (B) Survival chart of TraNac recipients of 2,000 cd41:GFP+ WKM cells. 3 experiments pooled, n =19. (C) Overview of protocol of experiments with c-myb bloodless mutants. (D) Survival of c-myb mutant recipients of 10^4 double negative (lyz- cd41-) WKM cells or 2,000 cd41:GFP+ WKM cells; 2 experiments (transplanted 2,000 cd41:GFP+ n=8, 10^4 double negative n=8). (E) Survival of c-myb fish, with and without a transplant of 2,000 cd41:GFP+ WKM cells (3 experiments, transplanted n=21, non-transplanted n=10).

Interestingly, there was no difference in the survival of c-myb mutant recipients of double negative cells and c-myb mutant recipients of cd41:GFP+ cells. Perhaps tracking these recipients for longer than 40 days, perhaps 6 months, would determine if the long term reconstitution is restricted to the cd41:GFP+ transplanted individuals.

3.3.4.3 Reducing cell dose reduces the rate of early post-transplant WKM repopulation rates but does not affect long term reconstitution

For this thesis, there was a need to establish a dose at which HSCs would be pushed to their limit so that reconstituting ability between HSCs from immune-challenged donors and healthy donors can be assessed. As shown above, 2,000 donor cd41:GFP+ cells can rescue irradiated TraNac recipients. In subsequent experiments, irradiated TraNac recipients received a transplant of 200, 50 or 20 cd41:GFP+ cells from Tg (cd41:GFP;lyz:dsRed) along with 10^5 PB cells (Figure 3.20A, B). Recipients were imaged 6 dpt as previous experiments had shown that recipients had visible cells in the WKM at this time point (Figure 3.18).

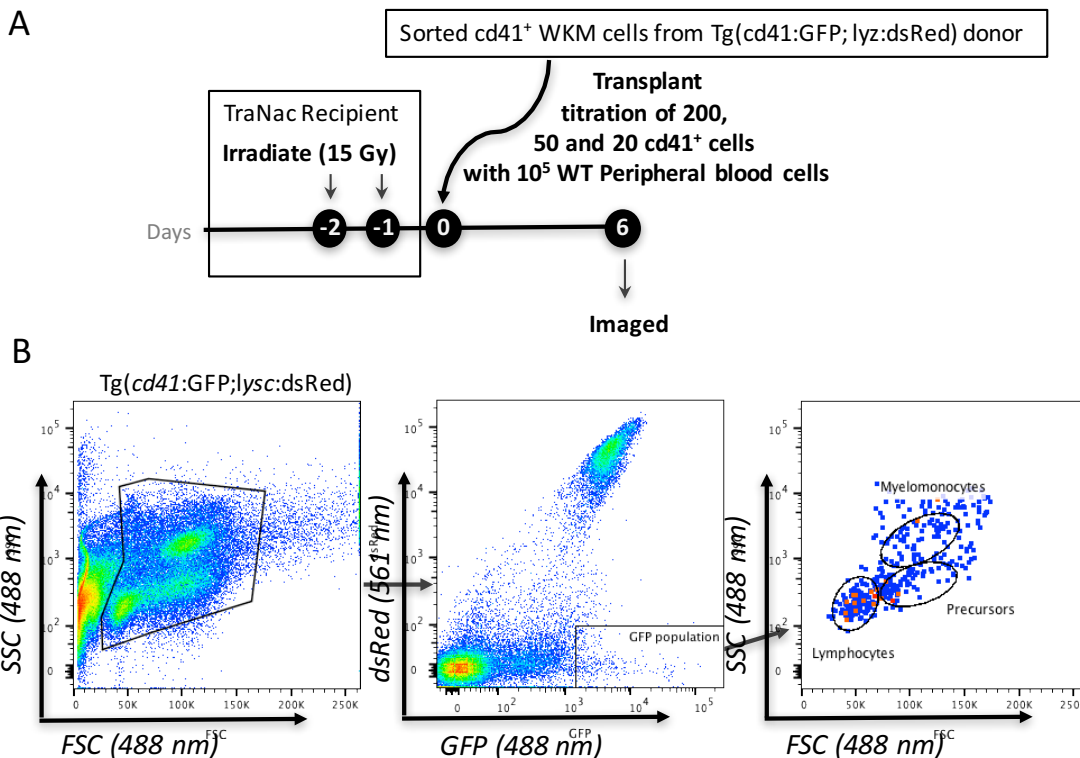


Figure 3.20: Titrating the number of transplanted cd41:GFP+ cells. WKM cells from Tg (cd41:GFP;lyz:dsRed) were sorted for GFP+ cells. (A) Procedure overview. (B) Flow cytometry scatter of donor WKM cells with gating used to obtain populations from donors for transplant. SSC indicates side scatter; FSC indicates forward scatter (both use 488 nm laser, 488/10 filter). The debris exclusion gated population is separated to obtain a lyz:dsRed- cd41:GFP+ population using GFP (488 nm laser, 530/30 filter), dsRed (561 nm laser, 610/20 filter). Far right scatter is a density plot of the sorted population (large dots).

The counts at 6 dpt show that the more cells that get transplanted, the more cells are visible in the WKM at this early time point post-transplant (Figure 3.21A, B). Fish receiving a transplant of 200 cells had on average 12 cd41:GFP+ cells (2 of 22 had none) and 26 lyz:dsRed+ cells (1 of 22 had none) at 6 dpt. However, fish receiving

only 20 cd41:GFP+ cells had on average 3 cd41:GFP+ cells (5 of 13 had none) and an average of 3 lyz:dsRed+ cells (9 of 13 had none) at 6 dpt (Figure 3.21A, B). Fish were imaged again at 12 dpt and 10 of 16 fish that received a transplant of 20 cd41:GFP+ cells had at least 1 fluorescent cell in the WKM. Most (29 of 32) surviving recipients of a transplant of 200 cd41:GFP+ cells had cells in the WKM by 12 dpt.

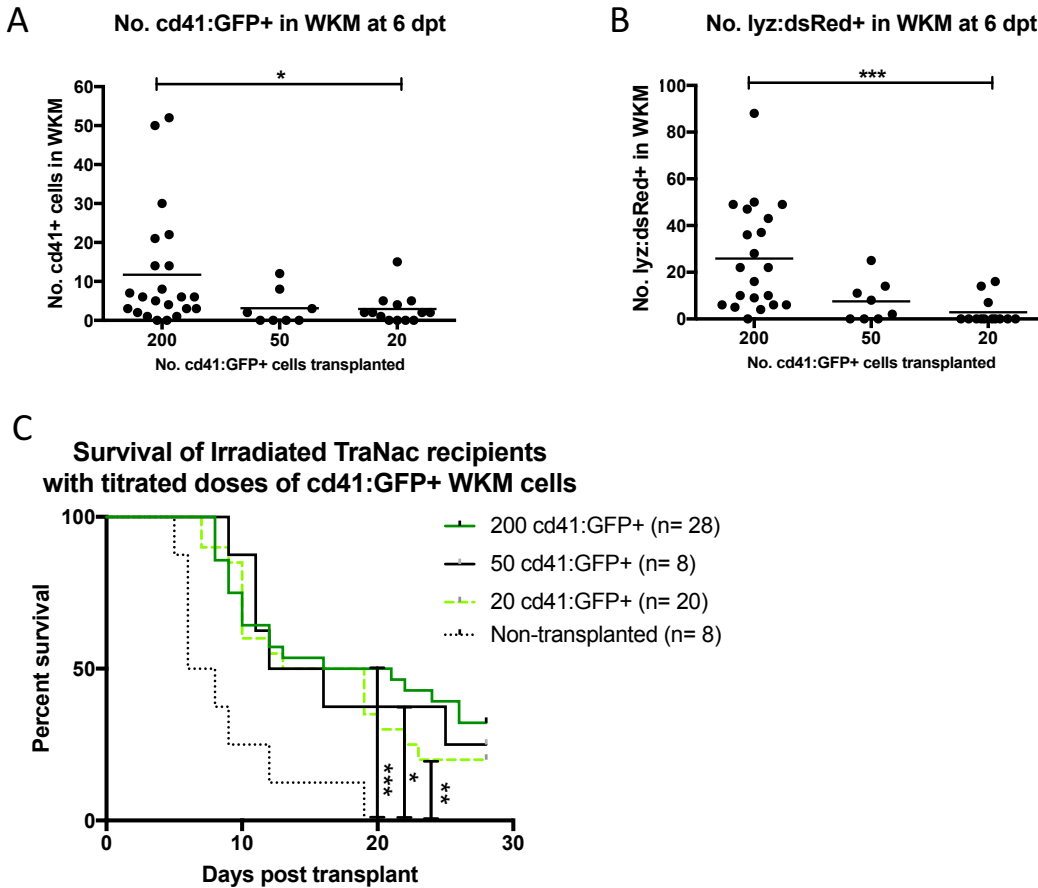


Figure 3.21: The presence of fluorescent cells in the WKM at 6 dpt is dependent on the number of transplanted cd41:GFP+ cells. WKM cells from Tg (cd41:GFP;lyzC:dsRed) donors were sorted for cd41:GFP+ cells. Irradiated TraNac were transplanted with 200, 50 or 20 cd41:GFP+ cells and 10^5 WT PB cells. Non-transplanted recipients did not receive 10^5 WT PB cells. The number of (A) cd41:GFP+ and (B) lyz:dsRed+ were counted in the WKM of the recipients. Each dot represents an individual recipient. Individual recipients were monitored for 28 days. (C) Survival of irradiated TraNac recipients transplanted with 200, 50 or 20 cd41:GFP+ cells. Four experiments pooled, Logrank test (*= $p<0.05$, **= $p<0.01$, ***= $p<0.001$).

Survival to 28 dpt was similar among the 3 groups (Figure 3.21C). Just 4 out of 20 of the recipients of 20 cd41:GFP+ cells survived to 28 dpt and their WKM was harvested for flow cytometry. They had too few cd41:GFP+ or lyz:dsRed+ cells to be detected by flow cytometry. A quarter (2 out of 8) of recipients of 50 cd41:GFP+ survived to 28 dpt. Both had lyz:dsRed+ cells but neither had cd41:GFP+. Surviving recipients of 200 cd41:GFP had both lyz:dsRed+ and cd41:GFP+ cells in the WKM at 28 dpt. Together, this suggests that the survival of the 4 recipients of 20 cd41:GFP+ cells was possibly due to the sub-lethal irradiation dose, and moreover, the PB carrier cells transplanted with the 20 cd41:GFP+ cells helped with their survival. A transplant of 20 or 50 cells did not yield adequate imaging data or survival for further studies. In order to ensure a robust imaging data and survival, a dose of 200 cells was chosen for future experiments.

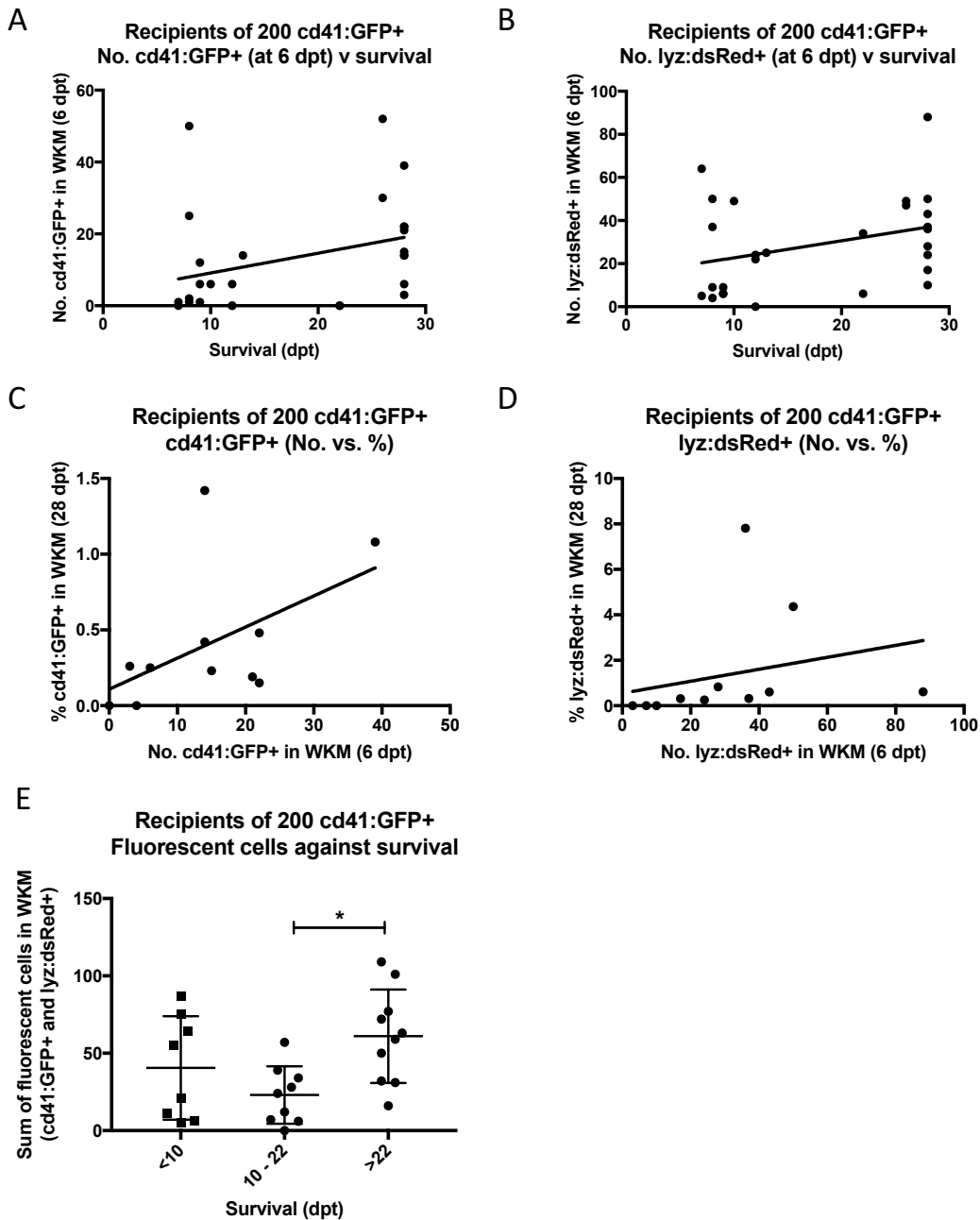


Figure 3.22: Transplanted cell behaviour in irradiation recipients of 200 cd41:GFP+ cells. WKM cells from Tg (cd41:GFP;lyzC:dsRed) donors were sorted for cd41:GFP+ cells. Irradiated TraNac were transplanted with 200, 50 or 20 cd41:GFP+ cells and 10^5 WT PB cells. Non-transplanted recipients did not receive 10^5 WT PB cells. Individual recipients were monitored for 28 days to correlate the number of (A) cd41:GFP+ and (B) lyz:dsRed+ in the WKM at 6 dpt with recipient survival, up to 28 dpt. At 28 dpt, recipient WKM was harvested for flow cytometry. The number of (C) cd41:GFP+ and (D) lyz:dsRed+ cells at 6 dpt was correlated with the percentage of cd41:GFP+ and lyz:dsRed+ cells (in the WKM, after debris exclusion). (E) Fish were split into 3 survival groups (<10 days, 10 to 22 days and > 22 dpt) to compare the total number of cd41:GFP+ and lyz:dsRed+ cells at 6 dpt with survival. Five experiments pooled. Includes data from controls in PolyI:C recipient experiments. Recipients transplanted with 200 (n=22), 50 (n=8) or 20 (n=13) cd41:GFP+ WKM cells.

One of the aims of this thesis was to determine whether transplant recipient survival could be predicted by observing post-transplant cell behaviour. Irradiated TraNac fish were transplanted with 200 cd41:GFP+ cells, imaged 6 dpt and tracked so that early imaging data could be matched with survival (Figure 3.22A,B). Neither the number of cd41:GFP+ (R square = 0.1099; p=0.0912), nor the number of lyz:dsRed+ (R square =0.1097; p=0.0914) cells in the WKM, significantly correlated with survival of the fish.

A third (35 %) of recipients died in the first 10 dpt (Figure 3.21C). Remarkably, these fish had a range of cd41:GFP+ (9 out of 11 had <10; 1 recipient had 50 and 1 had 25) and lyz:dsRed+ (7/11 had <10; 4 recipients between 37 and 64 cells) cells in the WKM at 6 dpt (Figure 3.22A,B). After this point, 50 % of the remaining fish (35 % of the overall) survived to 28 dpt (Figure 3.21C). Interestingly, the fish that survive to 28 dpt also had a range of cd41:GFP+ (7 of 9 recipients had between 14 and 39; 2 had <10 cells) and lyz:dsRed+ (8 of 9 recipients had between 17 and 88; 1 had 10 cells) cells at 6 dpt. The 30 dpt survival of fish with <10 fluorescent cells (Figure 3.22A,B) at 6 dpt could reflect the low number of fish that survive despite not receiving a transplant (Figure 3.7). It would appear that deaths occurring prior to 10 dpt cannot be predicted by the number of cells present at 6 dpt. However, the deaths after this period can be anticipated (Figure 3.22E). Recipients that died between 10 and 22 dpt had on average 66 % fewer fluorescent cells than recipients which survived beyond this point (Figure 3.22E). Thus, culling fish with fewer than <25 cells total at 6 dpt would reduce suffering in this protocol, without impacting the amount of data that could be obtained from the experiment (as these fish would not survive to 28 dpt anyway).

Flow cytometry was used to detect the number of fluorescent cells in the surviving recipients' WKM at 28 dpt (Figure 3.22C, D). There was no correlation between the number of cd41:GFP (R square = 0.0995; p=0.4466) cells, or the number of lyz:dsRed+ cells (R square = 0.001; p=0.9304), between 6 and 28 dpt.

3.3.5 Identifying dormant HSPCs in the zebrafish

3.3.5.1 BrdU labelling could not identify a dormant HSC population in the zebrafish

The transplantation experiments reported so far have relied on HSPC markers cd41 and runx. Both have been good markers for enrichment of the HSC population. However, in mice, the population of HSCs containing the best long-term self-renewal capacity are slow cycling “dormant” cells, also known as LT-HSCs. These have not yet been identified in zebrafish. In mice, they were discovered using BrdU in a “label retention assay”. In brief, cells are “pulsed” with BrdU, a thymidine analog that integrates into proliferating cells by replacing the thymidine residues. The BrdU is then removed from the system to start the “chase”. Proliferating cells lose BrdU as the replicated genome integrates thymidines again instead of BrdU. Non-proliferating cells do not lose the label as the genome is not being replicated (Figure 3.23B).

The BrdU label retention assay was used in order to identify if zebrafish have slower cycling cells with the objective of using this label in conjunction with cd41+ or runx1+ populations in transplantation assays. Firstly, it was established that BrdU labelling of WKM cells could be achieved by adding BrdU to tank water or injecting fish with the BrdU solution. WT and Tg (cd41:GFP) fish were either injected with 10mM BrdU twice a day for 4 days or BrdU was added to the tank water (10mM BrdU) for 4 days. The fish were then humanely killed, the WKM harvested, and the cells fixed and stained with anti-BrdU antibody. The samples were then analysed by flow cytometry (Figure 3.23A).

Injecting fish with BrdU labelled fewer WKM cells than swimming fish in a 10 mM BrdU solution (18 % and 25 % respectively; Figure 3.23C). This is probably because there is a chase period between injections when the cells that have taken up the BrdU are dividing and the thymidine analogue is being lost. Also, the percentage of cells labelled was more consistent between fish that bathed in the chemical.

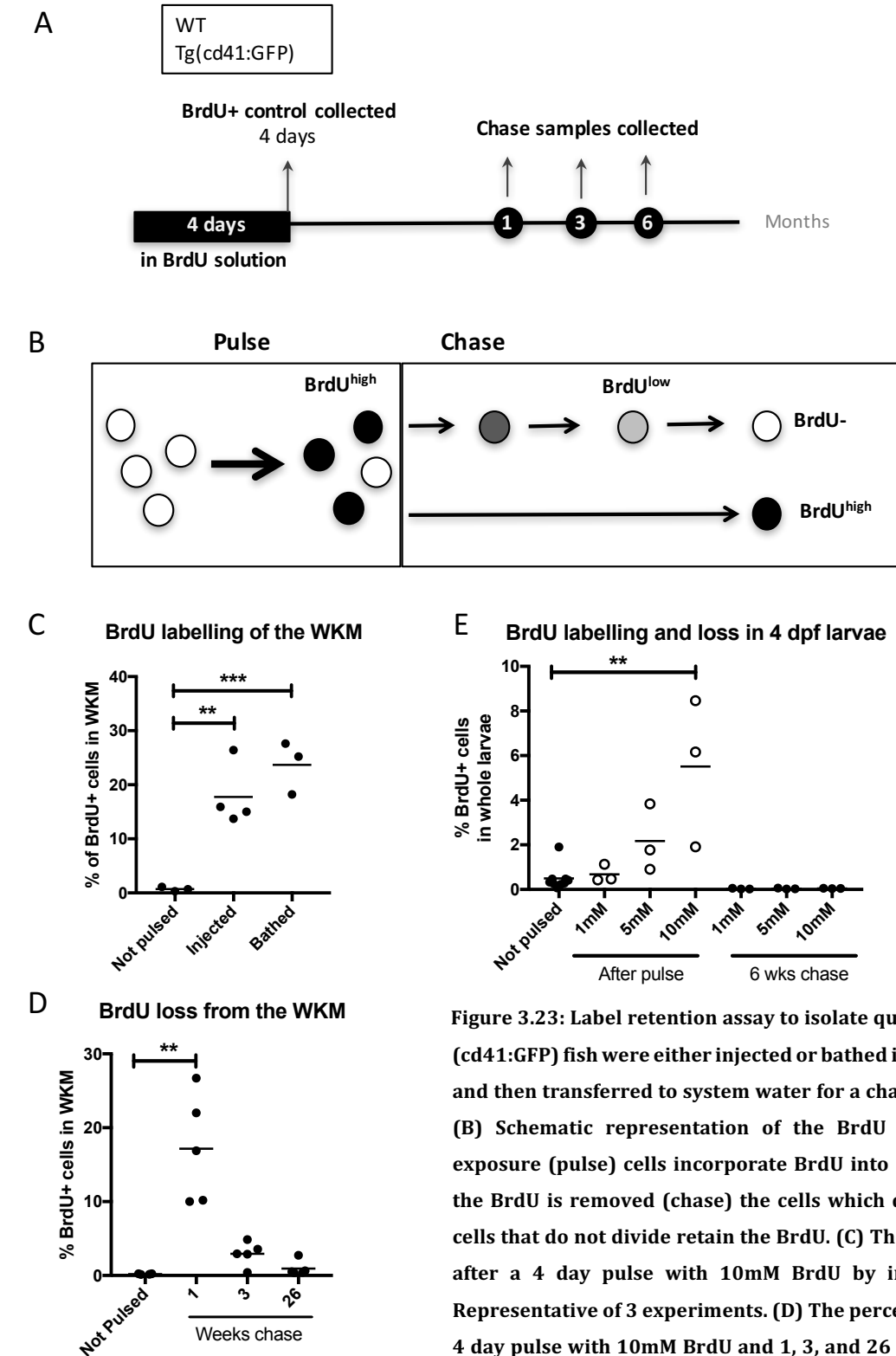


Figure 3.23: Label retention assay to isolate quiescent stem cells. TraNac and Tg (cd41:GFP) fish were either injected or bathed in 10mM BrdU for a pulse of 4 days and then transferred to system water for a chase period. (A) Protocol overview. (B) Schematic representation of the BrdU labelling process. During BrdU exposure (pulse) cells incorporate BrdU into DNA during DNA synthesis. After the BrdU is removed (chase) the cells which divide more will lose BrdU while cells that do not divide retain the BrdU. (C) The percentage of BrdU+ WKM cells after a 4 day pulse with 10mM BrdU by injection or bathing in solution. Representative of 3 experiments. (D) The percentage of BrdU+ WKM cells after a 4 day pulse with 10mM BrdU and 1, 3, and 26 weeks chase. Representative of 2 experiments. (E) The percentage of BrdU+ cells in Tg (cd41:GFP) larvae (4 days post fertilisation) after a 4 day pulse in 1, 5 and 10mM BrdU and after 56 days chase. Representative of 2 experiments. Statistical significance using one-way ANOVA and Tukey post test. **= $p < 0.01$, ***= $p < 0.001$

In subsequent experiments, BrdU was added to the water for a 4 day “pulse” period. Immediately after pulsing, fish had 25% of their WKM cells labelled with BrdU. However, 7 days later, only 18 % of WKM cells were BrdU+, and there was a wide range of labelling between fish (Figure 3.23D). After 3 weeks, only 5 % of WKM cells were BrdU+, and this population had disappeared completely (in 3 out of 4 fish) by 26 weeks (6 months). In one of the samples, 5 % of cells were BrdU+ after 26 weeks chase. The experiment needs to be repeated with more fish to determine if this result is an outlier or a true result. Due to the time requirement, this was not possible in the scope of the thesis. As GFP is quenched by fixation of cells, an anti-GFP antibody was used in conjunction with the anti-BrdU in order to correlate cd41 expression with slow cycling cells. No overlap between these two populations was seen (data not shown).

The original experiments were done in adult fish (>3 mpf; (Figure 3.23C, D) but no BrdU+ population was successfully identified in the WKM. It was reasoned that pulsing at 4 dpf, when HSCs have emerged from the aorta and are proliferating in the CHT, could help to label cells destined to become slowly dividing at a time when they are still highly replicating. Thus, 4 dpf larvae were pulsed for 3 days by adding BrdU to the E2 solution (1, 5 and 10 mM) used to raise embryos. At 7 dpf, these larvae are too small to dissect, therefore the whole organism was mashed, digested and filtered to run through the flow cytometer and assess BrdU incorporation after pulse. At the highest dose (10mM), BrdU labelled the largest percentage of cells (up to 9 % of the whole larvae). No BrdU+ cells were detected after 6 week chase (Figure 3.23E).

3.4 Discussion

Although the aim of this thesis is to assess the effect of inflammation on HSCs, it was first necessary to establish the baseline reconstitution ability of hematopoietic cells from healthy donors into WT adult recipients (pre-treated with irradiation). This chapter recapitulates previously described research and then builds upon zebrafish HCT by determining a method for assaying early post-transplant hematopoietic cell behaviour.

3.4.1 Summary of results

The findings of this chapter were:

1. Flow cytometry analysis of fish conditioned with 2 doses of 15 Gy *gamma*-radiation showed that, at the time of transplant, they had a significantly decreased erythrocytes, lymphocytes and HSPCs in the WKM (although the decrease was found in both runx1+23:NLSmCherry and cd41:GFP+ populations, but not the runx1+23:GFP+ population which was not significantly affected). Furthermore, qPCR data supported these findings as the cell lineage-associated transcripts for erythrocytes and lymphocytes were also decreased. Flow cytometry and qPCR data both suggest the myelomonocytes were less affected by the treatment.

2. Transcript levels of cytokines *il-1 β* , *il-6*, *tnf α* and *ifn γ 1.1* were all increased (2-4 fold) in irradiated fish at the time of transplant. Furthermore, transcript levels of chemokines *cxcl-c1c* and *cxcl12* were also increased.
3. Previously reported transplant protocols were replicated and confirmed previous findings. HCT was performed with 10^6 , 10^5 and 5×10^4 heterogeneous ubi:GFP+ WKM cells. The reconstitution of recipients was successful, as gauged by the significant survival and the significant percentage of ubi:GFP+ cells in the WKM of these recipients at 28 dpt. There were no differences between recipients of different doses, in terms of the percentage of ubi:GFP+ cells in the recipient at 28 dpt. Flow cytometry analysis suggested that the percentage of precursors in the WKM was greater at 28 dpt, compared with non-transplant naïve fish.
4. Live imaging of small optically transparent recipient fish revealed that transplanted cd41:GFP+ cells behave in 2 different ways: highly mobile and sedentary. Furthermore, these transplanted cells appeared to colonise the head WKM first (4 dpt), and subsequently the mid and tail WKM (5 dpt).
5. Donor cd41:GFP+ cells were not visible in recipients of transplanted 10^4 double negative (cd41:GFP-lyz:dsRed-) cells or 20 cd41+GFP+ cells. Some cd41:GFP+ cells were visible in recipients of 50 cd41:GFP+ cells but the best conditions for visualisation were in recipients of 200 cd41:GFP+ fish.
6. One third of transplant recipients of 200 cd41:GFP+ cells survived to 28 dpt. The highest number of deaths occurred between 8 and 12 dpt, even when donor cells were visible in the recipient by 8 dpt, meaning these deaths cannot be predicted. However, in successful transplants (survival and hematopoietic reconstitution by 28 dpt), fluorescent cells would be visible in the recipient WKM by 6 dpt and therefore any recipients without fluorescent cells should be humanely killed at this time point. In addition, survival was significantly lower (died before <22 dpt) when fewer than 25 fluorescent donor cells (sum of cd41:GFP and lyz:dsRed) were visible in the WKM at 8 dpt and therefore these fish should be humanely killed if studies persist beyond this range.
7. A 4 day chase with BrdU labelled ≈ 30 % of WKM cells. After a 3 week chase the BrdU+ population was < 5 % of WKM cells and by 6 months there were <0 % BrdU+ (1 or 2 events) cells in the WKM. Ultimately, attempts at labelling a dormant hematopoietic population in the zebrafish were unsuccessful.

3.4.2 Irradiation causes inflammation in the zebrafish

For HCT, recipients are depleted of resident hematopoietic populations to allow donor cells to engraft. In zebrafish models, this can be achieved through radiation (135, 386). The literature uses a variety of radiation doses (20-40 Gy). Traver *et al.* showed that a dose of 40 Gy was too high as a HCT (5×10^5) could not rescue recipients (135). A dose of 30 Gy (split into 2 doses of 15 Gy) was recommended as it was shown to result in good engraftment (45, 47). The split dose of 15 Gy is thought to be more effective for 2 reasons: firstly, it minimises the damage to less radiosensitive tissues (47); secondly demand for hematopoiesis is driven by the first dose, and thus the second dose of irradiation will target the activated cells. Finally, as de Jong *et al.* showed that the sensitivity (survival) to irradiation was dependent on the line of zebrafish (47), it was decided that a single dose of 30 Gy and 2 doses of 15 Gy would be tested on multiple WT and transgenic lines. However, in this study the

sensitivity of the lines was not determined by survival, therefore this factor cannot be compared. The Tg (cd41:GFP), Tg (runx1+23:GFP) and Tg (runx1+23:NLSmCherry) were used to assess whether HSPCs were depleted by the dose.

The effect of 30 Gy irradiation on hematopoietic cells of the WKM was assessed 24 hours and 5 days after treatment, based on the time course published by Traver *et al.* in which they show the myeloid, lymphoid and precursor populations are most significantly affected in WT fish after exposure to 20 Gy (135). Our results showed a similar decrease following 30 Gy in all 3 of these blood populations, both 24 hpIR and 5 dpIR (Figure 3.1C and Figure 3.2C). There was no difference between a single 30 Gy dose or when this was split into 2 doses of 15 Gy 24 h apart in WT fish (Figure 3.1C). The significantly depletion of WKM at 5 dpIR may be the reason why survival only begins to decline from 7 dpIR (Figure 3.7).

Traver *et al.* excluded the erythrocyte population because they found too much variation (135), which they explain is due to the limitations of dissecting the kidney separate to the dorsal aorta (although, they do include this population when they examine the recipients post-transplant). The flow cytometry results presented here showed an increase in erythrocytes (Figure 3.1C and Figure 3.2C). However, subsequent qRT-PCR data showed a significant drop in the erythrocyte gene *gata1* in irradiated samples at 24 hpIR (Figure 3.4B). This suggests that the increase in erythrocytes is due to debris scatter and the relative decreases in percentages of myeloid, lymphoid and precursors populations. Altogether, this data shows that the hematopoietic cells of the WKM are depleted following irradiation. These results gave cause to add PB in transplants that did not include a heterogeneous WKM mix or which contain a reduced number of donor cells.

Traver *et al.* also reported a slight increase in the myeloid population, which they suggest was due to the relative decrease in lymphoid and precursor populations (135). The results of the experiments done with a variety of fish lines suggest the myeloid population did not change. These results are supported by qRT-PCR data from whole fish samples harvested at 24 hpIR (Figure 3.4B), in which transcript levels of *l-plastin*, *mpeg* and *mfap4* remained unchanged. However, the increase in myeloid cells observed by Traver *et al.* are supported by a significant increase in transcript levels of monocyte and macrophage receptor *csf1ra* (Figure 3.4B).

The difference between Traver *et al.* findings, and the results presented here could be due to the differences in irradiation doses (30 Gy, while Traver *et al.* used 20 Gy). It may also be due to the limited number of fish examined in this study (n=3, while Traver *et al.* used n=21), or finally it may be the difference in zebrafish lines as reported by de Jong (47). It did not seem necessary to pursue these results further as it was clear the irradiation was having an effect on the WKM.

In mice and humans, radiotherapy is used to kill leukemic stem cells, and therefore HSCs are likely to be affected by the treatment. The effect of irradiation on the zebrafish HSPC population was assessed by irradiating reporter lines Tg (cd41:GFP), Tg (runx1+23:GFP) and Tg (runx1+23:NLSmCherry). To our knowledge, the effect of

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irradiation on HSPCs in the zebrafish has not been studied. The HSPC populations marked by cd41:GFP and runx1:mCherry were significantly decreased after 24 h (Figure 3.3B, C, D). However, the runx1:GFP population was not. This might be due to the difference in sensitivity between the labelled populations. For instance, cd41:GFP+ marks both thrombocytes and HSPCs. The significant decrease may be due to the sensitivity of the thrombocyte cells rather than the HSPCs. Distinguishing between cd41^{high} and cd41^{low} has been difficult (Supplementary Figure 7.4), however all attempts at splitting this population show that the decrease in cd41:GFP+ cells is significant for both cd41^{high} and cd41^{low} populations. Thus, the HSPC population is likely to be affected.

The difference between Tg (runx1+23:GFP) and Tg (runx1+23:NLSmCherry) is the NLS which sequesters the fluorescent protein in the nucleus, possibly preventing the fluorophore from being degraded, and thus, although cells no longer express *runx1*, they still contain the dsRed fluorophore (42). It may also be the difference in the half-life of these proteins. In any case, the runx1:NLSmCherry population is significantly bigger than the runx1:GFP population (possibly because the mCherry protein is retained in cells after they have differentiated). Runx1+23:NLSmCherry+ cells probably include a multipotent progenitor population as well as HSCs. The significant decrease in the runx1+23:NLSmCherry population following irradiation may be due to the sensitivity of the MMP population, which likely makes up a higher proportion of the runx1+23:NLSmCherry+ population.

Additionally, both the cd41:GFP+ and runx1+23:NLSmCherry+ populations were significantly decreased 5 dpIR (Figure 3.3B, D) but the runx1+23:GFP population was not (Figure 3.3C). This result might indicate that the runx1+23:GFP+ population is more resistant to irradiation. Together these results indicate that the HSPC population is decreased after irradiation. This could be because the population is radiosensitive, or it could be due to hematopoietic production (demand driven hematopoiesis) following the depletion of progeny myeloid, lymphoid and precursors cells. However, demand driven hematopoiesis is usually accompanied by HSPC self-renewal, which would cause this population to increase.

As previously reported in the mouse (367-369), the transcript levels of cytokines *il-1 β* , *il-6*, and *tnf α* were all increased in irradiated fish 24 h after two doses of 15 Gy (Figure 3.5). In addition, transcript levels of *cxcl-c1c*, a neutrophil chemokine, were increased. CXCL-c1c shares 40-50 % sequence similarity with many human proteins of the CXCL family, with CXCL10 (47.5 %) and CXCL8 (47.9 %) being the most similar (387). To our knowledge, high transcript levels of this gene have not been previously reported in association with irradiation. However, irradiation is known to cause recruitment of immune cells (388) and therefore it is perhaps not surprising that the neutrophil chemokine should be increased in irradiated fish. An ancestral genome duplication in the zebrafish lineage means that many zebrafish genes are present twice. Because of this, the duplicated genes sometimes become functionally or temporally distinct. This could explain why the cytokine *ifn γ 1.1* was increased, but the analogous *ifn γ 1.2* remained unchanged. Interestingly, with view of this work involving HSCs, the chemokine *cxcl12a* was increased in irradiated fish. The chemokine CXCL12 is a potent ligand expressed by CXCL12-abundant reticular (CAR) cells in the mammalian HSC niche that is essential for maintaining HSCs in the

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niche through binding with its receptor CXCR4 (237). Zebrafish HSPC maintenance is also regulated by an analogous CXCL12a-CXCR4 axis (307). High transcript levels of *cxcl12a* in the irradiated fish could have a bearing on the behaviour of transplanted HSPCs into the irradiated recipient environment. One way to avoid the confounding factor of a high cytokine milieu would be to use *bloodless* mutants (46). These fish could also be used to determine if HSPC behaviour is affected by the chemokine levels in irradiated recipients.

Preliminary work with these *bloodless* mutants is presented in this chapter (Figure 3.12). As described by Hess, transplanted *c-myb*^{t25127} mutants were indistinguishable from WT fish (46). Like Hess *et al.* (46), the histological sections performed here show the kidney qualitatively devoid of blood cells in the *bloodless* mutant (Figure 3.11C). The post-transplant reconstitution of the mutant system was compared to that of the non-mutant siblings (preconditioned with irradiation). It was hypothesised that *c-myb*^{t25127} mutants would be superior recipients as the entire hematopoietic compartment of the would be donor derived. However, the flow cytometry results show that these recipients do not necessarily reconstitute a comparable hematopoietic profile to that of their donor (Figure 3.13C). For instance, 1 of these recipients did not have any *lyz:dsRed*⁺ cells, while another had 25 % *lyz:dsRed*⁺, similar to the percentage in the WKM of Tg (*lyz:dsRed*) fish. These results are difficult to explain but indicate a loss of the *lyz:dsRed*⁺ subset of the myeloid population in this recipient. Perhaps the transplanted donor cells have lost or silenced this gene. This work was important as it revealed the possibility of using juvenile TraNac to visualise single cells in the transplant recipient.

3.4.3 Optimising HCT to obtain information on early post-transplant cell behaviour

Current “best-practise” of zebrafish HCT is done by RO injection. This protocol was amended to use a micro-injector and the technique verified was using dextran, a glucan used as a tracer because it marks the vasculature by binding to the endothelium and erythrocytes. Typically, researchers use dextran systematically when transplanting into zebrafish recipients of HCT. However, due to the long lifetime of this tracer, it was interfering with accurate counting of cells in the WKM at early time points post-transplant and therefore had to be omitted from the work done in this thesis. Because of this, the success of the method could not be verified in each transplant recipient and so it is possible that some recipients in which transgenic cells cannot be observed post-transplantation, could be due to a failure in transplanting the cells correctly. On the other hand, in a preliminary experiment, dextran was added to donor cells for transplantation. All 32 recipients were correctly injected, as demonstrated by the spread of dextran throughout the recipient, and showed similar survival and reconstitution to fish in subsequent experiments, transplanted without dextran for verification.

Similar to the experiments presented here, transplanting TraNac with 10⁵ WKM cells from Tg (*ubi:GFP*)), White *et al.* had transplanted optically transparent mutant line casper fish with 10⁵ β -actin:GFP⁺ WKM cells from Tg (β -actin:GFP) (379). They found that it took 2 weeks before they could observe β -actin:GFP⁺ cells in the WKM of these fish. In the experiments presented here, individual *ubi:GFP*⁺ cells were not visible in the WKM, although the area of the WKM was GFP⁺ under a fluorescent microscope at 14 dpt. Furthermore, White *et al.* found that

survival to 24 dpt was 50 %. Similarly, survival of transplant recipients of 10^5 ubi:GFP+ cells was 45 % at 24 dpt (Figure 3.9D).

It is worth noting that all transplants presented here were done without immune matching (genetically determining MHCs). De Jong *et al.* found that matching class I MHC genes on chromosome 19 between donors and recipients increased engraftment and percentage donor chimerism at 14 to 16 wpt ($47.86 \% \pm 30.9 \%$ and $10.51 \% \pm 19.88 \%$ for myeloid and lymphoid cells respectively compared with $6.45 \% \pm 1.77 \%$ and $1.28 \% \pm 0.38 \%$ for myeloid and lymphoid cells in unmatched fish) (45). The same group expanded these initial studies, matching class I genes on chromosome 19 (as before) and chromosome 3, as well as matching class II genes on chromosome 8 (47). The matching of genes on chromosomes 3 and 8 did not improve on the donor chimerism ($52.36 \% \pm 25.43 \%$ and $9.51 \% \pm 12.32 \%$ for myeloid and lymphoid cells respectively compared with $11.58 \% \pm 7.03 \%$ and $3.47 \% \pm 4.60 \%$ for myeloid and lymphoid cells in unmatched fish) found by just matching MHC genes on chromosome 19. As the transplant experiments presented here were not protracted for 14 to 16 weeks, and de Jong *et al.* used 50 to 75 000 β -actin:GFP+ WKM, it is not possible to compare the results.

Having established the transplantation technique with large cell doses (up to 10^6 WKM cells) from Tg (ubi:GFP) donors, transplants were done using cd41:GFP+ WKM cells from Tg (cd41:GFP;lyz:dsRed) fish. The Tg (cd41:GFP;lyz:dsRed) donors allowed sorting of a HSPC enriched population for transplantation, and to determine the contribution of cd41:GFP+ donor derived myeloid cells to the recipient's reconstituted immune system.

Small (<1 cm) recipients were used to facilitate imaging. Preliminary experiments found that the lyz negative population produced hematopoietic cells in the WKM by 8 dpt (Figure 3.15D). As cd41:GFP+ cells make up approximately 0.5 % of the WKM, this transplanted population contained approximately 1000 cd41:GFP+ cells (0.5 % of 2×10^5 cells). Subsequently, irradiated TraNac fish were transplanted with 2,000 cd41:GFP+ cells.

Live imaging of the recipient fish revealed the cd41:GFP+ cells behave in 2 different ways. One population is highly mobile, (potentially thrombocytes) and the other is quite sedentary (potentially HSPCs or TEPs). These hypotheses are supported by morpholino experiments by Lin *et al.* in which knock down of stem cell factors (*scl* and *c-myb*) could abolish the non-mobile cd41:GFP+ population (29). In addition, morphologically, cd41:GFP^{high} cells resemble mature thrombocytes while cd41:GFP^{low} cells have an immature phenotype (29).

Ma *et al.* also reported the circulation of cd41:GFP+ cells in 15 % of recipients surviving to 30 dpt (51). However, their observations were based on visualising cells in the tail fin at 30 dpt, and not throughout the organism at early time points. In the experiments presented here, cd41:GFP+ cells could be visualised from 3 dpt in recipients of 2,000 cd41:GFP+ cells (Figure 3.17B). This is particularly important as in some cases mobile cd41:GFP+ cells disappeared in recipients that had these cells at earlier time points. Thus, Ma *et al.* underestimated the frequency of recipients with circulating cd41:GFP+ cells.

While recipients of the lyz negative population (cd41:GFP^{+/−} lyz:dsRed[−]) of Tg (cd41:GFP;lyz:dsRed) donors had both cd41:GFP⁺ cells and lyz:dsRed⁺ cells present post-transplant, the recipients of the double negative population (cd41:GFP[−] lyz:dsRed[−]) contained very few cd41:GFP⁺ cells post-transplant. On the other hand, both groups of recipients contained lyz:dsRed⁺ cells. This suggests that the double negative population contains myeloid precursors, that can differentiate from cd41:GFP[−] precursors, but do not contain precursors to the cd41:GFP⁺ population. Although the cd41:GFP⁺ population produced lyz:dsRed cells, there is no evidence whether these cells represent a myeloid bias population as they do in mice (28). In order to investigate bias in this population, these fish would need to be crossed with fish containing a transgenic marker for lymphoid cells (possibly lck or rag).

All fish in all groups presented with lyz:dsRed⁺ cells around the site of transplant (recipients' eye). These cells were observed earlier (3 dpt) in recipients of the double negative population. This may be because the double negative population contains more myeloid progenitors than the cd41:GFP⁺ population. The cd41:GFP⁺ cell must first differentiate into a myeloid progenitor and then a mature (lyz:dsRed⁺) myeloid cell. Lastly, recipients of 2,000 cd41:GFP⁺ cells showed similar numbers of lyz:dsRed⁺ to recipients of the double negative population at 5 dpt (Figure 3.18A, B). This suggests that the cd41:GFP⁺ population can give rise to myeloid precursors and mature myeloid cells.

Finally, both irradiated TraNac and c-myb *bloodless* mutants have improved survival if they receive a transplant of 2,000 cd41:GFP⁺ cells (Figure 3.19A, B, D, E). Survival of irradiated TraNac transplanted with double negative cells is extremely low, suggesting that ultimately this population does not contain enough stem cells to rescue irradiated recipients. However, this population can rescue bloodless mutants at least 42 dpt (Figure 3.19E). The difference in survival between the TraNac and *bloodless* recipients transplanted with 2000 cd41:GFP⁺ cells is possibly because the latter do not have irradiation-induced damage which can contribute to morbidity. It is worth noting that previously, transplants into *bloodless* recipients have used much higher cells numbers (2 to 10 × 10⁴) and all recipients were rescued with these doses. These experiments using < 2000 cd41:GFP⁺ cells test the limits of the cells' capabilities to reconstitute the system. Together, this data shows that cd41:GFP⁺ cells are not the only HSPC containing population in the zebrafish, and that the cd41 negative population also contains cells with short term reconstitution potential.

As no markers are available to select for cd41 negative HSPCs, experiments continued using cd41:GFP⁺ cells from Tg (cd41:GFP;lyz:dsRed) donors. The number of cd41:GFP⁺ cells was titrated to establish a minimal dose at which HSCs will be pushed to their limit (in order to challenge HSC reconstituting ability), but which would also yield good quality imaging data.

Recipients of 20 and 50 cd41:GFP cells had very low survival and few observable cells in the WKM at 6 dpt (Figure 3.21A, B). Ma *et al.* reported that 10 cd41:GFP^{low} cells were enough to rescue 21 % (20 of 95) of recipients, although only 3 % (3 of 95) survived and contained donor WKM cells (51). Similarly, the results presented here

show that 20 cd41:GFP cells (approximately 10 cd41^{low}) could rescue 20 % of fish (4 of 20) to 28 dpt, although none showed engraftment (0 of 4). Perhaps if a greater number of transplants were done, the results would reflect those reported by Ma *et al.* (51). However, these transplants were not performed as the purpose of this experiment was to optimising a protocol in which cells can be imaged colonising the WKM within 6 dpt. Fish receiving a dose of 200 cd41:GFP cells showed significant survival and cells were visible by 6 dpt. Therefore, this was the set up used for the next 2 results chapters.

3.4.4 Predicting transplant recipient survival

The most novel aspect of this chapter is the work correlating early post-transplant observations with survival. Previously, predicting transplant survival has been based on animal posture, behaviour and colouring. In these experiments, only a third of irradiated recipients receiving a transplant of 200 cd41:GFP+ cells survived to 28 dpt, therefore real time imaging was used to try and predict reconstitution outcome and reduce suffering. It is important to note that imaging causes stress to animals, so the benefits of identifying survival markers must outweigh this distress. It is noteworthy that there is a lot of variability between recipients within the same experiment, but the range of results between experiments is consistent.

In this work the period from 8 to 12 dpt was identified as one of high morbidity (35 %; Figure 3.21C) in the irradiated transplant recipient, regardless of the presence of fluorescent cells (Figure 3.22A, B). It is hypothesised that the procedural steps (radiation damage, injection and imaging) are possible causes for this early morbidity because of the physical stresses as well as the chances of catching an infection. It is proposed that interventions to increase survival be made in the days (4 to 6 dpt) prior to this period. Morbidity in the longer term (after a couple of weeks) can be attributed to failure of HSCs to perform hematopoiesis or due to graft versus host (GvH). De Jong *et al.* have reported increased donor contribution and reduced GvH by matching histo-compatibility complexes between donor and recipient (45, 47).

When attempting to correlate survival with the number of fluorescent cells present in the WKM at 6 dpt, it was found that 80 % of fish that die before 10 dpt have fewer than 10 cd41:GFP+ cells, while 80 % of fish that survived beyond this had between 14 and 39 cd41:GFP+ cells. Although survival and the number of cd41:GFP+ cells did not correlate, these data suggest that surviving recipients tend to have more cd41:GFP+ cells in the WKM early post-transplant. Similarly, 70 % of recipients that died before 10 dpt had fewer than 10 lyz:dsRed+ cells in the WKM, while 88 % of those that survived longer had >17 fluorescent cells in the WKM. The survival of fish with fewer than 10 fluorescent cells at 6 dpt (Figure 3.22E) could reflect the low number of fish that survive despite not receiving a transplant (Figure 3.7).

Together these data suggest that there may be a case for humanely killing transplant recipients that do not have visible cells in the WKM by 6 dpt. These fish may survive, but those that did survive did not show donor hematopoietic contribution at 28 dpt. In addition, recipients with fewer than 25 cells (sum of cd41:GFP and lyz:dsRed) can be humanely killed after 10 dpt as they are the least likely to survive to 22 dpt. Fish with at least

70 fluorescent cells are projected to survive beyond 22 dpt (Figure 3.22E). This indicates that early occupation of the WKM is important for recipient survival. The reason this does not always happen is not yet clear. It may be because the cd41 marker isolates a population that does not contain enough true HSCs so that there are too few of these cells in a mix of 200 cells.

3.4.5 Cycling kinetics in the cd41:GFP+ population

Despite the zebrafish being an important model in the study of malignant hematopoiesis and in the study of hematopoietic stem cell generation at the onset of definitive hematopoiesis, difficulty remains in the isolation of HSCs from the kidney marrow of this organism. Previous studies have attempted to enrich the HSPC population by partitioning the cd41:GFP+ population into high and low, based on GFP expression. It was found that both cd41^{high} and cd41^{low} could reconstitute the immune system (Supplementary Figure 7.4) and therefore I chose to do all experiments without splitting this population. In order to improve upon the limited markers available, a BrdU label retention assay was explored to determine if a distinction could be made between high and low expression of cd41:GFP+ cells based on the cycle kinetics of the HSPC population isolated by cd41 expression. Various attempts (injection, swimming, timing, fish maturity) were made at labelling a dormant population in the zebrafish. However, none of the combinations tried were successful (Figure 3.23). The major problem with the method is that BrdU is cytotoxic and causes cells to die. Labelled cells lose BrdU during the chase period due to mitotic divisions. However, cells may also be removed if they are targeted for phagocytosis. Another problem was that too few cells were labelled during the pulse. Kiel *et al.* have also reported difficulties using BrdU in neonatal mice (75). They found that only 0.5 % of the 6 % of BrdU labelled cells were expressing HSC markers. Other methods for labelling slow cycling cells is using an inducible histone 2B-GFP method (72, 73).

3.5 Conclusion

The aim of this thesis is to assess the response of HSCs under conditions of inflammation. This will be done using side-by-side comparisons of donors and recipients in transplantation studies. HSCs will be exposed to inflammation either prior to (in donors) or post (in recipients) transplantation. This chapter outlines the groundwork necessary to address this question; essentially establishing a transplant and imaging procedure to obtain early post-transplant information.

The data present here demonstrates that cells can be imaged in the WKM of irradiated recipients of 200 cd41:GFP cells at 6 dpt. For this reason, fish will be assessed 4, 6 and 8 dpt to determine if HSC behaviour changes in treatment conditions. Through the establishment of this protocol, factors such as *time* (for acquiring imaging data), *space* (required to house fish individually over the course of the experiment), and *high morbidity* have meant that, for future experiments, data should be acquired by multiple experimental repeats with low numbers of fish.

This chapter also acknowledges that post-transplant deaths before 10 dpt are not predictable by imaging cells in the WKM at 6 dpt. However, it seems that deaths that occur between 10 and 20 dpt can be reduced by culling fish that have fewer than a total of 25 cells in the WKM by 6 dpt. This information is useful for reducing animal suffering and the severity of this protocol under UK Home Office regulations.

4 Post-transplant behaviour of HSCs from donors with systemic inflammation

4.1 Introduction

Previous studies have shown that hematopoiesis is affected by inflammation and infection, and that HSCs sense and respond to these signals directly and indirectly (194, 389, 390). It is necessary to understand the molecular mechanisms that control HSC self-renewal and differentiation in order to fully understand the potential of HSCs in clinical applications. Although examining HSCs in the endogenous host provides information about the systemic effects of poly I:C, it does not yield information about the effect of this stimulation on the self-renewal capacity of the stimulated HSCs. Examining HSC behaviour in the transplant recipient effectively separates the direct effects on this population from the indirect effects on the niche and the hematopoietic system. In this results chapter, irradiated recipients were transplanted with cd41:GFP⁺ HSPCs from poly I:C injected or saline injected donors. Transplantation is used as a regenerative challenge to assess HSPCs taken from stimulated and non-stimulated environments. This results chapter benefits from the imaging protocol developed in the previous chapter and reports on the early-post transplant hematopoietic cell behaviour.

4.1.1 The effect of inflammation on HSCs

As previously discussed in the introduction chapter, immune cells are responsible for resolving infection and injury and cytokines play a major role in the regulation of the response. There is growing evidence that HSCs also respond to infection through direct and indirect mechanisms and this profoundly affects how they behave. Direct mechanisms include direct infection and direct recognition of a pathogen. Indirectly, HSCs respond to cellular demand, macrophage egress from the BM and cytokine detection (358, 359).

4.1.2 Choosing a model to induce inflammation in the zebrafish

For this study it was necessary to choose an inflammatory model to cause systemic inflammation throughout the whole hematopoietic compartment. As mentioned in the introduction chapter 1.7, many lifestyle factors including smoking and diet (for which there are zebrafish models (391, 392)), appear to detrimentally affect hematopoiesis. However, these models require weeks of exposure to develop a chronic inflammation. Furthermore, they require extensive time to optimise. For these reasons, a simpler model was sought. There are a number of publications which use poly I:C (76, 333, 393), a synthetic viral mimic, to induce a controlled anti-virus-like inflammatory response in the mouse. This viral mimic model was chosen as the amount of poly I:C could easily be manipulated and the findings would be supported by and build on literature in the field.

4.1.3 Inflammation with Poly (I:C)

Poly I:C is a synthetic double stranded structure of inosinic and cytidylic polymers used to mimic viral RNA and, therefore, viral infections. A variant of this compound is the more stable Poly ICLC. Viral components, such as

double stranded RNA (dsRNA) are recognised by membrane bound TLRs or RLHs (394). This results in the production of cytokines, in particular type I interferons (76, 333, 393), which induce apoptosis of infected cells, cellular resistance to infection and activation of NK and T cells (394).

TLR3 recognises both dsRNA and the synthetic mimic poly I:C (395). In zebrafish, poly I:C is also recognised by TLR22, a TLR only in aquatic animals (396). Phylogenetic analysis reveals the zebrafish genome contains at least one homolog of each of the human TLRs, including TLR3 (397). Clustering of human, pufferfish and zebrafish TLR sequences showed a highly conserved TLR3 sequence across the 3 species. Gene expression analysis by M. Wane (Dallman lab, personal communication) shows, at the transcript level at least, TLR3 is expressed in the gill, heart, spleen, liver, gut and WKM of the zebrafish.

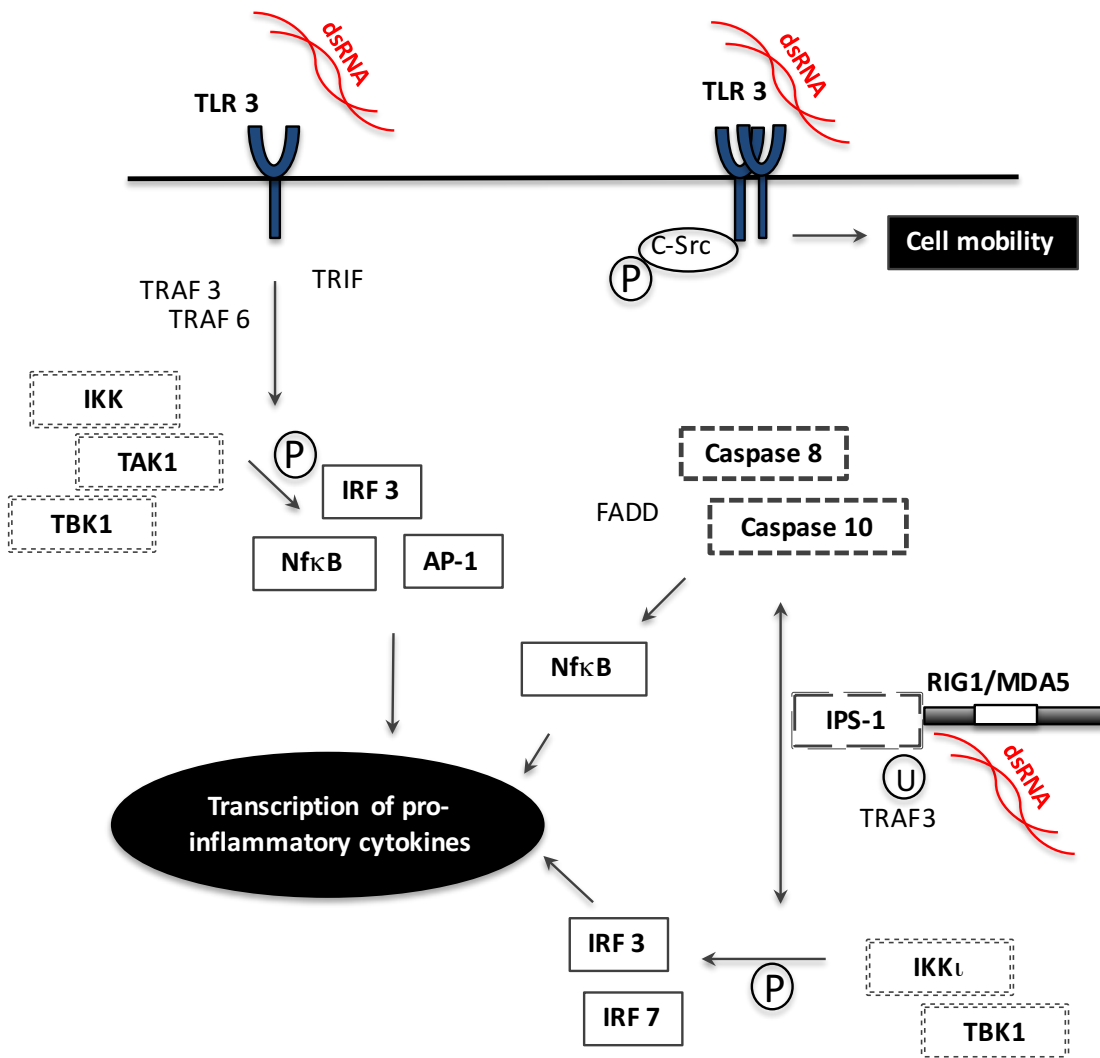


Figure 4.1: Cell detection of double stranded RNA or poly I:C through TLRs or RLHs. Adapted from Takeuchi *et al.* and DuFour *et al.*; Acronyms described in text. Membrane bound TLR 3 recognises intra and extra-cellular double stranded RNA. Adaptor protein TRIF transmits signal to kinases (IKK, TAK, TBK1) to phosphorylate transcription regulators (IRF-3, NFκB, AP-1). TLR 3 also dimerises and recruits c-Src which causes cell mobility when phosphorylated; Cytosolic RLHs (RIG-I and MDA5) also bind dsRNA and recruit IPS-1. Ubiquitination and TRAF3 are required for activation of IPS-1 signalling pathways. On one hand, TRAF3 recruits kinases (TBK1, IKK-i) to phosphorylate transcriptional regulators (IRF-3 and IRF-7). IPS-1 and TRAF3 also regulate a FADD and caspase-8/caspase-10-dependent pathway for the activation of NFκB.

Ligand binding to TLR3 causes activation of two pathways (Figure 4.1). One of these is through the adaptor protein TIR-domain-containing adapter inducing interferon β (TRIF) to signal to kinases TGF- β activated kinase (TAK1), I κ B kinase (IKK), and TANK-binding kinase 1 (TBK1). Together these induce transcription factors IRF-3, Nf κ B and AP-1 which induce transcription of many inflammatory genes. In a second pathway, reported by Johnsen *et al.*, TLR3 also recruits the oncogene c-Src (398). C-Src is a non-receptor tyrosine kinase regulated by phosphorylation. Yamashita *et al.* reported that *in vitro* poly I:C treatment of various cell types (including bone-marrow derived primary macrophages) caused c-Src activation which led to biphasic cell mobility; initially cell migration was increased, but following 4 h exposure was significantly suppressed (395). They showed that the suppressed migration was due to the translocation of active c-Src from the cytoplasm to lipid rafts. Together this suggests that poly I:C could have a measurable effect on the early post-transplant behaviour of HSCs.

Cytosolic sensors include PKRs (protein kinase R; dsRNA activated protein kinase) and RLHs (retinoic acid-inducible gene-I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5)), which are also involved in detecting viral infections. Specifically, MDA5 recognises poly I:C. RLHs bind with dsRNAs through the helicase domain and this stimulates the interaction with IFN- β promoter stimulator 1 (IPS-1). IPS-1 signalling is mediated by ubiquitination of TNF-receptor associated factor 3 (TRAF3). TRAF3 recruits and activates TBK1 and IKK to phosphorylate IRF-3 and IRF-7. Phosphorylated IRFs allows nuclear translocation and transcription of type I interferon genes. IPS-1 signalling is also mediated by FAS-associated death domain-containing protein (FADD) and caspases 8 and 10 which activate Nf κ B and its downstream targets (399). The endonuclease RNase L is activated by the virus to cleave self RNA. These small RNAs are recognised by the RLHs and responsible for interferon production.

Single strand RNA virus infection models support a role for TLRs, and not RLHs, in instructing the adaptive immune system. For instance, production of B- and CD4⁺ T-cells against the influenza virus is dependent on the presence of MyD88 but not IPS-1 (400). Further studies are required to determine if this applies more generally to all viruses.

4.1.4 The effect of interferons on HSC

Type I IFNs (predominantly IFN α and β in mammals, IFN ϕ in zebrafish) can be produced by most cells and are responsible for inducing transcription of a large number of genes which activate the immune system by binding to a specific cell surface receptor IFNAR. Type II IFNs (IFN γ in mammals and zebrafish) are produced by adaptive immune cells (T and NK cells) and bind IFNGR. IFN γ activates macrophages and induces class II MHC expression on APCs.

4.1.4.1 Type I Interferons

Type 1 IFNs paradoxically induce cell cycle arrest in human HSPCs (CD34⁺) *in vitro* (401) and cell cycling of mouse HSPCs (LSK CD150⁺ CD48⁻) *in vivo* (76, 333). HSCs (CD34⁻ LSK) produce the highest levels of IFN-2, a

Post-transplant behaviour of HSCs from donors with systemic inflammation
negative regulator of type I IFN signalling, indicating that preventing type I IFN signalling is important for maintaining this population (360). IFN α has been shown to induce proliferation of dormant HSCs in mice and exhaustion of HSCs after 4-5 round of serial transplantation (76). Constitutive type I IFN signalling (in IFN-2 knock out mice (393) and in mice expressing IFN α under the control of a constitutive promoter on a adeno-associated viral vector (402)) causes a significant loss in LT-HSCs (LK CD150⁺ CD48⁻), ST-HSCs (LK CD150⁻ CD48⁻) and MPPs (LK CD150⁻ CD48⁺) (393, 402). In addition, IFN-2 knock out mice are more sensitive to the ablative effect of 5-FU, probably because IFN signalling has increased cell cycling in the hematopoietic compartment (393).

Pietras *et al.* found that the balance between proliferative and suppressive effects came from the magnitude of the exposure (107). Acute IFN treatment (induced by a single injection of 10 $\mu\text{g g}^{-1}$ poly I:C) causes proliferation, but with chronic exposure (10 $\mu\text{g g}^{-1}$ poly I:C daily up to 30 days) HSCs return to a quiescent state. They showed that repeated treatment did not exhaust HSCs unless these cells were forced to replicate in regenerative assays, for instance in cell culture or transplant conditions.

Essers *et al.* (76) found using a BrDU label retaining assay that *in vivo*, poly I:C increased HSC (LSK CD150⁺ CD48⁻) cell cycling in WT but not in IFN- α receptor knock out mice (INFAR1^{-/-}). Work by Sato *et al.* supported these findings. Sato *et al.* transplanted irradiated recipients of WT or INFAR1^{-/-} HSCs. They subsequently injected recipients with poly I:C (4 times 300 μg at 48 h intervals, then 2 weeks recovery) and found cell cycling of WT HSCs, but not of INFAR1^{-/-} HSCs, indicating a direct effect on HSCs (393). Furthermore, in competitive transplant assays, WT HSCs outcompete IRF2^{-/-} (constitutive type I IFN signalling) HSCs, but this phenotype is reversed when WT compete with IRF2^{-/-} INFAR1^{-/-} HSCs (constitutive type I IFN signalling but no receptor for type I IFN) (393). Together this shows that interferon signalling causes HSC cell cycling that is detrimental to HSC function.

Essers *et al.* ruled out a stromal effect by showing that transplanted WT HSCs were activated by poly I:C in INFAR1^{-/-} knock out recipients. To show that HSCs were functionally affected by IFN- α , the group transplanted 3×10^7 bone marrow cells from WT or INFAR1^{-/-} donors that had previously been injected 8 times, at 48 h intervals, with 10 $\mu\text{g g}^{-1}$ poly I:C into lethally irradiated recipients. They found that while both recipient groups were successfully reconstituted, donor WT HSCs were disadvantaged in competitive assays compared to donor INFAR1^{-/-} HSCs. At 22 dpt, only INFAR1^{-/-} HSCs (LSK CD150⁺ CD48⁻) were present in recipient bone marrow. These results indicated that poly I:C affected the biology of WT HSCs such that their regenerative capacity was inferior to the INFAR1^{-/-} HSCs which were advantaged because they did not respond to the poly I:C stimulation. The paper did not report on how INFAR1^{-/-} HSCs cope in the long term to hematopoietic challenges. It should be noted that the transplant is of a large heterogeneous bone marrow population so the IFN- α effect is on more than just HSCs. This paper was used as the basis for establishing the inflammatory model for poly I:C treated donors in this thesis.

4.1.4.2 Type II Interferons

Type II IFNs are expressed by activated T and NK cells and have also been shown to affect HSCs (334, 355, 403, 404). Both *in vitro* and *in vivo* label retaining assays (CFSE, BrdU) showed that IFN γ caused an increase in HSC proliferation (334, 355, 404). MacNamara *et al.* have studied the effect of *Ehrlichia muris*, an inducer of IFN γ , in HSCs of mice (257, 404). During the acute infection there is an expansion of the progenitor (LSK) compartment and HSC (LSK CD34⁻ CD135⁻) population. Similarly, Balbridge *et al.* used a model of *Mycobacterium avium* infection to study the effect of IFN γ signalling. After 4 weeks of *M. avium* infection, LT-HSCs (LSK CD34⁻ CD135⁻), ST-HSCs and MPP populations increased (355). In both cases, IFN γ signalling is important as the LSK and HSC populations do not expand in infected IFN γ receptor knock out mice (IFNGR^{-/-}). Together there is evidence that IFN γ causes cell cycling but not to the same detrimental extent as type-I IFNs.

De Bruin *et al.* studied the effect of the Armstrong strain of lymphocytic choriomeningitis virus (LCMV) on HSCs (LK CD150⁺ CD48⁻) in WT and IFNGR^{-/-} HSCs (334). Again, they showed IFN γ signalling is important as they found hematopoiesis in IFNGR^{-/-} mice recovered by 8 days post infection, while hematopoiesis took 12 days to recover in infected WT mice. These results support the notion that IFN γ has a temporary negative effect on hematopoiesis of HSCs *in vivo*.

Indeed, when these mixed chimeras were infected with LCMV, the frequency of IFNGR^{-/-} HSCs (LK CD150⁺ CD48⁻) was higher than WT HSCs at 8 and 12 days (334). On the other hand, a similar experiment by McCabe *et al.* found that when chimeric recipients (60 % IFNGR^{-/-} HSCs, 40 % WT HSCs) were infected with *E. muris* 6 weeks after transplant, there was no change in the frequency of WT or IFNGR^{-/-} HSCs (LK CD150⁺ CD48⁻) in these recipients (257). These confounding results may be due to the difference in the levels of IFN γ induced by the different modelling approaches.

In non-competitive transplant assays, WT donors contributed to 60 % of recipient PB while in recipients of HSCs from *E. muris* infected donors at 8 and 16 days post infection, 10 % and 35 % respectively were donor derived. Similarly, Balbridge *et al.* transplanted HSCs from mice injected with recombinant IFN γ and found a modest impairment in recipient reconstitution compared with recipients of HSCs from non-injected mice (355). Both of these results indicate that WT HSCs perform better than IFN γ -treated HSCs although they were not assessed in a competitive assay.

One study has compared IFN treated HSCs with untreated HSCs in a competitive transplant assay. In this study, HSCs (LK CD150⁺ CD48⁻) were cultured in media with and without IFN γ . They found that IFN γ treated HSCs could not compete against untreated HSCs (334). These results confirm the non-competitive transplant assays above and are supported by the finding that IFNGR^{-/-} HSCs have a competitive advantage over WT HSCs in competitive transplant assays (334, 355, 404). Altogether this suggests that IFN γ is detrimental to HSC function in regenerative assays.

4.1.5 Inflammation in the donor affects early post-transplant behaviour of HSPCs

Currently, not much is known about the early post-transplant behaviour of HSPCs from donors affected by infection and inflammation. One study, by Rashidi *et al.*, examined the effect of *Trichinella spiralis* infection in mice *in vivo* (122). They found that this natural pathogen caused short term changes to hematopoiesis (increasing lymphoid and reducing myeloid populations) but that homeostasis was re-established within 28 days. During the infection cycle, the LT-HSC, ST-HSC and MPP populations did not appear to be affected, indicating that the effect of inflammation cannot always be determined by changes in hematopoiesis. However, using competitive repopulation assays, they found that bone marrow mononuclear cells from infected donors had a reconstitution advantage over controls both in the short term and long term. They used intra-vital microscopy of the calvarium bone marrow to image the behaviour of DiD-labelled HSCs (LSK CD34⁻ CD135⁻) from control and infected donors *in vivo*. They found that HSCs from uninfected mice would stably engage with niche cells. However, HSCs harvested during acute infection were very motile and would interact with a more diverse population of niche cells. Most interestingly, the movement of control HSCs was restricted and circular while half of the stimulated HSCs migrated within the field of view, as though scanning the tissue. Finally, unlike the studies of infections resulting in interferon production (poly I:C, *E. muris*, LCMV, *M. avium*) they found that *T. spiralis* infection improved HSC reconstitution ability in the transplant recipient. Altogether, this data suggests that early post-transplant behaviour is linked to engraftment and transplant outcome.

4.2 Aims

The aim of this chapter is to assess the engraftment of HSCs after extended exposure to poly I:C. As described above, the effect of inflammatory stimuli cannot always be determined by the abundance of immature populations (LSK; LSK CD150⁺ CD48⁻) after infection or inflammation. Therefore, in this results chapter, transplantation was used as a regenerative stress to better assess the growth of the cd41:GFP precursor population. In these experiments, donors were injected 8 times, at 48 h intervals, with poly I:C so that HSCs were exposed to an inflammatory stimulus prior to transplantation into an irradiated recipient. As in chapter 4, the number of transplanted cells was restricted to 200 cd41:GFP⁺ cells to place an additional regenerative stress on the cells. Mobility, self-renewal and differentiation were assessed visually at early post-transplantation time points (4, 6 and 8 dpt) throughout the organism. In addition, survival was assessed and flow cytometry was used to compare engraftment and reconstitution at 28 dpt.

More specifically, the aims were to:

1. Investigate the acute inflammatory response to poly I:C in the WKM of zebrafish.
2. Examine the inflammatory and hematopoietic response following repeated exposure to poly I:C
3. Observe the behaviour of HSCs (cd41:GFP⁺) in the transplant recipient and assess how pre-treatment with poly I:C affects this early behaviour
4. Determine transplant success based on hematopoietic response and morbidity of recipients

4.3 Results

4.3.1 Establishing systemic inflammation in the zebrafish with poly I:C

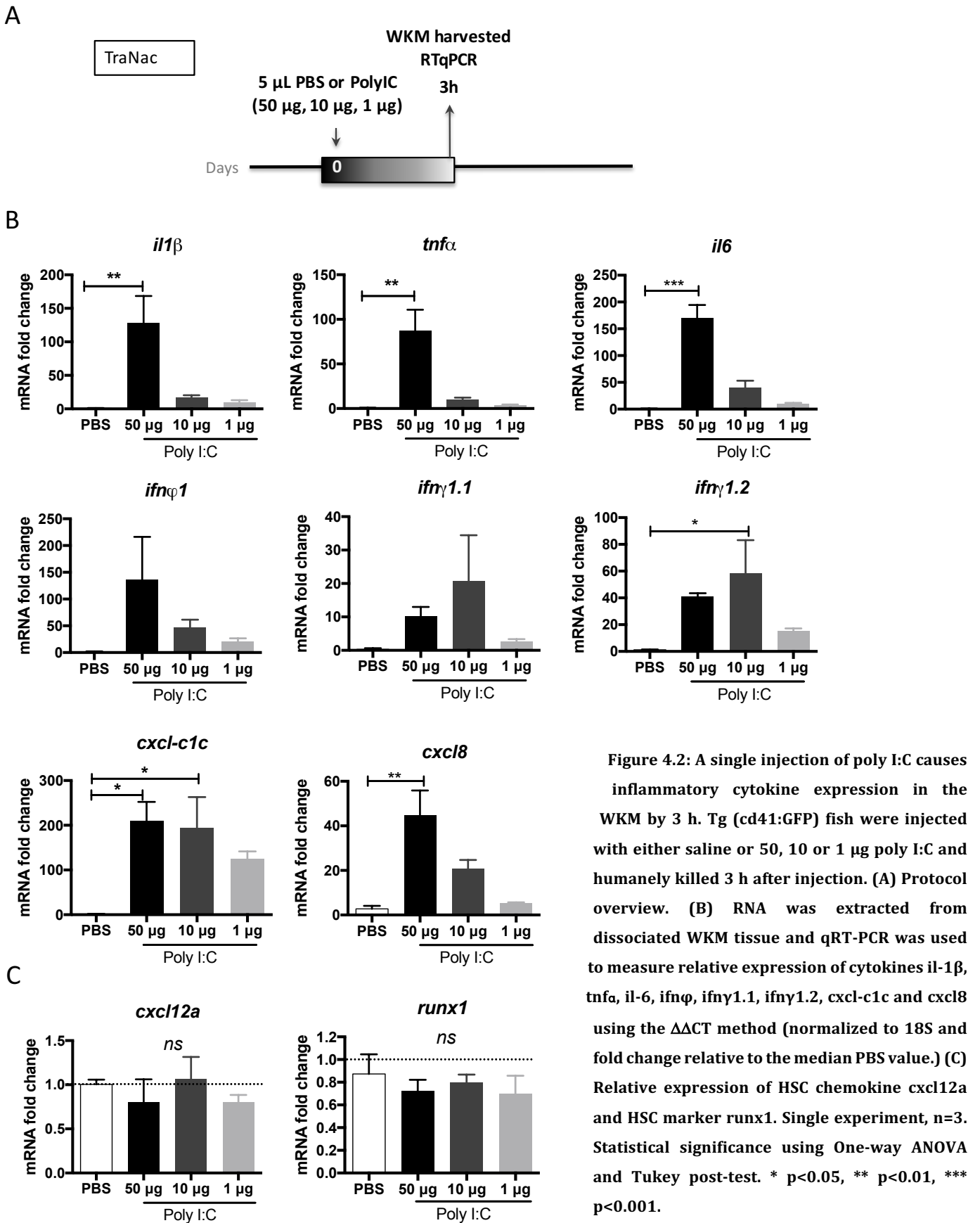
In order to begin studying the effect of inflammation on HSCs, it was first necessary to establish a protocol that would induce systemic inflammation in the zebrafish. There were a number of immune-stimulants available in the lab (diet, silica, cigarette smoke, tail transections). However, poly I:C was chosen as it is commonly used in mouse studies and easily regulated (the dose and method of delivery are easily adjusted).

Initially, TraNac fish were used to establish a dose which would cause a systemic response and permit recurring treatment. Fish received a single intra-peritoneal injection of 5 μ L containing 1, 10 or 50 μ g of poly I:C, or a 5 μ L injection of saline. Fish were harvested 3 h after the injection to compare with results in the literature (Figure 4.2A). mRNA levels were measured in various organs (405), including the WKM. As before, transcript levels were determined using the $\Delta\Delta$ CT method, normalised to 18S expression (see chapter 2.9.3).

The results show that all 3 doses of poly I:C induced a cytokine response in the WKM, and in general the greatest response was induced by the 50 μ g dose, with the exception of *ifn γ 1.1* and *ifn γ 1.2* (Figure 4.2B). *Il-1 β* was greater than 125 fold upregulated in fish injected with 50 μ g poly I:C, and 20 fold in recipients of 10 μ g poly I:C. *Tnf α* was almost 100 fold upregulated in fish injected with 50 μ g, and $\approx$ 15 fold in recipients of 10 μ g poly I:C. The cytokine *il-6* was also upregulated, with transcript levels over 150 fold higher than saline controls in response to 50 μ g (and almost 50 fold higher in response to 10 μ g), and the chemokine *il8* transcript level was increased $\approx$ 40 times in response to 50 μ g poly I:C (and $\approx$ 20 times in response to 10 μ g).

Poly I:C is also an inducer of type I and II IFNs. In zebrafish, the type I interferon is *ifn ϕ* and the type II IFNs are *ifn γ 1.1* and its homologue *ifn γ 1.2*. The results show that *ifn ϕ* is significantly upregulated (over 100 fold in response to 50 μ g poly I:C and $\approx$ 50 fold in response to 10 μ g poly I:C). Type 2 IFNs *ifn γ 1.1* and *ifn γ 1.2* were also slightly induced by the poly I:C stimulation. In both cases, the lower dose of 10 μ g poly I:C ($\approx$ 20 fold, $\approx$ 50 fold respectively) induced a similar level of cytokine transcription to the 50 μ g poly I:C dose ($\approx$ 10 fold, $\approx$ 40 fold respectively).

The HSC chemokine *cxcl12a* was also measured in the WKM as it is an important signalling molecule for the mobilisation of HSPCs. The results show that there was no change in the levels of *cxcl12a* in the WKM 3 h after poly I:C stimulation (Figure 4.2C). The level of *runx1* transcript, used as a marker for the HSPC population, also did not change.



4.3.1.1 A repeated stimulation with 10 µg of poly I:C causes an increase in erythrocytes in the spleen but does not affect blood populations of the kidney marrow

The subsequent experiments were done using a dose of 10 µg poly I:C as it caused a moderate cytokine response so that repeated administration would not likely be lethal but still cause systemic inflammation. Indeed, throughout the course of these experiments, there were no unexpected deaths following this significant treatment. Tg (cd41:GFP;lyz:DsRed) donors received an intra-peritoneal injection of either 5 µL saline or 5 µL of poly I:C (10 µg) eight times, at 48 h intervals, and then left to recover for a week prior to isolation of the WKM for transplantation (Figure 4.3A).

Poly I:C and saline injected donors were humanely killed and WKM cells were sorted for transplant studies. At the same time, the spleen was also harvested from these donors in order to measure specific transcript levels. In separate experiments, the WKM was harvested for transcript level measurements instead of transplantation. As the donors had a week to recover, it was not expected that chemokines and cytokines could still be detected and therefore this was not assessed. Instead, the level of transcripts for lineage-associated genes were quantified to assess relative abundance of blood populations.

Transcript levels of the immune cell gene *l-plastin* were not significantly changed in the spleen by the repeated poly I:C treatment (Figure 4.3B). Neither were the transcript levels of the progenitor cell-associated gene *runx1*. However, transcript levels of erythrocyte-specific gene *gata1* were significantly increased (~4 fold; Figure 4.3B) indicating poly I:C may induce erythropoiesis, or reduce loss of erythrocytes.

In separate experiments, fish treated repeatedly with poly I:C were humanely killed and the WKM harvested for qRT-PCR analysis. There was no significant difference in transcript levels of lymphoid genes *rag1* and *lck* (Figure 4.3C). Interestingly, transcript levels of erythrocyte gene *gata1* were not increased (Supplementary Figure 7.5), despite a significant increase in the spleen (Figure 4.3B). Transcript levels of immune cell gene *l-plastin* and macrophage gene *mpeg* were both significantly decreased (Figure 4.3C). Transcript levels of the progenitor cell genes *c-myb* and *runx1* (Supplementary Figure 7.5) were unchanged and there was no difference in HSC chemokine *cxcl12a* mRNA levels (Supplementary Figure 7.5).

4.3.1.2 Poly I:C injected donors have the same percentage of cd41:GFP+ cells but a smaller cd41:GFP+ population with properties (granularity and size) of a progenitor population

Although qRT-PCR did not reveal significant differences in transcription of HSPC-associated gene *runx1* transcription between the saline injected and poly I:C injected donors, it was reasoned that the series of poly I:C injections could still have a biological impact on the hematopoietic cells that would distinguish them in transplantation studies. Flow cytometry data was collected on individual donors as the WKM samples were being sorted for cd41:GFP+ cells for transplant (Figure 4.4A). The flow cytometry profile of the WKM was analysed to select the 4 major blood populations, the GFP+ population (cd41:GFP) and dsRed+ population (lyz:dsRed; Figure 4.4B).

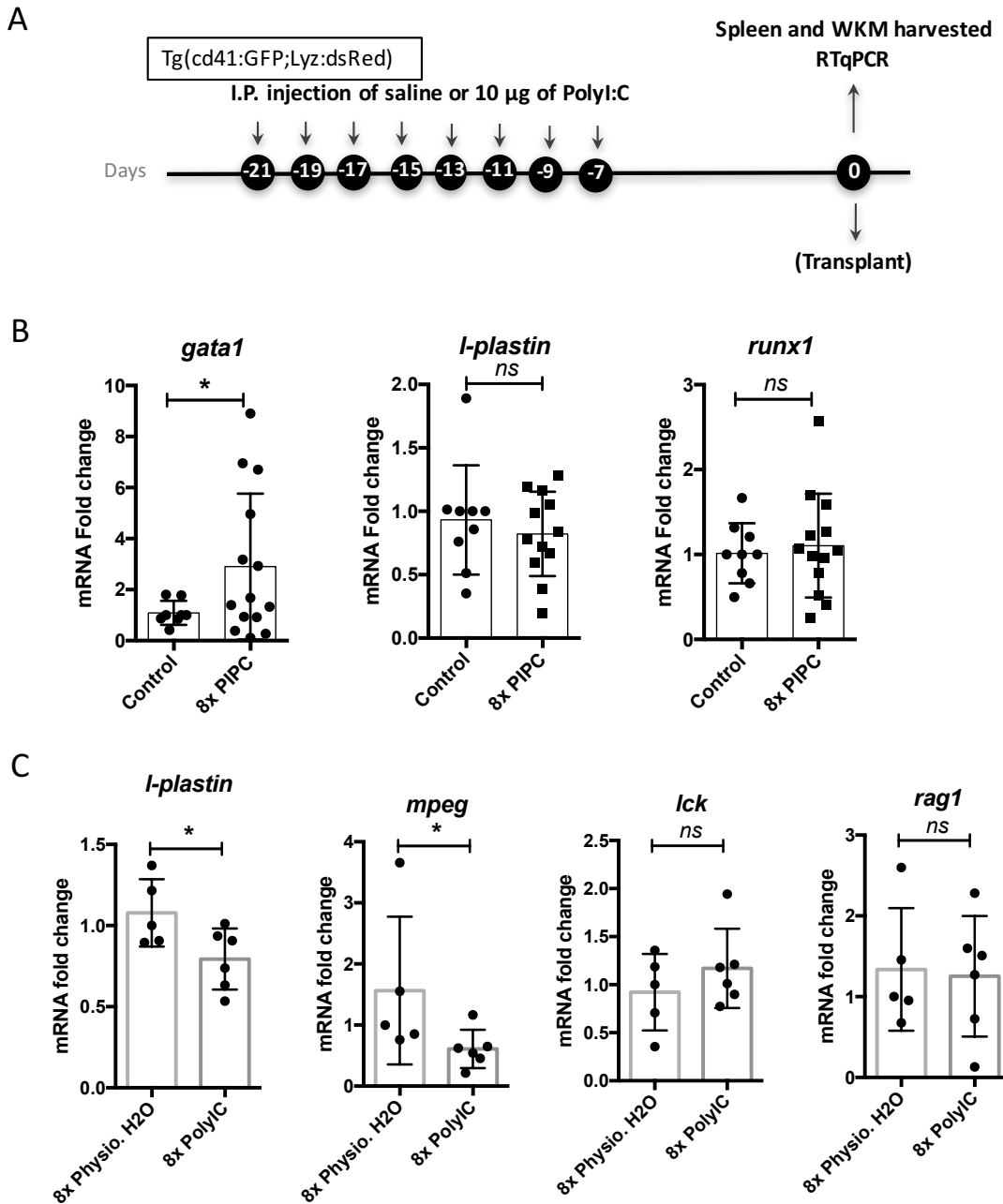


Figure 4.3: Sequential injections of poly I:C cause increased expression of *gata1* in the spleen and decreased expression of *l-plastin* and *mpeg* in the WKM at the time of transplant. Tg (cd41:GFP;lyz:dsRed) fish were injected 8 times with either saline or 10 µg poly I:C. One week post injection, fish were humanely killed and harvested of their spleen and WKM. The WKM and the spleen were used to transcript levels of cell-specific genes by qRT-PCR. (A) Procedure overview. Adapted from mouse protocol by Essers et al. (B) Expression of *gata1*, *l-plastin*, *mpx* and *runx1* in the spleen. Three experiments pooled ($n > 9$). (C) Expression of *l-plastin*, *mpeg*, *lck*, and *rag1* in the WKM. Two experiments pooled ($n > 5$). Relative gene expression level calculated using $\Delta\Delta CT$ method, normalising to 18S expression. Fold change normalised to the median “Saline” control value. Statistical significance using student t-test. * $p < 0.05$.

Analysis of the flow cytometry data analysis shows that, while the percentage of myelomonocyte and precursor populations did not change, the percentage of lymphocytes in the WKM increased from a mean of $\approx 22\%$ in saline injected fish to a mean of $\approx 27\%$ in poly I:C injected fish. This is a relative increase (calculated by the difference

Post-transplant behaviour of HSCs from donors with systemic inflammation over the control; 27-22 %/22 %) of ≈ 23 % (Figure 4.5A). This suggests that repeated poly I:C treatment may cause a lymphoid bias in the hematopoietic progenitor cells of donors in the short term that is detectable 1 week after the last poly I:C stimulation. It is note-worthy that stem cells scatter in the lymphocyte gate. Thus, an increase in the percentage of cells in the lymphocyte gate could be reflecting an increase in stem cells as well as T and B cells.

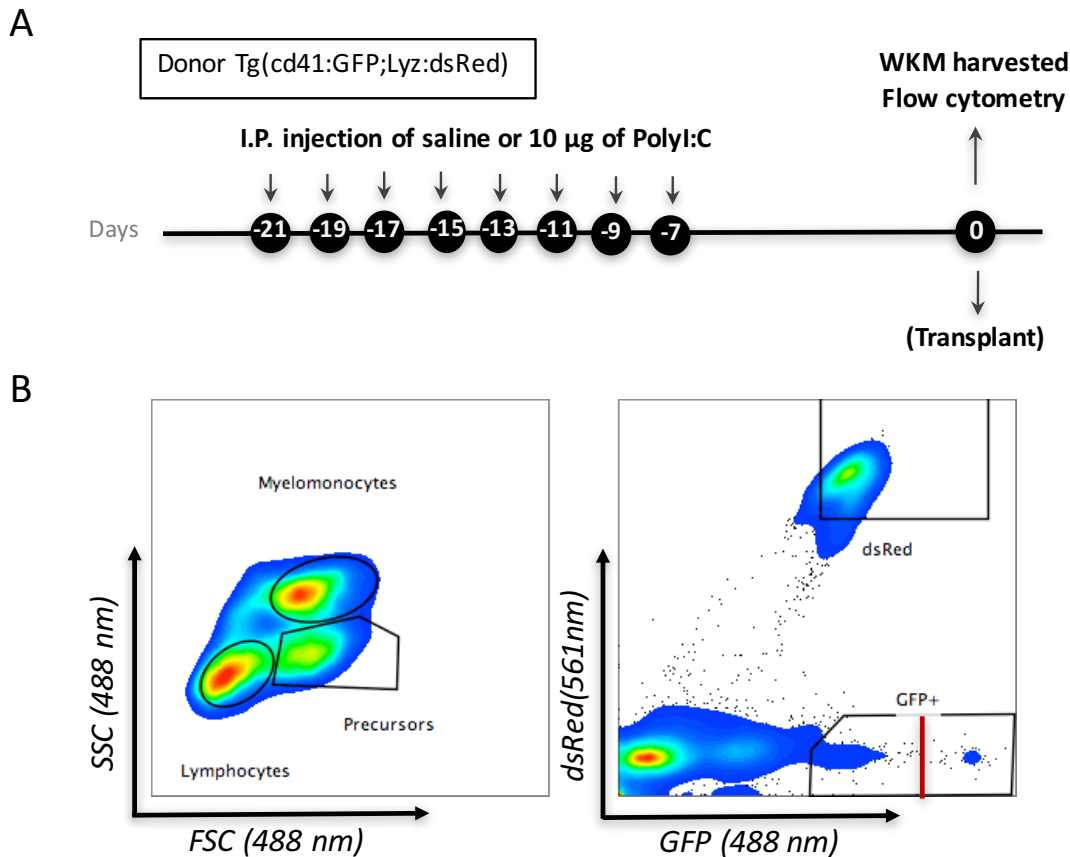


Figure 4.4: Experiment set up for flow cytometry analysis of the WKM of saline injected and poly I:C -injected donor fish, at the time of transplant. Tg (cd41:GFP;lyz:dsRed) fish were injected 8 times with either saline or 10 µg poly I:C. One week post injection, fish were harvested of their WKM and sorted for cd41:GFP+ cells for transplant. Flow cytometry data was collected for individual donors and analysed. Red line separate GFP+ gate into CD41high (right) and CD41low (left). (A) Procedure overview. (B) Flow cytometry gating of all WKM cells from saline injected Tg (cd41:GFP;lyz:dsRed) at day 0. Left graph; SSC indicates side scatter, FFC indicates forward scatter. (Both use 488 nm lazer, 488/10 filter). Right graph; GFP (488 nm lazer, 530/30 filter); dsRed (561 nm lazer, 610/20 filter).

Interestingly, the percentage of cd41:GFP+ cells and the number of cd41:GFP+ events in 60 000 events does not differ significantly between poly I:C and saline injected donors (Figure 4.5C). Furthermore, there was no difference in the percentage of GFP^{low} cells, a gate which roughly determines an enriched HSPC population (Figure 4.5C). As stem cells are found in the lymphoid fraction, the cd41:GFP+ population was back gated to determine the percentage of cd41:GFP+ cells in the lymphocyte gate. This population was significantly lower in poly I:C injected donors compared with saline injected donors (Figure 4.5C). This could suggest that poly I:C treated donors have a smaller HSPC population than saline treated donors. It also indicates that the increase in

lymphocytes in poly I:C treated donors is most likely due to an increase in adaptive immune cells and a lymphoid bias.

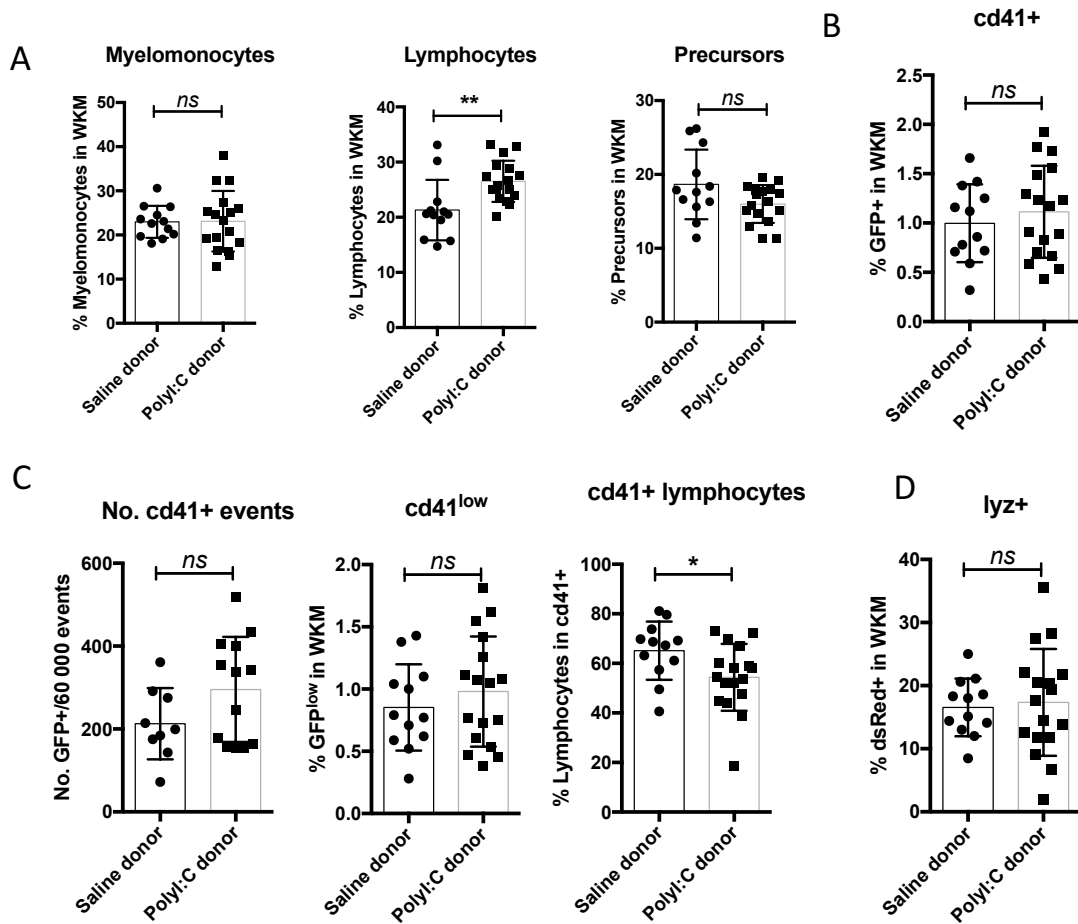


Figure 4.5: Poly I:C injected donors have an increased lymphocyte population but a smaller cd41:GFP+ lymphocyte population at the time of transplant, as determined by flow cytometry. Tg (cd41:GFP;lyz:dsRed) fish were injected 8 times with either saline or 10 μ g poly I:C. One week post injection, WKMs were harvested and sorted for cd41:GFP+ cells for transplant. Flow cytometry data was collected for individual donors and analysed. (A) The percentage of myelomonocytes, lymphocytes and precursors. (B) The percentage of cd41:GFP+ cells. (C) The number of GFP+ events in 60 000 events, the percentage of cd41low and the percentage of cd41:GFP+ cells that gated in the lymphocyte gate (back gating). (D) The percentage of lyz:dsRed+ cells. Individual dots represent individual donors. Four experiments pooled (n>12). Statistical significance using student t-test. * p<0.05, ** p<0.01.

Finally, poly I:C treatment did not affect the percentage of lyz:dsRed+ myeloid cells (Figure 4.5D), a result which is consistent with the data showing that poly I:C did not affect the percentage of myelomonocytes (Figure 4.5A). This finding further supports a lymphoid bias and suggests that poly I:C most likely causes an increase in adaptive immune cells in zebrafish.

Taken together, these results present the possibility that repeated poly I:C exposure results in changes to hematopoietic cell frequencies of the WKM. In particular, increases in the lymphoid gate.

4.3.2 Transplantation of 200 cd41:GFP+ cells from saline and poly I:C donors

Having established a poly I:C model of inflammation with repeated injections of poly I:C into donor Tg (cd41:GFP;lyz:dsRed) fish, the next step was to assess the effect poly I:C has on the biology of HSCs. HSC biology includes the ability to migrate, engraft and differentiate. These behaviours can only be studied in a regenerative assay such as transplantation. Based on imaging studies by Kunisaki *et al.* (406) and Rashidi *et al.* (122), it was hypothesised that early post-transplant migration, engraftment and/or differentiation would be affected by the poly I:C priming of cells. The increased percentage of cells in the lymphoid gate seen in the poly I:C injected donors at the time of transplant suggests that the differentiation of the cd41:GFP+ cells may be along the lymphoid lineage in the transplant recipient. However, as the donors did not have a transgenic marker for the lymphoid population (for instance rag or lck), it would not be possible to assess lymphocyte differentiation without killing the recipient.

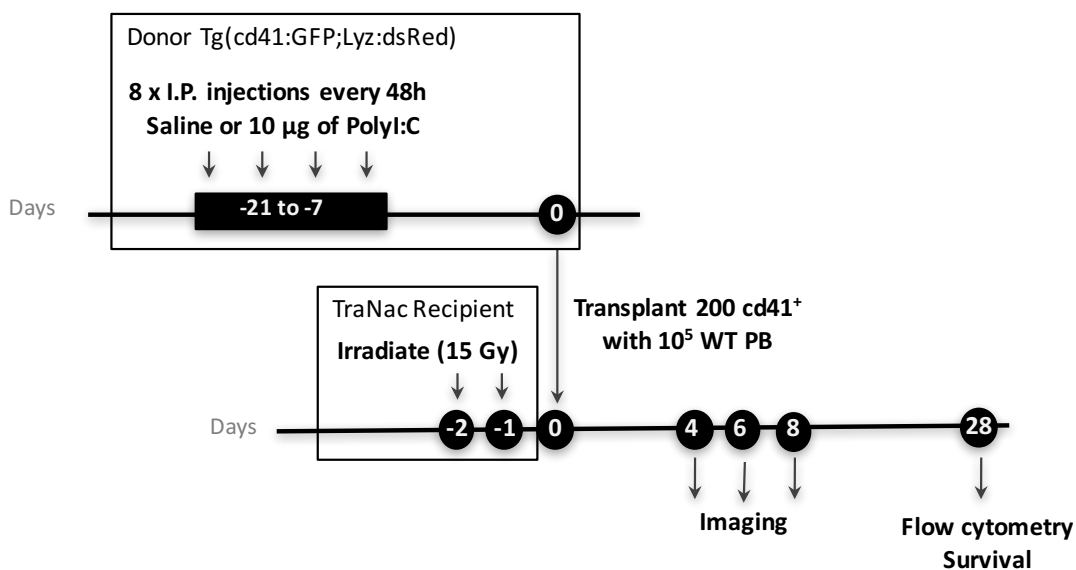


Figure 4.6: Schematic overview of the experiment to transplant 200 cd41:GFP+ cells from poly I:C or saline injected donors into irradiated TraNac recipients. Tg (cd41:GFP;lyz:dsRed) fish were injected 8 times with either saline or 10 µg poly I:C. One week post injection, WKM were harvested and sorted for cd41:GFP+ cells for transplant. TraNac recipients were irradiated with 15 Gy 48 and 24 h prior to RO injection of 200 cd41:GFP+ cells from these donors (and 10⁵ WT PB cells; Non-transplanted recipients did not receive 10⁵ WT PB cells). Recipients were imaged using Zeiss axiovert 200 inverted microscope (wide field) and fluorescent cells were counted in the WKM at 4, 6, and 8 dpt. Fish were housed individually and monitored for 28 dpt. Survival was recorded and flow cytometry was performed on the WKM of surviving recipients.

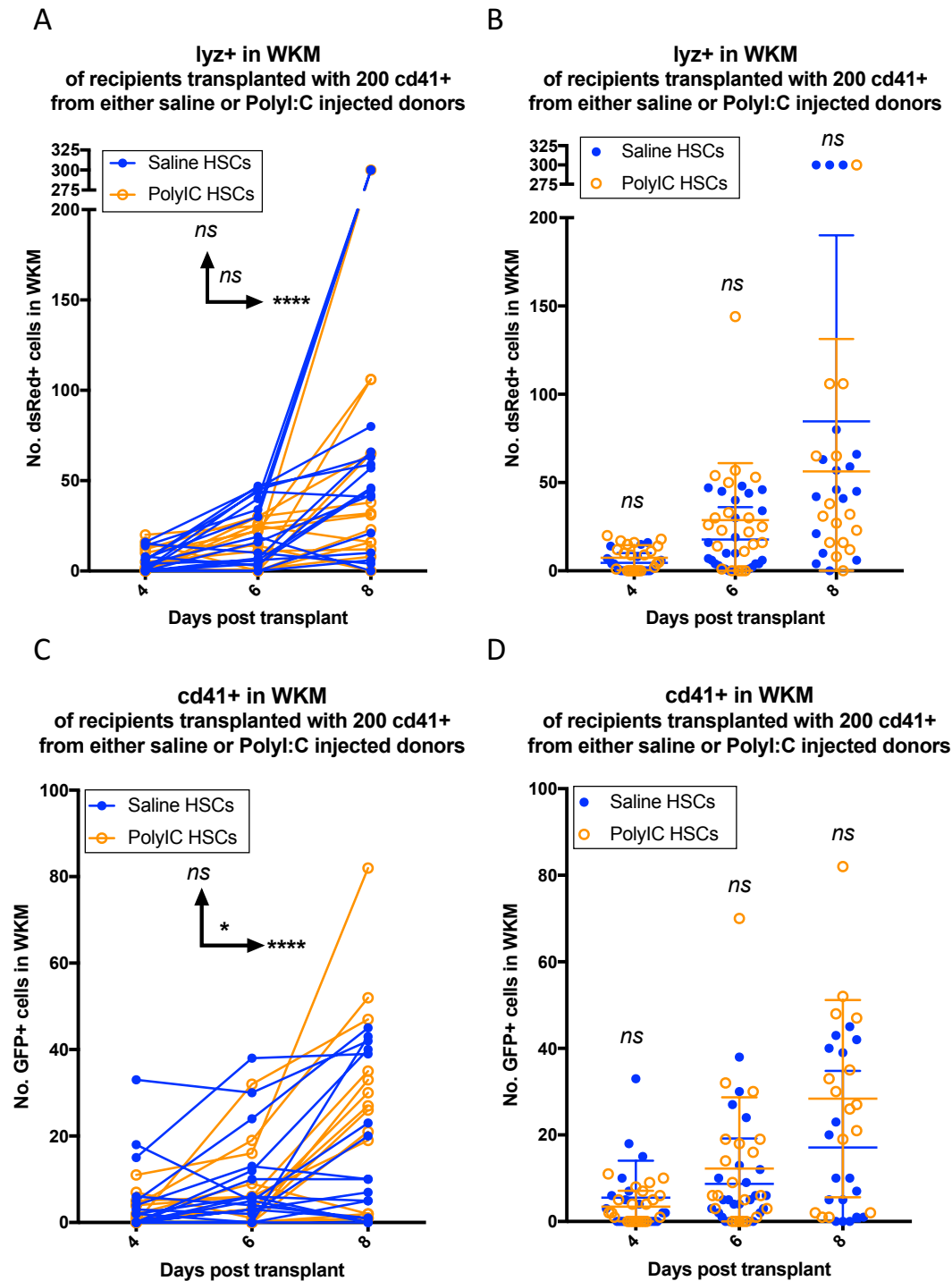


Figure 4.7: There is a significant difference in the rate of cd41:GFP+ accumulation in the WKM of recipients of HSCs from saline-injected donors and HSCs from poly I:C-injected donors. Tg (cd41:GFP;lyz:dsRed) fish were injected 8 times with either saline or 10 µg poly I:C. One week post injection, WKM were harvested and sorted for cd41:GFP+ cells for transplant. TraNac recipients were irradiated with 15 Gy 48 and 24 h prior to RO injection of 200 cd41:GFP+ cells from these donors (and 10⁵ WT PB cells; Non-transplanted recipients did not receive 10⁵ WT PB cells). Groups were blinded and recipients were imaged using Zeiss axiovert 200 inverted microscope (wide field) and fluorescent cells were counted in the WKM at 4, 6, and 8 dpt. The number of (A) lyz:dsRed+ and (C) cd41:GFP+ cells in the WKM of recipients was counted. The data from the same fish is connected by a line (4 experiments pooled; Recipients of HSCs from saline-injected donors in blue, n= 19; recipients HSCs from poly I:C-injected donors in orange, n= 17). Statistical significance tested using Two-way Repeated Measures ANOVA. Significance demonstrated with L-shaped arrows: significance over time indicated by the horizontal arrow, significance between treatment groups indicated by vertical arrow. Interaction indicated in the angle between the arrows. (B and D) The same data is represented as in A and C to show data spread. Each dot represents a fish. Student t-test at each time point.

The subsequent transplant experiments were designed to obtain multiple readouts from the same animal in order to reduce animal numbers and improve data quality and statistical power (Figure 4.6). Donor Tg (cd41:GFP;lyz:dsRed) were injected 8 times with 10 µg of poly I:C, at 48 h intervals, and left for 1 week prior to humane killing and WKM cell harvesting. Recipient TraNac were irradiated with 15 Gy 48 and 24 h prior to transplant. On the day of the transplant (day 0) donor cells were harvested and cd41:GFP+ cells were sorted from the WKM. As demonstrated in chapter 3, transplantation of 200 cd41:GFP+ cells from Tg (cd41:GFP;lyz:dsRed) donors (with 10⁵ PB from WT donors) was enough to rescue irradiated TraNac recipients and reconstitute their hematopoietic system. Thus, each irradiated TraNac recipient was transplanted this way and imaged at 4, 6 and 8 dpt. Experiments were blinded and individual fish were monitored to correlate observed cell counts with recipient survival to 28 dpt and with donor contribution to the recipient hematopoietic system at 28 dpt.

4.3.3 Early post-transplant behaviour of HSCs (cd41:GFP+) from poly I:C-injected donors

Early post-transplant behaviour of donor cells cannot be easily monitored in most transplant systems. However, the method devised and outlined in chapter 1 was used to quantify the presence of donor cells in these transplant recipients. The same recipients were tracked individually for 28 dpt, but imaged at 4, 6 and 8 dpt. The number of cd41:GFP+ and lyz:dsRed+ cells was counted throughout each transplant recipient and in the WKM in particular (Figure 4.7).

The results show that the number of lyz:dsRed+ cells significantly increased over time ($p < 0.0001$; Figure 4.7A). Although there was a significant increase in the mean number of lyz:dsRed+ cells over time, interestingly, the number of lyz:dsRed+ in the WKM of individual fish did not always increase from 4 to 6 to 8 dpt. The number of lyz:dsRed+ in the WKM of recipients did not differ between saline-treated and poly I:C treated recipient groups on any particular day (Figure 4.7B). Furthermore, there was no cumulative effect (interaction) between the donor treatment and over time (assessed using two-way repeated measured ANOVA).

Recipients were simultaneously studied for the presence of cd41:GFP+ cells in the WKM, including the presence of mobile cd41:GFP+ cells. Again, the number of cells in both groups of recipients increased significantly over time ($p < 0.0001$; Figure 4.7C). Also, the number of cd41:GFP+ cells in the two recipient groups did not differ significantly on any particular day (Figure 4.7D). However, as the data from the same fish was coupled, the two way repeated measured ANOVA revealed a significant interaction ($p < 0.05$) between changes over time and treatments. Thus, while the cd41:GFP+ population in recipients of cells from saline injected donors remained stable over time, the cd41:GFP+ population in recipients of cells from poly I:C injected donors started lower at 4 dpt and increased more by 8 dpt (Figure 4.7C). These kind of data cannot be obtained using other models of transplantation.

The data was also analysed to determine if a relationship exists between the number of cd41:GFP+ and lyz:dsRed+ cells in the WKM at 8 dpt (Figure 4.8A). Using the same data as before (Figure 4.7), the dependence

Post-transplant behaviour of HSCs from donors with systemic inflammation of these two variables was tested using Pearson's product-moment correlation. There was no correlation between the number of cd41:GFP+ and lyz:dsRed+ in either group of recipients at 8 dpt (recipients of HSCs from saline-injected donors $r = 0.3016$; recipients of HSCs from poly I:C-injected donors, $r = 0.2885$).

The analysis was also refined to include only recipients which survived to 28 dpt to determine if these fish have a correlation between cd41:GFP+ cells and lyz:dsRed+ cells at 8 dpt which would predict engraftment success (Figure 4.8B). There was no correlation in these fish either. However, it is noteworthy that the recipients with the highest number of cd41:GFP+ in the WKM at 8 dpt survived to 28 dpt (5 of 6 recipients of HSCs from saline-injected donors; 4 of 6 recipients of HSCs from poly I:C-injected donors).

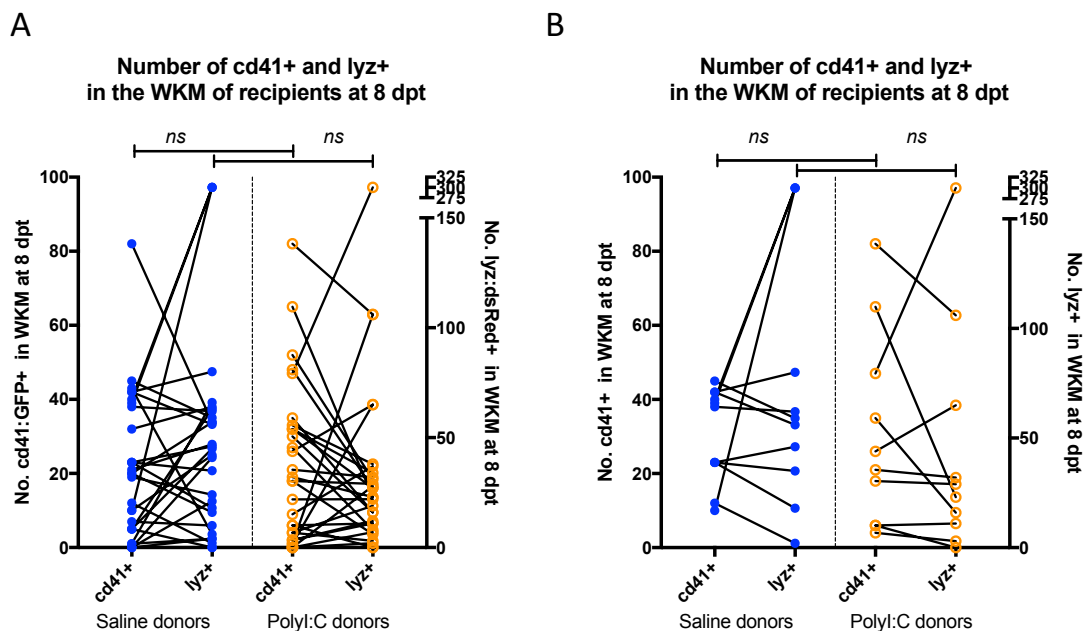


Figure 4.8: There is no relationship between the number of cd41:GFP+ cells and lyz:dsRed+ cells in the WKM of recipients of HSCs from saline-injected donors and HSCs from poly I:C-injected donors. Tg (cd41:GFP;lyz:dsRed) fish were injected 8 times with either saline or 10 µg poly I:C. One week post injection, WKM were harvested and sorted for cd41:GFP+ cells for transplant. TraNac recipients were irradiated with 15 Gy 48 and 24 h prior to RO injection of 200 cd41:GFP+ cells from these donors (and 10⁵ WT PB cells; Non-transplanted recipients did not receive 10⁵ WT PB cells). Groups were blinded and recipients were imaged using Zeiss axiovert 200 inverted microscope (wide field) and fluorescent cells were counted in the WKM at 4, 6, and 8 dpt. Both graphs link cd41:GFP+ counts with lyz:dsRed+ count in the same recipient, connected by a line. (6 experiments pooled, $n > 27$; same data as above plus 2 experiments imaged 8 dpt only). (A) Includes all recipients which had fluorescent cells in the WKM at 8 dpt. (B) Only includes recipients that survived to 28 dpt and had fluorescent cells as assessed by flow cytometry (6 experiments pooled, $n > 9$).

4.3.4 Transplant recipients of poly I:C cd41:GFP+ cells survive equally well as recipients of saline cd41:GFP+ cells

One of the measures of HSC transplant success is the survival of recipients, which is often associated with donor cell engraftment, differentiation and proliferation. To determine if inflammation affected the ability of HSCs to engraft, differentiate and proliferate, recipients were monitored for 28 dpt and then harvested for flow cytometry analysis (Figure 4.6). Altogether, 26 % of recipients of HSCs from saline injected donors survived to 28 dpt

Post-transplant behaviour of HSCs from donors with systemic inflammation (Supplementary Figure 7.6). However, when this group of recipients was split based on the observable presence of cells at 8 dpt, the survival to 28 dpt of recipients with observable cells (37 %) was significantly higher than those with no observable cells at 8 dpt (4 %; blue and broken blue line; Figure 4.9A). This suggests that early cell behaviour in these recipients could predict survival of these recipients. Recipients without cells observable at 8 dpt had the same morbidity as non-transplanted (irradiated) recipients.

On the other hand, the survival of recipients of HSCs from poly I:C-injected donors was 22 % (Supplementary Figure 7.6). This did not change significantly when the group was split into recipients with observable cells at 8 dpt (25 %) and those with no observable cells at 8 dpt (18 %; orange and broken orange line; Figure 4.9B). This shows that these early cell behaviour observations were not enough to separate recipients into groups with better or worse survival. The survival of both groups was significantly higher than non-transplanted (irradiated) recipients (0 %; broken black line; Figure 4.9B).

Altogether, the survival did not differ ($p=0.129$) between recipients of saline injected or poly I:C injected donors (orange and blue lines; Figure 4.9C). Both groups had better survival than irradiated recipients that did not receive a transplant ($p<0.001$ and $p<0.01$). As seen in chapter 3 (Figure 3.22), due to the stress of the procedure and the low number of HSCs used, morbidity is highest in the first 10 dpt in both recipient groups. However, survival continues to decline after this, in particular in recipients of HSCs from poly I:C-injected donors.

Survival of recipients was compared with recipients of cd41:GFP+ from non-injected (naïve) donors. These data were taken from control recipients in work which will be described later (see chapter 5.3.4). In all cases the recipients were irradiated 48 and 24 h before transplant. The transplanted cd41:GFP+ cells came from different donors. The results suggest that there is no difference between recipients of cd41:GFP+ cells from non-injected donor and recipients of cd41:GFP+ cells from saline injected donors. However, there is a greater, though non-significant ($p=0.0531$) difference in survival of recipients of cd41:GFP+ cells from poly I:C donors and non-injected donors (Figure 4.9D). In order to confirm this finding, the experiment needs to be repeated to include all 3 groups in the same study. This would mean the recipients would come from the same clutch of larvae to reduce variation in clutch survival.

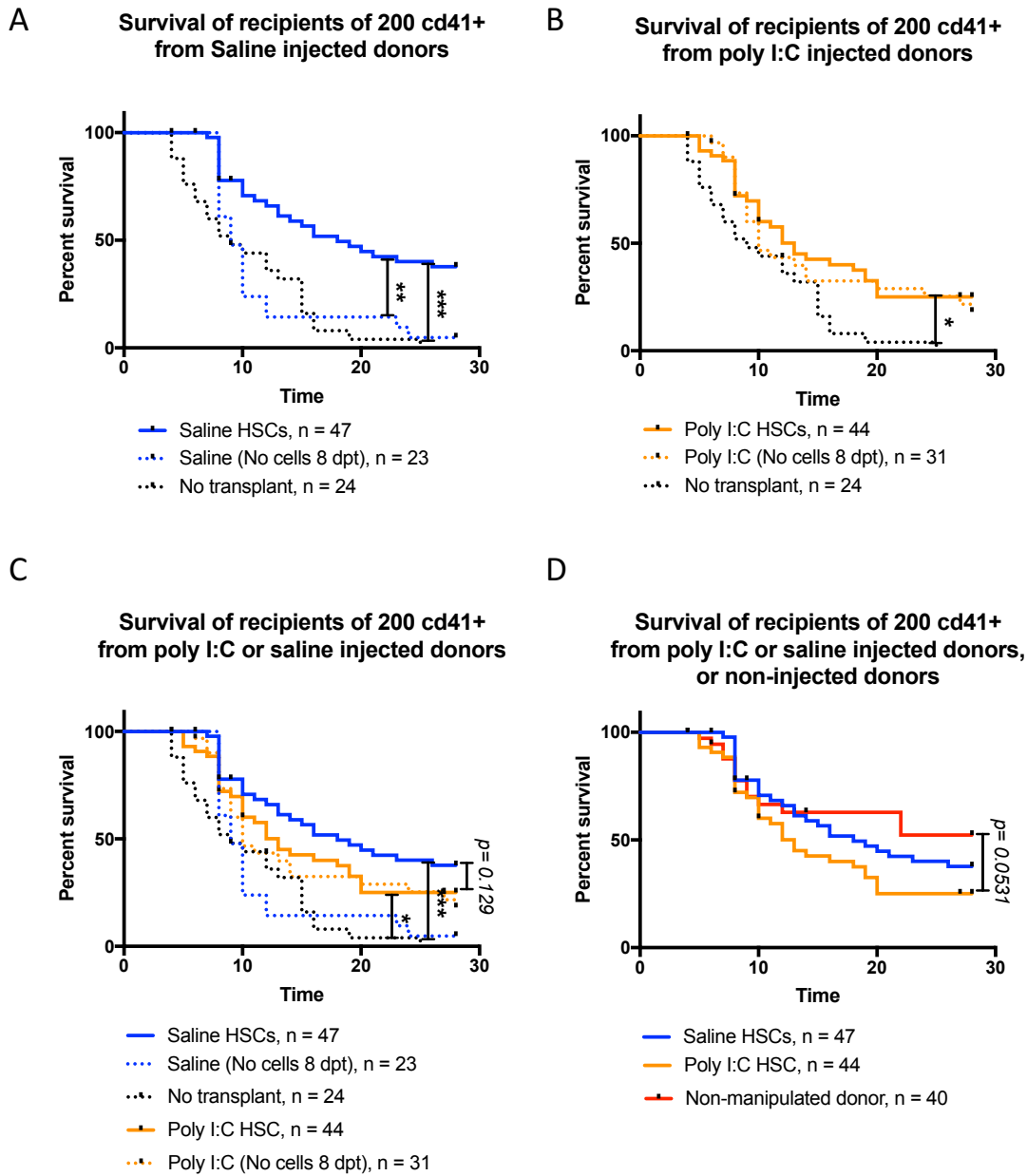


Figure 4.9: Transplanting 200 cd41:GFP+ cells from poly I:C treated donors rescues irradiated TraNac recipients to 28 dpt. Tg (cd41:GFP;lyz:dsRed) fish were injected 8 times with either saline or 10 μ g poly I:C. One week post injection, WKMs were harvested and sorted for cd41:GFP+ cells for transplant. TraNac recipients were irradiated with 15 Gy 48 and 24 h prior to RO injection of 200 cd41:GFP+ cells from these donors (and 10^5 WT PB cells; Non-transplanted recipients did not receive 10^5 WT PB cells). Groups were blinded and recipients were imaged and then monitored for survival to 28 dpt. Recipients were split into groups based on the presence of any fluorescent cells in the WKM at 8 dpt. 6 experiments pooled, n>24. (A) Survival of recipients of HSCs from saline-injected donors. (B) Survival of recipients of HSCs from poly I:C-injected donors. (C) Survival of recipients of HSCs from saline-injected donors and recipients of HSCs from poly I:C-injected donors. (D) Survival of recipients of HSCs from saline-injected donors, recipients of HSCs from poly I:C-injected donors and recipients of HSCs from non-injected donors. Data for recipients on-manipulated donors taken from controls in Poly I:C treated recipient experiments in chapter 5.

4.3.4.1 The number of lyz:dsRed+ cells in the WKM at 8 dpt correlates with increased survival in recipients of cd41:GFP+ from saline injected donors but not poly I:C injected donors.

In order to determine if survival can be predicted from the observable presence of fluorescent protein expressing cells (cd41:GFP and lyz:dsRed) in the WKM, survival of recipients was plotted against the number of lyz:dsRed+ cells in the WKM at 8 dpt. In both groups, the number of lyz:dsRed+ cells in the WKM varies enormously (from 0 to >300 cells) at 8 dpt (Figure 4.7A, B). The results show that there is a good correlation between the number of cells at 8 dpt and survival ($R^2 = 0.1334$; $p=0.0399$; Figure 4.10A) in recipients of HSCs from saline injected donors. This indicates that early deaths could be reduced by selecting recipients with fluorescent cells at 8 dpt in recipients of cd41:GFP from saline injected donors. However, it is important to note that due to the low R^2 value (<13 %), survival cannot be predicted from this model (a high R^2 is required for precise predictions).

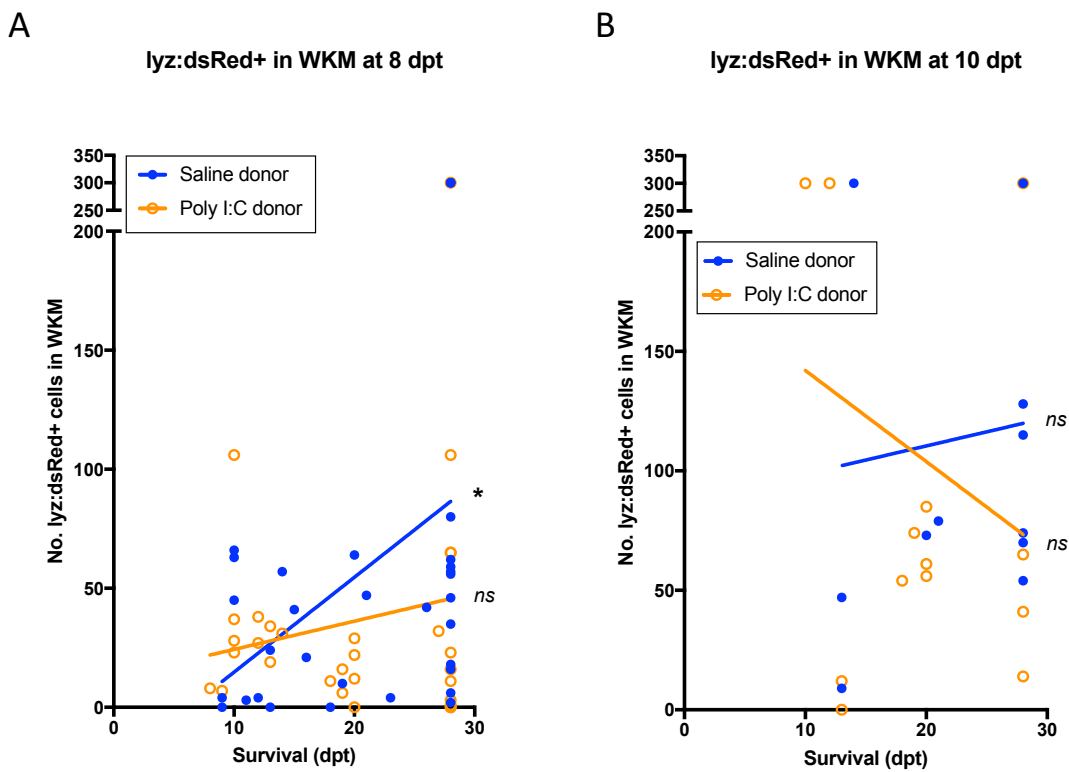


Figure 4.10: Survival of recipients of cd41:GFP+ cells from saline injected donors can be predicted by the observed presence of lyz:dsRed+ cells in the WKM at 8 dpt. Tg (cd41:GFP;lyz:dsRed) fish were injected 8 times with either saline or 10 µg poly I:C. One week post injection, donors were harvested of their WKM and sorted for cd41:GFP+ cells for transplant. TraNac recipients were irradiated with 15 Gy 48 and 24 h prior to RO injection of 200 cd41:GFP+ cells from these donors (and 10⁵ WT PB cells; Non-transplanted recipients did not receive 10⁵ WT PB cells). Recipient groups were blinded during imaging for cell counting and then monitored for survival to 28 dpt. (A) Number of lyz:dsRed+ cells at 8 dpt plotted against survival of recipient. 6 experiments pooled, $n>30$. (B) Number of lyz:dsRed+ cells at 10 dpt plotted against survival of recipient. (2 experiments pooled, $n>10$).

On the other hand, there is no correlation between survival and the number of lyz:dsRed+ cells in recipients of HSCs from poly I:C-injected donors ($R^2 = 0.0253$; $p=0.4011$) (Figure 4.10A). This suggests that successful transplant recipients cannot be selected for at 8 dpt in this group of recipients. As longer survival of recipients of

HSCs from poly I:C injected donors did not correlate with an increase in observable cell numbers at 8 dpt, the experiment was repeated to determine if there was a correlate between the number of observable cells at later time points. For this reason, recipient fish were imaged at 10 and 12 dpt. There was no correlation found between number of lyz:dsRed+ cells and survival for either group of recipients (recipients of HSCs from saline-injected donors ($p=0.8139$); recipients of HSCs from poly I:C-injected donors ($p=0.4689$); Figure 4.10B).

4.3.4.2 Reconstitution of recipients' WKM at 28 dpt

In order to determine if the surviving recipients' hematopoiesis was reconstituted, the WKM was harvested at 28 dpt for flow cytometry analysis (Figure 4.11A). Not unexpectedly, recipients that did not have any hematopoietic cells in the WKM by 8 dpt ($n=4$) but nevertheless survived to 28 dpt, did not have lyz:dsRed+ or cd41:GFP+ cells in their WKM as determined by flow cytometry. They were not included in the analysis as they had not successfully engrafted and restored recipient hematopoiesis.

The percentage of erythrocyte, lymphocyte, myelomonocyte, and precursor populations were determined from forward side scatter (Figure 4.11B). There was no significant difference in the reconstitution between the 2 groups of recipients.

Additionally, to assess donor contribution to the reconstituted recipient WKM, the percentage of lyz:dsRed+ and cd41:GFP+ events was determined. There was a lot of variation between recipients of each group but no statistically significant differences (Figure 4.12A, B). To determine if the recipients with a lower percentage of cd41:GFP+ also had a lower percentage of lyz:dsRed+, the data from the same recipient were paired (Figure 4.12C). There was no correlation between the two populations (Pearson's product-moment correlation: recipients of HSCs from saline-injected donors ($p=0.714$); recipients of HSCs from poly I:C-injected donors ($p=0.985$)).

4.3.5 The number of cd41:GFP+ and lyz:dsRed+ cells in the WKM at 8 dpt does not correlate with the percentage of those cells in the WKM at 28 dpt

Finally, although the number of fluorescent cells in the WKM at 8 dpt is not always an indicator of survival, it was still interesting to test whether the number of cd41:GFP+ and lyz:sRed+ cells observed in the WKM at 8 dpt relates to the percentage of cd41:GFP+ and lyz:dsRed+ events detected by flow cytometry at 28 dpt. As mentioned earlier, recipients with no cells at 8 dpt, did not have fluorescent cells by 28 dpt.

The recipients which survived to 28 dpt had between 10 and 80 cd41:GFP+ cells observable in the WKM at 8 dpt. However, in both recipient groups, the recipients with the highest number of cd41:GFP+ at 8 dpt did not have the highest percentage of cd41:GFP+ at 28 dpt (Figure 4.13A). In fact, there was no correlation between the number of cd41:GFP+ cells at 8 dpt and the percentage at 28 dpt (recipients of HSCs from saline-injected donors ($p=0.242$); recipients of HSCs from poly I:C-injected donors ($p=0.764$; Figure 4.13B).

The recipients which survived to 28 dpt had between 3 and 300 lyz:dsRed+ cells observable in the WKM at 8 dpt. Unlike the cd41:GFP+ counts, the number of lyz:dsRed+ at 8 dpt did correlate with the percentage of lyz:dsRed at 28 dpt in recipients of HSCs from saline-injected donors ($p=0.0213$), though not in recipients of HSCs from poly I:C-injected donors ($p=0.6077$).

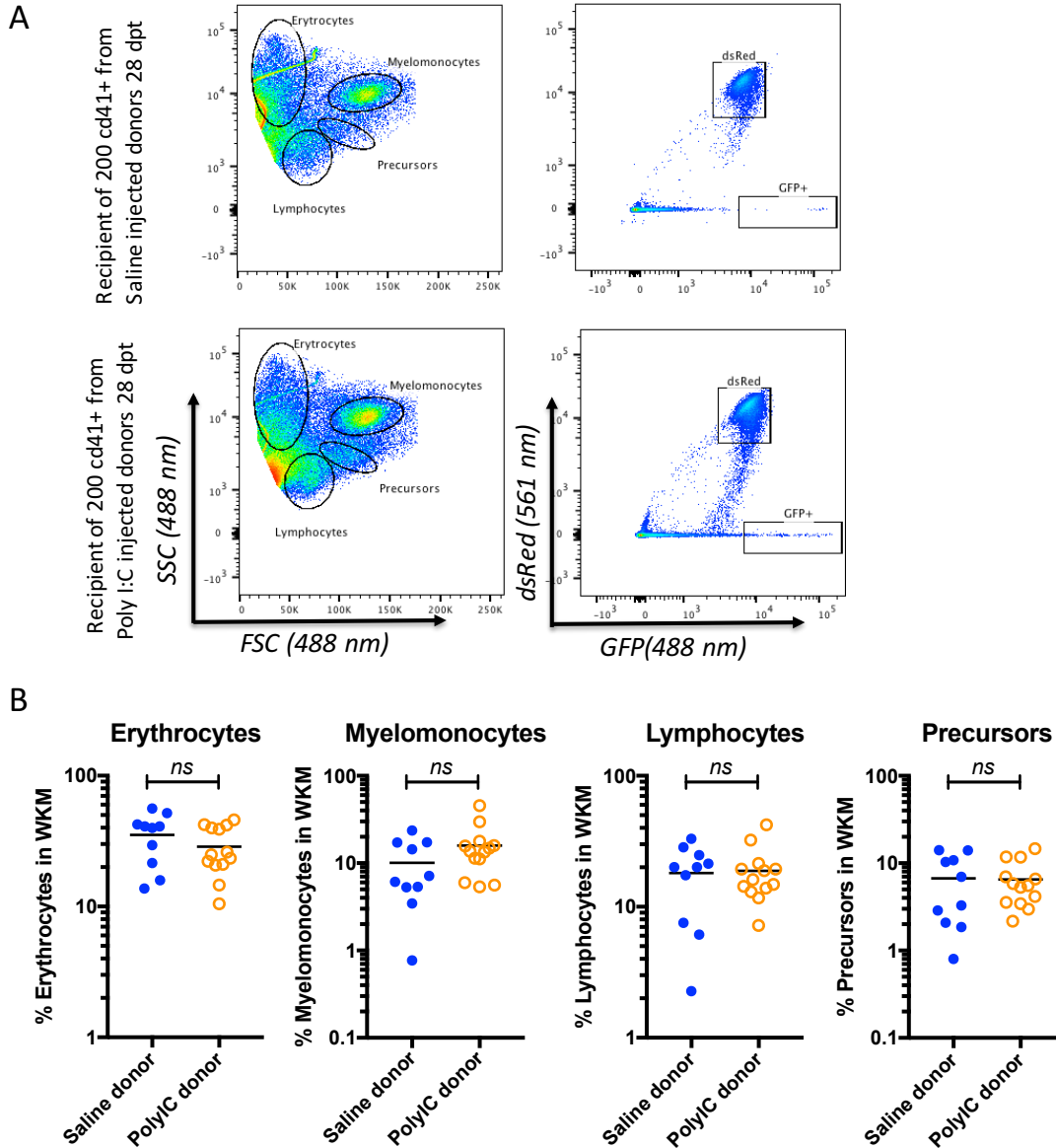


Figure 4.11: Reconstitution of the WKM in recipients of 200 cd41:GFP+ cells from saline and poly I:C donors. Tg (cd41:GFP;lyz:dsRed) fish were injected 8 times with either saline or 10 μ g poly I:C. One week post injection, donors were harvested of their WKM and sorted for cd41:GFP+ cells for transplant. TraNac recipients were irradiated with 15 Gy 48 and 24 h prior to RO injection of 200 cd41:GFP+ cells from these donors (and 10^5 WT PB cells; Non-transplanted recipients did not receive 10^5 WT PB cells). Recipients were humanely killed and their WKM harvested at 28 dpt for flow cytometry analysis. (8 experiments pooled; $n=11$; each dot represents a recipient). SSC indicates side scatter, FFC indicates forward scatter. (Both use 488 nm laser, 488/10 filter). GFP (488 nm laser, 530/30 filter); dsRed (561 nm laser, 610/20 filter). (A) Flow cytometry scatter of WKM cells from recipients. (B) Percentage of erythrocytes, myelomonocytes, lymphocytes, and precursors.

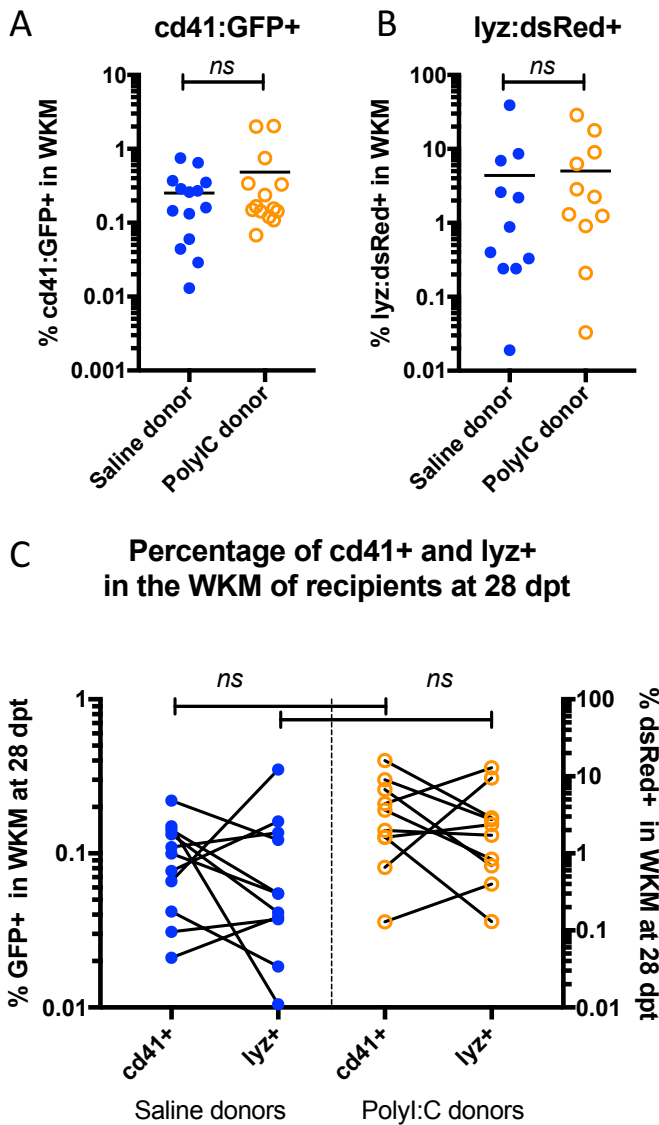


Figure 4.12: Donor cells in the recipient WKM in recipients of 200 cd41:GFP+ cells from saline-injected and poly I:C-injected donors. Tg (cd41:GFP;lyz:dsRed) fish were injected 8 times with either saline or 10 µg poly I:C. One week post injection, WKM was harvested from donors and sorted for cd41:GFP+ cells for transplant. TraNac recipients were irradiated with 15 Gy 48 and 24 h prior to RO injection of 200 cd41:GFP+ cells from these donors (and 10⁵ WT PB cells; Non-transplanted recipients did not receive 10⁵ WT PB cells). Recipients were harvested at 28 dpt for flow cytometry analysis. 8 experiments pooled; n=11; each dot represents a recipient. (A) Percentage of cd41:GFP+ and (B) lyz:dsRed+ events in the WKM. (C) The percentage of cd41:GFP+ is connected with the percentage of lyz:dsRed+ in the same recipient.

Altogether, the data indicates that, though the observable presence of cells in the WKM early post-transplant is a good indicator of fluorescent cells being present at 28 dpt (and therefore donor contribution to recipient hematopoiesis) in recipients of cd41:GFP+ cells from saline-injected donors, the observation of the cells in the WKM of recipients of cd41:GFP+ cells from poly I:C-injected donors does not guarantee fluorescent cells being present at 28 dpt. In these recipients, the data collected was not useful for predicting survival or donor contribution to recipient reconstitution.

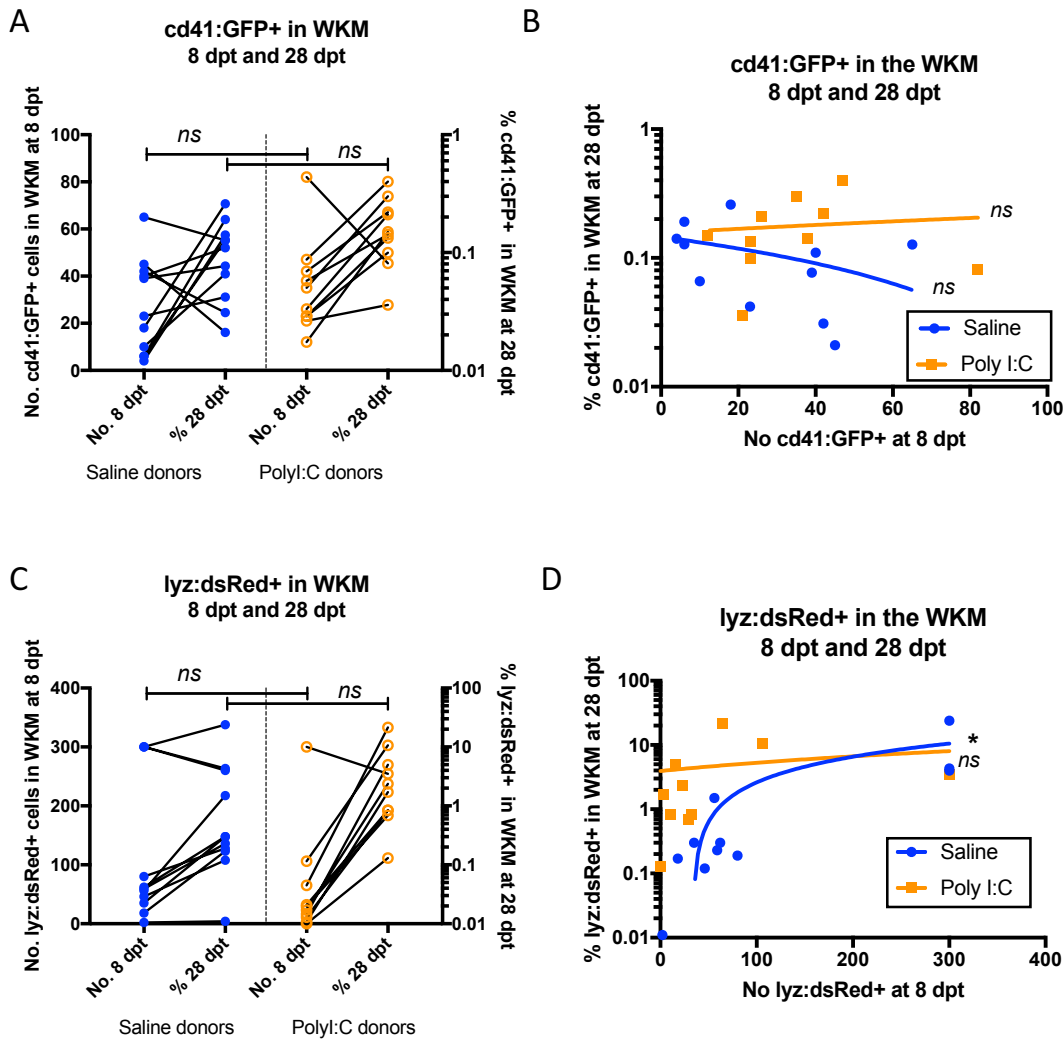


Figure 4.13: The number of lyz:dsRed+ cells in the WKM at 8 dpt correlates with the percentage of lyz:dsRed in the WKM of in recipients from saline-injected donors, at 28 dpt. Tg (cd41:GFP;lyz:dsRed) fish were injected 8 times with either saline or 10 µg poly I:C. One week post injection, WKM was harvested from donors and sorted for cd41:GFP+ cells for transplant. TraNac recipients were irradiated with 15 Gy 48 and 24 h prior to RO injection of 200 cd41:GFP+ cells from these donors (and 10⁵ WT PB cells; Non-transplanted recipients did not receive 10⁵ WT PB cells). Recipients were humanely killed at 28 dpt for flow cytometry analysis. 8 experiments pooled; n=11; each dot represents a recipient. (A) The number of cd41:GFP+ at 8 dpt plotted against the percentage of cd41:GFP at 28 dpt. Data from the same recipient is connected by a line. (B) The same data is presented in XY scatter. (C) The number of lyz:dsRed + at 8 dpt plotted against the percentage of lyz:dsRed+ at 28 dpt. (D) The same data is presented in XY scatter.

4.4 Discussion

The aim of this thesis is to use zebrafish to determine the early post-transplant effects of inflammation on the HSC. Although the effect of inflammation on HSCs was assessed using transplantation models before, this will be the first instance of using the zebrafish model and obtaining *in vivo* data on the early post-transplant behaviour of HSPCs.

In this chapter, HSCs are stimulated prior to transplantation. In order to control for inflammation in the recipient (induced by irradiation, as described in the previous chapter), in each experiment recipients came from the same

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clutch, were raised together and irradiated together. Recipients were randomly allocated to 2 groups and received a transplant. Donor cells came from adult Tg (cd41:GFP;lyz:dsRed), also randomly allocated to 2 groups, and injected with either saline or 10 µg poly I:C. The variation in individual response to treatment was reduced by pooling the harvested WKM from 3-4 adults prior to sorting. Sorted WKM cells were counted on a haemocytometer and 200 cd41:GFP+ cells per recipient were mixed with 2 x 10⁶ WT PB cells. Both donor groups were mixed with PB from the same WT donor to reduce variation. As the donor sets were distinct, there may be some variation in the number of cells aliquoted from the individual stocks. This error was reduced by repeating the experiment. Cells were transplanted using the same glass needle, washed repeatedly in deionised water. The order in which the donor WKM was harvested and cells sorted was alternated each experiment so as to alternate the order the recipient groups were transplanted.

4.4.1 Summary of results

The findings of this chapter were:

1. Flow cytometry and qPCR data indicated that poly I:C treatment (50 µg, 10 µg and 1 µg) significantly increased transcript levels of various (*il-1β*, *il-6*, *tnfα*, *ifnφ*, *ifnγ1.1*, *ifnγ1.2*, *cxcl-c1c* and *cxcl8*) cytokines. Importantly, the expected interferon response to poly I:C was generated.
2. A protocol for inducing systemic inflammation was devised (8 successive poly I:C injection of 10 µg every 48 h) and surprisingly, flow cytometry to assess the cd41:GFP+ population and qRT-PCR analysis of *runx1* and *c-myb* transcript levels indicated that fish treated with this protocol did not show the expected reduction in HSCs. Although there was no reduction in the cd41:GFP+ population, the percentage of cd41:GFP+ that were FSC^{low} SSC^{low} (like lymphocytes) was significantly lower. Furthermore, flow cytometry analysis indicated an increase in the lymphocyte scatter (FSC^{low} SSC^{low}) in poly I:C treated fish, but this increase was not reflected in the relative levels of *lck* transcript, therefore the increase maybe in HSCs, B or NK cells.
3. Analysis of data obtained by live imaging of recipients revealed the number of fluorescent cells (cd41:GFP+ or lyz:dsRed+) at each time point did not differ significantly between recipient groups. However, there was a greater increase in the number of cd41:GFP+ in the WKM from 4 to 8 dpt in recipients of cd41:GFP+ from poly I:C-injected donors, compared to recipients of cd41:GFP+ from saline injected donors.
4. The survival of recipients of cd41:GFP+ cells from saline-injected donors was not significantly different from recipients of cd41:GFP+ cells from poly I:C-injected donors (25 % and 22 % respectively). Interestingly, using live imaging data for each fish, analysis comparing recipients with observable fluorescent cells in the WKM revealed there is a more notable, though still not significant, difference between the recipient groups (37 % and 22 % respectively; p=0.129). This supports the hypothesis that the observable presence of fluorescent cells in the WKM is a good indicator of survival. In addition, there is an almost significant difference (p=0.0531) between recipients of cd41:GFP+ cells from poly I:C-injected donors and cd41:GFP+ cells from naïve donors. There is no significant difference between

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survival of recipients of cd41:GFP+ cells from saline-injected donors and survival of recipients of cd41:GFP+ cells from poly I:C-injected donors or naïve donors. This could suggest that the difference between the naïve donors and poly I:C-injected donors is a combination of the effects of the poly I:C and the intra-peritoneal injection.

5. The number of observed lyz:dsRed+ cells at 8 dpt correlated with survival of recipients of cd41:GFP+ cells from saline-injected donors. However, there was no correlation in recipients of cd41:GFP+ cells from poly I:C-injected donors.
6. There was no significant difference in the reconstitution of the recipient immune system between the two recipient groups. A similar number from each group of transplant recipients survived to 28 dpt (11 of 70 recipients of HSCs from saline-injected donors (15.7 %); 10 of 75 recipients of HSCs from saline-injected donors (13.3 %)). Interestingly, in recipients of cd41:GFP+ cells from saline-injected donors, the number of observed lyz:dsRed+ cells at 8 dpt correlated with the percentage of lyz:dsRed in the WKM at 28 dpt.

4.4.2 Systemic inflammation and its effect on the donor prior to transplant

The first part of this chapter presents work establishing systemic inflammation. Poly I:C doses were titrated to confirm an inflammatory response by the zebrafish immune system, and determine an appropriate dose for repeated stimulation (Figure 4.2). As expected, the most significant response was with the highest single dose (50 µg poly I:C). A non-significant (using one-way ANOVA) response was seen with the 10 µg poly I:C dose. It is important to note that one-way ANOVA takes into consideration variation among the groups as well as variation within each group to determine statistical significance. Variability within a group is based on standard deviation (quantified as the sum of the squares of the differences between each value and its group mean), and variation between groups is determined using this and the overall mean (quantified as the sum of the square of the difference between the group mean and the overall mean). This means that one-way ANOVA did not indicate statistically significant differences between saline and 10 µg poly I:C because the significant variation in the 50 µg poly I:C group. One could argue that any increase in transcript levels is significant in biological terms. Thus, although most of the upregulated genes are not significant according to the ANOVA test, personally I would consider anything above 2.5 fold (admittedly an arbitrary cut-off) as biologically significant.

All 3 doses of poly I:C had high transcript levels of inflammatory cytokines. Further support of the inflammatory response to a dose of 10 µg was published with Jha *et al.* (405). In this paper we injected zebrafish with 10 µg of either poly I:C, poly I:C LC, or TLR 7/8 agonist R848 (resiquimod). Fish injected with poly I:C had a significant increase in transcript levels of *tnfα*, *il-1β*, *ifnφ*, *ifnγ1.1* and *ifnγ1.2* in both the WKM and the gill. Altogether, these data support the conclusion that 10 µg poly I:C induces systemic inflammation in the zebrafish.

A 3 h post injection time point was chosen for analysis because previously Varela *et al.* had examined this time point and shown a significant upregulation of inflammatory genes in zebrafish injected with 10 µg poly I:C (407). As in this paper, *tnfα*, *il-1β* and *il-6* were all upregulated in the kidney, although fold change was twice as high as previously reported (Figure 4.2). To my knowledge, neither *cxcl-c1c* nor *cxcl8* (an important chemokine of the

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innate immune response) have previously been associated with injection of poly I:C. Thirdly, it is well established that poly I:C is an inducer of the interferon response and this data shows that both type I and II interferon responses (*ifn ϕ* , *ifn γ 1.1* and *ifn γ 1.2*) were strongly induced in zebrafish after 3 h.

Having established a dose of poly I:C for further experiments, the protocol for these experiments was put in place (Figure 4.6). Donors were treated 8 times with poly I:C and then harvested for transplantation. However, before beginning transplantation experiments, donor spleen and WKM was assayed qRT-PCR to determine if any hematopoietic changes could be used to validate the effect of the treatment. qRT-PCR could not be used to measure cytokine expression because of the short half-life of these transcripts (in support of this, a time-course measuring relative transcript levels in nasal lavage samples obtained from human volunteers challenged with TLR7/8 agonist R848 found that mRNA levels of TNF- α , IL-1 α , and IFN- α 2a peaked at 2, 5, and 4 h respectively and importantly, all had returned to basal levels within 24 h (405)). Analysis of the spleen revealed a significant increase in *gata1* expression in poly I:C injected donors. The spleen is the major site for erythropoiesis. Increased erythropoiesis has not been reported as an effect of chronic interferon stimulus. The spleen also contains lymphocytes and myelomonocytes (408), although the level of I-plastin transcripts was not altered (Figure 4.3).

The protocol for these experiments was adapted from a paper by Essers *et al.* (76) in which 8 successive poly I:C (5 $\mu\text{g g}^{-1}$) injections caused a significant loss in WT HSCs (LSK CD150⁺) in the bone marrow of chimera mice (50 % WT HSCs, 50 % type I interferon receptor knock out HSCs). Based on these reports, it was hypothesised that poly I:C injected donors would result in a significant loss of HSCs in the zebrafish and thus a smaller cd41:GFP⁺ population. However, the results show that there is no change in the cd41:GFP⁺ population (Figure 4.5), although the percentage of cd41:GFP⁺ that were FSC^{low} SSC^{low} (like lymphocytes) was significantly lower. As stem cells are found in the FSC^{low} SSC^{low} population, it can be used in conjunction with cd41 for HSC selection. This raises the possibility that fewer cd41:GFP⁺ HSPCs get transplanted from poly I:C donors. Furthermore, qRT-PCR of the WKM found that neither *runx1* nor *c-myb* mRNA levels were altered by the poly I:C treatment (Supplementary Figure 7.5). Together this suggests that the decrease reported by Essers *et al.* in mice was not replicated by the same treatment in the zebrafish.

It is also interesting to note that Pietras *et al.* reported an increase in HSCs and *runx1* expression after 20 consecutive days of *il-1 β* injections (107). Furthermore, a number of studies suggest type II interferons induce HSC proliferation (334, 355, 404). Perhaps the difference can be explained by the timing and intensity of the protocols and highlights the need for alternative HSC markers in the zebrafish as CD41 represents a large population of progenitor cells. It would be interesting to apply this protocol to Tg (*runx:GFP*) fish, an alternative transgenic line which labels HSPCs in the zebrafish (42).

Analysis of donor WKM prior to transplantation also revealed a significant increase in the lymphocyte population (Figure 4.5). This is supported by data from AVV- IFN α mice (mice expressing IFN α under the control of a constitutive promoter on a adeno-associated viral vector) which had a significant production of CD8⁺ T-cells in

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the bone marrow and increased differentiation of hematopoietic precursors (LK cells) in the thymus (402). These mice also had a profound decrease in LT-HSCs (LK CD150⁺ CD48⁻), ST-HSCs (LK CD150⁻ CD48⁻) and MPPs (LK CD150⁻ CD48⁺) (402). In transplant assays, cells from AVV- IFN α mice were significantly lymphoid biased and only differentiated into CD4⁺ and CD8⁺ cells. Gene expression analysis showed that B cell transcription factors were significantly decreased in IFN α treated cells, suggesting a mechanism for the bias seen in these mice. Furthermore, the transplantation of IRF2^{-/-} HSCs (results in constitutive type I IFN signalling) into irradiated WT recipients produced fewer granulocytes than control transplants (393), indicating that type I IFNs shift the differentiation bias away from the myeloid lineage. Thus, although *il-1b* expression which has been shown to rapidly induce *pu.1* expression and expansion of the myeloid population (107) was detected, the high levels of ifn α (Figure 4.2) appears to have caused lymphopoiesis in these donors. This bias could be tested by applying the poly I:C protocol to a transgenic zebrafish line in which the adaptive immune system is labelled such as transgenic *lck* or *rag* fish (in which the T cell and B cell populations are labelled respectively).

qRT-PCR of the donor WKM did not reflect the flow cytometry data. For instance, although the percentage of cells in the lymphocyte gate were significantly higher in poly I:C injected fish, transcript levels of *lck* were not significantly higher. This might be because *lck* is expressed by T-cells and the increase in lymphocytes was partially due to an expansion of B-cells, NK or HSCs as well. Transcripts of HSPC genes *runx1* and *c-myb* were not significantly higher indicating the increase in the lymphocyte gate is due to B or NK-cells. It would be interesting to examine transcript levels of B-cell specific *rag1* and NK specific levels of *NK-lysins*. Furthermore, transcript levels of leukocyte gene *l-plastin* and myeloid gene *mpeg* were decreased in the WKM of poly I:C treated donors at the time of transplant (Figure 4.3C). However, flow cytometry did not show a significant change in either the myelomonocyte gate, nor the dsRed (lyz⁺) populations. Together this indicates a discrepancy between flow cytometry and qRT-PCR data. As qRT-PCR data is a single readout of a specific parameter across a population while flow cytometry produces readouts for every member of the population, I would therefore suggest that in this case the flow cytometry data is more reliable. Especially considering that gene fold change is measured by normalising to constitutive gene (in this case 18S) required for basic cell maintenance and it is possible that poly I:C causes a change in 18S expression that would affect calculations of relative expression. However, I did check that cycle threshold values for 18S were consistent in both saline and poly I:C injected fish, suggesting that 18S expression was not affected. It would be interesting to use a different gene such as β -actin for normalisation.

As these results indicate an increase in lymphopoiesis in poly I:C treated donors, it would be interesting to use transgenic *lck* or *rag* line crossed with Tg (cd41:GFP) as donors for transplantations. Due to the absence of these lines (labelled with an appropriate fluorophore), this strategy was not possible within the time frame of this thesis. Future work could use Tg (runx1+23:NLSmCherry;lck:GFP) fish for this study.

While the expression of HSC genes *runx1* and *c-myb* did not change between donor groups in either the spleen or the WKM (Supplementary Figure 7.5), it is noteworthy that the cd41:GFP⁺ population with FSC^{low} SSC^{low} was significantly reduced in Poly I:C treated recipients at the time of transplant (Figure 4.5). This suggests that there

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may be a greater ratio of thrombocytes to HSPCs in the cd41:GFP+ cell population that gets sorted from poly I:C donors. Future work could clarify this by sorting these cells and using single cell transcriptome analysis.

The expansion of the cd41:GFP+ population was greater in recipients of poly I:C stimulated HSCs (Figure 4.7). Although many papers report an increase in HSC cycling in response to *il-1* and type I and II IFNs, this is the first example of these cytokines affecting cell proliferation after a delay. As the cells are transplanted into an irradiated recipient in which these cytokines are highly expressed, the expansion maybe due to the hyper sensitivity of treated HSCs over untreated HSCs. In fact Pietras *et al.* have found that the prior stimulus does weaken the self-renewal capacity of HSCs and is responsible for the exhaustion of these cells in a regenerative assay (107).

4.4.3 The rescue potential of HSCs from a donor with systemic inflammation

Survival is an important readout of the ability of a cell to self-renew and differentiate but is not often assessed as it requires death to be the end point of the licensed protocol. In previous studies, such as by Rashidi *et al.* (122), a higher number of transplanted cells (10^5 BM mononuclear cells in competitive repopulation studies, $1-1.5 \times 10^4$ labelled LT-HSCs in imaging studies) meant that the transplant was always successful. By studying transplants which fail, the transplanted cells are under an extreme regenerative pressure and therefore any potential differences are highlighted.

We obtained permission to assess the survival of recipients in this experiment. The survival of all recipients of cells from saline injected donors (n=70) was 26 % (Supplementary Figure 7.6). However, when the groups were split based on whether there were fluorescent cells observed in the WKM by 8 dpt, the survival of the recipients with cells (47 recipients out of 70) was higher (37 %) than the survival of recipients where cells were not observable by 8 dpt (23 out of 70; 4 %; Figure 4.9A). This suggests that transplanting 200 cells is an appropriate number for studying HSCs, as it was possible to study recipients at the boundary between hematopoietic success and failure.

Interestingly, 31 out of 75 recipients (41 %) of HSCs from poly I:C-injected donors did not have observable cells by 8 dpt, higher than in recipients of HSCs from saline donors (33 %) and may contribute to why survival cannot be predicted for recipients of cells from poly I:C-injected donors. Furthermore, the survival of recipients of cells from poly I:C-injected donors (22 %) was not significantly different if the population was split based on whether they had observable fluorescent cells in the WKM by 8 dpt (25 % and 18 %; Figure 4.9B). This could be indicative of a less competitive population from poly I:C treated donors as recipients of cells from saline-injected donors which have cells in the WKM by 8 dpt have a higher probability of survival ($p < 0.01$) compared to those without, but recipients of HSCs from poly I:C-injected donors which have cells in the WKM by 8 dpt do not have a significantly different chance of survival compared to those without. It is notable that without these early observations, the recipients could not be split into 2 groups and the survival of the groups would be much more similar. These data support the hypothesis that early post-transplant behaviour can be used to forecast survival.

Although early post-transplant data was a good predictor of the survival of recipients of cd41:GFP+ cells from saline-injected donors, it was not a good predictor of the survival of recipients of cd41:GFP+ cells from poly I:C-injected donors. In order to determine if there was a delay in the colonisation of the WKM in these latter recipients, the study was extended to 10 dpt. However, because of a number of early deaths in recipients with very populated WKMs, the correlation was almost negative. Perhaps a larger sample size would yield more information, but these experiments were not repeated as the preliminary study did not indicate a correlation ($p=0.4689$).

Cd41:GFP+ cells from poly I:C donors are exposed to interferon signalling prior to transplantation which may cause a lymphoid bias (Figure 4.5). This might explain why lyz:dsRed+ cell counts do not correlate with survival. Perhaps transplanting cd41:GFP+ from a transgenic donor in which lymphoid cells are also marked could produce data which fits better for poly I:C donors. Saline-injected donors produce myeloid and lymphoid cells equally and therefore either readout would correlate well with survival.

Altogether, the data from the recipients of cd41:GFP+ cells from saline-injected donors supports the findings by Rashidi *et al.* (122) and indicate that early post-transplant behaviour is linked to engraftment and transplant outcome. However, the behaviour of cd41:GFP+ cells from poly I:C-injected donors does not show a link between early post-transplant behaviour and survival or hematopoietic reconstitution.

4.5 Conclusion and future work

In conclusion, this chapter began to examine the effect of systemic inflammation in the donor on the migrating, engrafting and differentiation ability of cd41:GFP+ HSCs. Essentially, poly I:C treated HSCs are less predictable in the transplant recipient. I hypothesise that the population of cd41:GFP+ cells from the poly I:C injected fish have fewer HSPCs (based on flow cytometry evidence that relatively fewer cd41:GFP+ cells scatter in the lymphoid gate; Figure 4.5C) and therefore fewer HSPCs initially colonise the recipient WKM, thus their expansion needs to be greater (based on evidence that the increase in cd41:GFP+ cell numbers is greater in recipients of cd41:GFP+ cells from poly I:C injected donors; Figure 4.7C). The early post-transplant behaviour of HSCs from poly I:C-injected donors did not correlate with survival suggesting that there is a deficiency in colonisation of the WKM (either fewer cells or difficulty in migration), so there are more failed transplants in this group.

The data presented here demonstrates that cells from saline-injected donors behave in a more consistent manner. In recipients of these cells, the number of lyz:dsRed+ cells observed at 8 dpt correlates with survival and the percentage of lyz:dsRed+ cells at 28 dpt. On the other hand, the behaviour of cells in recipients of HSCs from poly I:C-injected donors did not correlate with survival or reconstitution at 28 dpt. Poly I:C stimulated HSCs did not behave consistently in the recipient and this in itself is an indication that behaviour of these cells was affected by the repeated donor stimulation.

Altogether this data supports the general consensus (76, 107) that inflammation has a detrimental effect on HSCs. Furthermore, it provides observational data that show a change in HSC behaviour due to prior exposure to poly I:C.

Like much of the work studying type II IFNs (334, 355, 403, 404), the fitness of the poly I:C stimulated HSCs have only been assessed in non-competitive transplant assay, measured by donor contribution to the WKM of the recipient. In these experiments, both recipient groups had equivalent percentages of donor derived cd41:GFP+ and lyz:dsRed+ in the WKM (Figure 4.11B). This suggests that they are equal in their ability to compete with endogenous HSCs. Ultimately, the best measure of stem cell fitness will be by competitive transplantation assay. The cells from both donors are transplanted into the same recipient and the population which successfully outcompetes the other in terms of populating the niche is the fittest. This cannot be done using our current transgenic donor because there are no markers available to distinguish donors (such as CD45.1 and CD45.2 used to identify donors in mice).

Recently, the Dallman lab started using Tg (runx1+23:GFP;lyz:dsRed) and Tg (runx1+23:NLSmCherry;lyz:GFP) in competitive repopulation assays. However, it was determined by flow cytometry that the number of fluorescent protein expressing cells in these 2 transgenic lines are very different, indicating the populations will be biologically different. Furthermore, the double positive cells in both lines overlap in the flow cytometry analysis. Therefore, I suggest the experimental setup shown in Figure 4.14B. Tg (runx1+23:GFP) donors would be injected with either poly I:C or saline. The runx:GFP+ cells from these donors will then be mixed with runx:mCherry+ from untreated Tg (runx1+23:NLSmCherry) donors and transplanted into irradiated TraNac recipients groups (recipients of HSCs from saline-injected donors and recipients of HSCs from poly I:C injected donors). In this way, the fitness of the runx1:GFP+ population will be assessed against a consistent runx1:mCherry+ population (Figure 4.14A).

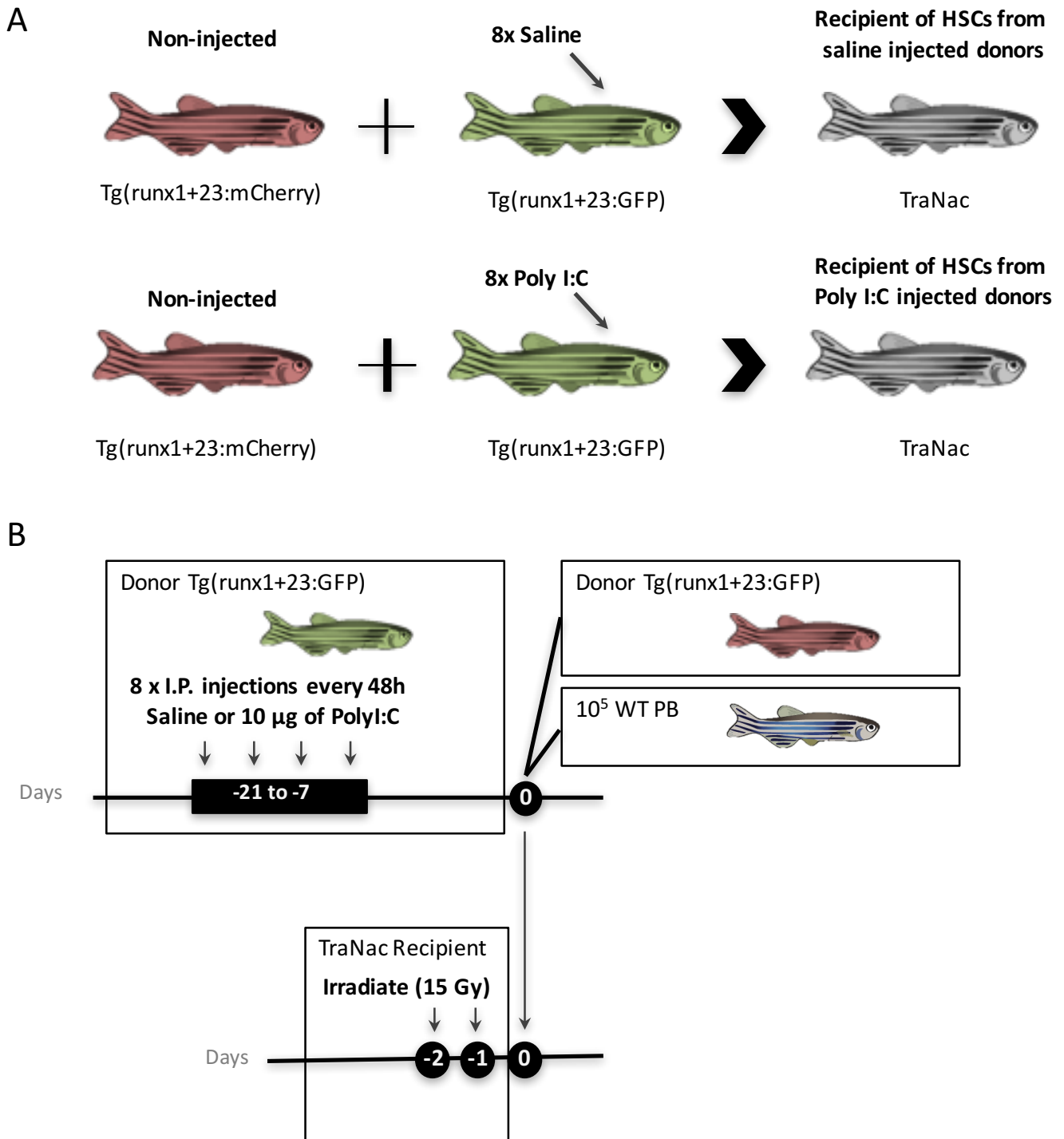


Figure 4.14: Schematic of suggested competitive transplant experiments. The Tg (runx1+23:GFP) donors are injected 8 times, at 48 h intervals, with either 5 µL saline or 10 µg Poly I:C. One week post injections, the WKM is harvested and sorted for runx1:GFP+ cells. These cells are mixed with runx1:mCherry+ cells from non-injected Tg (runx1+23:mCherry) donors and 10⁵ PB cells from non-injected WT. The mix is retro-orbitally delivered into irradiated TraNac recipients. The ratio of runx1:GFP+ cell to runx1:mCherry+ cells would determine which of the donors (poly I:C or saline-injected) were more competitive against non-injected donors. (A) Simple schematic diagram. (B) Procedure overview.

5 Post-transplant behaviour of HSCs in recipients with systemic inflammation

5.1 Introduction

The background to this chapter is carried on from previous chapters. In brief, there is mounting evidence that HSCs respond both directly and indirectly to infection and inflammation. This chapter mainly examines the response of HSPCs to indirect stimulation from the recipient niche.

The HSC niche is a complex milieu of different stromal cells that preserve the stem cell properties of HSCs while also regulating self-renewal, differentiation and trafficking to and from the niche. The mechanisms regulating HSC niche maintenance during homeostasis include both cell intrinsic and extrinsic factors. However, the factors that regulate the regenerative process that takes place after hematopoietic injury are less well understood. In fact, when this thesis began, the effect of inflammation in the recipient on transplanted HSCs had not yet been experimentally tested (409). However, just recently, Ishida *et al.* published the first study examining how pre-conditioning of the mouse recipient, and specifically the effect of $TNF\alpha$, could affect donor HSCs (410). They transplanted untreated HSCs (LSK CD34⁺) into irradiated recipients at various time points after irradiation in order to expose these cells to a range of inflammatory environments (there is a peak in inflammation at 3 dpIR). There was no difference in chimerism between primary transplant recipients transplanted at 0 or 3 dpIR. However, chimerism in secondary recipients was significantly lower in recipients of HSCs from donors initially transplanted 3 dpIR (when inflammation was at its peak; high cytokine and ROS milieu) compared with recipients of HSCs from donors initially transplanted 0 dpIR (when inflammation had not yet begun; low cytokine and ROS milieu). Thus, HSCs transplanted during the peak of inflammation in the primary transplant were less competitive than HSCs obtained from a time point with lower inflammation. Subsequently, Ishida *et al.* transplanted irradiated recipients at various time points (0, 1, 2 and 3 dpIR) with naïve HSCs and harvested cells 24 h after the transplant for secondary transplantation; for example, mice transplanted at 3 dpIR, were humanely killed at 4 dpIR and HSCs were harvested for a secondary transplant. Chimerism in secondary recipients was decreased, though not significantly, in recipients of HSCs from donors initially transplanted 3 dpIR and harvested 4 dpIR. The difference in chimerism between recipients of HSCs from a lower inflammatory milieu (transplanted 0 dpIR, harvested at 24 h) and recipients of HSCs from a higher inflammatory milieu (transplanted 3 dpIR, harvested at 4 dpIR) was most significant in tertiary transplant recipients. Together, this data shows that HSC reconstitution ability was significantly impaired after exposure to an inflammatory bone marrow environment, even for just 24 h. The inflammatory environment of the recipients in the study were created by changing the interval between irradiation and transplant so that the difference between recipients was in the levels of ROS and cytokines (*il-1 β* , *TNF α* and *IFN γ*). Although irradiation caused higher expression of multiple cytokines in the recipient, using single cell colony assays with the various cytokines induced, Ishida *et al.* showed that $TNF\alpha$ had the most significant effect on colony formation. They then showed that HSCs reconstituted better in an irradiated $TNF\alpha$ -KO recipient BM than in an irradiated WT recipient BM. Furthermore, they showed that incubating HSCs

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in N-Acetyl-L-Cysteine to inhibit TNF-mediated ROS accumulation prior to transplant allowed these cells to better reconstitute irradiated recipients than non-N-Acetyl-L-Cysteine treated HSCs.

5.1.1 The inflamed recipient environment

In this chapter, poly I:C stimulation was used to create an inflammatory environment in the recipients prior to irradiation and transplantation. As described in chapter 4.1.3, poly I:C is a ligand of TLR3 (and TLR22 in zebrafish) and induces pro-inflammatory cytokines, especially interferons. Acute interferon exposure causes proliferation (76, 333, 355) and chronic interferon stimulation leads to loss of *stemness* and exhaustion (334, 393). These studies have examined the effect of interferon stimulation (through poly I:C injection or infection models) on the endogenous population of HSPCs, through both direct and indirect stimulation. The loss of *stemness* was assessed using competitive and non-competitive transplantation of stimulated HSPCs.

Only a few experiments have examined the effect of inflammation in the recipient, with a view to understanding the role of interferon in the niche. Essers *et al.* showed that transplanted WT HSCs (LSK CD150⁺) were activated by poly I:C in IFN- α receptor knock out (IFNRA^{-/-}) recipients (76). This showed that HSCs could respond to interferons directly but does not exclude possible indirect stimulation from the niche. In fact, recently the Essers group examined the effect of poly I:C on the BM niche (411). They found that treatment of WT mice with poly I:C or IFN α increased BM vascularity (via VEGF) but compromised vessel integrity. The stimulation of endothelial cells (Lin⁻ CD45⁻ CD31⁺) caused an increase in the expression of junction molecules *endothelial cell-selective adhesion molecule*, *vascular endothelial-cadherin* and *laminin*. Interestingly, the stimulation was transient as continuous treatment (at 48 h intervals, 8 times) did not cause continuous inflammatory effects. They showed that the stimulation of endothelial cells occurs via hematopoietic and non-hematopoietic pathways as junction molecules increased in IFNAR^{-/-} recipients transplanted with WT HSCs (thus hematopoietic stimulation) and in WT recipients transplanted with IFNAR^{-/-} HSCs (thus non-hematopoietic stimulation). While these results indicate that poly I:C does in fact alter the BM environment, there is no indication that this had an effect on the reconstitution capacity of HSCs.

More substantially, Sato *et al.* found that transplantation of WT HSCs (LSK CD150⁺) into IRF2^{-/-} recipients (which have constitutive interferon signalling) did not affect reconstitution (393). This indicates that high levels of interferon signalling in the recipient is not important for reconstitution of the transplant recipient. Unlike Ishida *et al.*, these results indicate that environmental interferons do not significantly alter HSC survival(410).

Inflammatory stimulation of the recipient has also been used to establish a pre-conditioning regimen for transplantation. Sato *et al.* and Essers *et al.* used poly I:C and 5-FU as a pre-transplant conditioning of recipient mice (76, 412) as poly I:C induces proliferation (406) and 5-FU kills proliferative cells. Essers *et al.* injected mice with poly I:C twice (10 μ g g⁻¹, 48 h apart) and then twice with 5-FU (1 week apart). They found that without poly I:C exposure (injected PBS), 5-FU treatment alone did not affect survival. However, 2 injections of poly I:C prior to 5-FU severely affected mouse survival (none of the mice survived past 12 days post the 1st 5-FU treatment/5

Post-transplant behaviour of HSCs in recipients with systemic inflammation days post the 2nd 5-FU treatment). There were no fatalities when the same treatment was applied to IFNRA^{-/-} mice. These results indicate that IFN- α stimulation of the bone marrow made the hematopoietic cells sensitive to ablation. This supports findings by Ishida *et al.* who demonstrated that environmental inflammation exposure reduced HSC competitiveness (410).

Similarly, Sato *et al.* injected recipient mice with poly I:C twice (10 μ g g⁻¹, 48 h apart), then injected with 5-FU (48 h later) before transplantation with HSCs. They found that type I IFN-based pre-transplant conditioning permits the engraftment of multi-lineage, long-term repopulating donor HSCs without total body irradiation and reduced DNA damage (gamma-histone 2AX was not induced). It is Sato's protocol that was adapted for the studies in this chapter. 5-FU treatment was replaced with irradiation as the previous work (see chapters 3.3 and 4.3) had been done with irradiated recipients.

5.1.2 The control recipient environment

For this study, inflamed recipients were compared with "control" recipients, treated like those in the previous chapters (simply irradiated 48 and 24 h prior to transplant). Interestingly, this is also the inflammatory stimulus used by Ishida *et al.* in the experiments described above (410). As mentioned in chapter 3.1.3, ionising radiation (IR) causes significant damage to the environment that can affect the dynamics of bone marrow cell production, maturation, trafficking and lifespan (413). The effects of irradiation are highly dependent on dose and biological characteristics (microenvironment and proliferative state) of the cell (414). Although stromal cells such as MSCs are relatively resistant to exposure, mature mesenchymal cells undergo rapid cell death (415-417). Global gene expression studies with B10 cells (immortalised human MSCs) show time and dose dependent changes in > 5000 transcript levels ($p < 0.5$), highlighting the complexity of the response to irradiation stress (418).

Ishida *et al.* demonstrated time-dependent changes in conditions following radiation (410). There are mounting cytokine levels from the time of exposure to a peak about 3 dpIR which gradually reduces by 5 dpIR. Incomplete or delayed engraftment results in longer periods of immune-compromise. During this time, recipients are at a high risk of fatal infection or hematopoietic failure which is why transplants at 5 dpIR were ultimately lethal. However, Lo Celso *et al.* found that radiation damage of the microenvironment played a role in stimulating proliferation of HSPCs (121). When non-irradiated recipients are transplanted, cells typically could not engage with the endosteal region, which typically supports HSC quiescence, and cell engraftment was significantly reduced. However, when recipients were irradiated before being transplanted with WT HSPCs, both Lo Celso *et al.* and Xie *et al.* saw proliferating HSPCs located nearer to endosteal regions and donor cells could engraft (121, 211). Thus, despite the toxic effects of immune-ablation, removing or lack of BM resident hematopoietic cells is important for survival and engraftment of donor cells.

Irradiation is known to create an inflammatory environment where injured and dying cells produce DAMPs. Inflammatory molecules are also generated when macrophages respond to TLR ligands produced by the intestinal flora, released when the gastrointestinal lumen is damaged by irradiation (419). Phagocytic cells

Post-transplant behaviour of HSCs in recipients with systemic inflammation remain in irradiated tissue for months after exposure, possibly due to retention signals. High doses of radiation also cause the production of ROS which oxidate proteins and lipids and exacerbate DNA damage through the activation of cell cycle inhibitors (420). The loss of HSC senescence, and thus of *stemness*, alters HSC repopulating ability (417, 421).

5.1.3 A recipient environment that is not inflamed

The previously mentioned *bloodless* *c-myb*^{t25127} mutant (see chapter 3.3.3) is used as a recipient without induced inflammation in this chapter. Due to a transversion mutation, these mutants lack a functional *c-myb* transcription factor, thus affecting expression of downstream gene targets required for definitive haematopoiesis (46).

5.1.4 Inflammation in the recipient affects early post-transplant behaviour of HSPCs

Currently, not much is known about the behaviour of HSPCs in inflamed environments. Previous studies have used imaging techniques to understand the effect of infection on endogenous HSC behaviour with a view of understanding the role of the niche. For instance, Kunisaki *et al.* used *ex vivo* whole mount confocal immunofluorescence to examine HSC associations with small arterioles in the sternal bone marrow of mice (406). They found that injecting mice with poly I:C (5 µg g⁻¹, twice 48 h apart) increased HSC (LSK CD150⁺ CD48⁻) cell cycling and reduced the percentage of HSCs within 20 µm of arterioles, as compared with saline injected mice. As they used endogenous models and not transplantation, it is not possible to distinguish the direct effect of poly I:C on subset populations of the hematopoietic compartment (LT-HSC compared to ST-HSC etc.). It was also not possible to separate the direct effect on the hematopoietic cells from the indirect effect of poly I:C on the niche cells (MSC, vascular etc.). However, this data supports the notion that HSC behaviour is influenced by inflammatory signalling.

There is currently no *in vivo* imaging data on the post-transplant behaviour of HSPCs in inflamed conditions. The only studies available have examined the movement of the transplanted hematopoietic population by flow cytometry assessment of the organ of interest. For instance, Kašeta *et al.* have shown that transplanted progenitors (Lin⁻ cells) preferably relocate to the ischemic foot in a mouse model of contact hypersensitivity (422). Similarly, in a model of ischemic kidney, Stroo *et al.* used flow cytometry to show an increase of progenitor cells (DiI labelled LSK cells) in the damaged kidney 24 h post-transplant (423). Stroo *et al.* did image sections of the kidney *ex vivo*, but they did not focus on the associations of these cells with the environment. Although these studies were done using flow cytometry and not *in vivo* imaging, they do suggest that inflammatory signalling can influence post-transplant HSPC localisation.

Finally, a study by Bastianutto *et al.* used bioluminescent imaging to track transplanted HSPCs (Sca1⁺ c-Kit⁺ Thy1.1^{low}) following right leg irradiation (388). They found that HSPCs would preferentially relocate to the irradiated tissue following transplantation (388). They found that this recruitment was CXCL12, MMP2 and MMP9 dependent.

5.2 Aims

The aim of this chapter is to assess the effect of inflammation in the niche on HSCs. As described in chapter 4, a decrease in HSC *stemness* is most obvious under regenerative stress, such as transplantation. In this chapter, WT HSPCs (cd41:GFP+) were transplanted into either “control recipients” (irradiated with 15 Gy 48 and 24 h prior to transplant; albeit these recipients will have some irradiation induced inflammation) or recipients with additional inflammation (injected twice with 10 µg poly I:C, 6 and 4 days prior to transplant, and treated with 15 Gy irradiation 48 and 24 h prior to transplant). The number of transplanted cells was restricted to 200 cd41:GFP+ cells to cause additional regenerative stress on the cells. As in the previous chapter, cell behaviour (mobility, self-renewal and differentiation) was observed at early post-transplantation time points (4, 6 and 8 dpt) throughout the organism. Survival of each recipient was monitored for 28 days and then flow cytometry was used to compare engraftment and reconstitution. These data were compared with data from *bloodless* (c-myb^{t25127} mutant) recipients. These fish are not irradiated and do not have a hematopoietic system, therefore it is hypothesised that these recipients would have considerably lower inflammation (although will need to be determined). As they are not optically transparent, they cannot be used to obtain early imaging data. However, they could be used for non-imaging techniques such as survival and reconstitution (as measured using flow cytometry).

More specifically, the aims were to:

1. Compare the inflammatory environments created in “control” (irradiated) and “inflamed” (poly I:C and irradiated) recipients.
2. Observe the behaviour of naïve HSPCs (cd41:GFP+) in the transplant recipient to assess how an inflamed environment affects the early behaviour of these cells.
3. Determine if hematopoietic reconstitution and recipient survival are affected by the inflamed environment by comparing “control” (irradiated), “inflamed” (poly I:C injected and irradiated) and “non-inflamed” (c-myb mutant; no poly I:C and no radiation) recipients.

5.3 Results

5.3.1 Two intra-peritoneal injections of poly I:C cause an increase in inflammatory cytokines in the recipient

In order to study the effect of an inflamed environment on HSCs, it was first necessary to establish a protocol that would induce systemic inflammation in the zebrafish recipient. As in the previous chapter, poly I:C was used as a stimulus. The protocol used for inducing inflammation in donors (Figure 4.3) could not be used in this case because the length of the treatment (23 days) would mean that the recipients would be too large for acquisition of high quality images. Instead, a protocol from Sato *et al.* was adapted (412); Recipients were injected twice, 48 h apart, with 10 µg of poly I:C and subsequently irradiated with 15 Gy 48 h and 72 h later (equivalent to 48 h and 24 h prior to transplant). Since it is necessary to irradiate transplant recipients to make space for transplanted cells (121) control recipients were also irradiated 48 h and 24 h prior to transplant (Figure 5.1). In order to limit inflammation in controls, these recipients were not injected prior to irradiation as the intra-peritoneal injection

Post-transplant behaviour of HSCs in recipients with systemic inflammation

would cause significant trauma. Thus, differences between controls and Poly I:C treated recipients may not be poly I:C specific and could be due to inflammation caused by the procedure.

To ensure the protocol was effectively causing inflammation, 3 recipients were selected at random from each experimental repeat at 3 h post first injection “poly I:C 10 µg” and at 3 h post second injection, “poly I:C 2x 10 µg”. Three random samples were also taken at the time of transplant from each experimental repeat, from inflamed “poly I:C + IR” recipients (after 2 poly I:C injections and 2 doses of irradiation) and from control “IR” recipients (after 2 doses of irradiation; Figure 5.1). Non-irradiated, non-injected “No IR, No poly I:C” siblings were taken as untreated negative controls for measuring mRNA cytokine levels. The results from irradiated “IR” and non-irradiated “non-IR” groups were presented previously in chapter 3 and are shown again here for ease of comparison. Although previously *il-1β*, *tnfα*, *il-6*, *ifnγ1.1*, and *cxcl-c1c* mRNA transcripts were significantly increased in control “IR” recipients relative to untreated samples (Figure 3.5), they are not significantly different here due to the nature of the statistical test (One-way ANOVA). As I mentioned in the discussion of the previous chapter (4.4.2), this statistical test takes into consideration variation among the groups as well as variation within each group to determine statistical significance. This means that one-way ANOVA does not detect statistically significant differences between “IR”, “No IR, No poly I:C” and “poly I:C + IR” because of the significant variation in the “poly I:C 10 µg” and “poly I:C 2x 10 µg” groups. Therefore, in this chapter I present the one-way ANOVA analysis of all the groups, but one-way ANOVA analysis of just the “IR”, “No IR, No poly I:C” and “poly I:C + IR” groups can be found in the appendix (Supplementary Figure 7.7), and will be discussed at the end of the chapter.

In general, cytokine (*il-1β*, *tnfα*, *il-6*, *ifnφ*, *ifnγ1.1*, *ifnγ1.2* and *cxcl-c1c*) transcript levels were significantly higher at 3 h post poly I:C injection and 3h post 2nd poly I:C injection than at the time of transplant in either inflammatory “poly I:C + IR” recipients or control “IR” recipients. For instance, mRNA levels of *il-1β* were ≈25 fold higher than untreated (No IR, no Poly I:C) samples 3h after the 1st poly I:C injection and ≈30 fold higher 3h after the 2nd injection (Figure 5.2). However, in both control and poly I:C irradiated recipients, *il-1β* levels were not significantly higher than untreated controls (≈3.5 and ≈2 fold respectively) at the time of transplant.

Systemic levels of *tnfα*, *il-6* and *cxcl-c1c* transcripts followed the same profile as *il-1β*. Relative *Tnfα* transcript levels were ≈10 and ≈20 fold higher at 3 h post poly I:C injection but were not significantly elevated in irradiated control recipients (≈4 fold increase) or in irradiated Poly I:C treated recipients (≈2 fold increase) at the time of transplant, relative to untreated controls (Figure 5.2). Likewise, *il-6* increased ≈10 and ≈15 fold 3h after poly I:C injections but were at basal levels in both recipient groups following irradiation (≈2 fold in controls and ≈1.7 fold in Poly I:C treated recipients; Figure 5.2). Again, *il-6* levels in control and Poly I:C treated recipients are not significantly different to untreated samples. Also, *cxcl-c1c* increased by ≈40 fold 3 h after poly I:C injection but irradiated recipients did not have significant levels at the time of transplant (≈5.5 fold in control “IR” and ≈3.5 fold in Poly I:C + IR recipients; Figure 5.2).

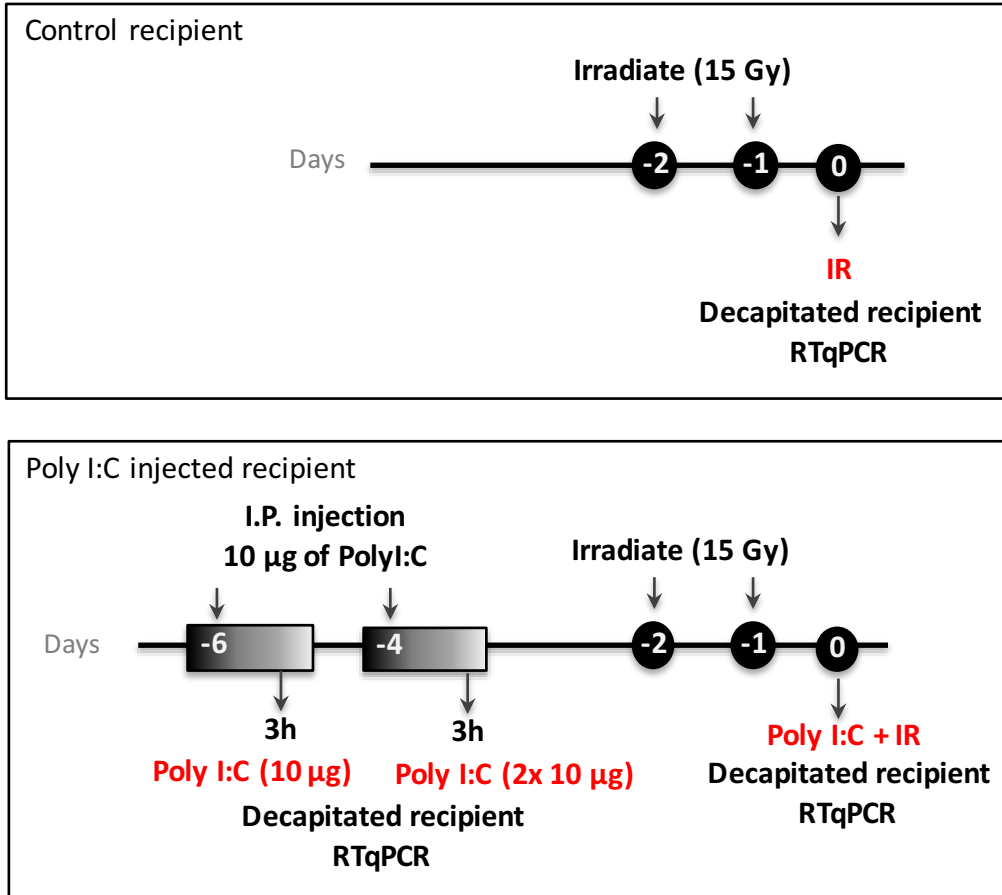


Figure 5.1: Schematic overview of sampling points for measuring gene expression in poly I:C stimulated recipients. Control recipients were irradiated twice with 15 Gy (48 h and 24 h before would-be transplant). Samples were collected 24 h after 2 doses of irradiation (IR). Poly I:C treated recipient TraNac fish were injected with 10 µg poly I:C twice, 48 h apart (samples were collected 3 h after the 1st injection of 10 µg poly I:C (Poly I:C 10 µg) and 3 h after 2 injections of 10 µg poly I:C (Poly I:C 2x 10 µg). Injected fish were then irradiated with 15 Gy 48 h and 72 h later (48 h and 24 h before would-be transplant). Samples were collected 24 h later (Poly I:C + IR). mRNA was extracted after collected samples were humanely killed and decapitated.

As described in chapter 4.1.3, transcript levels of type I and II IFNs are significantly higher after poly I:C injection. Thus, unsurprisingly, transcript levels of *ifnφ* rose ≈30 fold 3 h after each poly I:C injection (Figure 5.2) but were not significantly increased in irradiated recipients (control and poly I:C injected) at the time of transplant. Type II IFNs *infy1.1* and *ifnγ1.2* were also increased after poly I:C stimulation, although the profiles of the two transcripts were slightly different. *Infy1.1* increased ≈10 fold 3 h after each poly I:C injection and levels were ≈5.5 fold in irradiated recipients and ≈4 fold in irradiated Poly I:C treated recipients at the time of transplant (Figure 5.2). On the other hand, transcript levels of orthologue *ifnγ1.2* almost doubled between the 1st and 2nd poly I:C injection (from ≈17 to ≈30 fold) but had returned to basal levels in recipients at the time of transplant.

The transcript levels of HSC chemokine *cxcl12a* were not affected by the poly I:C injections (Figure 5.2). However, while transcript levels of *cxcl12a* doubled in irradiated control “IR” recipients relative to untreated “No IR, No Poly I:C” fish ($p < 0.01$; Figure 3.5), levels of *cxcl12a* were not significantly increased in inflamed “poly I:C + IR” recipients relative to untreated “No IR, No Poly I:C” fish.

Cumulatively, these results show that there are high levels of inflammatory cytokine transcripts in recipients 3 h after each poly I:C injection. They also indicate that the inflammatory profile in both recipient groups (control “IR” and inflamed “Poly I:C + IR”) are not significantly different to each other or “No IR, No poly I:C” samples at the time of transplant. From these measurements, there is a possibility that an inflamed recipient was not created and this is addressed in the discussion. The lower than expected levels of cytokine transcripts in Poly I:C treated recipients (poly I:C +IR) might be explained by the greater loss of cytokine producing cells from ionising irradiation, due to the proliferative effect of poly I:C, compared to control recipients.

In chapter 3.3.1.1, the effect of irradiation on the death of cells of the WKM was assessed using flow cytometry and the qRT-PCR data had supported the flow cytometry data. Due to their small size, the recipients in these experiments were too difficult to dissect therefore qRT-PCR was used to assess the prevalence of blood cells from the mRNA extracted from the whole body.

In general, the results show that blood-related transcripts increased after 2 injections of poly I:C and decrease following irradiation (Figure 5.3). However, radiation did not have a different effect on poly I:C injected fish (“Poly I:C +IR”) and non-injected fish (“IR”). It is not surprising that a single poly I:C injection would not cause major changes to blood transcript production within 3 h. However, there is evidence that a second injection of poly I:C, and 51 h after the 1st injection of poly I:C, there has been a major shift in hematopoiesis. Transcript levels of the erythroid-associated gene *gata1* were not affected within 3 h of poly I:C injection but had increased significantly (≈ 1.7 fold) at the second time point (51 h after the 1st injection, 3 h after the 2nd injection; Figure 5.3). Transcript levels of *gata1* in both control (IR) and Poly I:C treated recipients (Poly I:C + IR) were significantly lower than poly I:C injected fish ($p < 0.001$), and “No IR, No poly IC” fish ($p < 0.05$). However, there was no difference in *gata1* transcript levels between control and Poly I:C treated recipients at the time of transplant.

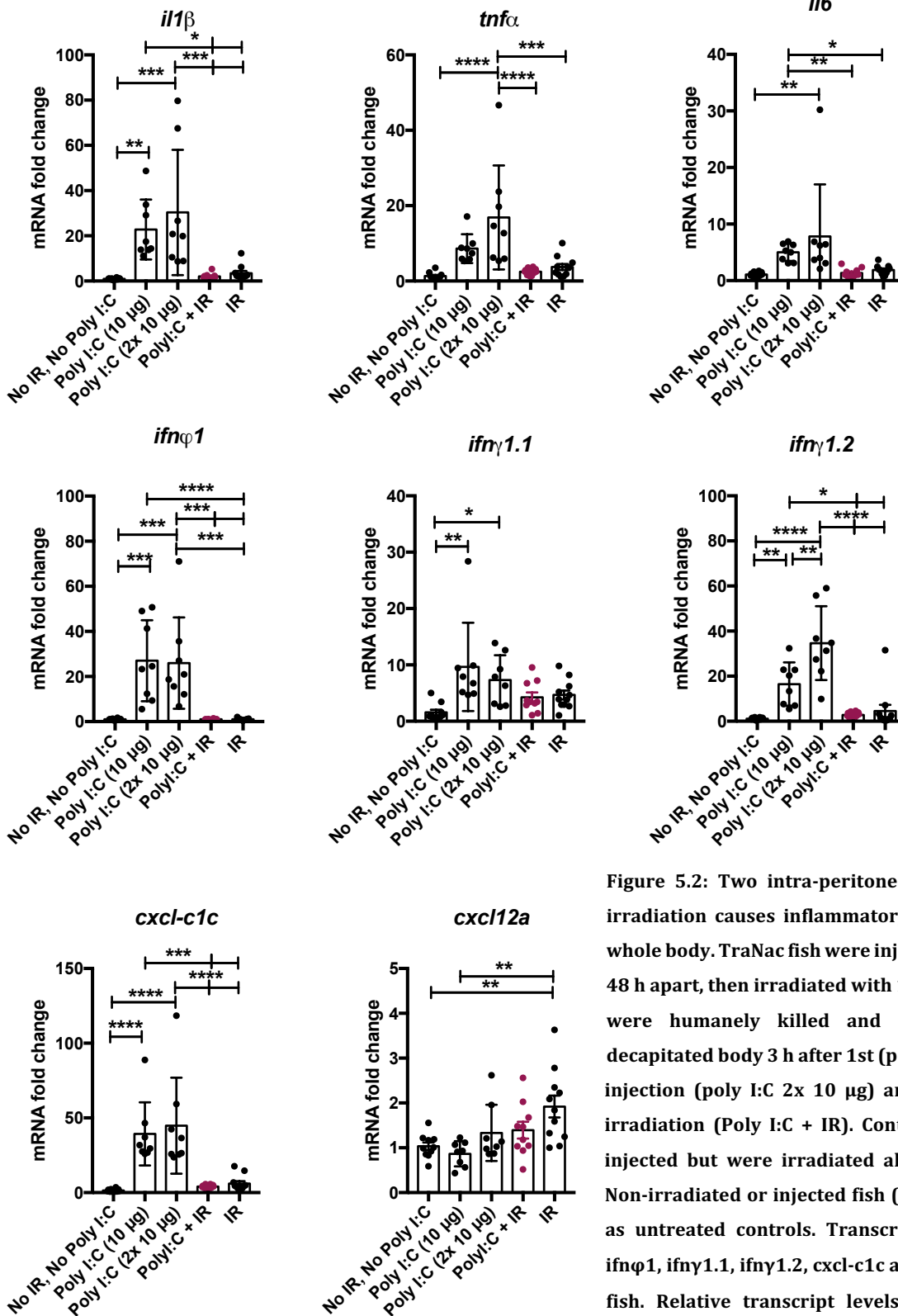


Figure 5.2: Two intra-peritoneal injections of poly I:C and irradiation causes inflammatory cytokine expression in the whole body. TraNac fish were injected with 10 µg poly I:C twice, 48 h apart, then irradiated with 15 Gy 48 h and 72 h later. Fish were humanely killed and mRNA extracted from the decapitated body 3 h after 1st (poly I:C 10 µg) and 3h after 2nd injection (poly I:C 2x 10 µg) and 24 h after the 2 doses of irradiation (Poly I:C + IR). Control recipients (IR) were not injected but were irradiated alongside inflamed recipients. Non-irradiated or injected fish (No IR, No poly I:C) were used as untreated controls. Transcript levels of *il1β*, *tnfa*, *il6*, *ifnφ1*, *ifnγ1.1*, *ifnγ1.2*, *cxcl-c1c* and *cxcl12a* in the decapitated fish. Relative transcript levels calculated using the $\Delta\Delta CT$ method, normalising to 18S expression. Fold change relative to the median “untreated” value. The experiment was repeated 3 times and results pooled, $n > 8$. “No IR No Poly I:C” and “IR” groups previously presented in chapter 3.3.1.3. Statistical significance using One-way ANOVA and Tukey post-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Like *gata1*, transcript levels of lymphoid-associated gene *lck* also increased after poly I:C treatment and decreased significantly following irradiation. Levels of *lck* transcript were doubled 3 h after the first poly I:C injection. The levels of *lck* almost tripled ($p < 0.05$) after the 2nd injection (51 h after the 1st injection, 3 h after the

Post-transplant behaviour of HSCs in recipients with systemic inflammation 2nd injection; Figure 5.3). Subsequent irradiation caused a significant decrease in *lck* transcript levels in both control and poly I:C injected fish relative to poly I:C injected fish ($p<0.05$) but not significantly different relative to untreated “No IR, No poly I:C” fish ($\approx 50\%$ and $\approx 25\%$ respectively).

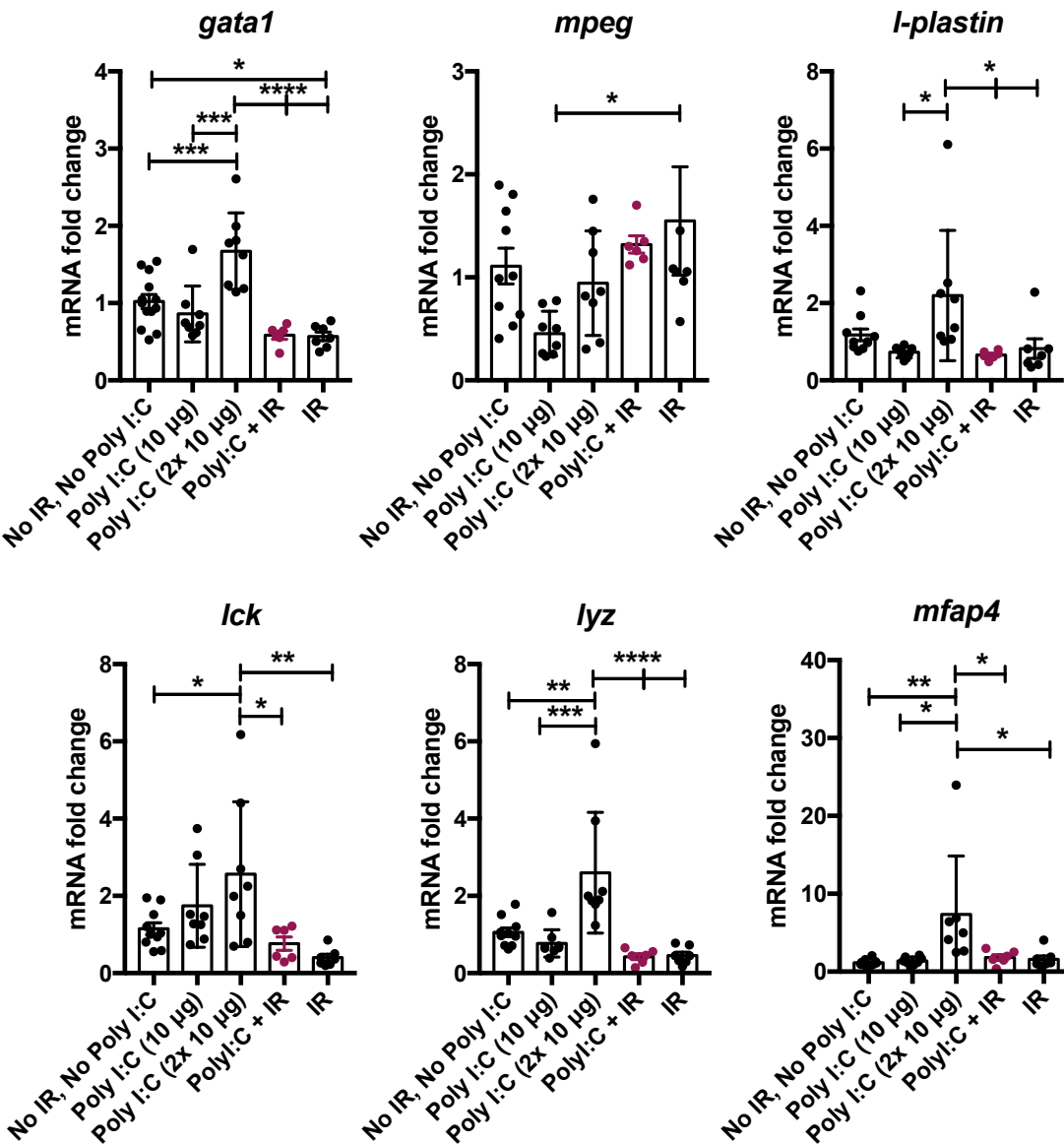


Figure 5.3: Two intra-peritoneal injections of poly I:C increase blood production and irradiation decreases blood populations in the whole body. TraNac fish were injected with 10 µg poly I:C twice, 48 h apart, then irradiated with 15 Gy 48 h and 72 h later. Fish were humanely killed and mRNA extracted from the decapitated body 3 h after 1st (poly I:C 10 µg) and 2nd injection (poly I:C 2x 10 µg) and 24 h after the 2 doses of irradiation (Poly I:C + IR). Control recipients (IR) were not injected but were irradiated alongside inflamed recipients. Non-irradiated or injected fish (No IR, No poly I:C) were used as untreated controls. RTqPCR was used to measure relative expression of *gata1*, *mpeg*, *l-plastin*, *lck*, *lyz* and *mfap4*, normalised to 18S, using the $\Delta\Delta C_T$ method. Repeated three times and experiments pooled, $n>6$. “No IR, No Poly I:C” and “IR” data presented in in chapter 3.3.1.3. Statistical significance using One-way ANOVA and Tukey post-test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Similarly, transcripts of myeloid associated genes *lyz* and *mfap4* (macrophage transcript) also increased after 2 poly I:C injections, relative to untreated fish ($p<0.01$; Figure 5.3). On the other hand, the transcript levels of immune cell-associated gene *l-plastin*, and myeloid-specific gene *mpeg* did not change significantly, relative to

Post-transplant behaviour of HSCs in recipients with systemic inflammation

untreated fish. It is interesting to note that the myeloid-associated transcript *mpeg* did not change, but macrophage transcript *mfap4* increased 8 fold after 2 injections of poly I:C. This might indicate that poly I:C could stimulate the differentiation of the *mfap4*⁺ subset of myeloid cells, or perhaps poly I:C might somehow intervene in the degradation of *mfap4* transcripts. Again, due to the nature of the one-way ANOVA statistical test, with the exception of *gata1*, there were no significant differences in transcript levels of cell-associated genes between untreated “No IR, No poly I:C” and irradiated groups (“IR” or “poly I:C + IR”).

5.3.2 A transplant of 200 cd41:GFP⁺ cells from Tg (cd41:GFP;lyz:dsRed) rescues both control recipients and poly I:C injected recipients

The next step was to determine whether transplanted cd41:GFP⁺ HSPCs behaved differently early post-transplant in a moderately inflamed environment, using the same imaging and counting method used in the previous results chapters.

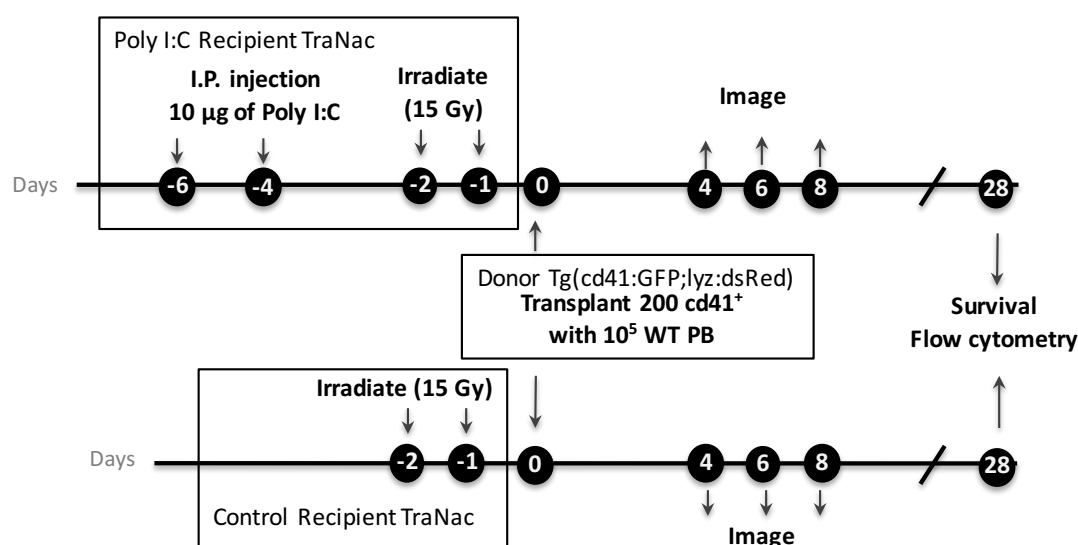


Figure 5.4: Schematic overview of experiment timeline. Poly I:C TraNac recipients were injected twice with 10 µg poly I:C, 48 h apart, and irradiated twice 48 h and 72 h later (48 h and 24 h before transplant). Control recipients were not injected but were irradiated twice, 48 h and 24 h before transplant. Donor Tg (cd41:GFP; lyz:dsRed) fish were harvested for WKM cells which were sorted for cd41:GFP⁺ cells. Control and Poly I:C treated recipients received a transplant of 200 cd41:GFP⁺ cells and 10⁵ WT PB cells from the same donor pool. Non-transplanted recipients did not receive 10⁵ WT PB cells. All recipients were imaged 4, 6 and 8 dpt and survival was monitored up to 28 dpt.

Inflamed recipients were prepared in the same way as before (Figure 5.4); recipients were injected twice with 10 µg of poly I:C (48 h apart) and irradiated 48 h and 24 h before transplant. “Control” recipients were not injected but were irradiated at the same time as poly I:C injected recipients, 48 h and 24 h before transplant. Naïve double transgenic Tg (cd41:GFP;lyz:dsRed) donors were humanely killed and cd41:GFP⁺ cells were selected from the WKM. Each recipient received a transplant of 200 cd41:GFP⁺ cells with 10⁵ wild-type PB cells delivered retro-orbitally. Since both groups of recipients received the same WKM and PB mix, transplants were delivered alternatively between the two recipient groups every 3 fish. This ensured that the time delay between

Post-transplant behaviour of HSCs in recipients with systemic inflammation
 experimental set-up and transplantation was evenly distributed across both groups. Recipients were imaged at 4, 6 and 8 dpt and the number of cd41:GFP+ and lyz:dsRed+ cells were counted in the entire recipient and in the WKM. Survival was monitored for individual fish up to 28 dpt, at which point the recipients were humanely killed for analysis by flow cytometry.

5.3.3 Early post-transplant behaviour of cd41:GFP+ cells in “control” and poly I:C treated recipients

A key outcome of this thesis has been the development of a method to monitor fluorescent protein expressing donor cells in the transplant recipient. Observations and quantification of lyz:dsRed+ and cd41:GFP+ cells were made in the same recipient at 4, 6 and 8 dpt (Figure 5.5).

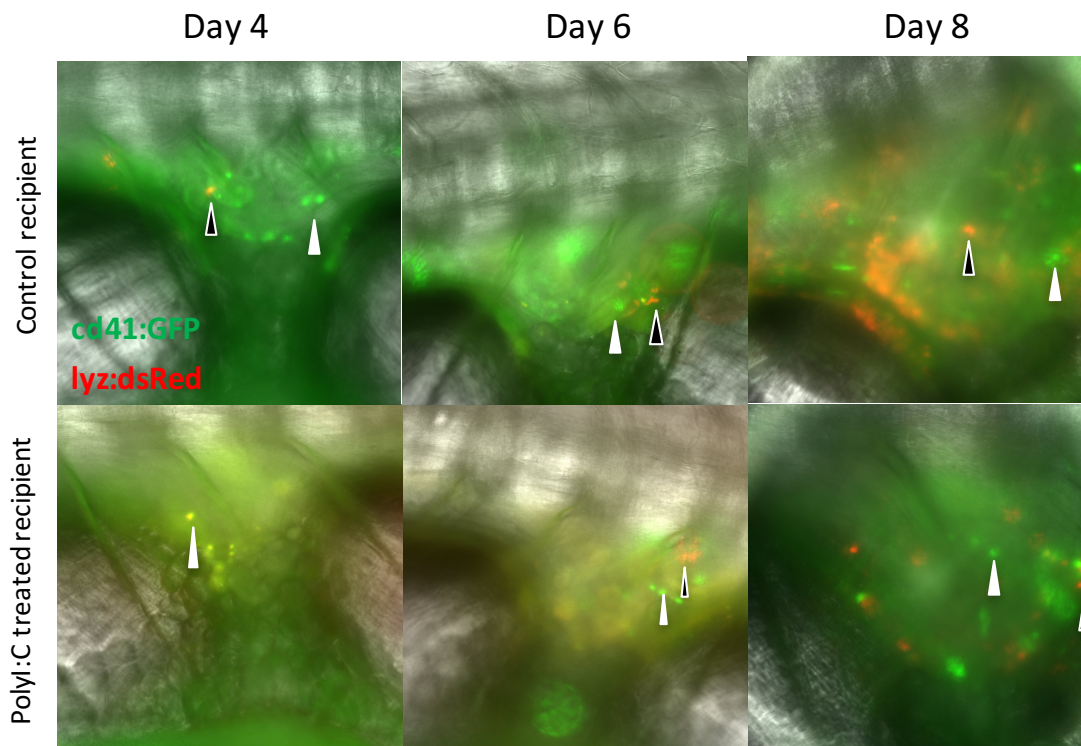


Figure 5.5: Visualising fluorescent cells in the mid WKM at 4, 6 or 8 dpt in control and poly I:C treated recipients. Poly I:C treated TraNac recipients were injected twice with 10 µg poly I:C, 48 h apart, and irradiated twice 48 h and 72 h later (48 h and 24 h before transplant). Control recipients were irradiated twice, 48 h and 24 h before transplant. WKM was harvested from humanely killed donor Tg (cd41:GFP; lyz:dsRed) and sorted for cd41:GFP+ cells. “Control” and poly I:C treated recipients received a transplant of 200 cd41:GFP+ cells and 10⁵ WT PB cells from the same donor pool (non-transplanted recipients did not receive 10⁵ WT PB cells). The same recipient was imaged 4, 6 and 8 dpt using a Zeiss Axiovert 200 inverted confocal microscope with 488 and 506 nm lasers. Representative images taken from a control (top) and a poly I:C (bottom) recipient over time (4, 6 and 8 dpt). White arrows indicate GFP+ cells, black arrows indicate dsRed+ cells.

The results show that the number of lyz:dsRed+ cells increased significantly over time ($p < 0.0001$) in both recipient groups, although the rate of increase was not consistent across fish, even in the same recipient group (Figure 5.6A). There was no difference between poly I:C and control groups and no significant interaction, a combination of recipient effect and time effect (Figure 5.6A). At each time point, the number of lyz:dsRed+ in the WKM of recipients was not significantly different between groups (Figure 5.6B).

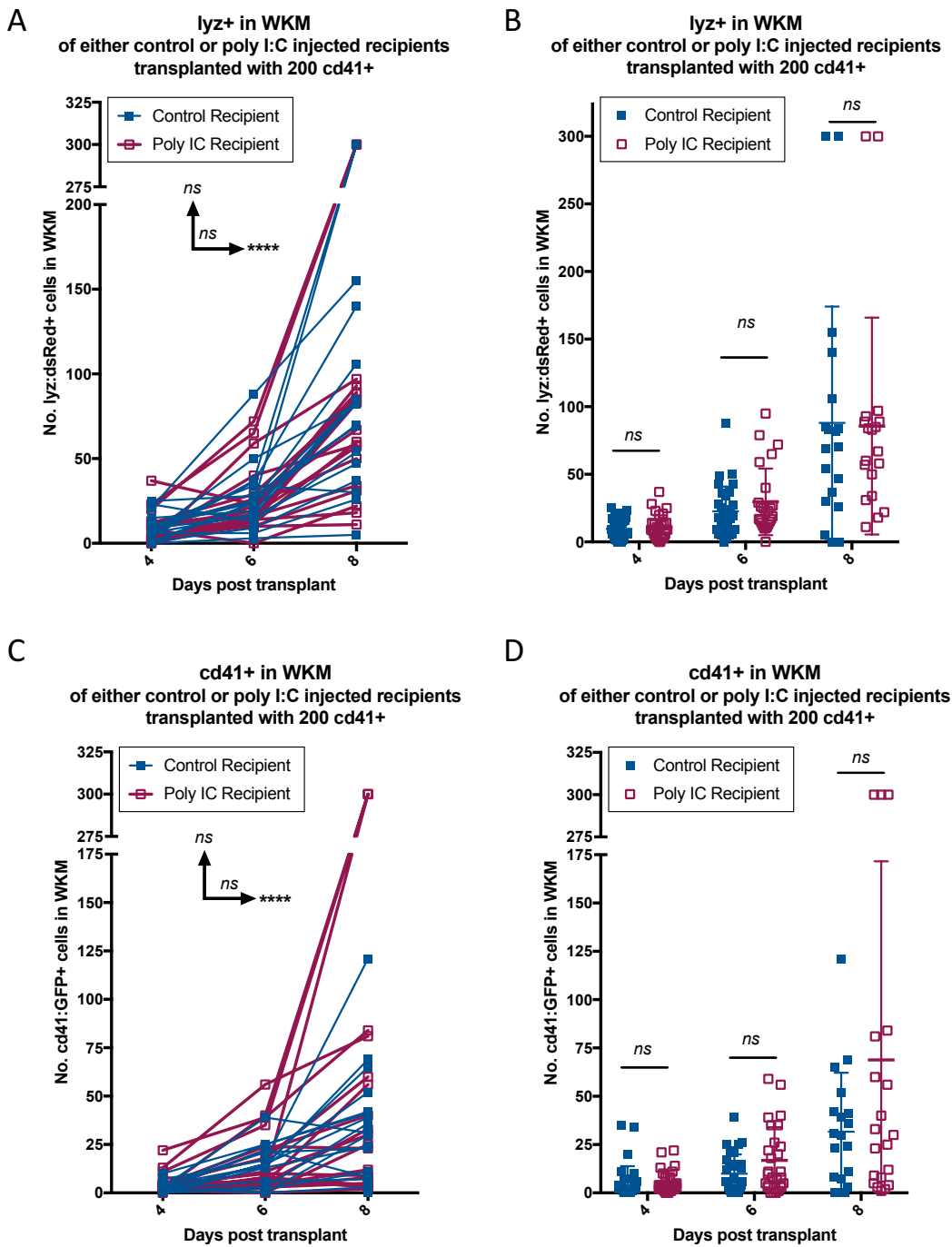


Figure 5.6: There is no difference in the accumulation of cd41:GFP+ and lyz:dsRed+ donor cells in poly I:C injected recipient WKM and non-injected recipient WKM. Poly I:C TraNac recipients were injected twice with 10 µg poly I:C, 48 h apart, and irradiated twice 48 h and 72 h later (48 h and 24 h before transplant). Control recipients were not injected but were irradiated twice, 48 h and 24 h before transplant. Donor Tg (cd41:GFP; lyz:dsRed) fish were humanely killed and WKM cells were harvested and sorted for cd41:GFP+ cells. “Control” and poly I:C treated recipients received a transplant of 200 cd41:GFP+ cells (and 10⁵ WT PB cells; non-transplanted recipients did not receive 10⁵ WT PB cells) from the same donor pool. All recipients were imaged using Zeiss axiovert 200 inverted microscope (wide field) and fluorescent cells were counted in the WKM at 4, 6, and 8 dpt. 4 experiments pooled. The number of (A) lyz:dsRed+ and (C) cd41:GFP+ cells in the WKM of individual recipients. The data from the same fish is connected by a line (Control recipients in blue, n= 17; Poly I:C treated recipients in plum, n= 19). Two way repeated measures ANOVA. Significance demonstrated with L-shaped arrows: significance over time indicated by the horizontal arrow, significance between treatment groups indicated by vertical arrow. Interaction indicated in the angle between the arrows. (B and D) The same data is represented as in A and C to show data spread. Each dot represents a fish. Student test at each time point.

Likewise, the number of cd41:GFP+ cells in the WKM significantly increased over time ($p < 0.0001$) but there was no significant difference in the number of cells between the two recipient groups at any of the time points groups (Figure 5.6C, D). Again there was no cumulative effect (interaction) between time and recipient treatment (Figure 5.6C). Together these results indicate that transplanted cells behave in a similar way in both recipient groups.

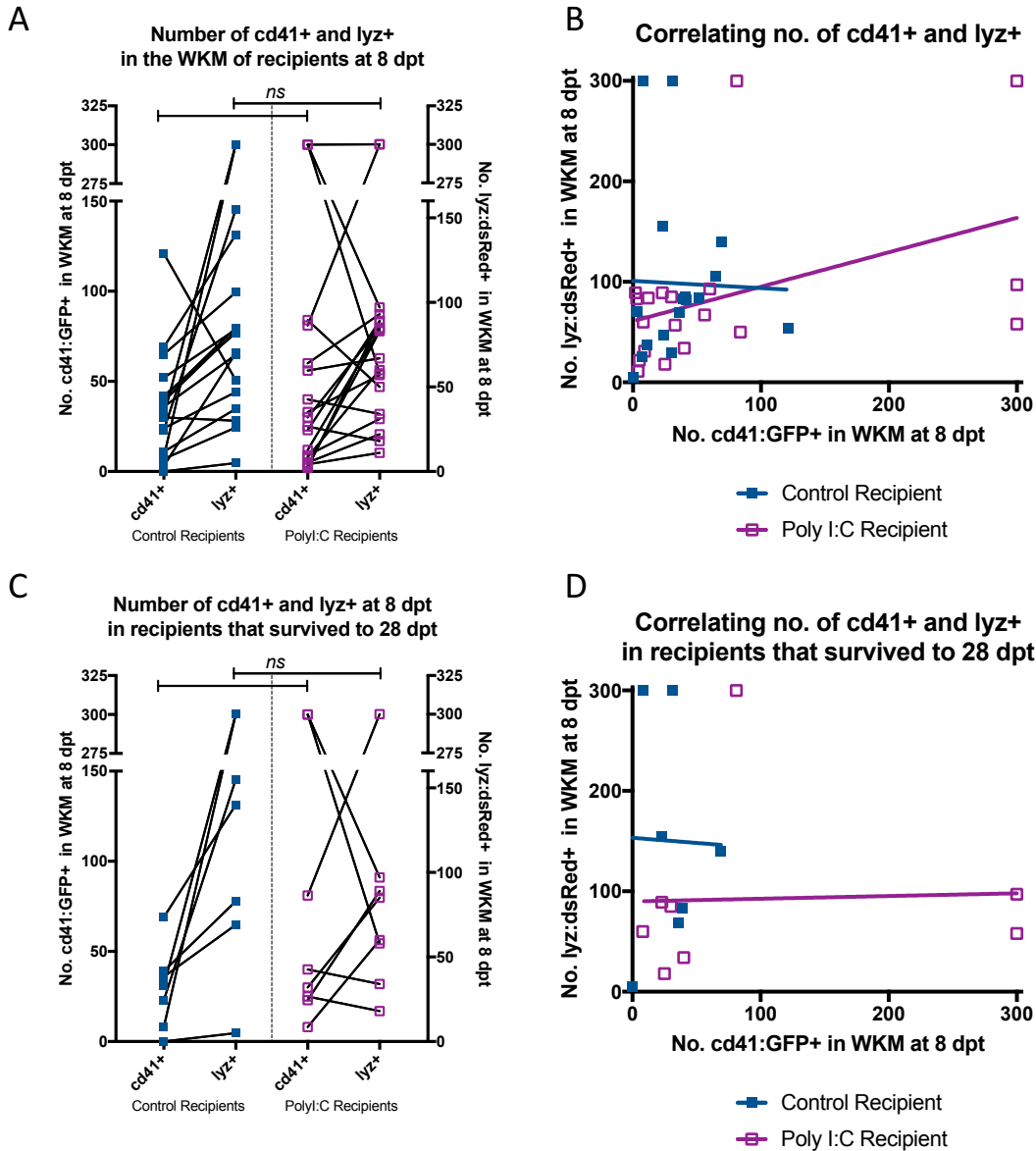


Figure 5.7: There is no correlation between the number of observed cd41:GFP+ cells and lyz:dsRed+ cells in the WKM at 8 dpt. Poly I:C TraNac recipients were injected twice with 10 µg poly I:C, 48 h apart, and irradiated twice 48 h and 72 h later (48 h and 24 h before transplant). “Control” recipients were not injected but were irradiated twice, 48 h and 24 h before transplant. Donor Tg (cd41:GFP; lyz:dsRed) fish were humanely killed and harvested for WKM cells which were sorted for cd41:GFP+ cells. “Control” and poly I:C treated recipients received a transplant of 200 cd41:GFP+ cells (and 10^5 WT PB cells; non-transplanted recipients did not receive 10^5 WT PB cells) from the same donor pool. Recipients were imaged 8 dpt using Zeiss axiovert 200 inverted microscope (wide field) and fluorescent cells were counted in the WKM. Experiment repeated 4 times and data pooled; the same recipient is connected by a line. The number of lyz:dsRed+ and cd41:GFP+ cells in (A) all recipients (“control” recipients in blue, $n = 17$; Poly I:C treated recipients in plum, $n = 19$) and (C) recipients that survived to 28 dpt (“control” recipients in blue, $n = 7$; Poly I:C treated recipients in plum, $n = 8$). (B and D) Same data as A and C presented as X and Y scatter with line of best fit (Pearsons correlation).

The same data were also analysed to determine if a relationship exists between the number of cd41:GFP+ and lyz:dsRed+ cells in the WKM at 8 dpt in either recipient group. In general, the number of lyz:dsRed+ exceeded the number of cd41:GFP+ in every recipient (Figure 5.7A). In fact, this trend was clear in fish that survived to 28 dpt. However, interestingly, 2 Poly I:C treated recipients with a higher number of cd41:GFP than lyz:dsRed+ also survived to 28 dpt. The dependence of these two variables was tested using Pearson's product-moment correlation. There was no correlation between the number of cd41:GFP+ and lyz:dsRed+ in either set of recipients at 8 dpt (control recipients, $r = -0.025$, $p = 0.922$; Poly I:C treated recipients, $r = 0.4458$, $p = 0.0558$; Figure 5.7B). Although the data from the poly I:C treated recipient group indicates a trend ($p = 0.0558$), the r value means that 55 % of the data does not fit the line and thus this model cannot be used to predict cd41:GFP+ counts from lyz:dsRed counts. The analysis was also applied to the recipients that survived to 28 dpt. These results showed even less correlation (control recipients, $r = -0.0203$, $p = 0.965$; Poly I:C treated recipients, $r = 0.0382$, $p = 0.929$) between the number of cd41:GFP+ and lyz:dsRed+ (Figure 5.7D).

5.3.4 Survival of poly I:C treated recipients is not affected

After imaging, individual recipients were tracked for 28 days to correlate survival with early post-transplant imaging data. There was a clear difference between transplant recipients (both "control" and poly I:C treated) and non-transplanted but irradiated controls and poly I:C injected and irradiated controls ($p < 0.01$). "Control" recipients had a 52 % chance of surviving to 28 dpt, while Poly I:C treated recipients had a slightly lower 42 % chance of surviving this long (Figure 5.8). This difference was not significant, indicating that the inflamed environment does not affect survival to 28 dpt. Interestingly, in chapter 4 many of the recipients did not have cells in the WKM by 8 dpt and this correlated with a significantly lower survival. However, in these experiments, only 2 transplanted controls did not have cells in the WKM and these fish died by 6 dpt. Likewise, just 4 poly I:C transplant recipients did not have cells in the WKM by 8 dpt and all of these fish died by 9 dpt. Thus, again the presence of cells in the WKM correlates with survival. Fish that did not have cells in the WKM by 8 dpt were omitted from the survival analysis.

Overall, there was no difference in survival between non-transplanted "control" recipients and non-transplanted Poly I:C treated recipients to 28 dpt (Figure 5.8). Although, at 10 dpt, the non-transplanted poly I:C group had better survival (≈ 60 %) than non-transplanted "control" (≈ 25 %). This shows that the poly I:C injections did not affect survival of recipients, independent of transplantation.

Survival of control and poly I:C injected transplant recipients of 200 cd41+ from Tg(cd41:GFP;lyz:dsRed)

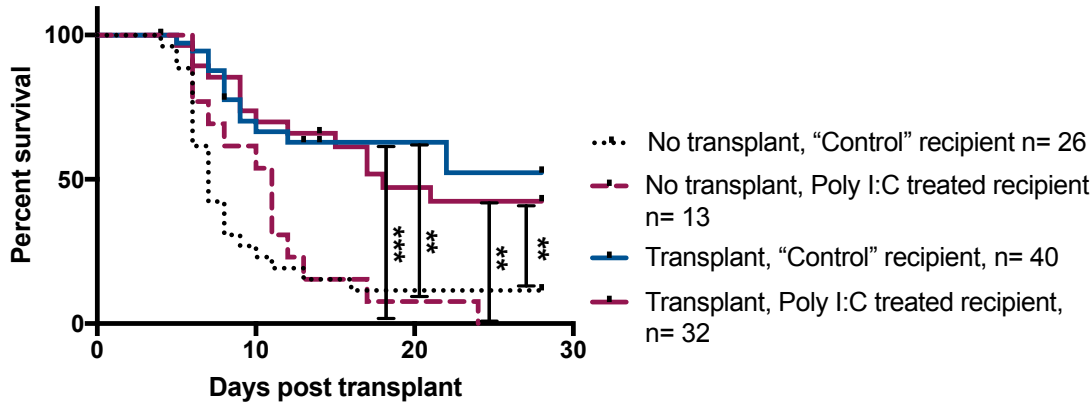


Figure 5.8: Survival of “control” and poly I:C treated recipients post-transplant. Poly I:C TraNac recipients were injected twice with 10 µg poly I:C, 48 h apart, and irradiated twice 48 h and 72 h later (48 h and 24 h before transplant). “Control” recipients did not get an I.P. injection but were irradiated twice, 48 h and 24 h before transplant. Donor Tg (cd41:GFP; lyz:dsRed) fish were humanely killed and harvested for WKM cells which were sorted for cd41:GFP+ cells. “Control” and poly I:C treated recipients received a transplant of 200 cd41:GFP+ cells (and 10⁵ WT PB cells) from the same donor pool. No transplant “control” recipients did not receive 10⁵ WT PB cells. All recipients were imaged and then tracked for survival to 28 dpt. 7 experiments pooled; “Control” recipients n= 40, data previously presented in Figure 4.9D; Poly I:C treated recipients n= 32. Mantel-cox test used to compare curves. *** p<0.001, ** p<0.01.

5.3.5 A transplant of 200 cd41:GFP+ cells from naïve Tg (cd41:GFP;lyz:dsRed) can reconstitute the WKM of “control” and poly I:C injected recipients

In order to determine if the recipient environment affected WKM reconstitution, recipients were harvested at 28 dpt to be analysed by flow cytometry (Figure 5.9). Firstly, there was no difference in the percentage of erythrocyte, myelomonocyte, lymphocyte and precursor populations as determined using forward and side scatter (Figure 5.10A). Secondly, there was no difference in the percentage of cd41:GFP+ between recipients groups (Figure 5.10B). As the recipients were individually monitored, the data can be paired for correlation studies (Figure 5.11A). The number of cd41:GFP+ cell at 8 dpt did not correlate with the percentage of cd41:GFP+ cells determined by flow cytometry at 28 dpt (Pearson’s product-moment correlation; “control” recipients, $r^2=0.0318$, $p=0.7351$; poly I:C treated recipients, $r^2=0.0902$, $p=0.6234$; Figure 5.11B).

There was large variation in the percentage of lyz:Red+ in recipients within the same group (Figure 5.10C, D). This indicates that while all recipients contained donor cells, there is a big variation in donor contribution to the WKM and myeloid population at 28 dpt. As *lyz* is a gene expressed by 57.4 ± 3.5 % of myeloid cells (56), the percentage of lyz:dsRed+ events in the myeloid gate was used to assess donor contribution. In some cases, the poly I:C treated recipients had more donor contribution (average 27 %, highest 87 %) than “control” recipients (average 9 %, highest 55 %; Figure 5.10D). However, the difference between the groups was not significant. Again the number of lyz:dsRed+ cell at 8 dpt was compared with the percentage of lyz:dsRed+ cells determined by flow

Post-transplant behaviour of HSCs in recipients with systemic inflammation cytometry at 28 dpt (Figure 5.11C) but no correlation was found (Pearson's product-moment correlation; "control" recipients, $r^2= 0.04464$, $p= 0.6878$; poly I:C treated recipients, $r^2= 0.198$, $p= 0.4527$; Figure 5.11D).

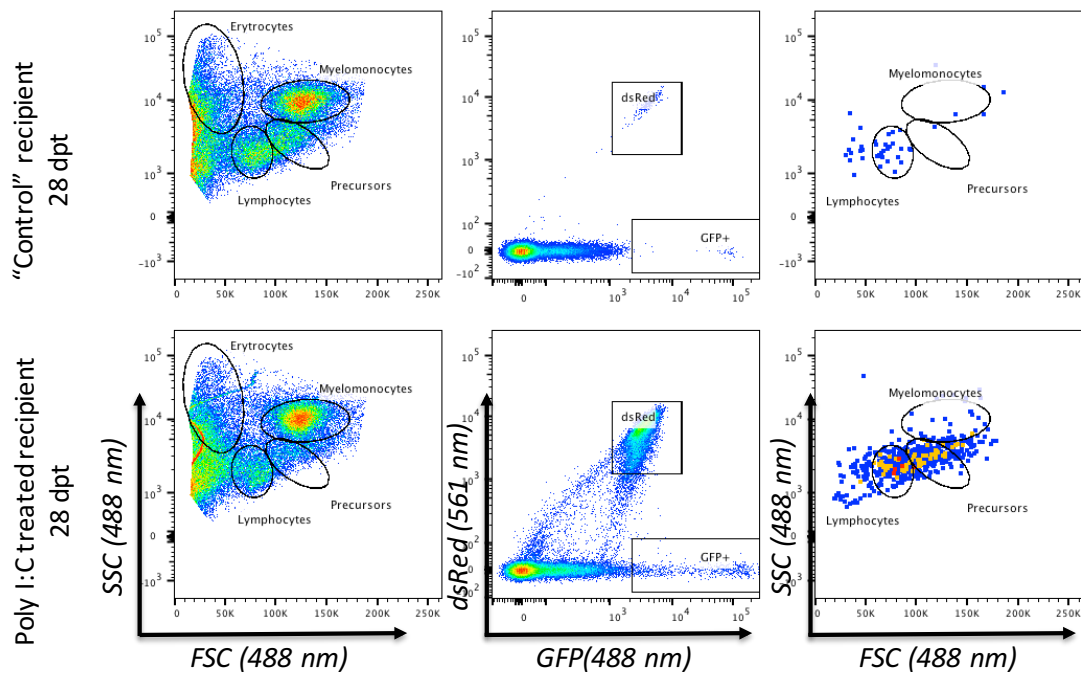


Figure 5.9: Flow cytometry scatter of a "control" and a poly I:C treated recipient at 28 dpt. Poly I:C TraNac recipients were injected twice with 10 µg poly I:C, 48 h apart, and irradiated twice 48 h and 72 h later (48 h and 24 h before transplant). "Control" recipients were irradiated twice, 48 h and 24 h before transplant. Donor Tg (cd41:GFP; lyz:dsRed) were humanely killed and WKM were harvested and sorted for cd41:GFP+ cells. "Control" and poly I:C treated recipients received a transplant of 200 cd41:GFP+ cells and 10⁵ PB cells from the same donor pool. Recipients were harvested at 28 dpt for flow cytometry analysis. All cells shown in first panel: SSC indicates side scatter; FSC indicates forward scatter (both use 488 nm laser, 488/10 filter). Events after debris exclusion shown in second panel: population is separated into dsRed+ (561 nm laser, 610/20 filter) and GFP+ (488 nm laser, 530/30 filter) events. Third panel shows GFP+ events only: SSC indicates side scatter; FSC indicates forward scatter (both use 488 nm laser, 488/10 filter).

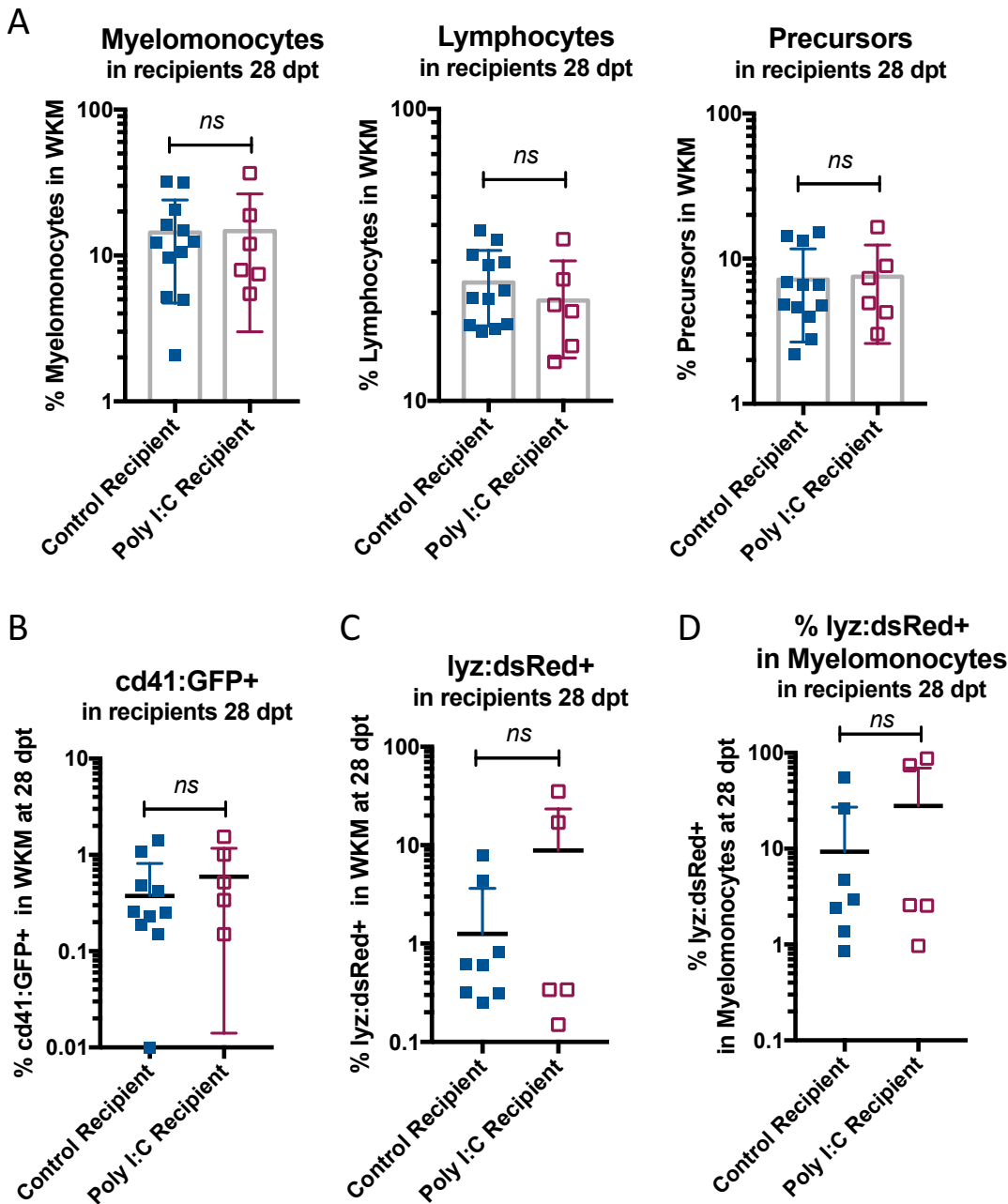


Figure 5.10: Reconstitution of the WKM in “control” and poly I:C treated recipients 28 days post-transplant. Poly I:C treated TraNac recipients were injected twice with 10 µg poly I:C, 48 h apart, and irradiated twice 48 h and 72 h later (48 h and 24 h before transplant). Control recipients were not injected but were irradiated twice, 48 h and 24 h before transplant. Donor Tg (cd41:GFP; lyz:dsRed) fish were humanely killed and WKM cells were harvested and sorted for cd41:GFP+ cells. “Control” and poly I:C treated recipients received a transplant of 200 cd41:GFP+ cells (and 10⁵ WT PB cells; non-transplanted recipients did not receive 10⁵ WT PB cells) from the same donor pool. Recipients were humanely killed at 28 dpt for flow cytometry analysis. (4 experiments pooled; “control” recipients n=12, Poly I:C treated recipients n=6). Each dot represents a recipient. (A) Percentage of myelomonocytes, lymphocytes, and precursors (Forward and by side scatter gating; Both use 488 nm laser, 488/10 filter). (B) Percentage of cd41:GFP+ (488 nm laser, 530/30 filter). (C) Percentage of lyz:dsRed+ (561 nm laser, 610/20 filter) in the WKM. (D) Percentage of myelomonocytes dsRed+.

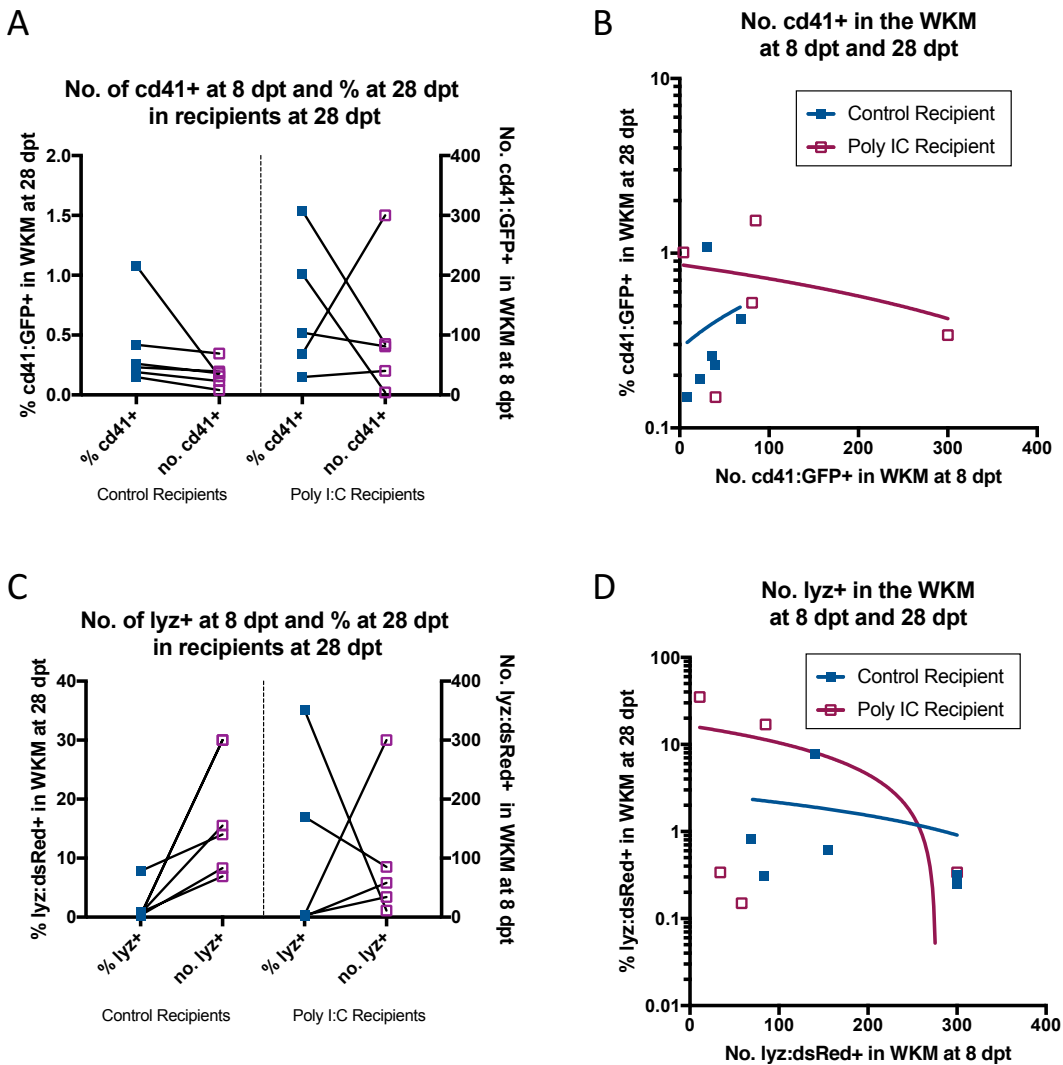


Figure 5.11: There is no correlation between the number of fluorescent cells observed at 8 dpt and the percentage of fluorescent cells at 28 dpt in the WKM of “control” or poly I:C treated recipients. Poly I:C treated TraNac recipients were injected twice with 10 µg poly I:C, 48 h apart, and irradiated twice 48 h and 72 h later (48 h and 24 h before transplant). “Control” recipients did not get an I.P. injection but were irradiated twice, 48 h and 24 h before transplant. Donor Tg (cd41:GFP; lyz:dsRed) fish were humanely killed and WKM cells were harvested and sorted for cd41:GFP+ cells. “Control” and poly I:C treated recipients received a transplant of 200 cd41:GFP+ cells (and 10⁵ WT PB cells) from the same donor pool. Non-transplanted recipients did not receive 10⁵ WT PB cells. All recipients were imaged 8 dpt and humanely killed at 28 dpt for analysis by flow cytometry. (2 experiments pooled; “control” recipients n=6, Poly I:C treated recipients n=5). (A) The number of cd41:GFP+ counted in the WKM at 8 dpt matched with the percentage of cd41:GFP+ (488 nm laser, 530/30 filter) in the WKM at 28 dpt. Data from the same recipients is connected by a line. (B) The same data as A is presented in an XY scatter with a line of best fit. (C) The number of lyz:dsRed + counted in the WKM at 8 dpt matched with the percentage of lyz:dsRed+ (561 nm laser, 610/20 filter) in the WKM at 28 dpt. (D) The same data as C is presented in an XY scatter with a line of best fit.

5.3.6 The percentage of precursor cells is lower in Poly I:C treated recipient WKM than “control” recipient WKM at 14 dpt

Altogether, the data indicates that there is no difference in reconstitution of “control” and poly I:C treated recipients’ WKM by 28 dpt. In order to determine if transplanted cells were reconstituting the recipient WKM differently before this stage, recipients were humanely killed at 14 dpt for analysis by flow cytometry.

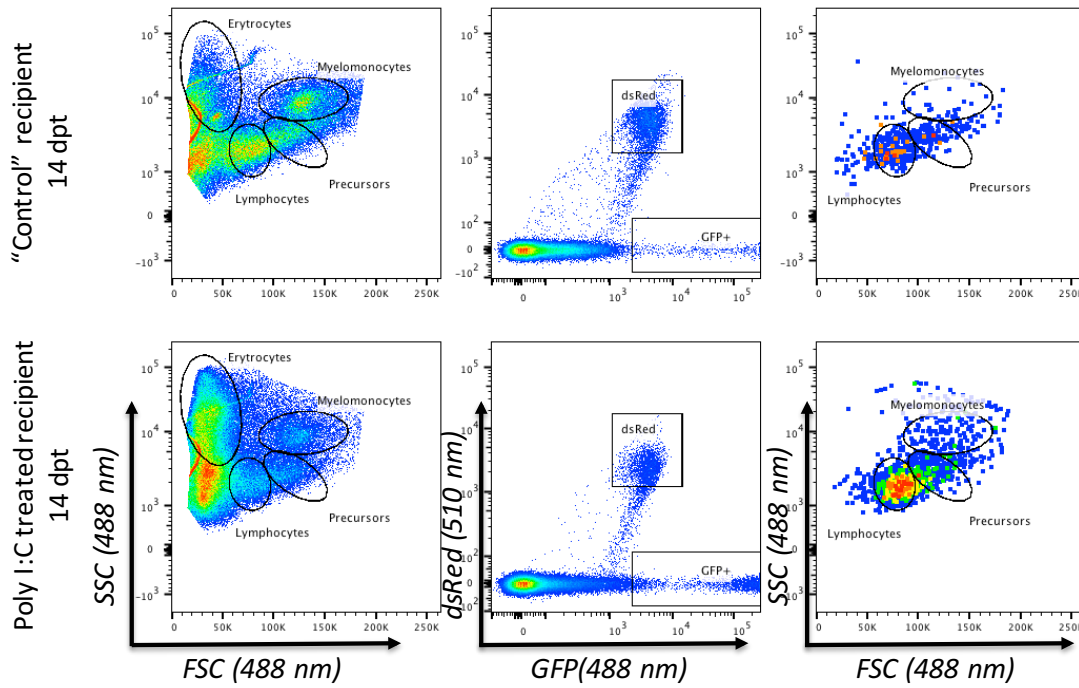


Figure 5.12: Flow cytometry scatter of “control” and poly I:C treated recipients at 14 dpt. Poly I:C treated TraNac recipients were injected twice with 10 µg poly I:C, 48 h apart, and irradiated twice 48 h and 72 h later (48 h and 24 h before transplant). Control recipients were irradiated twice, 48 h and 24 h before transplant. Donor Tg (cd41:GFP; lyz:dsRed) fish were humanely killed and the WKM was harvested and sorted for cd41:GFP+ cells. “Control” and poly I:C treated recipients received a transplant of 200 cd41:GFP+ cells and 10⁵ WT PB cells from the same donor pool. Non-transplanted recipients did not receive 10⁵ WT PB cells. Recipients were humanely killed at 14 dpt for analysis by flow cytometry. SSC indicates side scatter; FSC indicates forward scatter (both use 488 nm laser, 488/10 filter). The debris exclusion gated population is separated by dsRed (561 nm laser, 610/20 filter) and GFP (488 nm laser, 530/30 filter) scatter to obtain a lyz- population.

The major blood lineages were determined by forward and side scatter gating (Figure 5.12). The percentage of myelomonocytes and lymphocytes in the WKM did not differ significantly between the 2 groups (Figure 5.13A). However, the percentage of erythrocytes was significantly higher, and the percentage of precursors was significantly lower, in the poly I:C treated recipient group compared to the control recipient group. This might indicate that “control” recipients have a greater population of MPPs at this time, relative to poly I:C treated recipients.

In addition, the percentage of donor derived cells (cd41:GFP and lyz:dsRed) did not differ significantly between the groups at 14 dpt. These data support earlier observations at 8 dpt in which the number of these cells observed in the WKM did not differ significantly between the 2 recipient groups (Figure 5.6C, D).

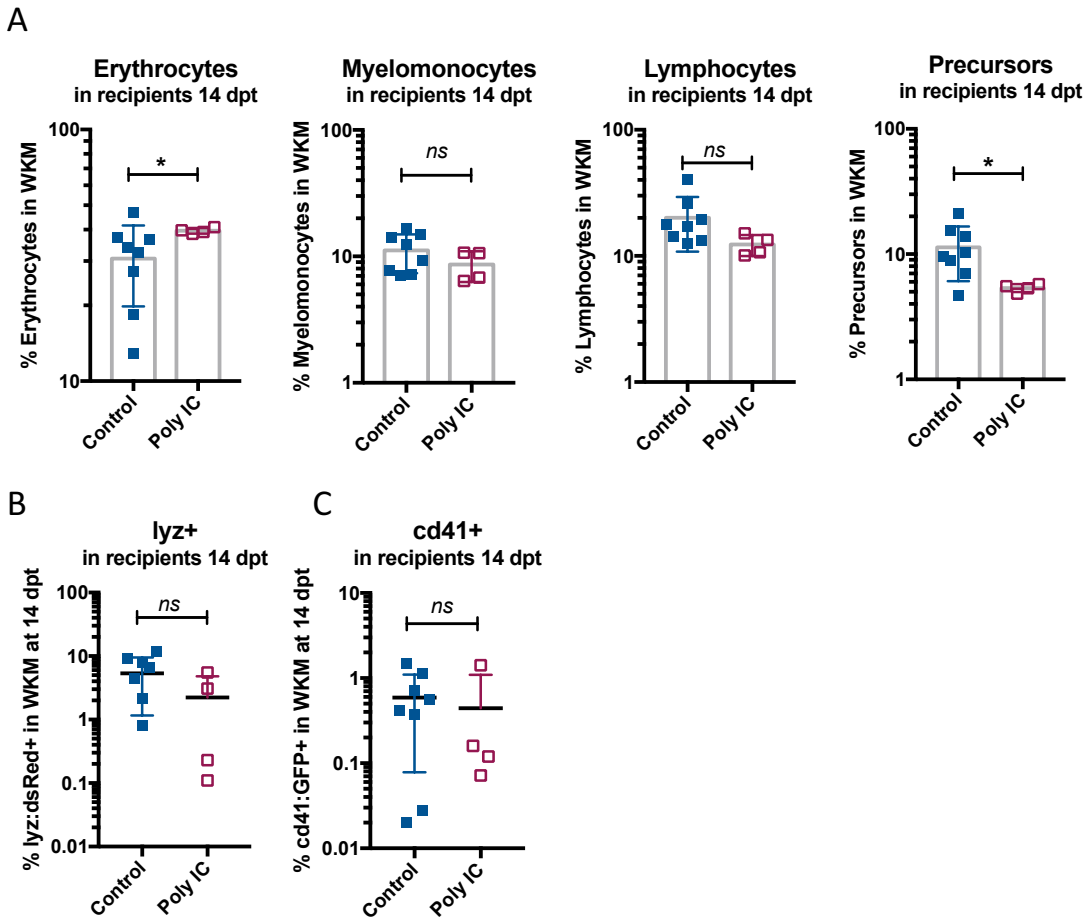


Figure 5.13: “Control” recipients have a higher percentage of precursors than poly I:C injected recipients at 14 dpt. Poly I:C treated TraNac recipients were injected twice with 10 µg poly I:C, 48 h apart, and irradiated twice 48 h and 72 h later (48 h and 24 h before transplant). “Control” recipients were not injected but were irradiated twice, 48 h and 24 h before transplant. Donor Tg (cd41:GFP; lyz:dsRed) fish were humanely killed and the WKM harvested and sorted for cd41:GFP+ cells. “Control” and Poly I:C treated recipients received a transplant of 200 cd41:GFP+ cells (and 10⁵ WT PB cells) from the same donor pool. Recipients were imaged 8 dpt and harvested at 14 dpt for analysis by flow cytometry. (2 experiments pooled; “control” recipients n=8, poly I:C treated recipients n=4). (A) The percentage of erythrocytes, myelomonocytes, lymphocytes, and precursors (Forward and by side scatter gating; Both use 488 nm laser, 488/10 filter). (B) The percentage of lyz:dsRed+ (561 nm laser, 610/20 filter) in the WKM. (C) The percentage of cd41:GFP+ (488 nm laser, 530/30 filter).

5.3.7 Using c-myb mutant recipients to examine a non-inflamed recipient environment

In order to study hematopoietic engraftment in a non-inflamed environment, a line of *bloodless* mutants with the c-myb^{t25127} mutation (46) was used. These fish were transplanted with either 200 cd41:GFP+ cells (in order to compare these recipients with the TraNac “control” and poly I:C treated recipients described previously in this chapter (Figure 5.8)) or 2,000 cd41:GFP+ cells from Tg (cd41:GFP;lyz:dsRed) donors, together with 10⁵ WT PB cells. The survival of mutants transplanted with 200 cells was very low (22 % to 28 dpt) compared to fish receiving 2,000 cells (p= 0.659; Figure 5.14B).

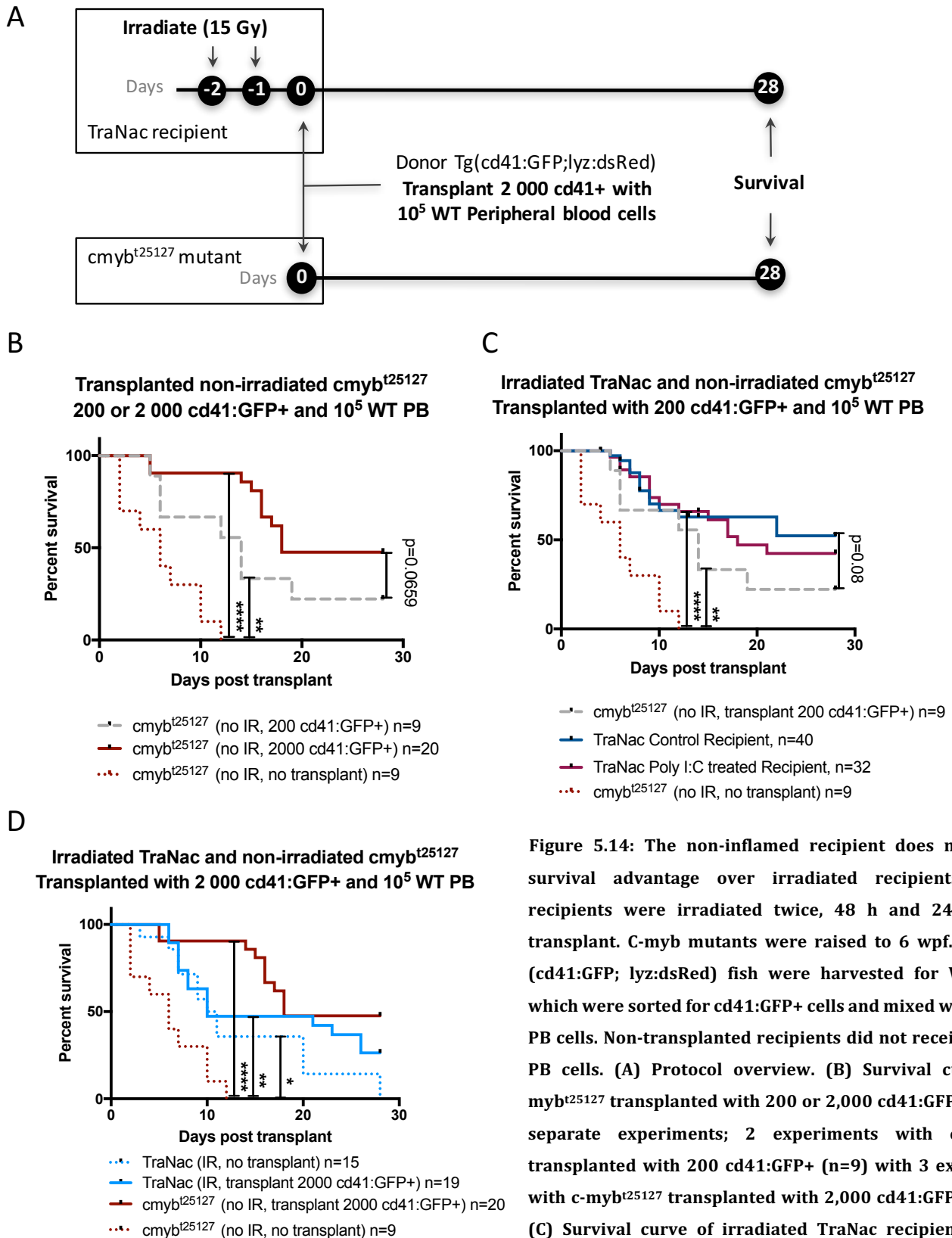


Figure 5.14: The non-inflamed recipient does not have a survival advantage over irradiated recipients. TraNac recipients were irradiated twice, 48 h and 24 h before transplant. C-myb mutants were raised to 6 wpf. Donor Tg (cd41:GFP; lyz:dsRed) fish were harvested for WKM cells which were sorted for cd41:GFP+ cells and mixed with 10⁵ WT PB cells. Non-transplanted recipients did not receive 10⁵ WT PB cells. (A) Protocol overview. (B) Survival curve of *cmyb*^{t25127} transplanted with 200 or 2,000 cd41:GFP+. (Pooled separate experiments; 2 experiments with *cmyb*^{t25127} transplanted with 200 cd41:GFP+ (n=9) with 3 experiments with *cmyb*^{t25127} transplanted with 2,000 cd41:GFP+ (n=20)). (C) Survival curve of irradiated TraNac recipients with *cmyb*^{t25127} recipients of 200 cd41:GFP+. (Pooled separate experiments; 3 experiments *cmyb*^{t25127} transplanted with 200 cd41:GFP+ (n=9), 7 experiments with TraNac “control” recipients (n= 40) and TraNac Poly I:C treated recipients (n= 32); data previously presented in Figure 4.9 and Figure 5.8). (D) Survival curve of irradiated TraNac recipients and *cmyb*^{t25127} recipients of 2,000 cd41:GFP+. (3 experiments; TraNac no transplant (n=15), TraNac transplanted with 2,000 cd41:GFP+ (n=19), *cmyb*^{t25127} no transplant (n=9), *cmyb*^{t25127} transplanted with 2,000 cd41:GFP+ (n=20).) Mantel-cox test, **** p<0.001.

experiments; 3 experiments *cmyb*^{t25127} transplanted with 200 cd41:GFP+ (n=9), 7 experiments with TraNac “control” recipients (n= 40) and TraNac Poly I:C treated recipients (n= 32); data previously presented in Figure 4.9 and Figure 5.8). (D) Survival curve of irradiated TraNac recipients and *cmyb*^{t25127} recipients of 2,000 cd41:GFP+. (3 experiments; TraNac no transplant (n=15), TraNac transplanted with 2,000 cd41:GFP+ (n=19), *cmyb*^{t25127} no transplant (n=9), *cmyb*^{t25127} transplanted with 2,000 cd41:GFP+ (n=20).) Mantel-cox test, **** p<0.001.

It is important to note that c-myb^{t25127} mutant survival was determined from transplant experiments performed independently of the TraNac transplants and therefore these experiments warrant repeating with all the groups included in the same experiment. However, the preliminary data here suggests that survival of the c-myb^{t25127} mutant transplanted with 200 cd41:GFP+ cells is lower than that of the irradiated TraNac (“control” or poly I:C treated) recipients transplanted with 200 cd41:GFP+ cells (

Figure 5.14C). The comparatively lower survival of the c-myb^{t25127} recipients might indicate that the inflammatory environment in the irradiated TraNac could be beneficial. However, I do not think this is likely. It is more likely that the c-myb^{t25127} mutants need to be rescued earlier than 6 wpf, and it also indicates that a dose of 200 cells is very low. Furthermore, the sub-lethal irradiation of the TraNac does not deplete the HSPC population completely and the remaining cells might be contributing to the recipient recovery.

Due to the low survival of the c-myb^{t25127} mutants transplanted with 200 cd41:GFP+ cells, the protocol was modified to a transplant of 2,000 cd41:GFP+ cells

(Figure 5.14A). The survival of transplanted c-myb^{t25127} recipients was not significantly different to survival of irradiated TraNac, also transplanted with 2,000 cd41:GFP+ cells (independent experiments;

Figure 5.14D). However, survival of transplanted c-myb^{t25127} mutants was significantly higher than non-transplanted c-myb^{t25127} mutant siblings ($p < 0.0001$;

Figure 5.14D). Taken together, these results indicate that c-myb^{t25127} recipients could be used independently of TraNac to compare an inflammatory and non-inflamed recipient environment.

5.4 Discussion

Studies suggest that infection and inflammation can modulate the HSPC compartment. In the previous chapter it was shown that inflammation pre-transplant can modulate the behaviour of HSPCs in transplant recipients. Indeed, repeated poly I:C treatment of zebrafish causes a post-transplant delay in the migration of cd41:GFP+ cells to the recipient WKM and subsequent proliferation in the recipient WKM.

In this chapter, HSPCs were transplanted into inflamed recipients. To understand the HSPC response, independently of the differentiated hematopoietic cell response, cd41:GFP+ cells from naïve donors were transplanted into non-injected irradiated “control” recipients and poly I:C injected irradiated recipients.

Unlike in the previous chapter, cells transplanted into both recipient groups came from the same mix of sorted cd41:GFP+ WKM cells and WT PB cells which significantly reduced the haemocytometer counting error within an experiment. In each experiment, recipients came from the same clutch and were raised together before being randomly allocated to 2 groups. Both groups were irradiated side by side in the same irradiation chamber. Variation in individual response to the pre-transplant treatment protocol was reduced by repeating the

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experiment with n >30 fish. Donor cells were transplanted using the same glass needle and transplant recipients were alternated every 3 fish.

5.4.1 Summary of results

The findings of this chapter were:

1. qPCR data indicated that after poly I:C treatment (10 µg) there was a significant increase in transcript levels of various (*il-1β*, *il-6*, *tnfα*, *ifnφ*, *ifnγ1.1*, *ifnγ1.2*, *cxcl-c1c* and *cxcl8*) cytokines. However, the high level of cytokine transcripts expected in the inflamed recipient “poly I:C + IR” fish at the time of transplant, relative to untreated “No IR, No poly I:C” or control “IR” recipient fish was not detected. The only transcript found to be significantly increased in irradiated recipients (“IR” and “poly I:C + IR”) was *cxcl12a*. There is a strong possibility that the protocol did not result in an “inflamed” recipient and this is likely to explain the rest of the results.
2. Analysis of data obtained by live imaging of recipients revealed the number of fluorescent cells (cd41:GFP+ or lyz:dsRed+) at each time point did not differ significantly between recipient groups.
3. Reconstitution of the recipient WKM was not significantly different in the two recipient groups at 28 dpt, although the percentage of precursors in the “control” recipients was higher than the percentage in poly I:C treated recipients at 14 dpt. This does not appear to have significantly affected the survival of poly I:C recipient group.
4. Finally, the survival of “non-inflamed” c-myb^{t25127} transplanted with 200 cd41:GFP+ was lower than irradiated TraNac transplanted with 200 cd41:GFP+ cells. However, survival of “non-inflamed” c-myb^{t25127} transplanted with 2,000 cd41:GFP+ was the same as irradiated TraNac transplanted with 2,000 cd41:GFP+ cells.

5.4.2 Creating an inflamed recipient environment

This chapter begins by establishing a protocol for inflamed recipients. The protocol was adapted from a paper by Sato *et al.* which used poly I:C and 5-FU as a pre-conditioning regiment for transplantation (412). As the previous chapters used irradiation pre-treatment, it was decided that this work would continue using irradiation. Poly I:C treated recipients were injected with 10 µg of poly I:C on days -6 and -4, then irradiated with 15 Gy on days -2 and -1. “Control” recipients were not injected days -6 and -4 but were irradiated with 15 Gy on days -2 and -1. qRT-PCR was used to measure systemic mRNA expression (whole body samples), normalised to 18S expression. Flow cytometry could not be used to determine the populations of the WKM because the size of the recipients meant they could not be dissected with precision. However, in chapter 3.3.1.1, qRT-PCR had supported flow cytometry data, indicating it is a reliable alternative to flow cytometry.

Importantly, the results of the one-way ANOVA with all the groups present in the analysis (Figure 5.2) concluded that there was no significant difference in transcript levels between untreated “No IR, No poly I:C” and irradiated

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("IR" and "poly I:C + IR") recipients. As I mentioned earlier, the one-way ANOVA test calculates p-values based on the F- statistic (the ratio of the variance among the means to the variance within the sample), and this reduces the statistical significance of the irradiated groups. Therefore, to understand if the protocol resulted in an "inflamed" recipient, the recipient groups ("No IR, No poly I:C", "IR" and "poly I:C + IR") were also tested for statistical significance separately (Supplementary Figure 7.7). Surprisingly, transcript levels for the tested cytokines (*il-1 β* , *il-6*, *tnf α* , *ifn γ* , *ifn γ 1.1*, *ifn γ 1.2* and *cxcl1*) were not significantly different between "IR" and "poly I:C + IR" recipients. Indeed, the "poly I:C + IR" recipient group had significantly higher levels of *il-1 β* ($\approx$ 2 fold) and *cxcl1* ($\approx$ 3.5 fold) transcripts relative to the untreated group, and not significant but > 2.5 fold higher levels of *ifn γ 1.1* ($\approx$ 4 fold). The "IR" recipient group had significantly higher levels of *il-1 β* ($\approx$ 3.5 fold), *il-6* ($\approx$ 2 fold), *tnf α* ($\approx$ 4.5 fold), *ifn γ 1.1* ($\approx$ 6 fold) and *cxcl1* ($\approx$ 6 fold) transcripts, relative to untreated samples. Additionally, there was no difference in transcript levels of blood cell-specific genes (Supplementary Figure 7.8). This analysis strongly indicates that the cytokine milieu of the "inflamed" recipient and "control" recipient groups were not significantly different and an "inflamed recipient" was not achieved by the protocol. I would suggest not using "inflamed" recipients in further studies, but instead focusing on "non-inflamed" recipients (such as the *cmyb* mutant, if this can be shown).

Finally, on the question of the difference between "control" and "inflamed" recipients, the chemokine *cxcl12a* was significantly upregulated in both these groups relative to poly IC injected and the non-injected, non-irradiated group (Figure 5.2). As in mice and humans (424), *cxcl12a* is a chemoattractant and in zebrafish attracts HSCs to the spleen, thymus and kidney marrow. Glass *et al.* found that *cxcl12a* is upregulated $\approx$ 3 fold in the tubule fraction of the WKM of fish irradiated with 30 Gy (307). As the experiment presented here took the whole fish for analysis, it is not clear whether the upregulation was specifically in the kidney or in other tissues such as non-hematopoietic tissues such as the skin and gills, also shown to express *cxcl12a* in the zebrafish (307). It is interesting that the increased expression is not as pronounced in the irradiated Poly I:C treated recipients "Poly I:C + IR" as it was anticipated that the injury caused by the intra-peritoneal injections would cause up-regulation of the chemokine to recruit HSPCs to the injured site. The lower than expected level of *cxcl12a* might be an indication that significant damage was done to the cells which would normally produce this chemokine.

5.4.3 HSPCs entering an inflammatory environment did not display distinct behaviours

As in the previous chapters, recipients were imaged 4, 6 and 8 days after transplantation with 200 cd41:GFP+ cells. There were no observable differences in the behaviour of cd41:GFP+ or lyz:dsRed+ cells in the WKM at any of these time points (Figure 5.6), despite the difference in expression of *cxcl12a*. This could suggest that the recipient environments of the two groups are too similar to observe behavioural changes at these time points. Indeed, this is supported by the previously discussed qRT-PCR data which is remarkably similar between "control" and poly I:C treated recipients at the time of transplant (Figure 5.2; Supplementary Figure 7.7). As the effects of radiation are most extreme between 4 and 8 dpt (135), it may be that the effects of the poly I:C treatment are comparatively unimportant. This idea is supported by the fact that the survival curves of these two groups overlap closely until 16 dpt (Figure 5.8).

There was a significant survival advantage to recipients of a transplant, whether treated with poly I:C or not. The survival of these recipients was identical for the first 16 days. After this point, the probability of survival of poly I:C treated recipients decreases slightly but not significantly. It would be interesting to see if this decrease persists in the longer term. Although, previously published data suggests pre-conditioning of recipients with poly I:C improves engraftment and recipient survival (412) when used with 5-FU, this study found no difference in the survival between pre-conditioning protocols with poly I:C and irradiation (Figure 5.8).

One of the main questions of this thesis was to determine if inflammation affected the behaviour of HSPCs, therefore it was important to examine a non-irradiated “non-inflamed” c-myb^{t25127} mutant recipient control. It is important to note that the inflammatory profile of the c-myb^{t25127} mutant recipient was not assessed and therefore is hypothetical. The survival of c-myb^{t25127} transplanted with 200 cd41:GFP+ was lower than irradiated TraNac, although comparable when transplanted with 2,000 cells (Figure 5.14C, D). The comparison with irradiated TraNac is not the most suitable as the organism development of cmyb mutants would have been affected by the absence of a hematopoietic system. Although Hess *et al.* argue the niche is not affected as the donor HSPCs are able to rescue the hematopoietic system (46), preservation of the niche signalling has not been demonstrated. Therefore, the results may indicate that the difference in survival is not due to the inflammatory environment but rather to the physiological weakness of the c-myb^{t25127} line. The survival of c-myb^{t25127} fish improved when these were transplanted with 2,000 cd41:GFP+ cells. This might be because there was less replicative stress on HSPCs when a larger population is transplanted.

Altogether, these results suggest that the recipient environment has a less significant impact on the behaviour of HSPCs than the more chronic exposure to inflammation described in chapter 4. This might be because the exposure is more transient, or it may be that the HSPCs have been affected but the effect on replication will only be determined by subsequent secondary transplants. Recently, Ishida *et al.* showed that the inflammatory environment of the irradiated mouse recipient does impact transplanted HSPCs, but the effect of this inflammation was only observed in secondary transplants (410).

5.4.4 Short term reconstitution of transplant recipients

As early survival and the behaviour of transplanted cells were comparable between the recipient groups in the first 2 wpt, it was hypothesised that differences might manifest just before the difference in survival is noted. Flow cytometry of recipients 14 dpt revealed a larger precursor population in “control” recipients than poly I:C treated recipients. This might be the means by which survival in the “control” group is improved. The environmental components which support/inhibit the growth of this population need further study.

Flow cytometry of recipients at 28 dpt shows that the percentage of donor contribution to the recipient WKM was not significantly different between control and poly I:C recipients. This is contrary to the report published by Sato *et al.* who found that poly I:C and 5-FU treatment of recipients lead to increased donor chimerism in recipient mice compared to 5-FU treated mice (7.66 % vs. 0.3 %), 2 months post-transplant (412). It is likely that

the difference in our conclusions is because the “inflamed” recipient in this work is not significantly different to the “control” recipient. It is worth noting that, when comparing pre-conditioning treatments, Sato *et al.* only assessed DNA damage (γ H2AX assay) and did not assess the inflammatory milieu. Thus, the difference in interferon signalling between irradiated, poly I:C treated and 5-FU treated recipients was not determined. It would be interesting to measure this component.

5.5 Conclusion and future work

In conclusion, the aim of this thesis is to assess the response of HSCs under conditions of inflammation. This chapter examined the acute effects of exposing naïve cd41:GFP⁺ HSPCs to an inflammatory environment. However, it is not clear that an inflamed recipient was created because the cytokine and cellular profiles of the “control” and poly I:C treated recipients were not significantly different. This may explain why the initial response of transplanted cells was not significantly different in the two environments. However, Sato *et al.* also concluded that environmental interferons did not affect post-transplant reconstitution in mice (393). It is important to remember that the zebrafish is a highly regenerative organism, capable of reforming amputated fins or brain lesions, spinal cord and heart damage (425). Furthermore, they are very resilient to chronic inflammation, capable of healing after 6 h exposure, 3 days a week over 6 weeks (426). This may have had an important role in the physiology of the zebrafish WKM as, unlike mammalian BM, the endogenous niche cells were actively regenerating. Indeed, there is an indication that the effects of the environment were observed a little later at 14 dpt, when the precursor population would be expanding in non-injected irradiated recipients. In poly I:C injected recipients, the precursor population was a smaller percentage of the WKM, and this could be an indication that the regeneration of HSPC supportive cells was affected by the poly I:C treatment. Furthermore, a reduced precursor population at 14 dpt may be the cause of a slightly lower survival of Poly I:C treated recipients.

Based on these findings, it would be interesting to examine the efficiency of WKM regeneration after irradiation (with and without prior poly I:C treatment). For instance, Cre-recombinase based methods or photo-convertible cells are used for lineage tracing experiments. It would be important to examine the cellular composition of the WKM and compare the composition at various time points to establish the regeneration rate the various cell types as this will have an important impact on the retention of HSPCs. In the study of heart regeneration, reporter lines were used to visualise the release of signals that facilitate regeneration over time (427). However, in order to carry out this study in the zebrafish, whole transcriptome analysis will be necessary to identify candidate genes required in the regeneration of the WKM.

To better understand inflammation in the recipient, future work could examine the effect of poly I:C injection in recipients after the transplantation of naïve HSPCs. These recipients would be prepared by irradiation, transplanted with 200 cd41:GFP⁺ cells and then injected with poly I:C or saline at an appropriate interval (perhaps 6 hpt). This might be able to cause an increased inflammatory milieu in poly I:C injected recipients that would be significantly different from the protocol described in this chapter.

Mechanistically, the migration of transplanted HSPCs must depend on signals. It was not possible to demonstrate a change in migration in the Poly I:C treated recipient, possibly because the signalling was not significantly different from irradiated controls. It would be interesting to test the involvement of specific inflammatory molecules in the process of migration. For instance, a transgenic *cxc12a* line exists and could be used in conjunction with the protocol established in this thesis to further the understanding of the role of this chemokine.

Furthermore, the effect of radiation on transplanted cells requires further investigation. Radiation damage could be limited using ROS reducing agents or inflammation reducing agents. In fact, a single dose of SCF, TPO and IL-3 within 2 h of radiation significantly reduced cytopenia and improved hematopoietic recovery in mice and nonhuman primates (428). This suggests that a greater understanding of the role of cytokines could potentially lead to fewer toxic radiation effects and greater hematopoietic recovery.

6 Final Discussion

While mouse models have contributed enormously to understanding hematopoietic regeneration, it has not been possible to examine HSPC behaviour *in vivo* without terminal anaesthesia. The zebrafish has been a central model for the understanding of early hematopoiesis and human hematopoietic disorders (150, 429-431). Importantly, immune cell types and inflammatory molecular pathways are conserved between zebrafish and mammals (432-434). This thesis has demonstrated that live imaging of young adult zebrafish can be used to obtain important data on the early-post-transplant behaviour of transplanted hematopoietic cells from transgenic donors. Indeed, the regenerative capacity and mobilisation behaviour of transplanted HSPCs has been assessed, in the context of inflammation generated by a viral mimic. There are many exciting directions this work could take in the future and these are discussed below.

6.1 Significance of the study and key findings

Chapter 3 presents the refinements made to the HCT protocol in zebrafish in order to qualitatively and quantitatively analyse the behaviour of transplanted cells. This meant developing a method to deliver the cells safely and without causing damage into small (<1 cm) optically transparent juveniles to provide quality images. This is first time that transplanted cells have been imaged individually in the WKM of the adult recipient organism and the method is central to answering subsequent questions about HSPC post-transplant behaviour in response to inflammation. Data correlating cell number and transplant recipient survival was presented. It revealed that post-transplant deaths that occurred between 10 and 20 dpt can be predicted and reduced by humanely killing fish that have fewer than a total of 25 fluorescent donor cells in the WKM by 6 dpt. If this protocol is applied, the number of animals suffering and dying because of failed HCT would be reduced by 50 %. This information is important for improving animal welfare and reducing the severity of this protocol under UK Home Office regulations.

In chapter 4, the HCT donors were repeatedly stimulated with IFN inducer poly I:C prior to harvest and transplant into irradiated recipients. Through the tracking of individuals over time, a statistically significant interaction was found between the treatment and time. The increase in the number of HSPCs in the recipient WKM was greater when transplanted from poly I:C injected donors. This underscored a change in HSPC (cd41:GFP+) behaviour in poly I:C treated cells that was not apparent by examining individual timepoints. However, the survival of recipients of HSPCs from poly I:C injected donors was not significantly different to recipients of HSPCs from saline injected donors and there was no evidence that treatment of the donor affected either early myeloid (lyz:dsRed+) output or long term (28 dpt) hematopoiesis in recipients. Together, the conclusion is that poly I:C injected donors have HSPCs that expand rapidly in the transplant recipient but cope equally to the reconstitution challenge as HSPCs from saline injected donors.

In chapter 5, the HCT recipients were stimulated with poly I:C prior to irradiation and receiving HSPCs from naïve donors. Essentially, the initial post-transplant behaviour of donor cells was not significantly different to

behaviour in “control” (non-injected, irradiated) recipients. Survival was not significantly different either. The limitation of this work is that the “inflamed” recipient was not demonstrably different to the “control” recipient at the time of transplant. Preliminary work with *bloodless* mutants (non-injected, non-irradiated) suggests that these could be a better model to examine the effect of the inflammatory environment on transplanted HSPCs. Together, the conclusion is that the inflammatory environment created by this protocol did not differentially affect transplanted HSPCs. Some suggestions for future work are discussed below.

In conclusion, this thesis has demonstrated the first use of *in vivo* imaging to quantify early post-transplant events in the adult zebrafish. The results indicate that the recipient environment has a much less significant impact on the survival of transplant recipients than the state of the donor HSPC prior to transplantation.

6.2 Future work

This project has developed the HCT method in a manner that can be applied to many questions. In the immediate, it would be important to examine the competitiveness of the pre-stimulated HSPC population against the non-stimulated HSPC population in the same recipient. An experiment design was outlined in Figure 4.14 in which donor Tg (*runx1+23:GFP*) were injected with either saline or poly I:C before harvesting *runx1:GFP+* HSPCs from their WKMs. These HSPCs would then be transplanted into recipients along-side HSPCs from untreated Tg (*runx1+23:NLSmCherry*) donors and PB from WT donors. Mobilisation, expansion and engraftment of *runx1:GFP+* cells from poly I:C injected donors would be measured relative to the *runx:mCherry+* HSPCs from the untreated donors; likewise the mobilisation, expansion and engraftment of *runx1:GFP+* cells from saline injected donors would be measured relative to the same population. Thus the poly I:C and saline simulated HSPCs would be measured relative to the same untreated population of *runx:mCherry+* HSPCs.

This protocol will be immensely useful for all kinds of work requiring the *in vivo* readout of cell potential and may even be applied to examining HSC behaviour in the absence/presence of specific niche components, as mentioned early for the 14 dpt expansion of the precursor population. For instance, Notch has an important role in the emergence of HSCs, partly because of its role in body segmentation. Using this protocol, HSPC behaviour could be observed in a heat shock induced knockout of Notch. This protocol could also be applied to examining leukemic cell behaviour and cells of xenogeneic transplant studies.

Another immediate question that could be answered is to measure the cytokine environment of the *bloodless c-myb*^{t25127} mutant using qRT-PCR as this group of recipients were regarded as a non-inflamed control, but cytokines can be produced by various cell types. In addition, the niche environment of the *c-myb*^{t25127} mutant should be examined to understand if it has been affected by the absence of hematopoiesis during development. Further experiments can take advantage of these mutants, independently of TraNac fish. For instance, crossing the mutant with a line of fish in which inducible promoters (eg. vascular specific gene or nephros specific gene) could drive cytokine (*il-1 β* , *tnf α* , *il-6* etc.) expression and would create a cytokine specific inflamed recipient niche without the use of poly I:C or irradiation. In this way, the *c-myb* mutant could be compared with and

without cytokine expression. The different segments of the kidney tubules express different ion transporters (311) and these could be used to drive the cytokine expression in distinct sections of the WKM.

Furthermore, the results of chapter 5 indicate that the environmental inflammation did not affect the transplanted HSPCs. However, previously in the mouse, Slayton *et al.* have found that HSCs help to repair the endothelial and vascular cells of the irradiated environment (435). Therefore, it might be of interest to study how HSPCs can affect environmental inflammation. Do HSPCs interact and repair the endothelial and vascular cells of the zebrafish? Perhaps the imaging techniques described for HCT could be used to examine the interaction of HSPCs and vascular cells and the molecules involved. With the progressing development of new imaging techniques, the zebrafish has advantages over rodent models to obtain high-resolution information of biological processes. For instance, the Dallman lab has developed FRET (*Förster Resonant Energy Transfer*)-sensors of Caspase 1 and 3 activation (436). Fluorescence-lifetime imaging and optical projection tomography of these fish will facilitate the dissection of molecular interactions in the whole organism in real time. For instance, if the promoter driving the construct were chosen from candidate genes from cells in the WKM, the caspase activity in those cells could be examined following irradiation and used to compare death in the non-injected and poly I:C-injected recipients used in Chapter 5.

A question which arose in this thesis was the cell-cycle kinetics of zebrafish HSPCs (see chapter 3.3.5). The BrdU labelling experiments did not reveal any WKM cells with slow cycling activity. This may be due to technical obstacles or this may indeed reflect that zebrafish do not have a slow cycling population. If the latter is the case, then the protections provided by the quiescent state (from mutations for instance) of the LT-HSC must be compensated for by other mechanisms (perhaps cell-autonomous) in the zebrafish. Future work could determine if the hematopoietic system of zebrafish can be exhausted as this would reveal if there is a limit to the supply of the most primitive population. Furthermore, the heterogeneity of the cd41:GFP⁺ HSPC population could be explored using single-cell lineage tracing experiments as recently published (55). With recent single-cell transcriptome data analysis, the field is accepting that individual cells of the LT-HSC population have significantly different transcriptomes and therefore the heterogeneity of this population must be taken into account when examining its behaviour (79, 80). With the recent development of the *Zebrabrow* system (55) which activates a unique heritable colour in each cd41:GFP⁺ cell, cells of the same clone could be examined for post-transplant behaviour, differentiation and cell cycle kinetics.

Finally, the developed method offers the possibility of integrated multi-organ analysis of pathophysiological responses, which will undoubtedly be the focus of future research. Indeed, there is emerging evidence that life-style choices such as diet and smoking have a significant impact on the immune system. There is a need for systematic reviews and meta-analysis to be performed to get an understanding of how these factors, as well as gender and donor age can affect the reconstitution capacity of their blood. Indeed, the Dallman lab has reported inflammatory responses to both acute and long term exposure to a high cholesterol diet (HCD) and cigarette smoke (391, 392) in the zebrafish and therefore this project could be launched relatively quickly. It would be

interesting to address the effect of chronic inflammatory responses due to a high cholesterol diet or cigarette smoke exposure on HSPC behaviour, both of which are of clinical interest (325, 437-440).

6.3 Implications and future directions

Lastly, the differences and similarities between the zebrafish, mouse and human HSPC populations are difficult to align. Therefore, to fully achieve the potential benefits of an immunological understanding for human wellbeing, more emphasis needs to be placed on human studies as it is key to learning more about these conditions from clinical samples. Studying stem cells in model organisms may give good insights but it is difficult to draw the line between the general and the animal specific findings. In order to understand more about human HSPCs, it is important to develop humanised animal models and to study patient samples.

7 Supplementary Data

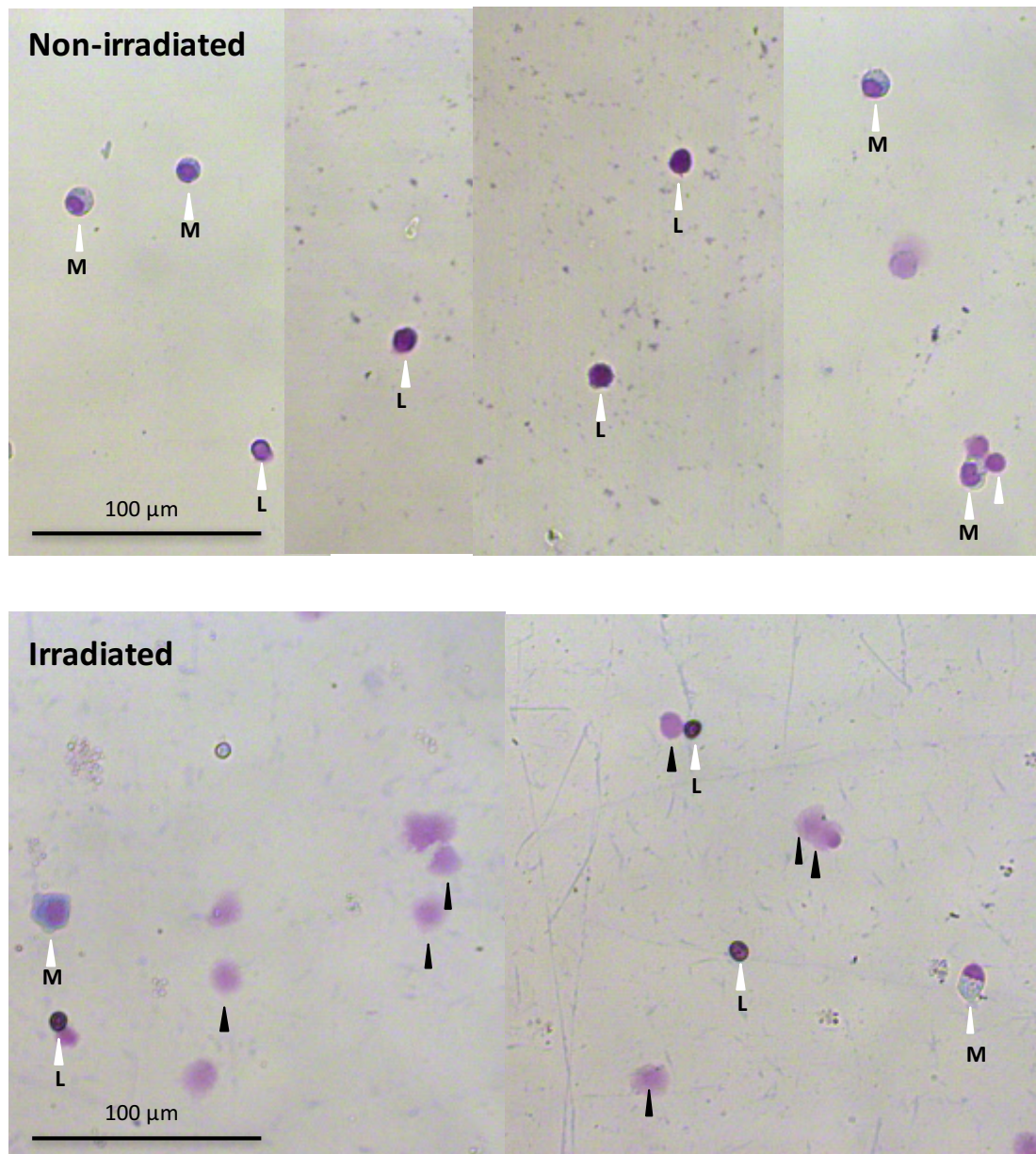


Figure 7.1: Cytospin of whole kidney marrow cells from irradiated and non-irradiated TraNac. Adult TraNac fish were irradiated twice with 15 Gy (24h apart) and their WKM harvested 5 days later. Live WKM cells were cytospun onto slides and stained with Wright-Geimsa. Representative images shown here. White arrows indicate nucleated cells (myeloid (M), lymphoid (L)); Black arrows indicate enucleate cells.

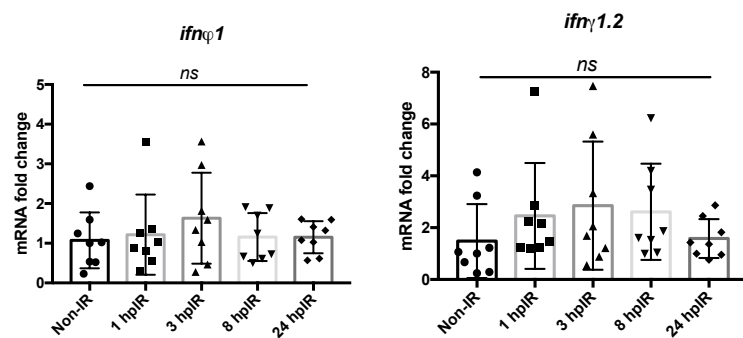


Figure 7.2: Irradiation did not change interferon gene expression in the whole kidney marrow. TraNac fish were left untreated (Non-IR) or irradiated (IR) with 2 doses of 15 Gy, 24 h apart, and the WKM was harvested 1, 3, 8, and 24 h after the 2nd dose. RNA was extracted and qRT-PCR was performed on samples. 2 experiments pooled. Work done in collaboration with D. Polos. Expression levels of *ifn̑* and *ifn̑1.2* were normalised to 18S using the $\Delta\Delta CT$ method. Fold change relative to the median control value. 2 experiments, n=8. Each dot represents WKM from an individual fish. Statistical significance using one-way ANOVA.

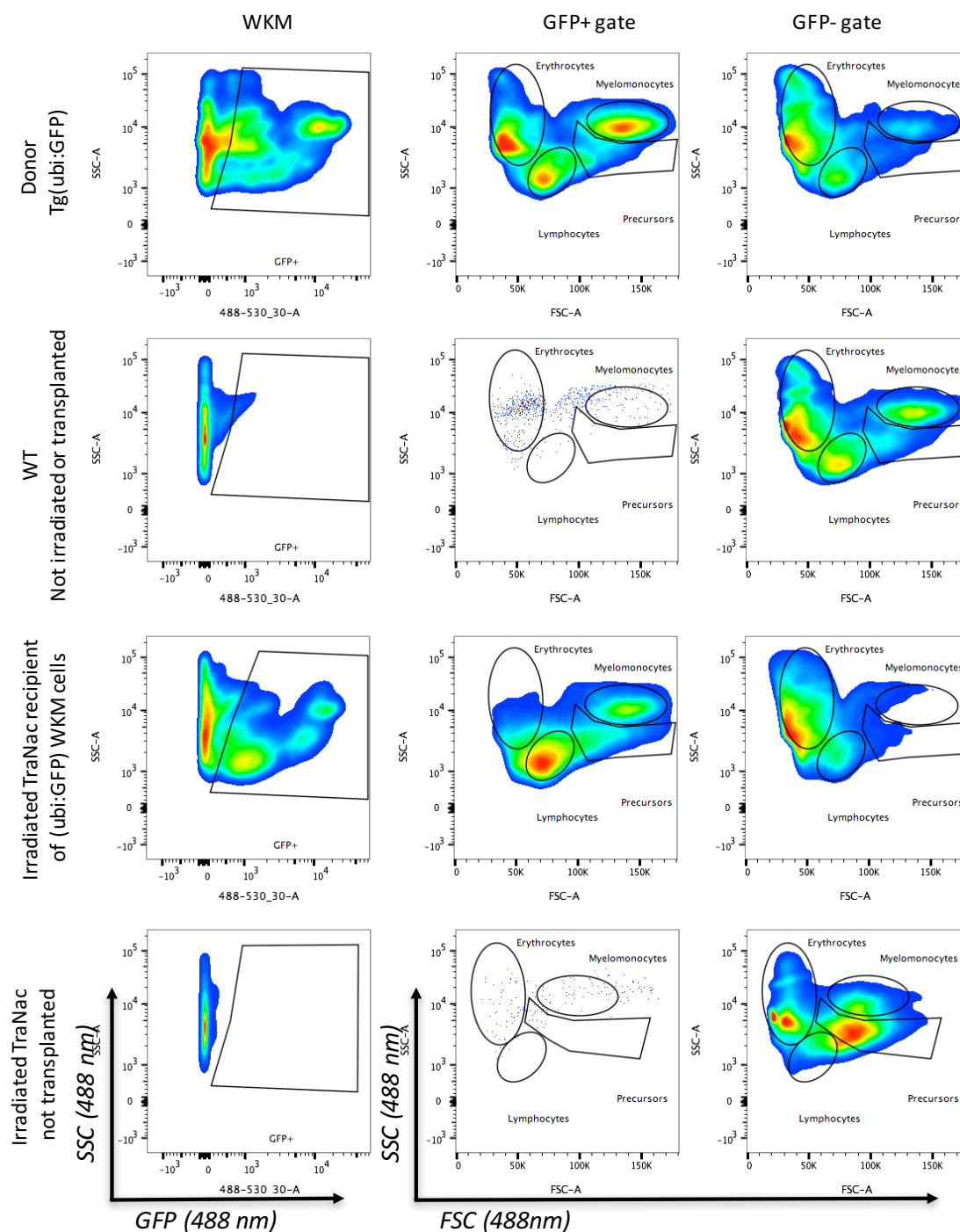
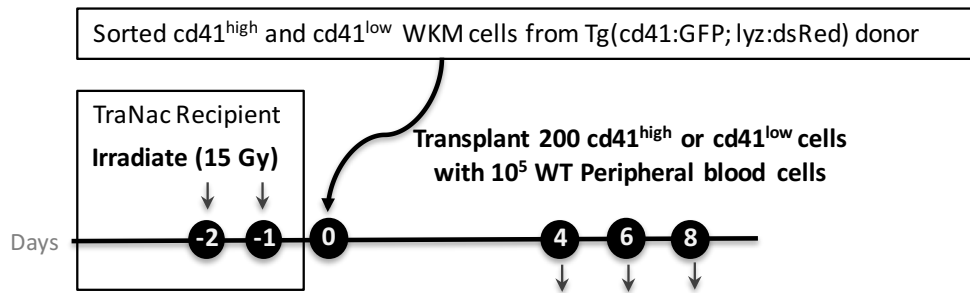
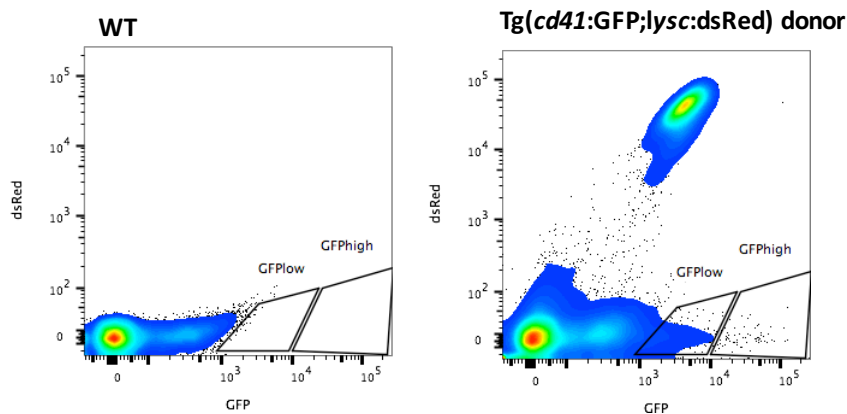


Figure 7.3: Flow cytometry scatter of WKM samples from transplant recipients and non-transplanted Tg (ubi:GFP) and WT fish. TraNac or WT fish were irradiated with 2 doses of 15 Gy and retro-orbitally transplanted with a whole Tg (ubi:GFP) donor WKM (1:1), 10^5 WKM cells or 5×10^4 cells from a Tg (ubi:GFP) donor. (7 experiments pooled; 3 (1:1) transplants, 2 (10^5) and 2 (5×10^4), $n > 11$). Flow cytometry scatter profile of Tg (ubi:GFP) donor, WT, transplant recipient TraNac 28 dpt and non-transplanted irradiated TraNac recipient. SSC indicates side scatter (488 laser, 488/10 filter); GFP indicates (488 laser, 530/30 filter).

A



B



C

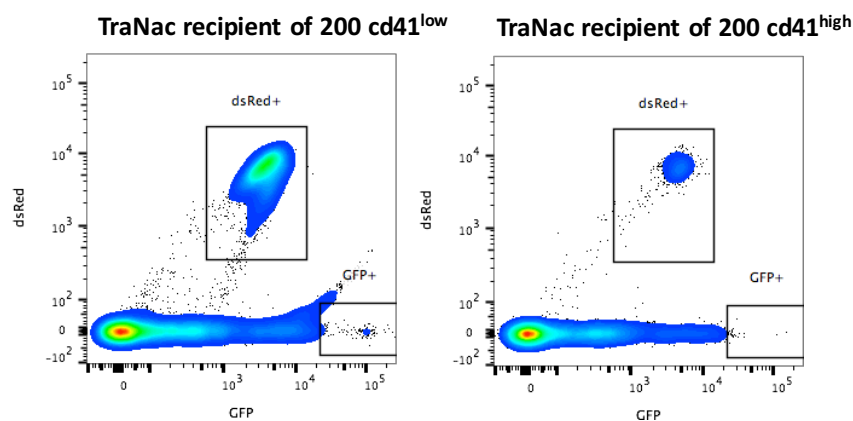


Figure 7.4: Both $cd41^{low}$ and $cd41^{high}$ contain HSPCs with reconstitution ability. WKM cells from Tg ($cd41:GFP$; $lysc:dsRed$) were sorted for $cd41^{high}$ or $cd41^{low}$ cells. (A) Procedure overview. (B) Flow cytometry scatter of donor WKM cells with gating used to obtain populations from donors for transplant. (C) Flow cytometry scatter of recipient WKM, 28 dpt. SSC indicates side scatter; FSC indicates forward scatter (both use 488 nm laser, 488/10 filter), GFP (488 nm laser, 530/30 filter), dsRed (561 nm laser, 610/20 filter)

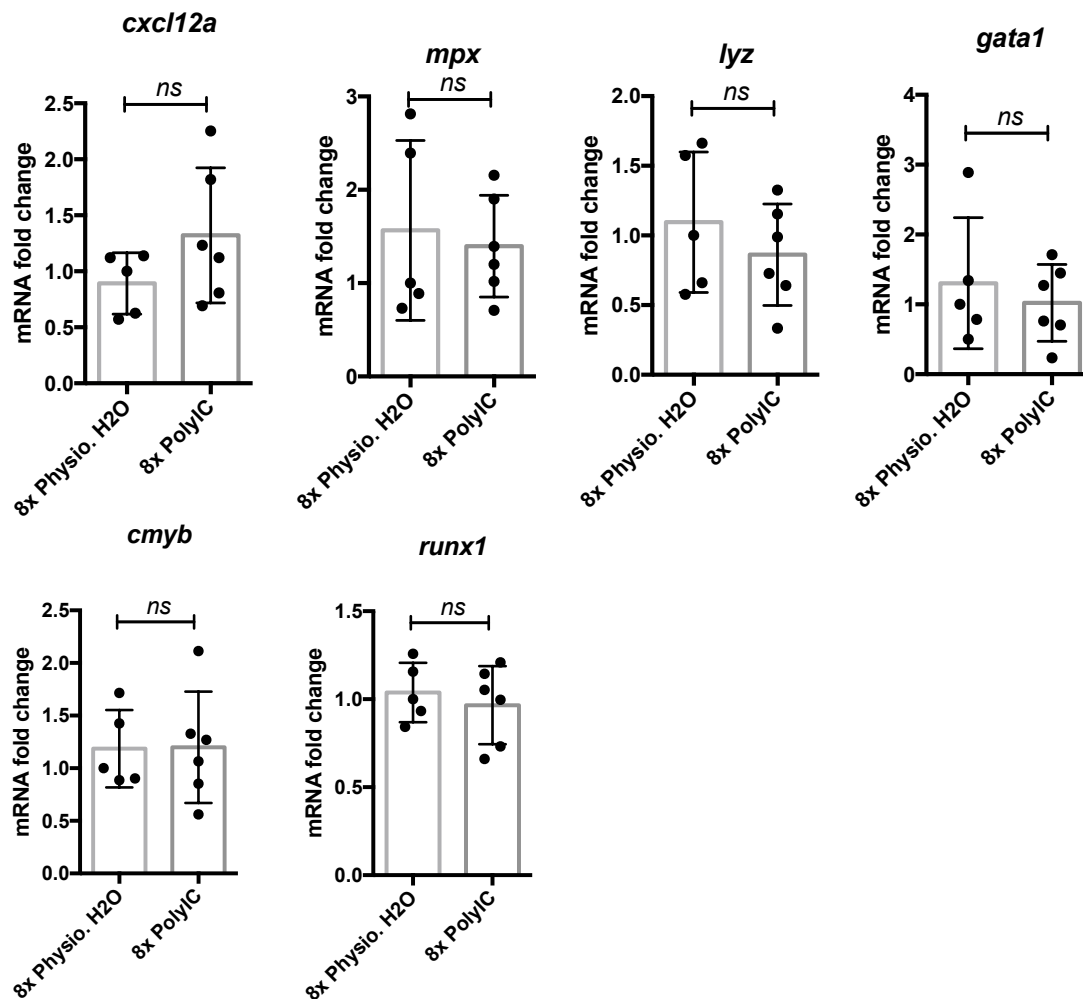


Figure 7.5: Eight sequential injections of poly I:C does not change blood populations significantly at the time of transplant. Tg (cd41:GFP;lyz:dsRed) fish were injected 8 times with either saline or 10 μ g poly I:C. One week post injection, WKMs were harvested to measure changes in blood populations by qRT-PCR. Expression of *cxcl12a*, *mpox*, *lyz*, *gata1*, *cmyb* and *runx1* were normalised to 18S, using $\Delta\Delta$ CT method. Fold change normalised to the median "Saline" control value. Two experiments pooled ($n > 5$). Statistical significance using student t-test. * $p < 0.05$.

Survival of recipients of 200 cd41+ from Saline or Poly I:C injected donors

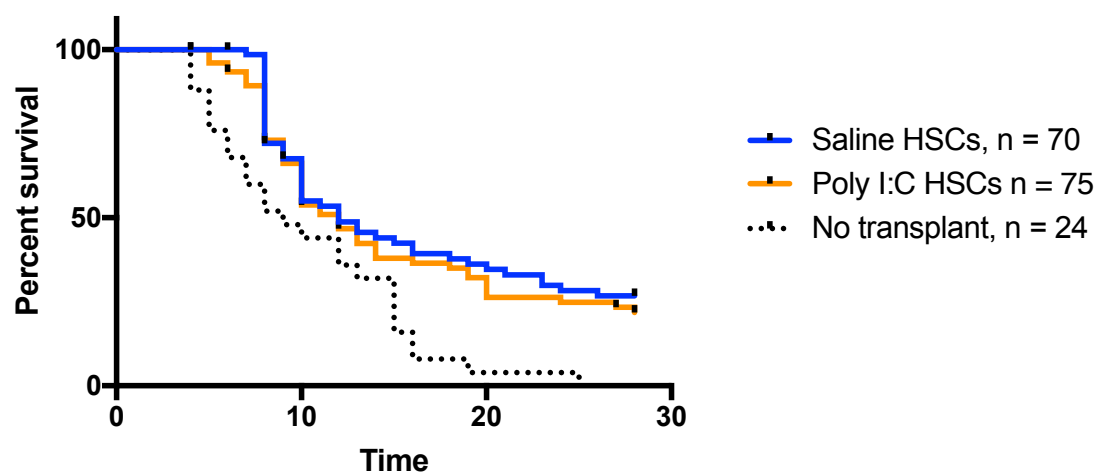


Figure 7.6: Pooled survival. Tg (cd41:GFP;lyz:dsRed) fish were injected 8 times with either saline or 10 μ g poly I:C. One week post injection, donors were harvested of their WKM and sorted for cd41:GFP+ cells for transplant. TraNac recipients were irradiated with 15 Gy 48 and 24 h prior to RO injection of 200 cd41:GFP+ cells from these donors (and 10^5 WT PB cells). Recipients were imaged and then monitored for survival to 28 dpt. (6 experiments pooled, $n > 70$).

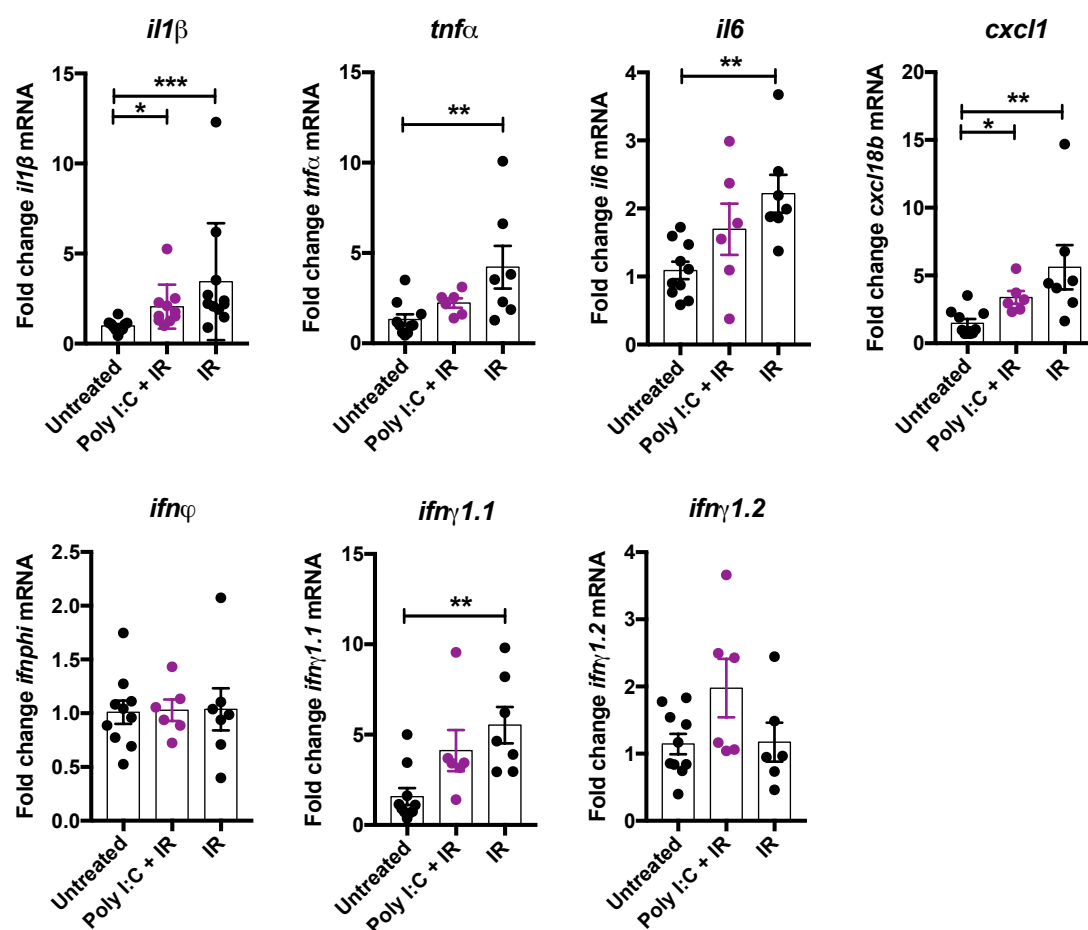


Figure 7.7: Cytokine levels in recipients after irradiation. Poly I:C treated recipient (Poly I:C + IR) TraNac fish were injected with 10 µg poly I:C twice, 48 h apart, then irradiated with 15 Gy 48 h and 72 h later. “Control” recipients (IR) were not injected but were irradiated alongside inflamed recipients. Non-irradiated or injected fish (No IR, No poly I:C) were used as untreated controls. Fish were humanely killed and the decapitated body harvested for analysis. RTqPCR was used to measure relative expression of *il-1β*, *tnfa*, *il-6*, *ifnφ*, *ifnγ1.1*, *ifnγ1.2*, and *cxcl-c1c*, normalised to 18S, using the $\Delta\Delta CT$ method. Three experiments pooled, $n > 8$. “No IR No Poly I:C” and “IR” data presented in in chapter 3.3.1.3. Statistical significance using One-way ANOVA and Tukey post-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

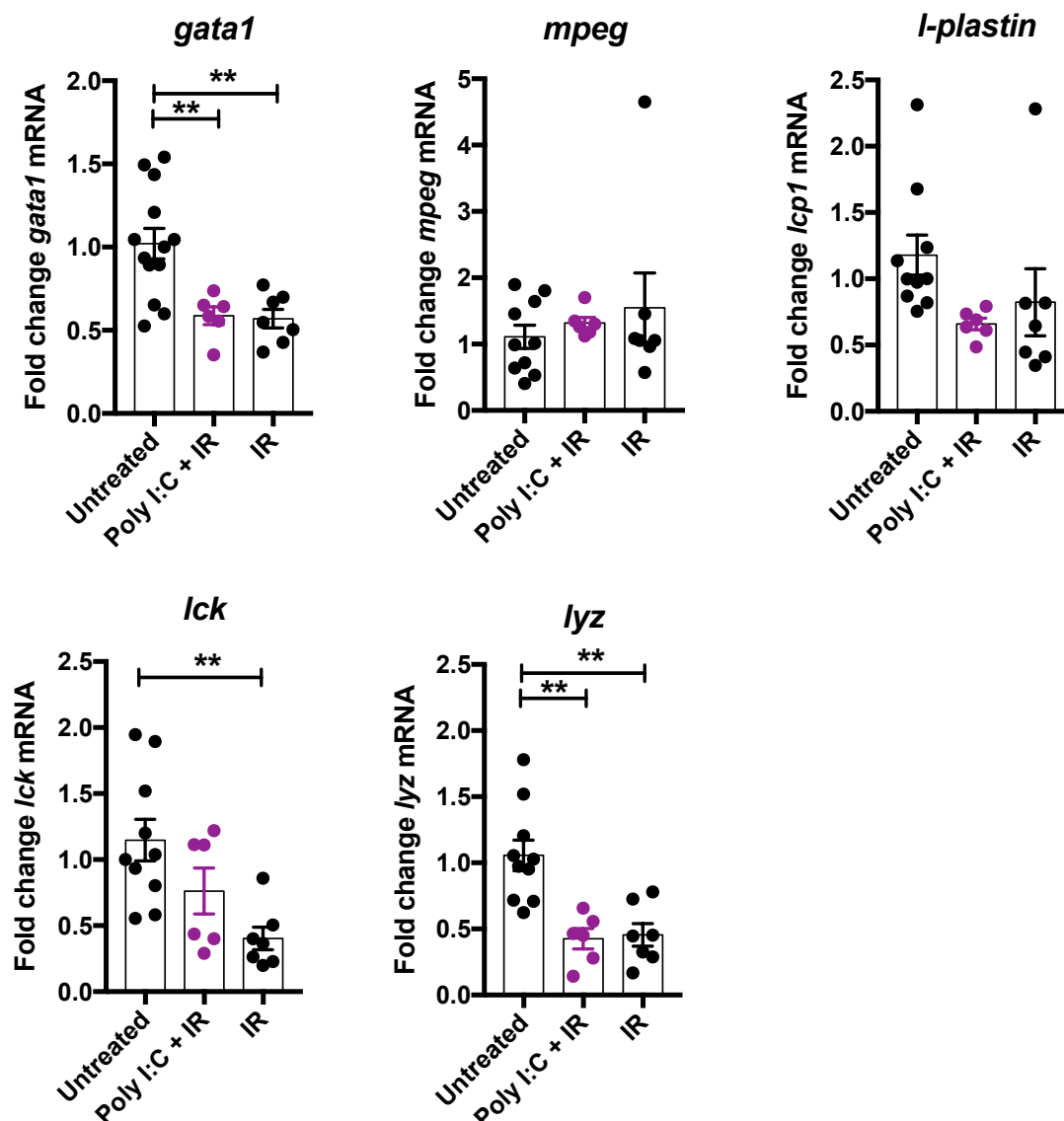


Figure 7.8: Relative transcript levels of specific blood genes in recipients after irradiation. Poly I:C treated recipient (Poly I:C + IR) TraNac fish were injected with 10 μ g poly I:C twice, 48 h apart, then irradiated with 15 Gy 48 h and 72 h later. “Control” recipients (IR) were not injected but were irradiated alongside inflamed recipients. Non-irradiated or injected fish (No IR, No poly I:C) were used as untreated controls. Fish were humanely killed and the decapitated body harvested for analysis. RTqPCR was used to measure relative expression of *gata1*, *lck*, *lyz*, *mpeg*, *mfap4* and *l-plastin*, normalised to 18S, using the $\Delta\Delta$ CT method. Three experiments pooled, n>6. “No IR No Poly I:C” and “IR” data presented in chapter 3.3.1.3. Statistical significance using One-way ANOVA and Tukey post-test. * p<0.05, ** p<0.01, *** p<0.001.

8 References

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