IMPROVEMENTS ON HUMANIZED NOD/SCID IL-2rg$^{-/-}$ MICE

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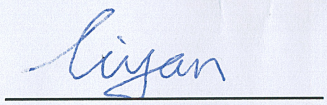
DECLARATION

I hereby declare that this thesis is my original work and it has been written by me in its entirety.

I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously



Li Yan

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In Loving Memory of my Father

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Summary

Humanized mice offer a great platform to study human diseases *in vivo* and to test new therapies. Although much progress has been made to improve human hematopoietic cell engraftment by generating new strains of immunodeficient mice, the limited number of hematopoietic stem cells (HSCs) in a single unit of cord blood, and the poor reconstitution and function of human monocytes/macrophages still remain as major limitations in current non-obese diabetic (NOD) /scid IL-2rg$^{-/-}$ mice (NSG mice).

To overcome these two problems, we first developed a robust HSC co-culture system wherein cord blood CD133^{+}CD34^{+} cells were co-cultured with mesenchymal stem cell (MSC) engineered to express angiopoietin-like-5 (Angptl5) in cytokine supplemented media. The number of double positive (DP) cells was expanded around ~60 fold after 11 days in this culture system, and expanded cells were capable of long-term engraftment in NSG mice. Further, the expanded cells supported multilineage reconstitution of human blood cells in NSG mice, including a more efficient T cell reconstitution. With this expansion method, large-scale experiments with humanized mice reconstituted with cord blood HSCs become possible, and the variation among different donors could be minimized.

The poor engraftment and function of human macrophages in NSG mice were corrected by a single injection of plasmid encoding human macrophage colony-stimulating factor (M-CSF). As the mouse M-CSF does not work on

human cells, we identified that the human macrophage development was blocked at promonocyte stage in the bone marrow (BM) of NSG mice. This is unexpected because M-CSF is believed to play a role at a much earlier stage in mouse macrophage development. After the induction of human macrophages by human M-CSF, we observed engraftment of human macrophages in various organs, such as liver and lung. This is the first direct evidence for human tissue macrophage reconstitution in humanized mice. Moreover, these human tissue macrophages responded to infections with several natural human pathogens *in vivo,* and were able to slow down disease progression. With the engraftment of human tissue macrophages in NSG mice, we not only facilitate the study of human macrophages *in vivo* but also make the humanized mice one step closer to clinical and preclinical applications.

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List of Abbreviations

Angptl5	Angiopoietin-like 5
BCG	Bacillus Calmette-Guérin
BFU-E	Erythroid burst-forming unit
BM	Bone marrow
BSA	Bovine serum albumin
CBA	Cytometric bead array
cDNA	Complementary deoxyribonucleic acid
CD40L	Cluster of differentiation 40 ligand
CSF-1	Colony stimulating factor 1
C/EBP	Ccaat-enhancer-binding protein
CFU	Colony-forming unit
CFU-E	Erythroid colony-forming unit
CFU-G	Granulocyte colony-forming unit
CFU-GEMM	Granulocyte, erythrocyte, monocyte, megakaryocyte colony-forming unit
CFU-GM	Granulocyte, macrophage colony-forming unit
CFU-M	Macrophage colony-forming unit
DAPI	4', 6-diamidino-2-phenylindole
DC	Dendritic cell
DNP-KLH	2,4- Dinitrophenyl-keyhole limpet hemocyanin
DP	Double positive
EBV	Epstein-barr virus
EF1a	Elongation factor-1 alpha
EGR-1	Early growth response protein 1
ELISPOT	Enzyme-linked immunosorbent spot
EPO	Erythropoietin
FACS	Fluorescence-activated cell sorting
FCF	Feeder cell-free

FCS	Fetal Calf Serum
Flt3	Fms-like tyrosine kinase receptor-3
FGF	Fibroblast growth factors
FSC	Forward scatter characteristics
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HBSS	Hank's balanced salt solution
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cell
H&E	Hematoxylin and eosin
IFN	Interferon
Ig	Immunoglobulin
IGFBP2	Insulin-like growth factor binding protein 2
IL	Interleukin
LPS	Lipopolysaccharide
LZM	Lysozyme
M-CSF	Macrophage colony-stimulating factor
Min	Minute
MHC	Major histocompatibility complex
MLR	Mixed lymphocyte reaction
MMM	Mature macrophage marker
MMR	Macrophage mannose receptor
MNC	Mononuclear cell
MOI	Multiplicity of infection
MPO	Myeloperoxidase
mRNA	Messenger ribonucleic acid
MSC	Mesenchymal stem cell

NK	Natural Killer
NOD	Non-obese diabetic
NSG	NOD/scid IL-2rg$^{-/-}$
OD	Optical density
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PMA	Phorbol 12-myristate 13-acetate
PNAS	Proceedings of the National Academy of Sciences
RAG	Recombination-activating gene
RBC	Red blood cell
RT-PCR	Reverse transcription polymerase chain reaction
SCF	Stem cell factor
Scid	Severe combined immunodeficiency
SIRPa	Signal regulatory protein alpha
SRC	Scid repopulating cell
SSC	Side scatter characteristics
TLR	Toll-like receptors
TNF-a	Tumor necrosis factor-alpha
TPO	Thrombopoietin
TT	Tetanus toxoid
UCOE	Ubiquitous Chromatin Opening Element
VDR	Vitamin D receptor
VSVG	Vesicular stomatitis virus glycoprotein

CHAPTER 1

INTRODUCTION

1.1 Introduction to the humanized mice

The term ‘humanized mice’ is used to describe several experimental systems. In this thesis, it refers to immunodeficient mice with components of the human immune system. As a small animal model, humanized mice allow versatile experimentation and are easily accessible to the research community. More importantly, the knowledge obtained from this model may be more relevant and applicable to humans. Because of the differences between human and murine immune systems, the direct extrapolation of data from studies of mouse models to the clinic becomes complicated, sometime inappropriate. For example, TGN1412, a humanized monoclonal antibody binding to CD28 receptor of T cells, was originally developed for treatment of B cell chronic lymphocytic leukemia and rheumatoid arthritis. In its first clinical trial in 2006, it caused catastrophic systemic organ failure in the treated subjects at the administered dose, which was 500 times lower than the dose found safe in animal tests (Hansen and Leslie, 2006; Kenter and Cohen, 2006; St Clair, 2008; Stebbings et al., 2009; Suntharalingam et al., 2006). In a recent study from our lab, cytokine storm similar to what has been reported in clinical trials was observed in improved humanized mice (manuscript in preparation). Hence, the humanized mice offer a tool to bridge the bench and bed gap without putting the patient into risk.

Despite calls for humanized mice, the development of a mouse strain permissive for good human cell engraftment has been slow. In the past 20 years, the increasing use of clinical hematopoietic cell transplantation in genetic malignant diseases and immunodeficiencies, as well as the emergence of threatening pandemics caused by infectious diseases, motivated many scientists to improve humanized mice from

extremely low human hematopoietic cell reconstitution to a functional immune system. Thus, several major breakthroughs in this field are reviewed below.

1.1.1 The development of humanized mice

The history of humanized mice began with the discovery of a Prkdc^{scid} mutation (protein kinase, DNA activated, catalytic polypeptide; severe combined immunodeficiency) mutation in CB17 mice (Bosma et al., 1983). The Prkdc^{scid} mutation results in the absence of the catalytic subunit of a DNA-dependent protein kinase (Araki et al., 1997; Blunt et al., 1996; Fried et al., 1996; Jeggo et al., 1996; Kirchgessner et al., 1995), which plays an indispensable role in activation of a DNA recombinase enzyme during V(D)J recombination of lymphocyte antigen receptors (Lieber et al., 1988; Malynn et al., 1988; Miller et al., 1995). Hence, this strain of mice lacks mature and functional T lymphocytes. This scid mouse model allowed only 0.5%-5% human cell reconstitution, but it was already a successful 'proof of concept'. The extremely low levels of human reconstitution are due to the presence of relatively intact murine innate immunity (e.g. murine monocytes, macrophages, and natural killer cells) and the leakiness of the mutation itself (Bosma et al., 1988; Shultz et al., 1995).

In an effort to overcome the rejection of human graft caused by murine myeloid cells and natural killer (NK) cells in CB17-scid mice, the scid mutation was introduced into NOD mice, which reached 5-10 fold higher levels of human peripheral blood mononuclear cell (PBMC) reconstitution (Hesselton et al., 1995). The improvement in NOD/scid mice was thought to be due to additional defects in innate immunity of NOD strain. For instance, NOD mice carry a polymorphism in signal regulatory protein (SIRP) alpha, which serves as the inhibitory receptor on mouse macrophage to

prevent the phagocytosis of human cells (Takenaka et al., 2007). The hemolytic complement pathways in NOD mice are also inactive due to the specific deficiency of C5 complement (Baxter and Cooke, 1993). Moreover, the NOD-scid mice are resistant to the leakiness seen in CB17-scid mice, as only 10% of mice have detectable immunoglobulin (Ig) in serum at 6 months of age compared to 90% of CB17-scid mice at the same age (Shultz et al., 1995). Hence, the NOD/scid mice became the gold standard for human cell engraftment studies and were widely used as xenotransplant recipient mice since 1995. The usefulness of this model, however, is limited by the short life span caused by the development of fatal thymic lymphomas around 8 months old (Prochazka et al., 1992; Shultz et al., 1995). In addition, although the engraftment of human cells is much improved in NOD/scid mice, high levels of human cell engraftment are still not seen. This is likely due to remaining NK cell activity in NOD/scid mice (Greiner et al., 1998).

To increase further the engraftment of human cells, several methods to genetically block the development of NK cells were tested. One major success was achieved in 2005, when Shultz and colleagues back-crossed immunodeficient mice lacking the gene for the common cytokine-receptor γ chain to the NOD/scid mice to create NOD/scid IL-2rg$^{-/-}$ (NSG) mice (Ishikawa et al., 2005; Shultz et al., 2005). The common γ chain is a critical component for interleukins (IL) 2, 4, 7, 9, 15, 21 receptors (Asao et al., 2001; Sugamura et al., 1996). These cytokines play myriad roles in differentiation, maturation, activation and homeostasis of T cells (IL-2, IL-7 and IL-15), B cells (IL-7, IL-21), and NK cells (IL-2, IL-15) (Janeway, 2001; Kovanen and Leonard, 2004). As a result of this deficiency, not only mouse T and B cells, but also NK cells do not develop in NSG mice (Shultz et al., 2005). The average human cell engraftment in this model is reported to be 6 times higher compared to

NOD/scid mice (Shultz et al., 2005). Furthermore, NSG mice are free of thymic lymphomas, which increase the lifespan from 37 weeks in NOD/scid mice to 90 weeks in NSG mice (Shultz et al., 2005). This much longer life span makes long-term experiments possible, especially for HIV studies. It is NSG mice reconstituted with human CD34+ cells that will be the focus of this thesis.

1.1.2 Remaining issues of humanized NSG mice

The previous efforts to improve humanized mice specifically aimed for high levels of human cell engraftment by the creation of various mouse strains with deficiencies in mouse immunity. This goal has been fulfilled by the creation of NSG mice. The current focus of humanized mice switched to establishing a functional human immune system and using this model for preclinical and clinical applications. Several critical issues that need to be addressed in order to achieve this aim will be discussed below:

1.1.2.1 Stable reconstitution and the availability of human HSC

At least $1x10^5$ human $CD34^+$ cells are needed to achieve >10% reconstitution in newborn pups or in adult NSG mice, and the reconstitution is only stable between 2 to 12 months. There are too few human cells before 2 months of reconstitution and one type of human blood cells, usually human T cells, dominates after 10 months of reconstitution. In contrast, single syngeneic mouse HSC could lead to more than 50% hematopoietic chimerism for at least 10 months, whereas 10 fold greater numbers of mouse HSCs are required for fully major histocompatibility complex (MHC)-mismatched recipients. In addition, homeostatic expansion of human $CD34^+$ cells is not observed in humanized mice, and the frequency of scid repopulating cells (SRC) and chimerism decline over time. This evidence suggests that human HSC are not maintained in humanized mice (Manz, 2007). This could be due to the difficulty of human HSC homing to mouse BM, or the insufficient support from mouse BM niches, or the residual mouse immunity against human HSCs. To improve this situation, co-transplantation of human MSC (Muguruma et al., 2006) or mouse MSC engineered to express human cytokines with human HSCs may benefit the homing and maintenance of human HSC. Genetically engineering human HSCs to express

mouse MHCs or to transiently express certain mouse chemokine receptors may also help the homing and long-term reconstitution. Till now, not much progress has been made on this issue of humanized mice.

The common sources for human $CD34^+$ cells are human cord blood, human fetal liver, and less frequently, cytokine mobilized adult peripheral blood and adult BM. Since the $CD34^+$ cells isolated from adult humans are less effective to reconstitute humanized mice, $CD34^+$ cells from cord blood or fetal liver are typically used (Lepus et al., 2009). As the availability of human fetal liver is uncertain, and the use of fetal samples for research purpose is strictly regulated, many laboratories working with humanized mice use cord blood as their routine source for human $CD34^+$ cells. However, the number of $CD34^+$ cells isolated from a single unit of cord blood is around $0.2\text{-}1x10^6$, which is only enough to reconstitute 1-5 newborn pups. The limited number of mice produced from one unit of cord blood makes it difficult to set up experiments with proper controls. Humanized mice made from different batches of cord blood or from pooled human $CD34^+$ cells may be used to increase the number to meet the requirement of experiments. However, the results obtained from different units of cord blood often make it difficult to draw conclusions because the reconstitution kinetics and ability vary among batches (Drake et al., 2011). In addition, the purchase of one unit of cord blood and the isolation process typically cost several hundred dollars. It would be prohibitively expensive for a lab to generate humanized mice routinely with this method. Thus, it would be desirable to expand a population of long-term reconstituting human HSCs *in vitro* so that an entire study or one set of experiments could be performed from a single unit of cord blood.

1.1.2.2 Unbalanced human hematopoietic cell reconstitution

8 to 12 weeks after injection, human HSC and progenitor cells differentiate and form a human hemato-lymphoid system in NSG mice. Almost all major cell populations of the human immune system, including dendritic cells (DC), natural-interferon producing cells, T cell, B and Ig-producing cells, could be found in organs and peripheral blood of NSG mice. However, the level of human cell reconstitution differs significantly among different lineages.

B cells: Human B cell differentiation in BM of humanized mice seems to resemble that in humans since several stages of B cell precursor populations exist, such as early B cells, pro-B cells, pre-B cells and immature B cells (Watanabe et al., 2009). However, the peripheral mature B cells in humanized mice express high levels of CD5 (Matsumura et al., 2003), which is markedly different from human B cells found in adult peripheral blood. CD5 was considered a marker for B-1 cells, a minor fraction of B cells characterized by three functions: spontaneous IgM secretion, efficient T cell stimulation, and tonic intracellular signaling (Griffin et al., 2011). The origin and development of B-1 cells are still unclear but evidence suggests that they may arise from precursors in fetal liver, and certain antigens are needed to shape the repertoire of the B-1 population (Bendelac et al., 2001). Recently, B1 population is defined by $CD20^{+}CD27^{+}CD43^{+}CD70^{-}$ in umbilical cord blood and adult peripheral blood and the overlap of this population with $CD20^{+}CD5^{+}$ is only ~34% (Griffin et al., 2011). As the majority of B cells in humanized mice are $CD5^{+}$ B cells, further characterization of this population is needed to understand the problem of human B cell development.

T cells: The major achievement in NSG mice is efficient intrathymic *de novo* human T cell development (Gimeno et al., 2004; Ishikawa et al., 2005; Shultz et al., 2005). T

cells in NSG mice, including CD4 and CD8 T cells, show a broad Vβ distribution. The ratio among CD4 T cells, CD8 T cells, $Foxp3^+$ $CD25^+$ regulatory T cells and γδ T cells is quite close to physiological level. The problem for human T cells in humanized mice is poor activation and function, not the development or reconstitution. That problem will be discussed separately.

NK cells: NK cells development could be detected in BM as early as 4 weeks after HSC transfer in humanized mice. However, the number of peripheral NK cells is generally low or undetectable (Chen et al., 2009). The poor reconstitution of human NK cells possibly results from inappropriate cytokine cross-reactivity between mouse and human (Manz, 2007). In 2009, hydrodynamic injection was used to express human IL-15 and Flt-3/flk2 required for NK cell development. This method successfully improved the reconstitution and function of NK cells in NSG mice (Chen et al., 2009). In other studies, human NK cells were also induced by *in vivo* supplementation of human IL-15/human IL-15Rα complex or by transgenic expression of human IL-2 in NOG mice (Huntington et al., 2009; Ito et al., 2012).

Monocytes/Macrophages and DCs: Although monocytes/macrophages and DCs develop in humanized mice, their frequencies are usually very low. In a study from our lab, expression of human granulocyte-macrophage colony-stimulating factor (GM-CSF) /IL-4 and human macrophage colony-stimulating factor (M-CSF) also resulted in significantly increased numbers of human DCs and monocytes in peripheral blood of humanized mice, respectively (Chen et al., 2009). Similarly, monocytes and alveolar macrophages were boosted in M-CSF and GM-CSF/IL-3 human cytokine knock-in NSG mice, respectively (Rathinam et al., 2011; Willinger et al., 2011). But to date, there is no report of human tissue macrophages in humanized

mice, except for alveolar macrophages in GM-CSF/IL-3 mice, and *in vivo* immune functions in NSG mice with improved monocyte reconstitution have not been reported.

Granulocytes and erythrocytes: Granulocytes and erythrocytes comprise the largest fraction of human hematopoietic cells. These two cell types, however, are the lowest reconstituted populations in humanized mice. Several groups attempted to improve these two types of cells but without much success. A slight increase in $CD15^+$ granulocytes was observed in the BM of stem cell factor (SCF), GM-CSF and IL-3 transgenic NSG mice (Billerbeck et al., 2011). Some human $CD66^+$ granulocytes also were produced in the BM of Thrombopoietin (TPO) knock-in mice (Rongvaux et al., 2011). In a study from our lab, a low but significant level of human erythrocytes in NSG mice could be induced by hydrodynamic injection of plasmid expressing erythropoietin (EPO) and IL-3 (Chen et al., 2009). However, the induction took more than one month. Collectively, the development of granulocytes and erythrocytes could be partially improved by the addition of human cytokines.

1.1.2.3 Weak antigen specific T cell and B cell responses

Upon unspecific stimulation, these generated human T cells are capable of proliferating and producing cytokines both *in vitro* and *in vivo*. However, human T cells have a limited ability to respond to antigen stimulation. To understand this problem, we have to unravel what MHC is to be expected from human T cells educated on a mouse background. As the positive selection of thymocytes preferentially occurs on epithelial cells, and no evidence of human thymic epithelial cells could be derived from reconstituted $CD34^+$ cells, human thymocytes should be positively selected on mouse MHC content. There has been a report of human DCs in the mouse thymic DC compartment, and negative selection of thymocytes occurs on

both epithelial cells and hematopoietic derived DCs. Hence, human thymocytes may be negatively selected on both mouse and human MHC content.

This hypothesis of human thymocyte selection in a mouse thymus was supported by the mixed lymphocyte reactions (MLRs), in which human T cells from NSG mice proliferated vigorously when stimulated with human allogeneic DCs, but proliferated weakly when stimulated with autologous human DCs or mouse DCs (Traggiai et al., 2004). In addition, we used humanized mice to establish Epstein-Barr virus (EBV) and dengue infection models in our lab, but human T cells in mice were unable to control the virus replication (unpublished data). There were also studies on influenza virus infection in humanized mice. They reported that human T cells specific for viral epitopes were only found in the context of mouse MHC (Legrand et al., 2006). Taken together, the evidence suggests that human T cells in mice are tolerant for both autologous human and mouse MHC. They frequently mount reactions towards allogeneic MHC but produce, to some extent, specific responses only in the context of autologous mouse MHC.

As a result of insufficient human T cell responses *in vivo*, antibody production in humanized mice is far from optimal. Human IgG concentration in serum increases over the course of reconstitution, and is on average 10-100 fold lower than in human adults and wild-type mice (Baenziger et al., 2006; Traggiai et al., 2004). Specific IgG responses against tetanus toxoid (TT), ovalbumin, and dinitrophenyl-keyhole limpet haemocyanin (DNP-KLH) are very low or undetectable (Baenziger et al., 2006; Ishikawa et al., 2005; Traggiai et al., 2004). To enhance the antibody response in humanized mice, the key is to establish a robust T cell response to adequately help B cells mature into antigen-specific antibody-producing cells. The obvious solution to this problem is to replace the mouse MHC with human MHC components. Till now,

NSG mice with human MHC class I have been created. Cytotoxic human T lymphocytes became functionally mature, and mounted human leukocyte antigen (HLA)-restricted cytotoxicity against EBV infected human B cells in NSG-HLA-A2 mice (Jaiswal et al., 2012). However, the antibody response in this mouse was not much improved. The NSG-HLA-DR mice and the NSG-HLA/A2-HLA/DR mice are still under development by the Jackson Laboratory (http://research.jax.org/collaboration/escell.html).

1.2 Focus and objectives of this thesis

Despite high human cell engraftment in humanized NSG mice, those issues discussed above greatly hampered its impact and application in basic and applied preclinical human immunology research. In this thesis, we only focused on three critical problems of humanized mice:

Limited supply of human cord blood HSCs and variations from small cohorts of cord blood HSCs in reconstituted humanized mice

Chapter 3 describes the efforts to develop a method for HSC expansion *in vitro*. Four goals were addressed: 1) Establish and optimize the conditions for human HSCs expansion. 2) Evaluate and compare the differentiation potential of expanded HSCs into hematopoietic lineages in NSG mice. 3) Evaluate the long-term reconstitution potential of expanded HSCs. 4) Investigate the functions of human immune system in humanized mice engrafted with expanded HSCs.

Poor understanding of human macrophage development and no tissue macrophage reconstitution in humanized mice

Chapter 4 shows the efforts to generate human monocytes/macrophages in humanized mice. Four goals were addressed 1) Investigate the problem of human monocyte/macrophage development in humanized mice. 2) Develop methods to restore the monocyte/macrophage population in peripheral blood and tissues of humanized mice. 3) Test the functions of induced monocytes/macrophages. 4) Evaluate the innate immunity in humanized mice with improved monocyte/macrophage reconstitution.

Weak antigen specific antibody response in humanized mice

Chapter 5 shows the efforts to increase antigen specific antibodies in humanized mice. Two approaches were designed: 1) Expression of human CD40 ligand (CD40L) on human T cells to promote the maturation and proliferation of antigen-experienced B cells. 2) Expression of NSG mouse MHC class II (IA-g7) on reconstituted human cells for proper T cell activation and antibody response.

CHAPTER 2

MATERIALS AND METHODS

2.1 Stem cell purification

Purified human $CD133^+$ cord blood cells were purchased from AllCells (California). Alternatively, umbilical cord blood was obtained from the Singapore Cord Blood Bank. Red blood cells (RBCs) were removed by Ficoll-Hypaque density gradient.

Human fetal liver samples were obtained from aborted fetuses at 15-23 weeks of gestation, in accordance with the institutional ethical guidelines of the National University Hospital of Singapore. All women gave written informed consent for the donation of their fetal tissue for research. Fetuses were collected within 2 h of the termination of pregnancy. A single cell suspension was prepared. To isolate human $CD34^+$ cells, fetal liver samples were excised into small pieces and digested with collagenase IV (2 mg/ml) at 37 °C for 15 min. A single cell suspension was prepared by passing the digested tissue through a 100 μm cell strainer (BD Biosciences).

$CD34^+$ cells were purified with the RosetteSep system using the CD34 positive selection kit (Stem Cell Technologies, Vancouver, BC). The purity of purified cells was 90 to 99% $CD34^+$. On average, 95% of the purified $CD34^+$ cord blood cells, and 30% of purified $CD34^+$ fetal liver cells were also $CD133^+$ (data not shown). Following *in vitro* expansion, $CD133^+$ cells were purified by staining cells with PE-conjugated anti-CD133 (Ebiosciences) followed by a PE positive selection kit (Stem Cell Technologies).

2.2 Feeder cell-free (FCF) culture of cord blood $CD34^+CD133^+$ cells

Cryopreserved or freshly isolated $CD34^+CD133^+$ cord blood cells were cultured *in vitro* as described (Zhang et al., 2008). Briefly, StemSpan medium (Stem Cell Technologies, Vancouver) were supplemented with 20 ng/ml SCF (R&D), 10 ng/ml TPO (R&D), 10 ng/ml fibroblast growth factors-1 (FGF-1) (Gibco), 100 ng/ml Insulin-like growth factor-binding protein 2 (IGFBP2) (R&D), 500 ng/ml angiopoietin-like 5 (Angplt5) (Abnova, Taiwan), 500 μg/ml of heparin (Sigma), 1x penicillin and streptomycin (Gibco) to obtain the expansion media. About 10^4 $CD34^+CD133^+$ cord blood cells were plated in a 96-well round bottomed plate, in 200 ml/well of the expansion media. Cells were transferred to a 6-well plate 4 days later, and fresh media were added to keep cells at 200,000 /ml one day after the transfer. Cells were supplemented with more fresh media 2-3 days later, in order to maintain them at 700,000 cells/ml and then allowed to expand until the end of the 11-day culture.

2.3 Angplt5-expressing MSCs

The human Angptl5 gene (DNA NM_178127.2) was excised from the pCMV6-XL5 vector (Origene, Rockville, USA) using Not I, and cloned into the pLB2 lentiviral vector at the Not I site. The resulting vector encodes both Angplt5 and GFP under the same EF1a promoter (provided by Dr. Patrick Stern of Massachusetts Institute of Technology). To produce lentiviruses, 293FT cells were co-transfected with lentiviral vectors, the HIV-1 packaging vector Delta8.9 and the VSVG vector. Supernatants containing lentiviruses were harvested on day 2 or 3, concentrated by

ultracentrifugation at 25,000g for 90 min, and frozen until use. Lentivirus titers were 10^7-10^8 /mL based on GFP expression in 293FT cells.

Human MSCs from the BM of adult donors were purchased commercially (Lonza or Stem Cell Technologies). MSCs were cultured in MesenCult MSC Basal Medium and Mesenchymal Stem Cell Stimulatory Supplements (Stem Cell Technologies, Vancouver) at a density of 5,000-6,000 cells per cm^2 in a T175 flask, and passaged before reaching 70% confluence. MSCs from 2nd to 4th passages were transduced with a lentiviral vector (pLB2) expressing GFP alone or both GFP and Angplt5 at MOI of 5. Four to five days post-transduction, 30-45% of cells were GFP^+ by flow cytometry analysis. The mixture of transduced and non-transduced MSCs were expanded and used for co-cultures.

2.4 Co-culture of cord blood $CD34^+CD133^+$ cells

MSC-A5 or MSC-GFP was plated in a 24-well plate at 5 x 10^4 cells/well overnight. $CD34^+CD133^+$ cord blood cells were added to the culture at a cord blood cell-to-MSC ratio of 1:5 (i.e., 10^4 cord blood cells for $5x10^4$ of pre-plated MSCs per well). The expansion media was used for co-culture except for the addition of Angptl5. The initial volume of culture media was adjusted to 300 µl/well, and fresh media were added every 2 days starting on the 3rd day of culture. At different time points of the culture, hematopoietic cells were resuspended by carefully pipetting 5-7 times (avoiding the detachment of the MSCs), counted and analyzed by flow cytometry to obtain the total cell number and the number of DP cells. After 11 or 14 days of culture, hematopoietic cells were resuspended thoroughly (to loosen strongly attached cells to the feeder layer), analyzed for CD34 and CD133 expression.

2.5 Mice and intracardiac injection

NSG mice were obtained from the Jackson Laboratory and maintained under specific pathogen-free conditions in the animal facilities at Massachusetts Institute of Technology, National University of Singapore and Nanyang Technological University. Pups within 48 h of birth were sublethally irradiated (100 rad) using a Cesium source, and engrafted with either expanded or unexpanded cells by intracardiac injection.

For engraftments, either 10^5 purified $CD34^+CD133^+$ unexpanded or expanded cells in 50 μl were injected per recipient, or total expanded cell suspension containing 10^5 $CD34^+CD133^+$ cells in 50 μl were injected per recipient. All research with human samples and mice was performed in compliance with the institutional guidelines.

2.6 Serial reconstitution assays

Serial reconstitution was performed as follows: Fourteen weeks after the reconstitution, BM cells were harvested from both femurs and tibias of the primary recipients. Human $CD34^+$ cells were stained with PE-conjugated anti-CD34 followed by a PE positive selection kit (Stem Cell Technologies). $CD34^+$ cells were pooled from different mice reconstituted with expanded cells from the same cord blood donor, and then 10^4 cells were injected into sublethally irradiated newborn pups.

2.7 Single cell preparation

Single cell suspensions were prepared from the spleen and BM by standard procedures. To isolate hepatic mononuclear cells (MNC) from humice, the liver was

pressed through a 200-gauge stainless steel mesh, and debris was removed by centrifugation at 50 g for 5 min. Supernatants containing MNCs were collected, washed in PBS, and resuspended in 40% percoll (Sigma) in RPMI medium 1640. The cell suspension was gently overlaid onto 70% percoll, and centrifuged at 750 g for 20 min. MNCs were collected from the interphase, washed twice in PBS. To isolate lung MNCs, lungs were minced, suspended in medium containing 0.05% collagenase (Sigma) and 0.01% DNase I (Sigma), and incubated at 37 °C for 20 min. The lung samples were passed through a 200-gauge stainless steel mesh, and MNCs were isolated by percoll centrifugation as described above. To isolate MNCs from humanized mice brain, the brain was homogenized in 7 ml RPMI 1640 and added 3 ml stock isotonic percoll to make 30% percoll. 10 ml cell suspension was gently laid onto 70% percoll, and centrifuged at 500 g for 30 min. MNCs were collected from the interphase, washed with HBSS, and centrifuged for 7 min at 500 g.

2.8 Flow cytometry and cell sorting

MNCs were analyzed by flow cytometry using FACScalibur, FACS-Canto or LSR II cytometers (Beckton Dickinson). Cell sorting of monocyte/macrophage progenitors in BM was performed using FACS Aria cell sorter (BD Biosciences). The following antibodies were used: CD45 (2D1), CD34 (581), CD38 (HIT2), CD209 (DCN46) from BD Biosciences; CD14 (HCD14), CD45.1 (A20), HLA-DR (L243), CD33 (WM53), MMR (15-2), CD209 (9E9A8), CD56 (MEM-188), CD3 (HIT3a), CD19 (HIB19), CCR5 (HEK/1/85a), CD40 (5C3), CD7 (CD7-687), CD116 (4H1), CD114 (LMM741), CD15 (HI98), TLR2 (TL2.1), CD11c (3.9), CD11b (ICRF44), CD36 (5-271), CD80 (2D10), CD86 (IT2.2), CD16 (3G8), CD64 (10.1), CD163 (GHI61), CD68 (Y1-82A) from Biolegend; CD13 (WM15), MMM (25F9), TLR4(HTA125)

from Ebiosciences. Dead cells were excluded from analysis by DAPI staining. Analyses were performed with the FACS Diva (BD Biosciences) or FlowJo (TreeStar Inc).

2.9 Immunization and ELISPOT assay

Sixteen weeks after engraftment, mice were immunized intraperitoneally (*i.p.*) with 10 µl of TT vaccine adsorbed on hydrated aluminum hydroxide (Teabag, Sanofi Pasteur, France) diluted in 90 µl PBS, representing 1/50 of the recommended vaccination dose for a human adult. Mice were boosted twice with the same dose at three-week intervals. Two weeks after the third immunization, mice were sacrificed and the frequencies of IFN-γ secreting cells in the spleen were measured by ELISPOT assay (Ebiosciences, San Diego, CA). Briefly, single cell suspensions were prepared from spleens, counted and analyzed by flow cytometry for the frequency of human $CD3^+$ T cells. $5x10^5$ $CD3^+$ cells were plated per well in a 96-well flat-bottomed plate (Multiscreen-IP, Millipore, MA, USA) that was coated overnight at 4 °C with anti-IFN-γ monoclonal antibody. For T cell stimulation, 10 ng/ml of PMA or 0.5 mg/ml of the tetanus toxin peptide (830-843) (Genscript, USA) were added to the culture. After 48 hours of incubation at 37 °C, 5% CO2, IFN-γ immunospots were detected according to the manufacturer's instructions. The spots were counted using an ImmunoSpot S5 Versa Analyzer (Cellular Technology Ltd. Ohio, USA) and analyzed with ImmunoCapture software (Analysis Software).

2.10 Hydrodynamic injections

pcDNA3.1 (+) vector was used to clone human cytokine genes. Plasmid DNA was prepared by Maxi-prep Kit (Promega) with endotoxin removal. For hydrodynamic injection, 50 μg plasmid of each cytokine gene in 2 ml PBS was injected into mice older than 8 weeks within 7 seconds using a 27-gauge needle.

2.11 *In vitro* differentiation assay and colony-forming unit (CFU) - assay

BM MNCs were flushed out from tibias of 12-week old humanized mice. Sorted monocyte/macrophage progenitors from BM MNCs were cultured in RPMI 1640, 10% FCS, supplemented with 50 ng/ml M-CSF for macrophage differentiation or 50 ng/ml GM-CSF, 50 ng/ml IL-4 for DC differentiation. All cytokines were purchased from R&D Systems.

Sorted monocyte/macrophage progenitors were plated at 2000, 5000, 10000 cells per dish in complete MethoCult media (MethoCult H4435 enriched; Stem Cell Technologies) according to the product manual. Cells were incubated in 35 mm culture dishes at 37 °C with 5% CO_2. The numbers of colonies were counted under a stereomicroscope after 14 days in culture.

2.12 RNA extraction and real-time PCR

Total RNA was extracted from lysed tissues or cells with the RNeasy Plus Mini kit (Qiagen) according to manufacturer's instructions. 1 μg of RNA was used for cDNA synthesis with ImProm-II Reverse Transcription System (Promega). Quantitative RT-

PCR was performed with the CFX96 Real-Time System (Bio-rad) with self-designed primers (supplementary Table 1). Expression values were calculated by the comparative threshold cycle method, and normalized to mouse L32 or human GAPDH.

2.13 Histology and Giemsa staining

For histology, each mouse was intracardiacally perfused with saline buffer for 10 min. Organs were harvested, placed in disposable molds, immersed in OCT (the embedding compound) and flash frozen in liquid nitrogen. The blocks were stored at -80 °C and cut at 10 μm per section in histology laboratory at National University of Singapore.

Hematoxylin and eosin (H&E) staining was performed on formalin-fixed, frozen spleen sections. Immunofluorescence staining was performed on 75% acetone/25% ethanol fixed, frozen spleen sections. Monoclonal antibody to CD20 (L26, Abcam, UK), CD68 antibody (KP1, Abcam), mouse CD68 antibody (FA-11, Abcam), and polyclonal antibodies to CD3 (Abcam) were used as primary antibodies. Alexa fluro647 donkey anti-mouse IgG (Invitrogen, USA) and Alexa fluro546 donkey anti-rabbit IgG (Invitrogen) were used as secondary antibodies. H&E stains and immunofluorescence stains were visualized with the MIRAX MIDI Fluorescence microscope (Zeiss).

For Giemsa staining, 10^5 cells in 100 μl 2% FCS-PBS were added to the cytospin cassette, and centrifuged 1000 rpm for 5 min. The cell smears on polylysine-coated slides were air-dried, fixed with methanol for 1 min, stained with Giemsa for 5 min

and washed with distilled water. The stained slides were examined under a microscope.

2.14 LPS challenge, influenza virus A infection and bacillus calmette-guérin (BCG) infection

10 μg LPS (Sigma) were injected *i.p.* into mice. Sera were collected 0 h, 2 h and 12 h after injection. Concentrations of human inflammatory cytokines in sera were analyzed by the Cytometric Bead Array System (CBA) (BD Biosciences) on LSRII according to the manufacturer's instructions, and data were analyzed by FCAP Array software (BD Biosciences).

300 pfu of influenza A/PR8 (H1N1) virus in 75 μl PBS were administered to anesthetized mice via intratracheal route. Lungs were harvested at 0 h, 24 h, 48 h, and 72 h after infection. For each mouse, the left lobe was used for the single cell preparation for flow cytometry analysis as described above. The superior lobe of right lobes was excised to extract RNA for quantitative RT-PCR analysis as described above. The rest of the three right lobes were weighted and homogenized in 100 mg tissue per 1 ml of ice-cold HBSS with 0.1% Bovine serum albumin (BSA). The lung homogenates were centrifuged for 10000 g, 10 min at 4 °C to collect the supernatants for CBA assay to measure the cytokine concentration.

Mycobacterium bovis BCG Pasteur strain bacilli (ATCC, Manassas, VA, USA) was grown in 7H9 culture medium at 37 °C in an 850 cm^2 polystyrene roller bottle (Corning) at 10 rpm. The culture reached an optical density (OD600) of 0.6, at which point the concentration of cells was ~$5x10^7$ /ml. Each mouse was injected *i.p.* with $5x10^6$ CFU of BCG in 0.5 ml. The peritoneal lavage was flushed out with 5 ml PBS,

and the spleen was homogenized in 10 ml PBS at 0 h, 24 h, and 72 h post-infection. To determine the numbers of recovered viable bacilli in a culture or tissue homogenate, colony counting was performed with serial dilutions of the samples in 7H9 with 100 μL of serial dilutions plated on 7H11 agar, and incubated at 37 °C with 5% CO_2. Serial dilutions and 7H11 plates were performed in triplicate, and the numbers of colonies on each plate counted after 4 weeks. The rest of the peritoneal lavage was centrifuged down to obtain cells for flow cytometry analysis, and to extract RNA for quantitative RT-PCR analysis as described above.

2.15 Statistical analysis

Data are presented as mean and standard error of the mean. The nonparametric Mann-Whitney U test was used to analyze the differences between two groups. A P-value of <0.05 was considered as statistically significant.

CHAPTER 3

IN VITRO EXPANSION OF HSC FOR HUMANIZED MICE RECONSTITUTION

Work has been published as (Khoury et al., 2011) and LI Yan is the co-author with Dr. Maroun Khoury on this paper. LI Yan contributed in all the experiments presented in this chapter and independently designed and performed the experiments in Figure 5 and Figure 7.

3.1 Introduction

3.1.1 The demand for expansion of HSCs for clinical and preclinical applications

HSCs give rise to all blood lineage cells, which function in diverse biological processes from immune responses to oxygen transport. Because of this unique property, there has been great interest in clinical and preclinical applications of HSCs. BM transplant is widely used to treat congenital immunodeficiencies, where the transfer of HSCs from normal to deficient individuals is the basis of the treatment. Transplantation of purified HSCs from BM, mobilized peripheral blood or umbilical cord blood is also a well-established treatment for certain hematologic malignancies. However, these treatments are often limited by the availability of HSCs in terms of finding suitable matching donors as well as obtaining sufficient numbers of cells.

In preclinical applications, there has been an enormous effort in developing mice with human blood lineage cells (humanized mice) for studying human blood cell diseases and immune responses to pathogens. Construction of humanized mice requires engraftment of human HSCs in scid mice. With the development of a new generation of scid mice, such as NSG mice, which also lack NK cells, it is now possible to routinely construct humanized mice with high levels of human blood cell reconstitution. The easiest accessible source of human HSCs is cord blood. However, only limited numbers (10^5~10^6) of $CD34^+$ cells can be isolated from a single cord to reconstitute a small number (1~10) of mice. Although HSCs from different cords can be used, to minimize variations due to differences in the genetic background of HSCs, humanized mice reconstituted with HSCs from the same source are ideally used in the same experiment. Large-scale application of humanized mice for research and

preclinical studies requires access to sufficient numbers of human HSCs (Giassi et al., 2008).

3.1.2 Methods for HSC expansion

One way to overcome the shortage of HSCs is via the expansion *in vitro*. In general, two approaches have been used: FCF culture in the presence of specific growth factors and co-culture with feeder cells, such as MSCs. In FCF culture, the best *in vitro* expansion of human HSCs achieved to date was reported recently by Delaney et al. 2010. Using a cocktail of growth factors, including human IL-3, IL-6, TPO, Flt-3 ligand, SCF and notch ligand, total and $CD34^+$ cells were expanded ~790 and ~200 fold, respectively, after 17-21 days of culture, resulting in a ~16 fold expansion of SRCs as assayed in NSG mice (Delaney et al. 2010). Using a different combination of growth factors, including TPO, SCF, FGF-1, insulin growth factor binding protein 2 (IGFBP2), and angiopoietin-like 5 (Angptl5), Zhang et al. 2010 reported a lower total cell expansion (~200 fold) as cells undergo senescence after 14 days. Nevertheless, a 20-fold expansion of SRCs was observed in NOD-scid mice following a 10-11 day culture (Zhang et al., 2008; Zhang et al., 2006).

Another way to expand HSCs is co-culture using a feeder layer that mimics the physiological microenvironment of HSCs. In particular, BM-derived MSCs have been shown to support the growth of HSCs from cord blood through an increased cell division and a better maintenance of the primitive phenotype (Walenda et al., 2009). MSCs from other sources, including Wharton's jelly of umbilical cord segment (Wang et al., 2004), cord blood (Lee et al., 2004), amnion-derived adherent cells (In 't Anker et al., 2004; Kita et al., 2009), and other fetal tissues (Campagnoli et al., 2001), have also been shown to support *ex vivo* expansion of HSCs (Bakhshi et al., 2008;

Campagnoli et al., 2001; Huang et al., 2007; Jang et al., 2006; Lee et al., 2004; Mizokami et al., 2009; Walenda et al., 2009; Wang et al., 2004; Wang et al., 2009). Many of these reports suggest that co-culture is superior to the FCF culture in terms of both total cell expansion and the yield of $CD34^+$ cells when compared under the same culture conditions. Depending on the co-culture conditions, 80 to 800-fold increase in total cell numbers, and 4 to 100 fold increase in the $CD34^+$ cells were reported (Alakel et al., 2009; Briquet et al. 2010; da Silva et al. 2010). The highest expansion was achieved by culturing HSCs with MSCs in a 3D configuration using a woven mesh (96 fold expansion $CD34^+$ cells in 10 days, (Zhang et al., 2006). However, most studies with co-culture did not rigorously examine the scid repopulating activity of expanded cells in mice using long-term reconstitution, limiting dilution or competitive reconstitution assays. In the few cases where expanded cells were tested in mice, only one dose of expanded progeny was injected in NOD-scid mice, and reconstitution was assayed 6 weeks later in the BM (Briquet et al. 2010; Fei et al., 2007; Zhang et al., 2006). Another report showed a 3.4-fold increase of SRCs when the progeny of $CD34^+$ cells co-cultured with human brain endothelial cells were injected into mice (Chute et al., 2004).

3.1.3 Expansion of HSC in angptl5 expressing MSC co-culture

One way to further enhance HSC expansion without promoting differentiation is to combine co-culture with growth factors that are shown to promote HSC maintenance and expansion. In this study, we co-cultured HSCs with engineered MSCs that express Angptl5 plus the addition of SCF, TPO, FGF1 and IGFBP2 in the media. Dramatically, we observed ~60 and ~150 fold expansion of $CD34^+$ $CD133^+$ DP cells 10 days and 14 days after co-culture, respectively. The expanded DP cells possess the

same repopulating and long-term HSC properties as uncultured cells using long-term and serial reconstitution. In addition, mice reconstituted with the expanded HSCs from the co-culture have an enhanced ability to differentiate into T cells, leading to a more normal T cell to B cell ratio in the reconstituted mice. These results suggest that co-culture in the presence of selected growth factors in the media is an efficient method to expand human HSCs *in vitro.*

3.2 Results

3.2.1 Combination of co-culture and selected growth factors enhance expansion of cord blood $CD34^+CD133^+$ cells.

To investigate whether MSCs and recently identified growth factors, such as IGFBP2 and Angptl5, synergize in HSC expansion, we tested expansion of $CD34^+CD133^+$ cells on an MSC feeder layer in the presence of additional growth factors. Because human Angptl5 was required at high concentrations (Zhang et al, 2010) and expensive, we expressed it in human primary BM MSCs using a lentiviral vector. The Angptl5 expressing MSCs are referred to as MSC-A5. As a control, GFP-expressing MSCs (MSC-GFP) were constructed following the same approach. For expanding HSCs, purified $CD133^+$ cord blood cells which were also $CD34^+$, were cultured on top of a confluent MSC-A5 or MSC-GFP feeder layer at a ratio of 1:5 in the presence of SCF, FGF, IGFBP2, and TPO in the culture media. For comparison, purified $CD133^+$ cells were also cultured in FCF culture, i.e., serum-free medium supplemented with Anglt5, SCF, FGF, IGFBP2, and TPO. At the start of the co-culture, hematopoietic cells were observed to attach to the MSC feeder layer (Figure 1A). By day 5, the density of hematopoietic cells was noticeably increased. By day 7, proliferation of hematopoietic cells was evidenced by cell density and cell counting (Figure 1A). By day 11, hematopoietic cells had completely covered the MSC-A5 feeder layer (Figure 1A) and were split into fresh feeder layers and medium. To monitor HSC expansion, cultured cells were assayed for CD34 and CD133 expression since we have shown previously that SRCs reside in the $CD34^+CD133^+$ DP fraction, and some of the DP cells possess the long-term HSC activity (Drake et al, submitted). At the beginning of the co-culture, more than 95% of the input cells were $CD34^+$ and

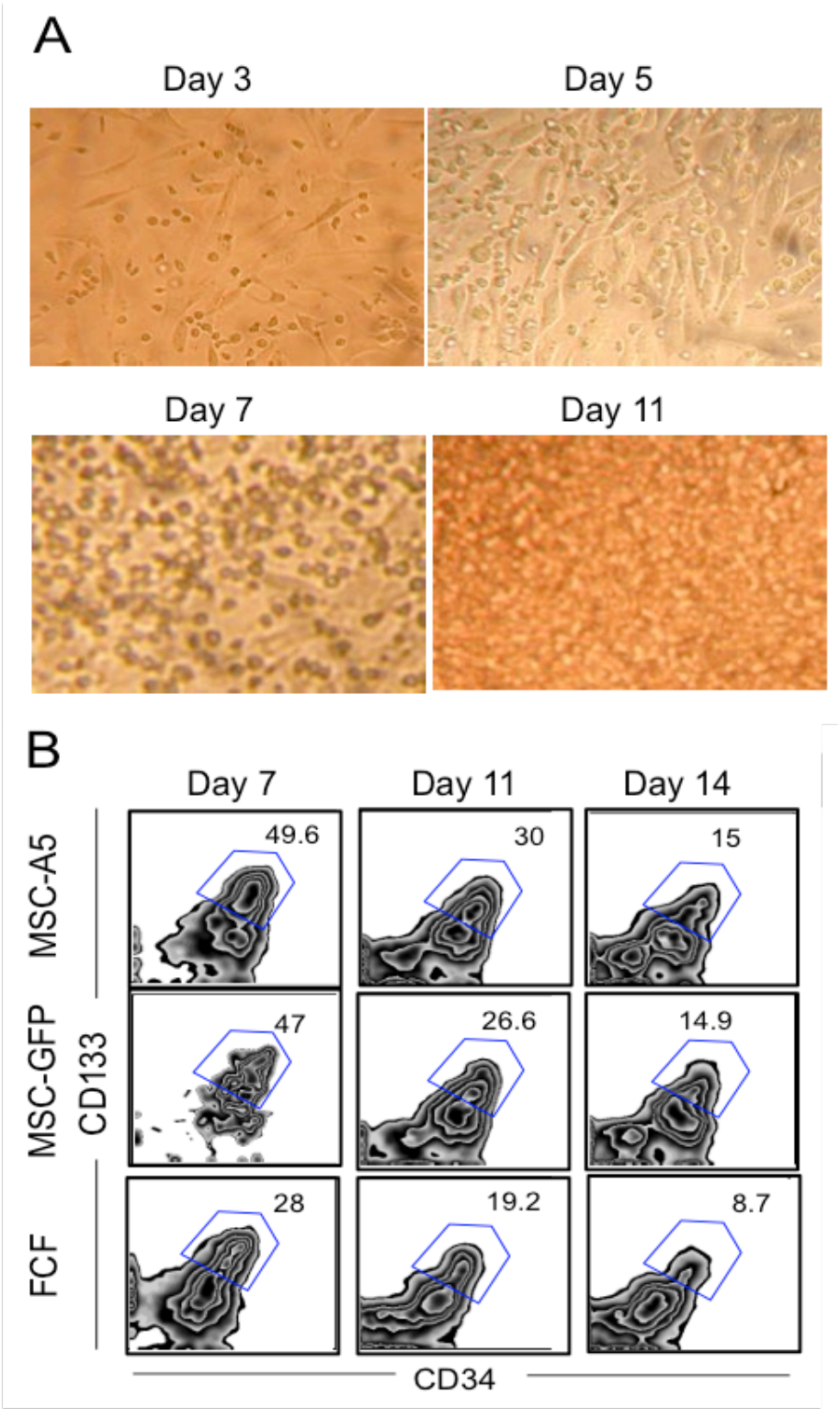

Figure 1. Combination of co-culture and growth factors enhance expansion of $CD34^+CD133^+$ cord blood cells.

Purified $CD34^+CD133^+$ cord blood cells were co-cultured with MSC-A5 or MSC-GFP in the presence of SCF, FGF, IGFBP2, TPO and heparin or cultured in the FCF culture. (A) Co-culture with MSC-A5 was visualized at indicated time points by an inverted microscope (10x magnification). Non-adherent cells in MSC-A5 and MSC-GFP co-cultures and FCF culture were harvested at 7, 11, and 14 days, enumerated, and stained for human CD34 and CD133, and followed by flow cytometry analysis. (B) Representative CD34 versus CD133 staining profiles of cultured cells. The number indicates the percentage of DP cells in the gated region.

$CD133^+$ (data not shown). After culture for 7 days, the percentage of DP cells decreased to ~50% in MSC-A5 or MSC-GFP co-culture, and ~30% in FCF culture (Figure 1B).

The percentages continued to decrease and by day 14, they were ~15% in co-cultures and ~10% in FCF culture. Despite the decrease of the percentages of DP cells in the cultures, because the total cell numbers increased dramatically (Figure 2A), the actual numbers of DP cells increased ~60 fold in MSC-A5 co-culture, ~29 fold in MSC-GFP co-culture, and ~20 fold FCF culture at day 11 (Figure 2B). By day 14, while the number of DP cells did not increase significantly in the FCF culture, the number of DP cells increased ~150 and ~60 fold in MSC-A5 and MSC-GFP co-cultures, respectively. When $CD34^+$ cells isolated from fetal liver and mobilized peripheral blood were cultured using the same conditions, expansion of DP cells in MSC-A5 co-culture was also significantly higher than that in the FCF culture (data not shown). These results show that the combination of MSCs and the different growth factors can support a much more robust $CD34^+$ $CD133^+$ HSC expansion.

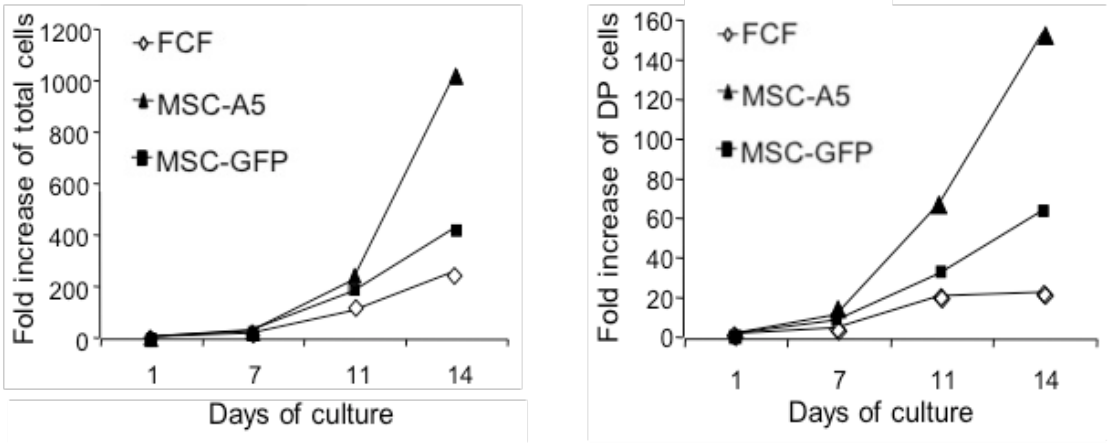

Figure 2. Fold increase of total cells and DP cells for 14 days co-culture
(A) Fold increase of total hematopoietic cells during the course of the cultures. (B) Fold increase of $CD34^{+}CD133^{+}$ cells during the course of the cultures. The experiments were repeated at least six times and representative data are shown.

		FCF		MSC-GFP		MSC-A5	
Cord blood #	% of purity post-selection	Fold increase total cell	Fold increase CD34+CD133+	Fold increase total cell	Fold increase CD34+CD133+	Fold increase total cell	Fold increase CD34+CD133+
1	98	100	20	174	31	220	66
2	95	140	26	163	37	210	62
3	97	95	19.2	145	29	198	58
4	98	89	16	128	22	167	46
mean	**97**	**106**	**20.3**	**152.5**	**29.75**	**198.75**	**58**
SD	1.4	23.1	4.2	20.2	6.2	23.0	8.6
P value		0.0006	0.0001	0.012	0.0009		

Table 1. Umbilical Cord Blood Expansion Characteristics
Purity of the starting cultured cells after isolation, fold increase of total hematopoietic cells, and fold increase of $CD34^{+}$ $CD133^{+}$ double positive cells at day 11 of the different conditions are shown. The MSC-A5 condition is the reference group for P values. MSCs, mesenchymal stem cells.

3.2.2 Evaluation of contributions of feeder cells and soluble factors to the enhanced expansion of cord blood $CD34^+CD133^+$ cells

To further assess the role of each of the five growth factors in the expansion of $CD34^+CD133^+$ cord blood cells, we performed the co-cultures without adding specific growth factors. For easy comparison, the expansion of DP cells in the different cultures was normalized to that under the standard condition where $CD34^+$ $CD133^+$ cord blood cells were co-cultured with MSC-A5 in the presence of SCF, TPO, FGF and IGFBP2 in the media. When $CD34^+CD133^+$ cord blood cells were co-cultured with MSC-A5 without the addition of any of the growth factors, they did not expand at all (Figure 3). Addition of SCF and TPO, which are well-established HSC growth factors and broadly used together (Liu et al., 1999; Ohmizono et al., 1997), into the above co-culture led to expansion of DP cells, reaching 19% of that obtained under the standard condition. With further addition of FGF or IGFBP2, the relative expansion was 36% and 61%, respectively. Without exogenously added Angplt5 (co-culture with MSC-GFP in the presence of SCF, TPO, FGF, and IGFBP2), the relative expansion was 43%. These results suggest that all five growth factors contribute to the expansion of $CD34^+CD133^+$ cord blood cells, with IGFBP2 and Angplt5 having the biggest impact.

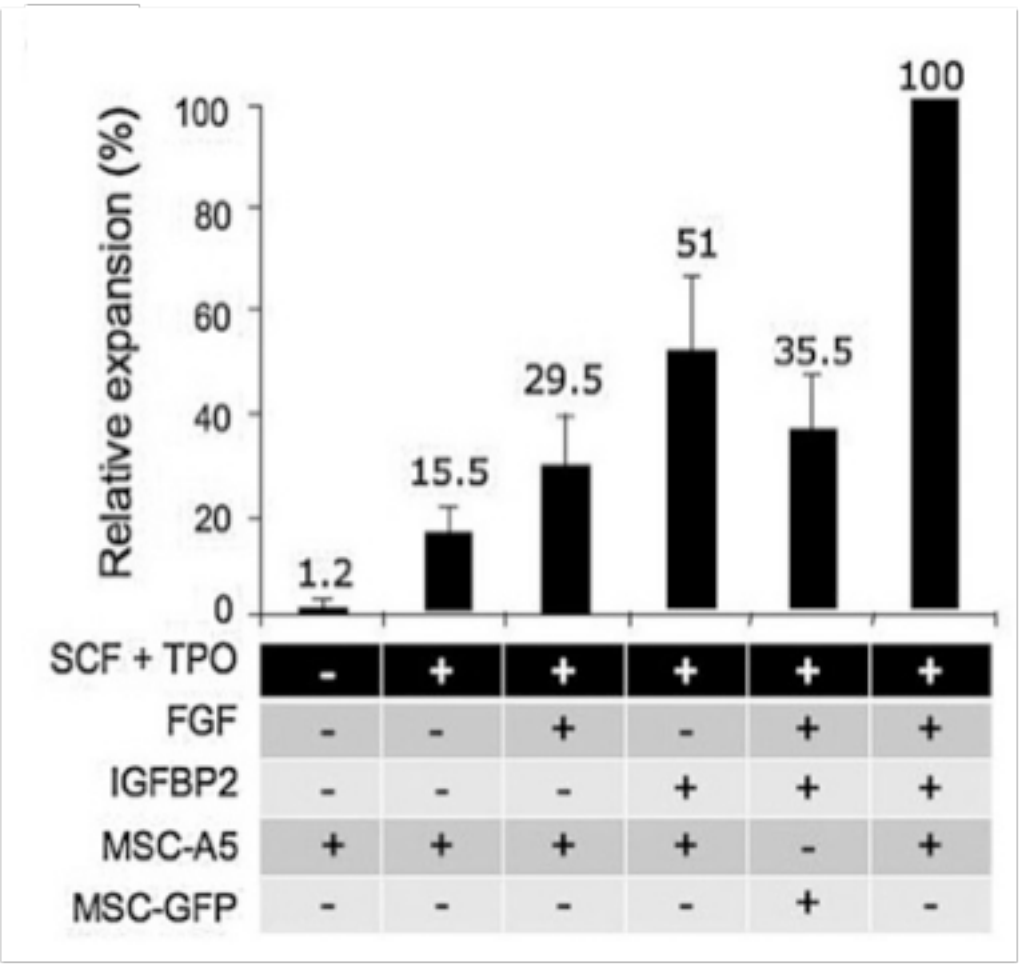

Figure 3. Evaluation of contributions of feeder cells and soluble factors to the enhanced expansion of $CD34^+CD133^+$ cord blood cells

$CD34^+CD133^+$ cord blood cells were co-cultured with MSC-A5 or MSC-GFP without or with addition of the indicated growth factors for 11 days. Hematopoietic cells from the cultures were analyzed as above. The expansion of DP cells in different cultures was normalized to that under the standard condition where $CD34^+CD133^+$ cord blood cells were co-cultured with MSC-A5 in the presence of SCF, TPO, FGF and IGFBP2 in the media. Shown are relative percentages of DP cells at day 11 of culture under the different conditions.

3.2.3 Expanded $CD34^+CD133^+$ cells give rise to multiple lineages of blood cells in NSG mice

We tested the ability of expanded cells from the co-cultures to reconstitute different lineages of human blood cells in NSG mice. $CD34^+CD133^+$ cord blood cells were either expanded in the MSC-A5 co-culture or the FCF culture for 11 days. Total expanded cells, containing 10^5 DP cells, were engrafted into sublethally irradiated NSG newborn pups. Fourteen weeks later, the presence of different human blood lineage cells in the peripheral blood, spleen and BM were analyzed by flow cytometry. Among various human blood lineage cells analyzed, T cells ($CD3^+$), B-lineage cells ($CD19^+$ or $CD19^+IgM^+$), macrophages ($CD14^+CD33^+$), myeloid precursor cells ($CD33^+$), hematopoietic stem/precursor cells ($CD34^+$ or $CD34^+CD133^+$), DCs ($CD11c^+$ $HLA\text{-}DR^+$), neutrophils ($CD15^+$), and NK cells ($CD56^+$) were detected in the blood, spleen or BM (Figure 4 and data not shown). Importantly, the levels of various human cell reconstitution, both in terms of median percentage and the range among different recipient mice, were similar regardless of the conditions used to expand $CD34^+CD133^+$ cord blood cells (Table 1), except significantly higher percentages of $CD3^+$ T cells were observed in the blood and spleen of recipient mice that were injected with cells from MSC-A5 co-culture than the FCF culture ($p<0.05$). H&E staining revealed a restoration of the splenic architecture with the appearance of the white pulp in mice reconstituted with co-cultured cells, but not in unreconstituted NSG mice (Figure 5). Furthermore, T cells and B cells were found in the same follicles, with $CD3^+$ T cells residing predominantly in the inner region of the follicles, whereas $CD20^+$ B cells resided in the outer region of the follicle. These results suggest that expanded cells from the co-cultures are capable of differentiating into multiple lineages of blood cells in NSG mice.

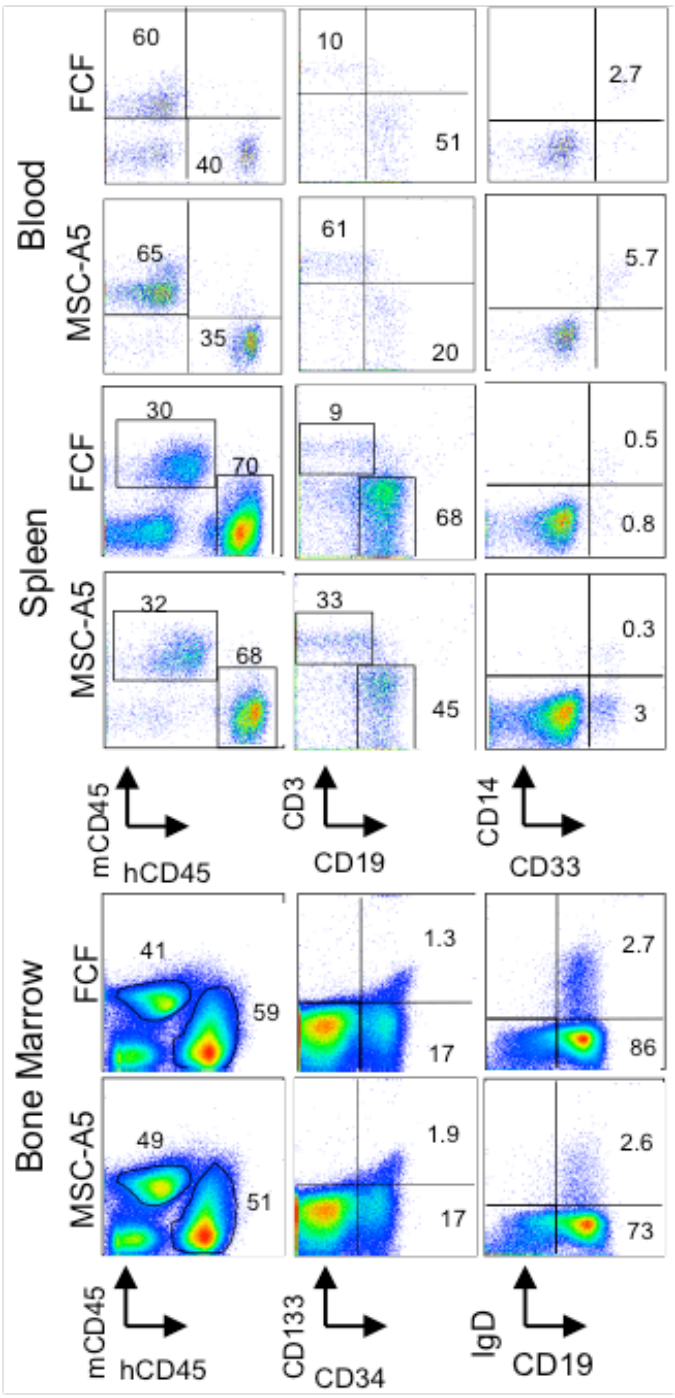

Figure 4. Expanded cells are capable of differentiating into multiple lineages of blood cells in NSG mice.

Ten days after expansion in the MSC-A5 or FCF cultures, expanded cells containing 10^5 DP cells, were injected into newborn NSG pups. Fourteen weeks after injection, the presence of various lineages of human blood cells in the blood, spleen and bone marrow were analyzed by flow cytometry. Except for mouse CD45 (mCD45) versus human CD45 (hCD45) staining profiles, which were gated on total live cells from the individual tissues, all other staining profiles were gated on human $CD45^+$ cells. The number indicates the percentages of positive cells in the gated region, except the percentages of human or mouse $CD45^+$ cells, which was calculated by dividing the percentage of human or mouse $CD45^+$ cells with the sum of percentages of human and mouse $CD45^+$cells .Representative dot plots are shown from mice reconstituted with three different cord blood cells.

	hCD45		CD33		CD3		CD19		CD33+CD14+		CD11c+HLA-DR+		CD15		CD56		CD34	
	Median	(Range)	Median	(Range)	Median	(Range)	Median	(Range)	Median	(Range)	Median	(Range)	Median	(Range)	Median	(Range)	Median	(Range)
Blood																		
FCF	40	(33-50)	5.6	(3-8)	11.5	(10-44)	36	(21-51)	4.6	(1.5-5.2)	5	(4.7-5.9)	5.67	(3.9-6.4)	1.12	(1-1.20)	ND	
MSC.A5	34	(26-46)	5.7	(3-9.2)	44	(21-68)	21	(7-54)	5.63	(3.8-9.7)	8.9	(3.2-16.5)	9.69	(3.9-12.7)	0.98	(0.5-1.16)	ND	
Spleen																		
FCF	80	(76-87)	1.1	(0.4-1.1)	17.18	(5.3-29)	54	(20-87)	0.13	(0.07-0.19)	1	(1-2.9)	1	(0.7-1)	1.4	(1.2-2.1)	ND	
MSC.A5	75	(63-87)	1	(0.8-3.2)	30	(8.3-54)	55	(25.8-85)	0.35	(0.2-1.1)	1.44	(0.7-2.3)	0.85	(0.5-1.5)	0.69	(0.6-1)	ND	
BM																		
FCF	44	(41-48)	3.35	(3-5.2)	2.68	(0.4-4.9)	69.5	(60-79)	1.9	(1-2.1)	1.3	(1.2-2)	3.7	(1.5-6)	ND		16.8	(8.6-33)
MSC.A5	45	(19-74)	4.7	(2.1-9.8)	2.57	(0.8-7.8)	72.3	(67-76)	1.8	(1.3-4.6)	1.5	(1.2-4.2)	2.33	(0.4-4.6)	ND		20.75	(10-32)

Table 1. Comparison of reconstitution levels in NSG recipient mice engrafted with expanded cells from either the MSC-A5 co-cultures or the FCF cultures.

See Figure 4 legend for detailed description of experimental procedures and analysis. Shown are the median percentages and the range of the percentages of different human blood lineage cells in the blood, spleen and bone marrow (BM). Data were obtained from analysis of 9 and 3 mice engrafted with expanded cells from the MSC-A5 co-culture and the FCF culture, respectively. Representative data from one of the two sets of analyses are shown.

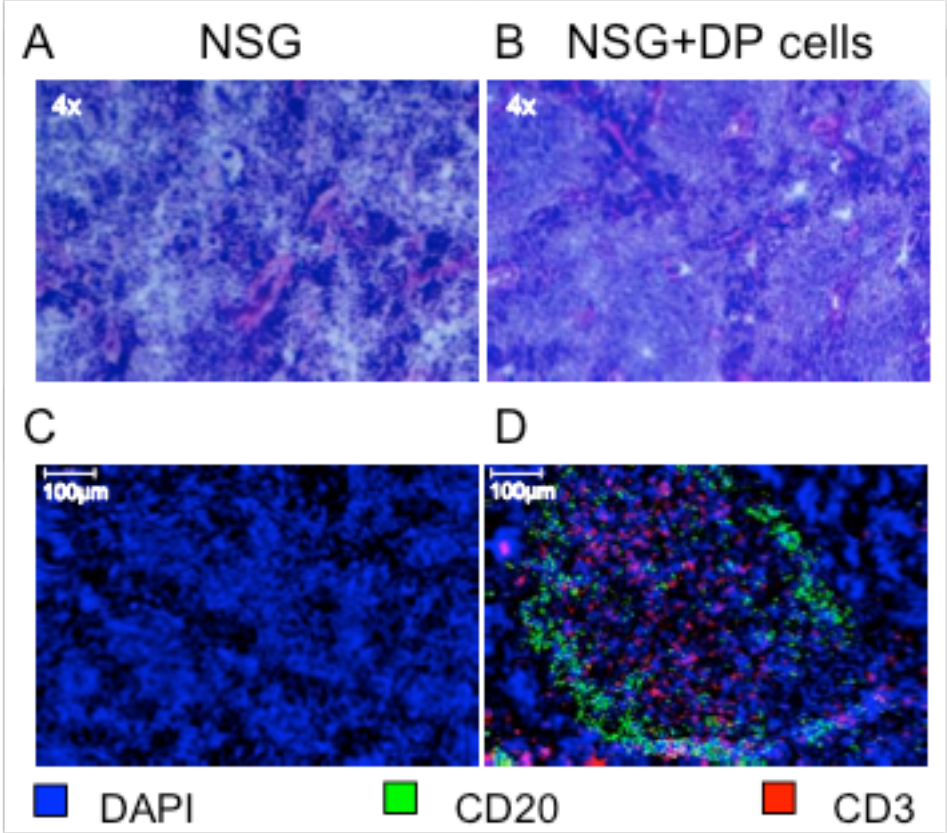

Figure 5. Comparison of spleen sections of NSG mice and NSG mice engrafted with expanded cells from MSC-A5 co-culture.

20 weeks after engraftment, (A, B) H&E Staining. Magnification 4x. (C,D) Anti-CD3 and anti-CD20 staining. Scale bar is 100µm.

3.2.4 Expanded cells are capable of stable long-term and efficient secondary reconstitution

The expanded cells were tested for their ability to give rise to stable long-term reconstitution in NSG recipients. Following 11-day expansion in either MSC-A5 co-culture or FCF culture, expanded cells were transferred into sublethally irradiated newborn NSG pups. By 12 weeks following engraftment, the percentages of human leukocytes in the blood were less than 50% (Figure 6A). These percentages increased slightly or were maintained at 24 weeks after engraftment (Figure 6A, B). No difference in reconstitution was observed between expanded cells from MSC-A5 co-culture or FCF culture in the blood and spleen (Figure 6A). The level of T cell reconstitution in both blood and spleen was higher with expanded cells from co-culture than FCF culture (data not shown). These results suggest that expanded cells from both protocols possess similar levels of long-term reconstitution activity.

We also assessed the presence of long-term HSC activity in the expanded cell population by serial transfer. Expanded cells from day 11 MSC-A5 co-culture were transferred into irradiated NSG pups. Fourteen weeks after engraftment, human $CD34^+$ cells were magnetically enriched from the BM of primary recipient mice, and injected into sublethally irradiated adult NSG mice. Twelve to 16 weeks after, BM cells of the secondary recipient mice were analyzed by flow cytometry for human and mouse $CD45^+$ cells. Among the 14 secondary recipient mice, 11 had 0.1-2% of human $CD45^+$ cells in the BM, one had 11% and the

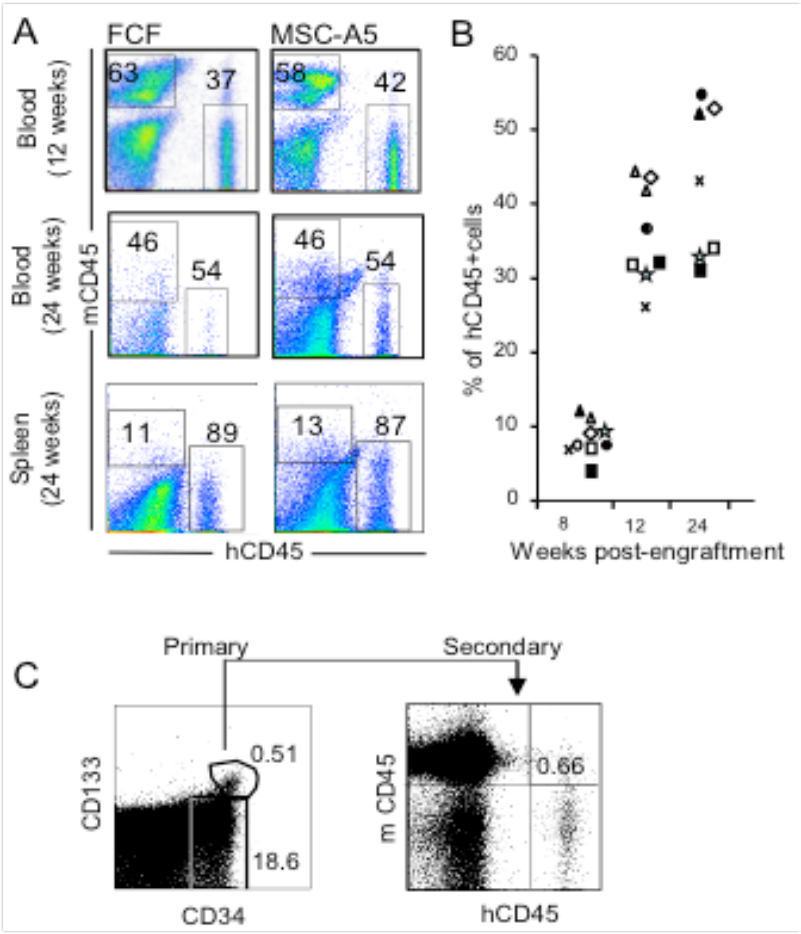

Figure 6. Expanded cells are capable of stable long-term and efficient secondary reconstitution

(A) Stable long-term reconstitution by expanded cells. Day 11-expanded cells from either MSC-A5 co-culture or FCF culture were engrafted into sublethally irradiated newborn pups (10^5 DP cells per recipient). Mice were bled at the indicated time points and PBMCs were analyzed for human CD45 and mouse CD45. Reconstitution in the spleen was also assayed in some mice 24 weeks after engraftment. Dot plots show mCD45 versus hCD45 staining profiles of PBMCs at 14 and 24 weeks, or spleen at 24 weeks of the same mouse engrafted with either expanded cells from co-culture or FCF culture.

(B) Comparison of percentages of human CD45 cells in PBMCs of mice reconstituted with expanded cells from MSC-A5 co-culture. One symbol represents one mouse. The same symbol represents the same mouse at different time points.

(C) Serial transfer. Day 11-expanded cells from MSC-A5 co-culture was transferred into irradiated newborn pups (10^5 DP cells per recipient). Fourteen weeks later, bone marrow cells were harvested from primary mice and human $CD34^+$ cells were enriched by magnetic sorting and transferred into sublethally irradiated newborn pups. Twelve weeks after secondary transfer, the presence of human $CD45^+$ cells in the bone marrow was analyzed by flow cytometry. mCD45 versus hCD45 staining profile is shown for a representative secondary recipient mouse. The experiments were done twice with expanded cells from two different cord blood sources.

other two had ~0.03%. A representative mouse is shown in Figure C. These data show that the expanded CD34+CD133+ cells from MSC-A5 co-culture retain a secondary reconstitution potential, suggesting the presence of long-term HSC activity.

3.2.5 Human T cells are functional in the reconstituted mice

The increased T cell development with co-culture expanded DP cells prompted us to test if the T cells are functional. Similarly reconstituted mice with expanded cells from either co-culture or FCF culture were immunized with TT and boosted twice at an interval of 3 weeks. Two weeks after the last immunization, splenocytes were assayed for IFN-γ expression by ELISPOT assay. Without immunization, significant numbers of IFN-γ immunospot were detected only if splenocytes were stimulated with PMA during the assay, regardless of whether the cells were from mice reconstituted with expanded cells from the co-culture or the FCF culture (Figure 7A). Similarly, significant numbers of spots were detected in PMA-stimulated splenocytes from immunized mice, regardless whether reconstituted with expanded cells from the co-culture or the FCF culture. As expected, with TT stimulation of splenocytes in the culture, significant numbers of spots were detected only in TT immunized mice. However, the average numbers of spots were significantly higher in mice reconstituted with cells from the co-culture than the FCF culture (104 ± 37 vs. 22 ± 14, $P < 0.005$). These results suggest that enhanced T cell development leads to an enhanced T cell response in mice reconstituted with co-culture expanded cord blood cells.

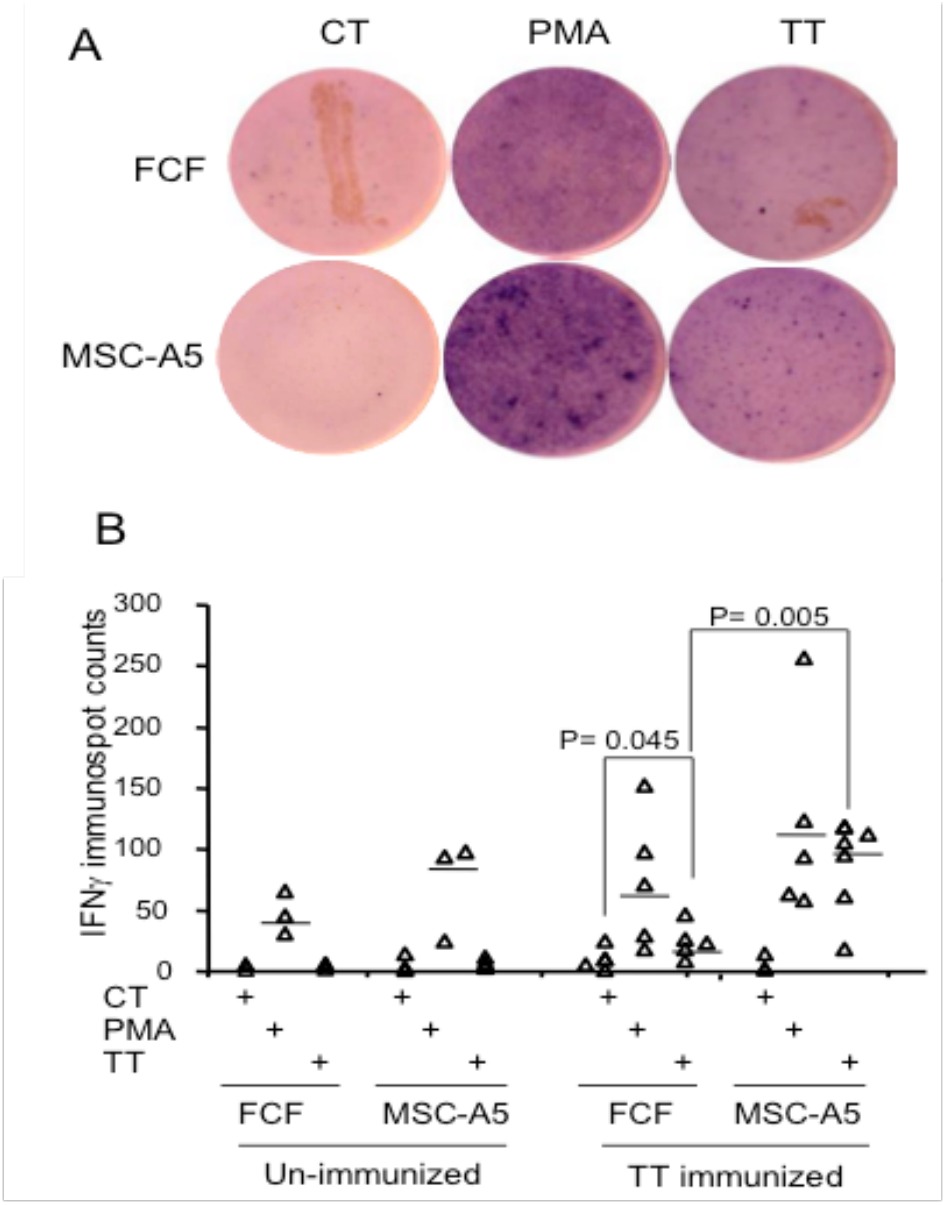

Figure 7. T cell responses in mice engrafted with co-culture expanded cells.

$CD34^+CD133^+$ cord blood cells were expanded for 11 days in either the co-culture or the FCF culture and injected into sublethally irradiated newborn pups. Sixteen weeks later, mice were screened for levels of reconstitution in the peripheral blood. Mice with similar human leukocyte reconstitution (20-55%) were immunized with tetanus toxoid (TT) three times at 3 weeks interval. Two weeks after the third immunization, spleens were harvested and the percentages of human T cells were determined by flow cytometry. For ELISPOT assay, the same number ($5x10^5$) of human T cells from different samples were seeded into wells coated with anti-human IFN-γ antibody and cultured for 48h under three conditions: medium alone (control), in the presence of PMA or in the presence of a TT-specific peptide. ELISPOT was developed and counted. (A) Representative ELISPOT wells with splenocytes from immunized mice. (B) Comparison the numbers of IFN-γ immunospots among different samples. Each symbol represents one mouse. Data shown are from one of two independent experiments.

3.3 Discussion

Here we report an improved method to expand HSCs from umbilical cord blood by combining the HSC growth factors recently identified by Zhang et al. 2010 with a BM-derived MSC feeder layer. We reasoned that by adding the growth factors into the co-cultures it might be possible to further enhance HSC expansion while minimizing differentiation. By all measures, this approach worked significantly better than the FCF culture or co-culture without the selected growth factors. In our experiments, $CD34^+$ $CD133^+$ cells from the same cord blood donor were expanded using the three different conditions, and the fold expansion of DP cells was monitored because we have shown previously that the SRCs reside in the DP cell fraction. Compared to FCF culture, which expanded DP cells 20-25 fold during 11-14 days, co-culture with MSC-A5 expanded DP cells 60-150 fold, whereas co-culture without Angptl5 (MSC-GFP) expanded DP cells 28-60 fold during the same period. These observations were further supported by results from experiments where different growth factors were added into the MSC-GFP co-culture. It is noticeable that the co-culture system expanded the SRCs, as measured by CD34 and CD133 expression, faster than FCF cultures and retained a higher proportion of $CD34^+$ (47% vs. 20% on day 14) and $CD34^+CD133^+$ cells (15% vs. 9% on day 14), producing 150 fold of expansion of DP cells by day 14. These findings suggest that both MSCs and the selected growth factors, especially Angptl5 and IGFBP2, contribute significantly to the expansion of $CD34^+$ $CD133^+$ cord blood cells in the co-culture.

Different from previous co-culture studies, we rigorously evaluated the reconstitution activity of expanded cells from the co-culture in long-term and serial reconstitution. We show that the expanded cells were able to support stable long-term (6 months)

reconstitution. When human $CD34^+$ cells were isolated from the BM of primary recipient mice and transferred into second recipient mice, 12 of 14 recipients had more than 0.1% human cells in the BM 12-16 weeks later. These results suggest that some of the expanded cells still possess the long-term HSC activity.

MSCs have been shown to stimulate differentiation of HSCs. With the recent development of the NSG mouse model that supports robust lymphoid engraftment, we examined the ability of the co-culture expanded cells for T and B cell reconstitution. While unexpanded or expanded $CD34^+CD133^+$ cells from the FCF culture give robust B cell but poor T cell reconstitution initially, we show that the co-culture expanded cells gave significantly enhanced T cell reconstitution, reaching the normal T to B cell ratio as in humans and mice. The observed T cell-bias was opposite to the effect reported by Giassi et al. (Giassi et al., 2008), further suggesting a critical role of MSC in regulating differentiation in the co-culture. Given that we did not observe any reduction in long-term repopulation capacity, the T cell-biased reconstitution suggests that MSCs also support progenitor cell expansion. Furthermore, the expanded T cell population significantly enhanced the antigen-specific T cell response in mice reconstituted with co-culture expanded cord blood cells as assayed by ELISPOT. However, whether the improvement in cytotoxic T cell response is due to the increased T cell number or improved functionality is still not known.

In both clinic and laboratory, the supply of HSCs is limiting with little prospect of finding significant new sources of cells. Strategies to make the existing sources of cells go further are essential for both improving treatments of hematologic malignancies, and our understanding of human hematology and immunology. While progress is being made with strategies to improve delivery of stem cells, *ex vivo* expansion of HSCs is the key to improving clinical outcomes. In the first clinical trial,

transfer of unexpanded and expanded cells from different donors together significantly reduced the period of neturopenia following transplantation (Delaney et al. 2010). However, over time the graft was universally dominated by unexpanded cells in all patients. In another clinical trial, HSCs from umbilical cord blood were expanded by approximately 12-fold on donor-related MSCs and injected in 6 cancer patients. Five out of the six patients were in complete remission after one year of the engraftment (Kelly et al., 2009). The improved approach for expanding HSCs reported here not only solves one bottleneck for the humanized mice research field to generate large numbers of humanized mice from the same HSC source for large-scale preclinical studies, but also provides a promising method to expand HSCs for transplantation.

CHAPTER 4

HUMAN M-CSF MOBILIZES PROMONOCYTES IN BM AND RESTORES THE FUNCTIONS OF HUMAN TISSUE MACROPHAGES IN HUMANIZED MICE

A manuscript is being prepared for this work and LI Yan is the co-first author with Dr. Qingfeng Chen on this paper. LI Yan designed and performed all the experiments, analyzed and interpreted the data, and wrote the manuscript.

4.1 Introduction

4.1.1 The problem of human monocyte/macrophage development in NSG mice

A major avenue for improvement of current humanized mice is to generate mice with a functional immune system of human origin. To achieve this, a complete hematopoietic system is a prerequisite. In NSG mice, human T and B cells can stably reconstitute the mice for more than one year while NK cell reconstitution, which was very poor in NSG mice, is also improved by expression of human IL-15 and Flt3 (or flk-2) (Chen et al., 2009; Hiramatsu et al., 2003; Ishikawa et al., 2005; Shultz et al., 2005; Yahata et al., 2002). Nevertheless, myeloid lineage cells, such as monocytes/macrophages, granulocytes, megakaryocytes and erythrocytes, are poorly developed and maintained in humanized mice (Ishikawa et al., 2005; Legrand et al., 2006; Manz, 2007; Shultz et al., 2007). The defect of human myeloid development in NSG mice has been speculated to be the result of an inefficient myelopoiesis, and a relatively intact mouse myeloid compartment (Macchiarini et al., 2005; Manz, 2007; Shultz et al., 2007). In view of the fact that myeloid cells play a vital role in oxygen transport (erythrocyte), blood coagulation (megakaryocytes) and development of efficient immunity against infectious pathogens (granulocyte and macrophages), generation of humanized mice with efficient human myelopoiesis is of paramount importance.

4.1.2 The role of human monocytes/macrophages in immune responses

In this part of the thesis, we will focus on correcting the defect of human monocyte/macrophage development in humanized mice. Monocytes/macrophages, along with DCs, form the mononuclear phagocytic system (Murray and Wynn, 2011).

The mononuclear system is derived from common myeloid precursors in BM, circulates in the blood as monocytes, and populates tissues as macrophages in the steady state and during inflammation. Monocytes function as immune effector cells, and do not proliferate in a steady state (Auffray et al., 2009a; Auffray et al., 2009b). The expression of chemokine receptors and adhesion receptors on monocytes facilitate their migration from blood circulation to sites of inflammation. In the course of migration to tissues, they produce inflammatory cytokines and differentiate into inflammatory DCs or macrophages. Even in the steady state, monocytes are believed to serve as a pool to replenish tissue macrophages and DCs (Serbina et al., 2008). Macrophages are resident phagocytic cells in both lymphoid and non-lymphoid organs. They participate not only in the initiation and resolution of immune response during tissue injury or infection, but also play a critical role in steady-state tissue homeostasis, via the clearance of apoptotic cells, and the production of growth factors (Geissmann et al., 2010). In addition, there is accumulating evidence that many diseases may be caused by dysregulated macrophage function, such as adipose tissue macrophages in metabolic disorders, M1-like macrophages in atherosclerosis, and Kupffer cells in liver fibrosis (Murray and Wynn, 2011). The study of macrophage biology would greatly help to develop therapies for the above diseases. Nevertheless, the developmental origin and the function of tissue macrophage subsets, such as microglia, dermal macrophages, alveolar macrophages, Kupffer cells, splenic marginal zone and metallophilic macrophages remain insufficiently understood (Geissmann et al., 2010).

4.1.3 Cytokines and monocyte/macrophage development

The development of a mononuclear phagocyte system is governed by cytokines: small secreted proteins that promote cell-cell communication, and can act as growth and differentiation factors. The generation of monocytes and macrophages, and to some extent DCs, is dependent on cytokine M-CSF (or CSF-1), which was originally defined by its ability to generate macrophage colonies when added to cultures of BM progenitor cells *in vitro (Stanley et al., 1975)*. Subsequent discovery of $CSF1^{op}/CSF1^{op}$ mouse, which carried a spontaneous mutant lacking a functional Csf1 gene, showed the indispensable role of M-CSF in the generation and maintenance of monocytes and tissue macrophages (Cecchini et al., 1994; Yoshida et al., 1990). At which point the M-CSF is critical in macrophage development is still debated (Lenzo et al., 2011). In the *op/op* mice, there is severe depletion of monocytes in circulation. The production of macrophage colony-forming cells and monocytic cells (monoblasts, promonocytes and monocytes) is also defective in BM, and various tissue macrophages are deficient (Felix et al., 1990a; Felix et al., 1990b; Wiktor-Jedrzejczak et al., 1992; Wiktor-Jedrzejczak et al., 1991; Wiktor-Jedrzejczak et al., 1982). This evidence together suggests that the absence of M-CSF causes the macrophage development to be blocked at an early myeloid progenitor stage. However, a few immature macrophages are present in *op/op* mice, which are believed to be M-CSF independent macrophages derived from granulocyte/macrophage colony-forming cells or earlier hematopoietic cells with the help from GM-CSF and IL-34 (Naito et al., 1991; Wei et al., 2010; Wiktor-Jedrzejczak et al., 1992). Another important cytokine for monocyte/macrophage development is GM-CSF, which was also found in the *in vitro* BM cell culture to generate both granulocyte and macrophage colonies. However, unlike M-CSF, the deficiency in GM-CSF does not

compromise the steady-state myelopoiesis and most tissue macrophage populations. The major phenomenon observed in GM-CSF deficient mice is the pulmonary alveolar proteinosis because of a defect in alveolar macrophage maturation (Stanley et al., 1994). Taken together, this evidence supports the argument that M-CSF plays a major role in monocyte/macrophage development and acts at the early stages of myeloid development, whereas the role of M-CSF, GM-CSF and IL-34 in human macrophage development is still unclear due to the lack of a proper model to study.

4.1.4 Past efforts to improve monocytes/macrophages in humanized mice

Of note, the understanding of mouse tissue resident macrophages does not often apply to human macrophages because homotropic pathogens are predominantly or solely found in humans, and different sets of molecules are employed by human macrophages to control infections (Murray and Wynn, 2011). To translate knowledge obtained from animal models to better understand human macrophages and their role in associated diseases, humanized mice may offer an important *in vivo* readout if the engraftment and function of human macrophages reach a comparable level to humans. Moreover, the "human" innate and adaptive immune responses in current humanized mice, which are measured without the participation of human macrophages, are not a natural response as it is in humans and may hinder the application of humanized mice for preclinical testing. Hence, there is a great need to improve the reconstitution of tissue resident macrophages in humanized mice.

As certain murine cytokines critical for development of monocytes/macrophages, such as M-CSF, GM-CSF and IL-34, do not act on human counterparts (Fixe and Praloran, 1997; Ginhoux and Merad, 2010; Manz, 2007; Metcalf, 1986; Stevenson and Jones, 1994; Wei et al., 2010), we used hydrodynamic tail vein injection to

introduce human GM-CSF or M-CSF into humanized mice and significantly improved the reconstitution of human DCs and monocytes in peripheral blood and various organs, respectively (Chen et al., 2009). However, to what extent this treatment would restore the population and function of human tissue macrophages are still uncharacterized. Billerbeck et al. 2011 created NSG mice transgenically expressing human SCF, GM-CSF and IL-3 (NSG-SGM3), and then humanized them with $CD34^+$ human fetal liver (FL) cells (Billerbeck et al., 2011). Although the frequencies were elevated, human myeloid cells were still mostly found in the BM, but few in the peripheral organs. Subsequently, several other groups created human GM-CSF/IL-3 or M-CSF gene knock-in humanized mice to improve the engraftment of monocytes/macrophages (Rathinam et al., 2011; Willinger et al., 2011). Despite much progress that has been made in improving the engraftment of human monocytes in peripheral blood of these cytokine-supplemented mice, there is lack of direct evidence of improved reconstitution and function of human tissue macrophages. Moreover, a clear explanation of the problem of monocytes/macrophage development in humanized mice is still lacking.

4.1.5 Improvements of human macrophage reconstitution and immune function in humanized mice

Based on the hypothesis that human monocyte/macrophage development is impaired by cytokine cross-reactivity, we analyzed the BM of humanized mice and identified a unique large population of $SSC^{high}CD14^{low}CD45^+$ human monocyte precursors. These cells could be differentiated *in vitro* and *in vivo* to monocytes and macrophages when cultured with human GM-CSF or M-CSF. By hydrodynamic injection of plasmids encoding human M-CSF, we were able to generate significant levels of tissue resident

macrophages in various organs in humanized mice compared to untreated mice. Furthermore, enhanced engraftment and functionality of monocytes/macrophages leads to substantial improvement in human immune responses in M-CSF-treated mice compared to untreated mice.

4.2 Results

4.2.1 Human monocyte and macrophage development is blocked in BM of humanized NSG mice

To generate humanized mice, sublethally irradiated newborn pups were injected via intracardiac route with $CD34^+$ HSCs isolated from human FL. Twelve weeks after injection, human monocyte and macrophage reconstitution was examined in peripheral blood and various organs. Among human $CD45^+$ MNCs, the levels of $CD14^+CD11b^+$ monocytes ranged from 0% to 6% in blood. Although monocytes were detected in blood and organs, their frequency was relatively lower than that in corresponding human tissues and murine tissues (Figure 8A) (Fluks, 1981). To determine the frequency of tissue resident macrophages, $CD14^+CD68^+$ cells were examined in spleen, BM, lung and liver (Figure 8B). Most $CD14^+$ cells did not express the macrophage marker CD68 in the spleen, lung and liver. This suggests that human monocytes cannot efficiently differentiate into macrophage in humanized mice.

One important observation is that a large $CD14^{low}$ population was present only in BM but not in other organs or blood. Along with this observation, we also found a unique population with high side scatter (SSC) in BM (Figure 9A). The average number of this population per mouse BM (one tibia) was ~3 million, which is ~10% of human cells in BM. To find out the relationship between these two populations, the $CD14^{low}$ population was gated, and traced back to forward and side scatter characteristics (FSC/SSC) gating. As a result, >80% of $CD14^{low}$ cells are SSC^{high} cells (Figure 9B). Since both the low expression of CD14 and high SSC are the characteristics of

promonocytes (van Lochem et al., 2004), we hypothesized that the monocyte development in BM stopped at the promonocyte stage and hence monocyte could not be released into the peripheral.

Figure 8. Poor human monocyte/macrophage reconstitution in humanized mice.

Single-cell suspensions of peripheral blood, spleen, BM, lung and liver from 12 week old humanized NSG mice were prepared, stained for human CD45, CD14, CD11b and CD68, and analyzed by flow cytometry. Cells that are human $CD45^+$ were pregated and discriminated based on CD14 and CD11b expression (A), or CD14 and CD68 expression (B). Data are representative of 3 independent experiments.

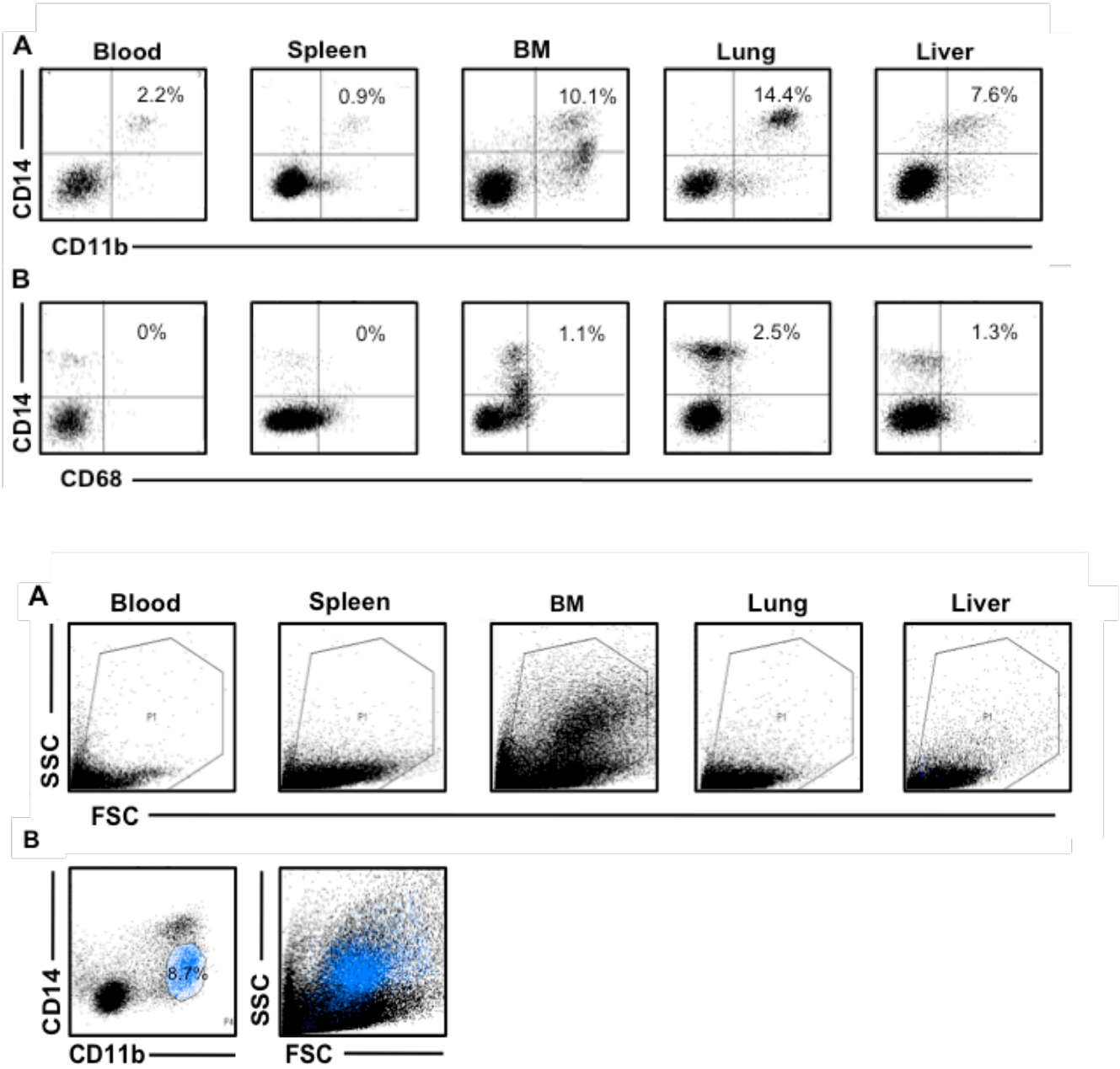

Figure 9. Unique human SSC^{high} myeloid cell population in BM of humanized mice.

Single-cell suspension of peripheral blood, spleen, BM, lung and liver from 12 weeks old humanized NSG mice were prepared, stained for CD45, CD14, and CD11b, and analyzed by flow cytometry. Dot plots show SSC versus FSC profiles gating on total cells (A). (B) CD14^{low}CD11b^{+} population in BM CD14 versus CD11b staining were gated (Left) and backgated to FSC versus SSC staining (Right). Data are representative of 3 independent experiments.

4.2.2 Human myeloid differentiation stops at monoblast to promonocyte stage in the BM of humanized mice

To further confirm the developmental stage of this unique population in BM, SSC^{high} cells were purified (>90%) to check their morphology by Giemsa staining (Figure 10A). The majority of these cells had an oval or indented nucleus with several nucleoli (Figure 10B). This is in agreement with the high SSC and suggests they are promonocytes. In addition, the phenotype of these cells was $CD45^{+}CD34^{-}CD13^{+}CD33^{+}CD15^{+}CD11b^{+}CD14^{low}$, which resembled the surface expression as promonocytes (van Lochem et al., 2004) (Figure 10C). Quantitative RT-PCR analysis of these cells also showed that they expressed myeloid genes (e.g. EGR-1, PU.1, VDR and LZM), and the levels of expression were lower than the levels in $CD14^{+}$ cord blood monocytes (Figure 11). This suggests that these BM $CD14^{low}$ cells belong to the myeloid lineage. Their low expression of the early myeloid commitment gene c-myb, and the expression of the differentiation gene C/EBP β further restrained the developmental progression to post-monoblast stage (Valledor et al., 1998). As myeloid cells are known to down-regulate the expression of MPO after maturing to monocytes (Lubbert et al., 1996), the relatively high expression of MPO suggests these cells are still at an immature stage. Based on these results, we conclude these immature cells in BM are $CD14^{low}SSC^{high}$ promonocytes. Hence, the accumulation of promonocytes and lack of mature human macrophages in peripheral blood strongly suggests that human monocyte/macrophage development in humanized mice may be blocked at the promonocyte stage.

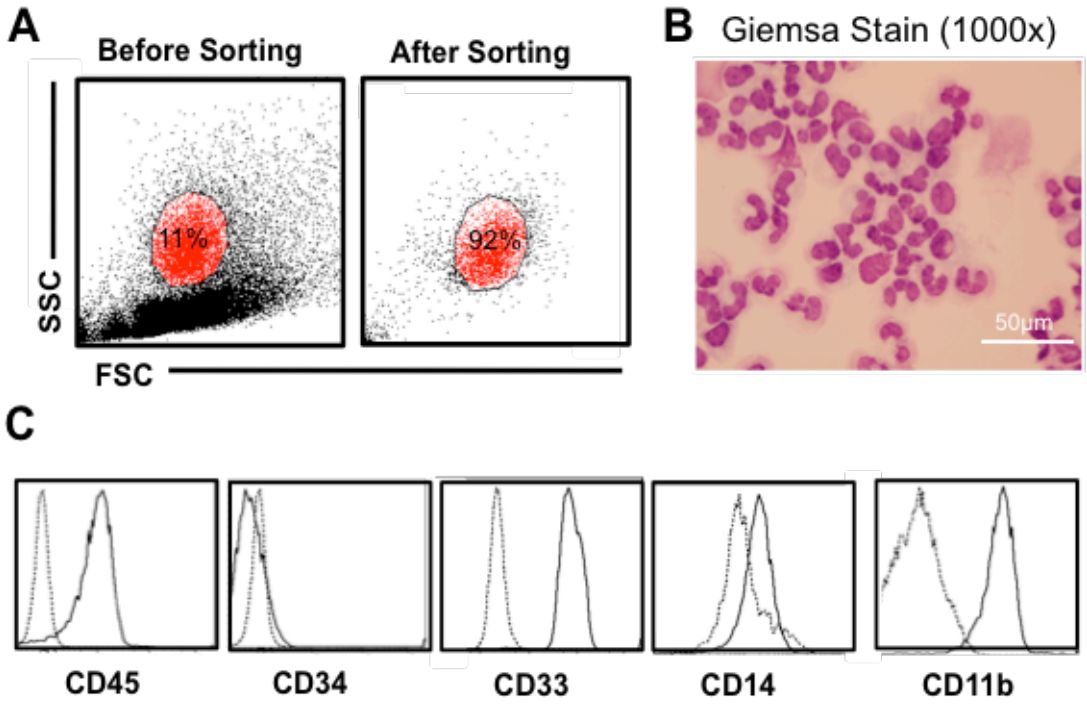

Figure 10. Characterization of SSC^{high} population in BM of humanized mice.

(A) Comparison of SSC versus FSC profiles of single cell suspensions from the BM of humanized mice (Left) and after purification by cell sorting (Right). Data are representative of 5 independent experiments. (B) Giemsa stain of sorted SSC^{high} population from BM. Sorted cells were cytospun, stained with Giemsa and analyzed by microscopy. A representative image is shown from one of three samples. (C) Sorted SSC^{high} population (black lines) from BM were stained for human CD45, CD34, CD33, CD14, and CD11b, analyzed by flow cytometry and compared to unstained controls (grey lines). Data are representative of 3 independent experiments

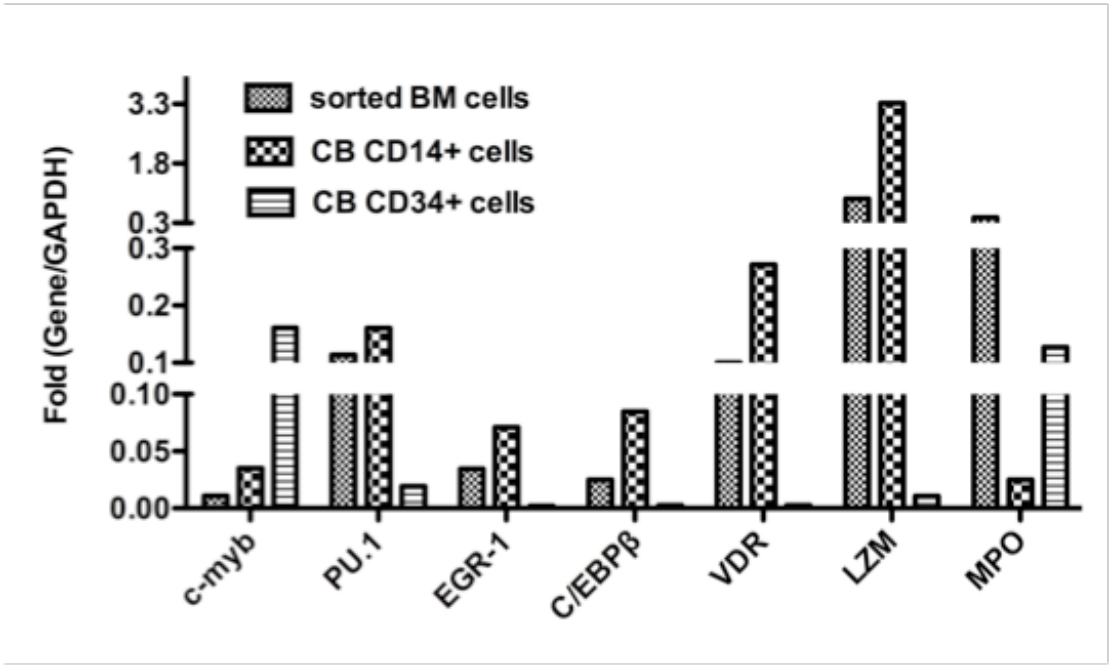

Figure 11. Gene expression profiles of SSC^{high} population in BM of humanized mice

RNA of Sorted SSC^{high} cells, cord blood CD14^{+} monocytes and cord blood CD34^{+} progenitor cells were extracted and quantitative PCR analysis was performed with human c-myb, PU.1, EGR1, C/EBPβ, VDR, LZM and MPO specific primers. Shown are the mean values of duplicate samples. Input cDNA was normalized according to GAPDH expression levels. Data are representative of 2 independent experiments.

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4.2.3 Human monocyte/macrophage development is restored by cytokine treatment

Human monocyte/macrophage lineage commitment and maturation rely on serial sets of cytokines. GM-CSF, M-CSF and IL-34 are one set playing a critical role in the development of granulocyte and monocyte precursor (CFU-GM) (Valledor et al., 1998). However, mouse GM-CSF, M-CSF and IL-34 are not cross-species active on human cells (Manz, 2007). Hence, we also performed the colony-forming cell assay to obtain information on the frequency and types of progenitors present in the SSC^{high} population, and their ability to proliferate and differentiate when supplemented with human cytokines. Cells were plated in enriched MethoCult, which supports the optimal growth of erythroid progenitors, including colony-forming-unit-erythroid (CFU-E) and burst-forming-unit-erythroid (BFU-E);

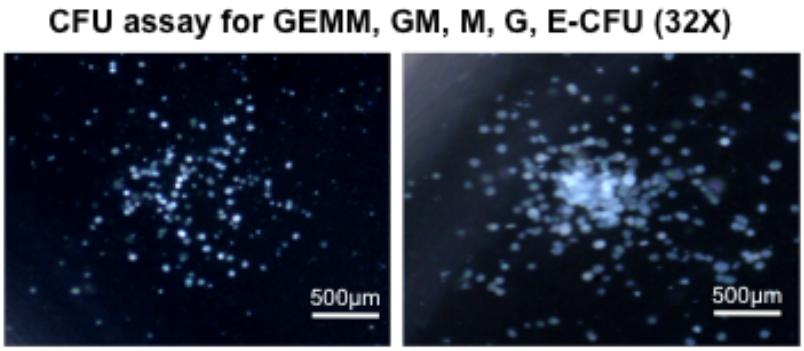

Figure 12. *in vitro* differentiation of SSC^{high} population from BM of humanized mice.

Sorted SSC^{high} cells were plated in triplicates in complete Methocult for 14 days. Colonies were numerated under a stereomicroscopy, and only CFU-M was detected. Magnifications are shown. Representative images are shown from 2 independent experiments.

granulocyte/macrophage progenitors, including colony-forming-unit-granulocyte macrophage (CFU-GM); colony-forming-unit-granulocyte (CFU-G) and colony-forming-unit-macrophage (CFU-M); and multipotential-colony-forming-unit-granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM). Two weeks later, only CFU-M was detected under the microscopy with a frequency of 1/220 (Figure 12B). Many small CFU-M like colonies with less than 20 cells were observed but not counted. These colonies may be derive from monoblasts and promonocytes, which have limited proliferation potential. Hence, it is likely that SSC^{high} population is predominantly promonocytes, but may also contain a few monoblasts.

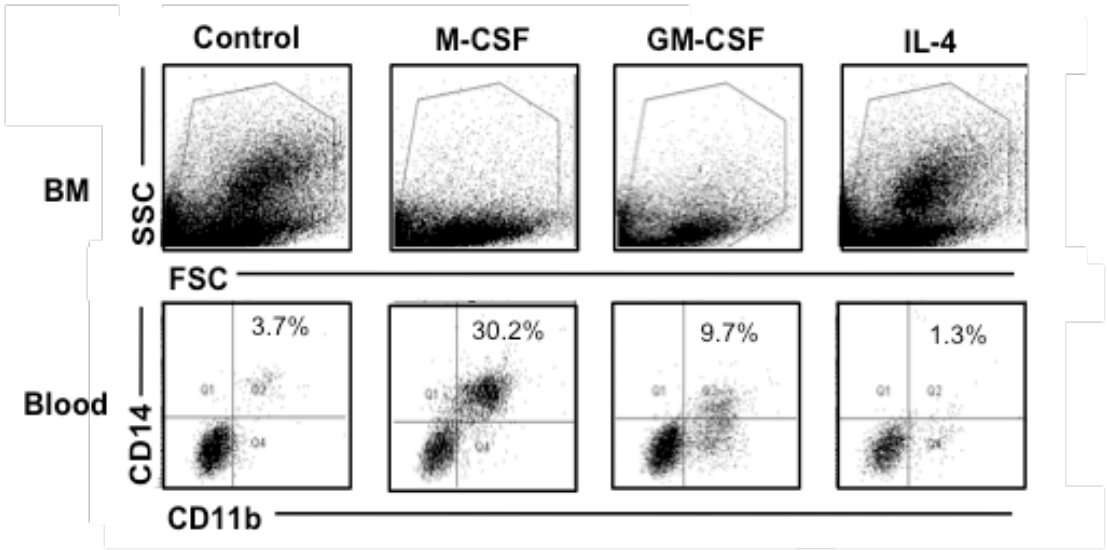

Figure 13. *in vivo* differentiation of SSC^{high} population from BM of humanized mice.

pcDNA vectors expressing M-CSF, GM-CSF, or IL-4 were hydrodynamically injected into humanized mice. 7 days after injection, single cell suspensions of blood and BM were collected from these mice, stained for human CD45, CD14 and CD11b human antibodies, and analyzed for SSC and FSC profiles for BM (Top) and CD14 versus CD11b profiles gating on $CD45^+$ cells for blood (Bottom). Data are representative of 3 independent experiments.

The finding that SSC^{high} population comprised monocyte/macrophage progenitors, and that human monocytes developed *in vitro* in the presence of M-CSF and GM-CSF/IL-4 suggests that these human cytokines may also drive the differentiation of SSC^{high} populations to mature monocytes *in vivo*. To introduce human cytokines into mice, we used hydrodynamic delivery of cytokine-encoding plasmids. 7 days after a single injection of 50 μg human M-CSF or GM-CSF encoding plasmids, promonocytes disappeared and abundant human $CD11b^+$ monocytes were presented in blood, while promonocytes and blood monocytes remained unchanged in untreated control mice and in mice injected with human IL-4 encoding plasmids (Figure 13). The percentages of human $CD11b^+$ monocytes also significantly increased in the spleen, BM, liver and lung in M-CSF or GM-CSF treated mice, but not in IL-4 treated mice (Figure 14A). Human monocytes/macrophages induced by M-CSF are known to express high CD14, whilst GM-CSF or IL-4 stimulated monocytes down-regulate the expression CD14 and exhibit accessory cell functions like DCs (Liu et al., 2001; Ruppert et al., 1991; Shalhoub et al., 2011). The same phenomenon was observed in our cytokine-treated humanized mice. To further explore whether the increased monocytes in M-CSF and GM-CSF treated mice were macrophages, we purified $CD45^+CD11b^+$ cells from splenocytes of GM-CSF treated mice and M-CSF treated mice, and stained these cells with Giemsa. These sorted cells from both GM-CSF treated mice and M-CSF treated mice exhibited typical macrophage morphology, i.e. large in size, round shape and heavily vacuolated (Figure 14B). Based on the results obtained, we found promonocytes could be mobilized by GM-CSF or M-CSF treatments in the human monocyte/macrophage development in humanized mice.

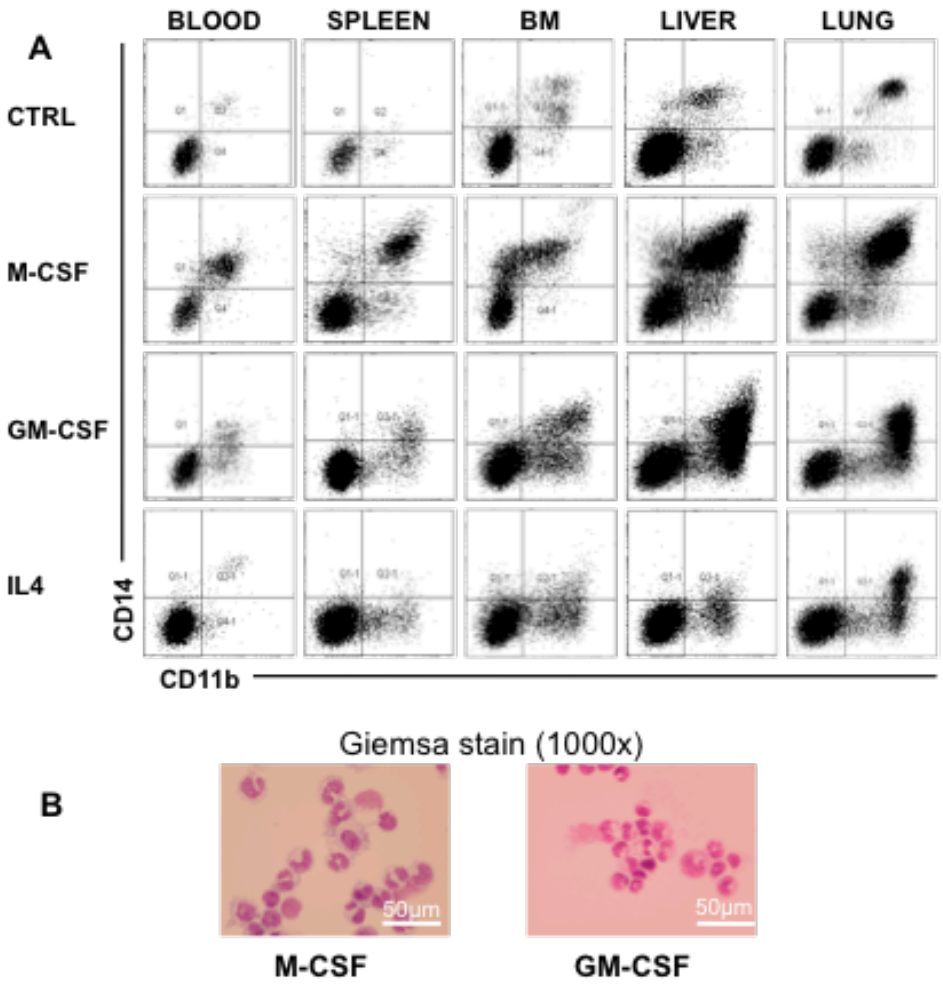

Figure 14. Expression of GM-CSF or M-CSF stimulates human monocyte/ macrophage development in vivo.

(A) pcDNA vectors expressing M-CSF, GM-CSF or IL-4 were hydrodynamically injected into humanized mice and untreated humanized mice from the same batch were used as controls. 7 days after injection, cells were prepared from the indicated tissues and stained for CD45, CD14 and CD11b human antibodies. Dot plots show CD14 versus CD11b staining profiles gating on $CD45^+$ cells. Data are representative of 2 independent experiments. (B) Giemsa staining of sorted $CD11b^+$ splenocytes from M-CSF or GM-CSF hydrodynamically injected mice. Shown are representative pictures of cytospin samples from n=3 mice per group. Magnifications are indicated.

4.2.4 Human tissue resident macrophages are generated in M-CSF treated mice

Since $CD14^+CD11b^+$ monocytes/macrophages were significantly increased in M-CSF treated mice, and the sorted $CD11b^+$ cells exhibited macrophage morphology in both GM-CSF treated mice and M-CSF treated mice, we further analyzed the M-CSF treated mice for tissue resident macrophages. M-CSF treated mice were perfused with saline, and their organs including brain, spleen, lung and liver were harvested. Single cell suspensions were prepared, and stained with tissue resident macrophage marker CD163 and CD68. Interestingly, untreated humanized mice did not reconstitute well with $CD14^+$ cells, and also most of $CD14^+$ cells did not express the macrophage marker CD68 and CD163. These results suggest that monocytes/macrophages naturally developed in humanized mice cannot mature into tissue resident macrophages. As expected, the frequencies of human $CD14^+CD68^+$ and $CD14^+CD163^+$ cells were significantly increased in all organs of M-CSF treated mice (Figure 15).

To further support the findings that tissue resident macrophages can develop in M-CSF treated mice, histology analysis of liver, lung and kidney was performed on saline-perfused M-CSF treated mice. Consistent with the flow cytometry data, untreated humanized mice showed no human $CD68^+$ cells in the examined organs, but a considerable number of mouse $CD68^+$ cells were observed in the lung and liver (Figure 16A). In contrast, the lung and liver of M-CSF treated mice were substantially reconstituted with human $CD68^+$ macrophages (Figure 16B). Of note, these M-CSF induced macrophages selectively reconstituted in the liver and lung, but not in the kidney. Their frequency and distribution patterns were similar to their mouse counterparts. Taken together, these data demonstrate that tissue resident macrophages

successfully reconstitute M-CSF treated mice and were strategically located in selected target organs.

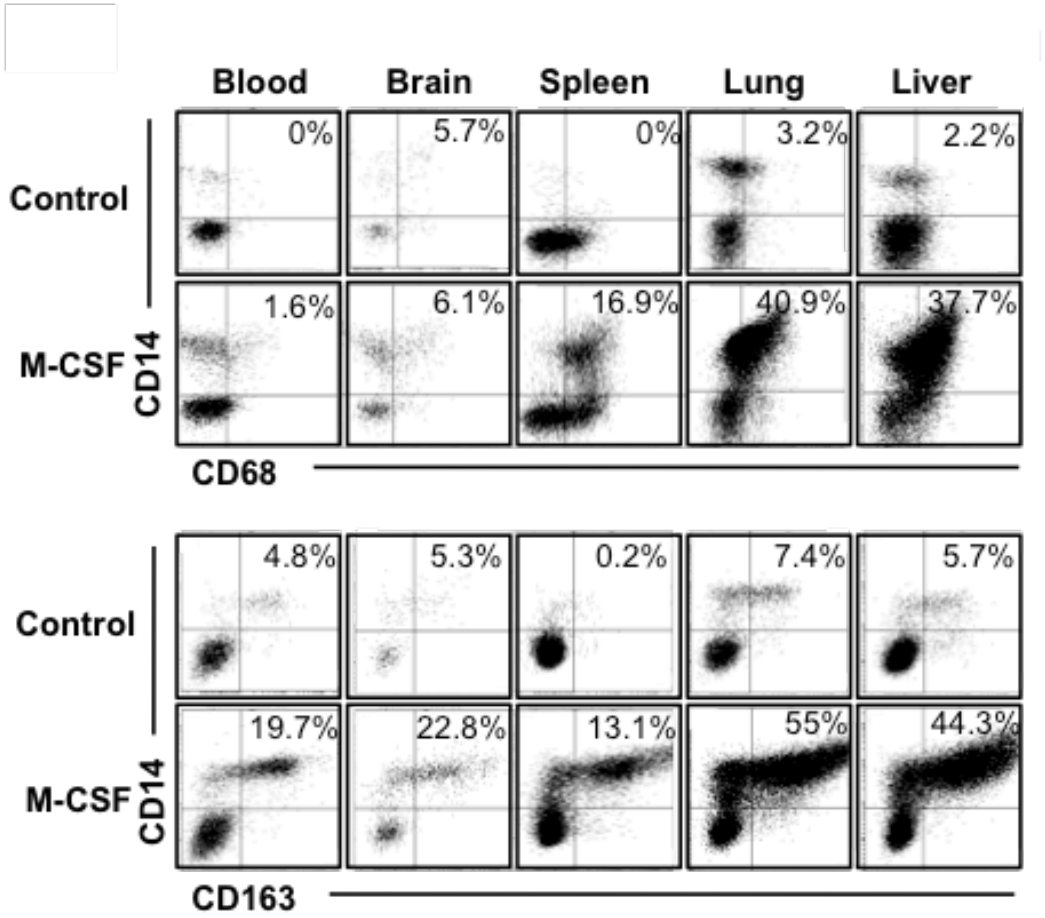

Figure 15. Tissue resident macrophages in M-CSF treated mice.

Improved reconstitution of tissue resident macrophages. 12 weeks old humanized mice were hydrodynamically injected with pcDNA vectors expressing human M-CSF and untreated humanized mice from the same batch were used as controls. 7 days after injection, single cells suspension was prepared from indicated tissues of M-CSF treated humanized mice and untreated humanized mice. Cells were stained for human CD45, CD14, CD68, and CD163. Shown are CD14 versus CD68 (Top) and CD14 versus CD163 (Bottom) staining profiles gating on $CD45^+$ human cells. Data are representative of 3 independent experiments.

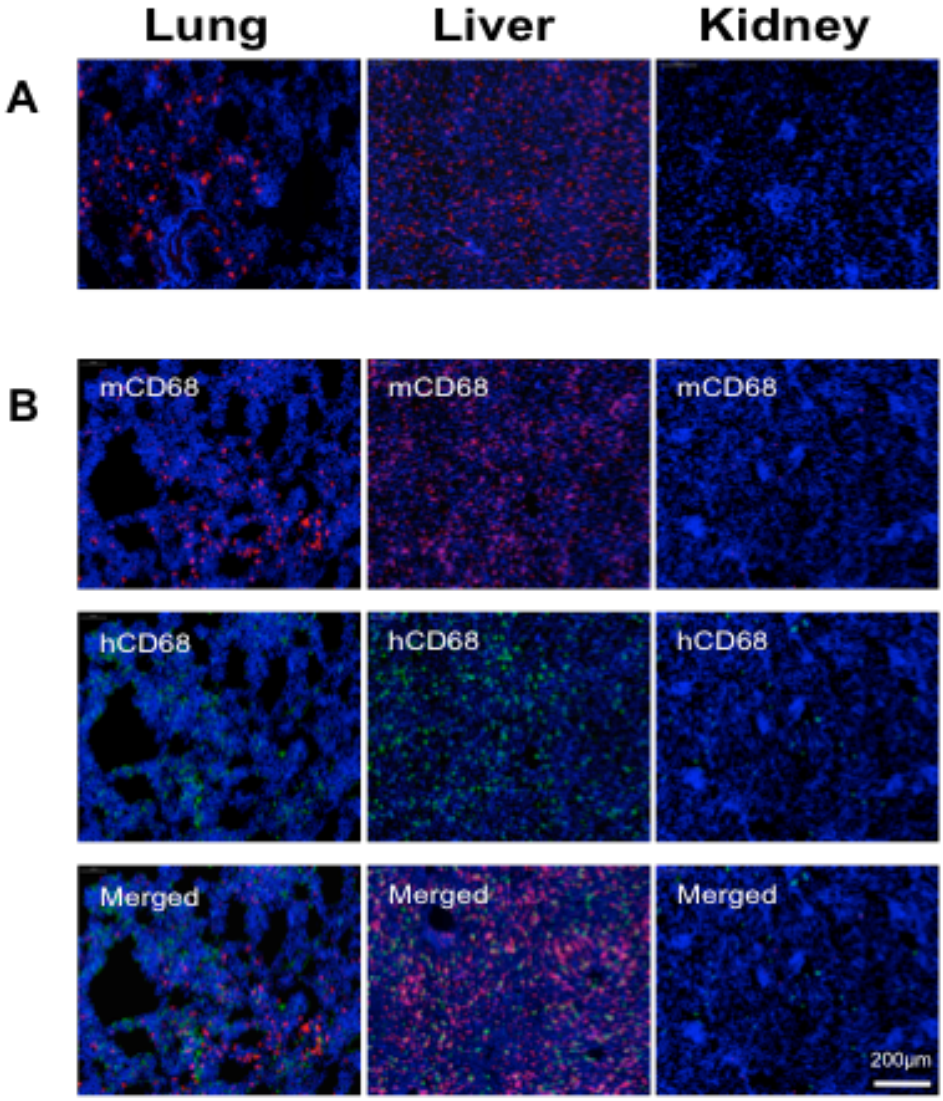

Figure 16. immunofluorescence staining on mouse frozen sections

Immunofluorescence staining of mouse CD68 and human CD68 on cryosections of organs from untreated humanized mice and M-CSF treated mice organs. Lung, liver and kidney samples were snap-frozen, cryosectioned, stained for human CD68 (Green) and mouse CD68 (Red) and scanned by microscopy. Merged images are shown for sections from untreated humanized mice (A). Single color and merged images are shown for sections from M-CSF treated mice (B). Representative images are shown from one of 5 mice per group.

4.2.5 Human inflammatory responses are enhanced in M-CSF treated mice

To investigate whether the induced monocytes and tissue macrophages in M-CSF treated mice were functional, we injected LPS i.p. to induce systemic inflammation. 2 hours and 12 hours after injection, mice were bled and the serum levels of human proinflammatory cytokines were determined by CBA assay. Consistent with the increased frequencies of monocytes and macrophages in M-CSF treated mice, significantly elevated levels of IL-6, TNF-α, IL-10, IL-1β and IL-8 were detected in the M-CSF treated mice (Figure 17). Of note, the basal levels of these cytokines were the same between M-CSF treated mice and untreated mice, which indicated the quiescent status of newly generated monocytes and macrophages in M-CSF treated mice. Overall, human inflammatory responses mediated by human monocytes and macrophages were enhanced with M-CSF treated mice.

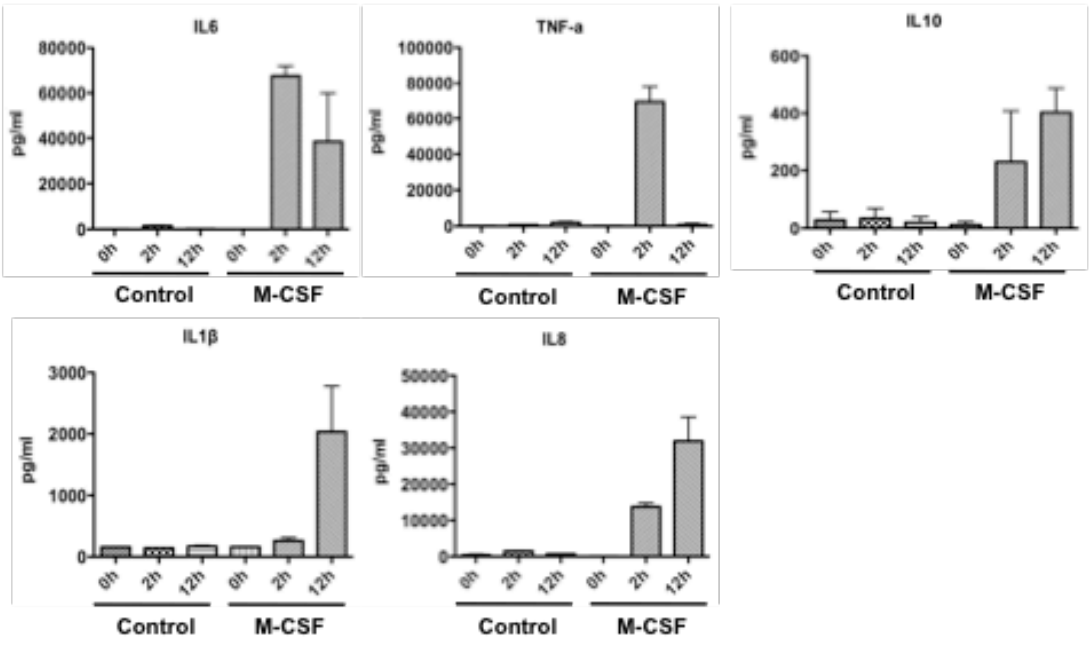

Figure 17. Enhanced human inflammatory response in M-CSF treated mice after LPS challenge.

CBA assay of human IL-6, TNF-α, IL-10, IL-1β and IL-8 were performed on sera from M-CSF treated mice (n = 3-5) and untreated humanized mice (n = 3-5) 0 h, 2 h, 12 h after LPS injection. Untreated humanized mice from the same batch were used as controls. Error bars indicate SEM. Data are representative of 2 independent experiments.

4.2.6 M-CSF treated mice mount enhanced immune response against influenza A virus

After we have shown the presence of tissue resident macrophages and their responses to LPS, we further used influenza A virus infection as a model to test whether the human alveolar macrophages can provide innate immunity to a natural pathogen in M-CSF treated mice. We infected mice intratracheally with 300 pfu influenza A/PR8 (H1N1) virus, and sacrificed them at 24 h, 48 h, and 72 h post-infection for various tests. The influenza virus RNA was examined at each time-point by quantitative PCR of lung homogenates and normalized to mouse gene L32 expression. The viral levels were significantly lower in M-CSF treated mice than untreated mice during the course of infection (Figure 18A). However, the difference of virus load between M-CSF treated mice and untreated mice became smaller as the infection progressed. To investigate if the decreased virus load was the results of improved human macrophage reconstitution in the lung, we carried out several experiments to assay human macrophage response to influenza infection. First, the human alveolar macrophages obtained from M-CSF treated mice displayed enhanced activation as assessed by the upregulation of CD80 costimulatory molecules at 24 h post-infection (Figure 18B). Second, in addition to the increased frequency of human alveolar macrophages (as demonstrated in Figure 15 and Figure 16), their absolute numbers were significantly higher throughout the course of infection (Figure 18C). Third, consistent with the increased number and functionality of alveolar macrophages in M-CSF treated mice, significant levels of proinflammatory cytokines, such as IL-6, TNF-α and IL-1β, were detected both at protein and RNA levels in lung homogenates between 24 h and 72 h post-infection in M-CSF treated mice (Figure 19). In contrast, untreated mice showed no significant inductions of proinflammatory cytokine during the course of

influenza A infection. Similarly, as in LPS condition, the fact that only basal levels of proinflammatory cytokines were detected before infection supported that alveolar macrophages were in resting status in M-CSF treated mice. Taken together, these data show that M-CSF treated mice can exhibit enhanced immune response against influenza virus due to improved human macrophage chimerism and functions in the lung.

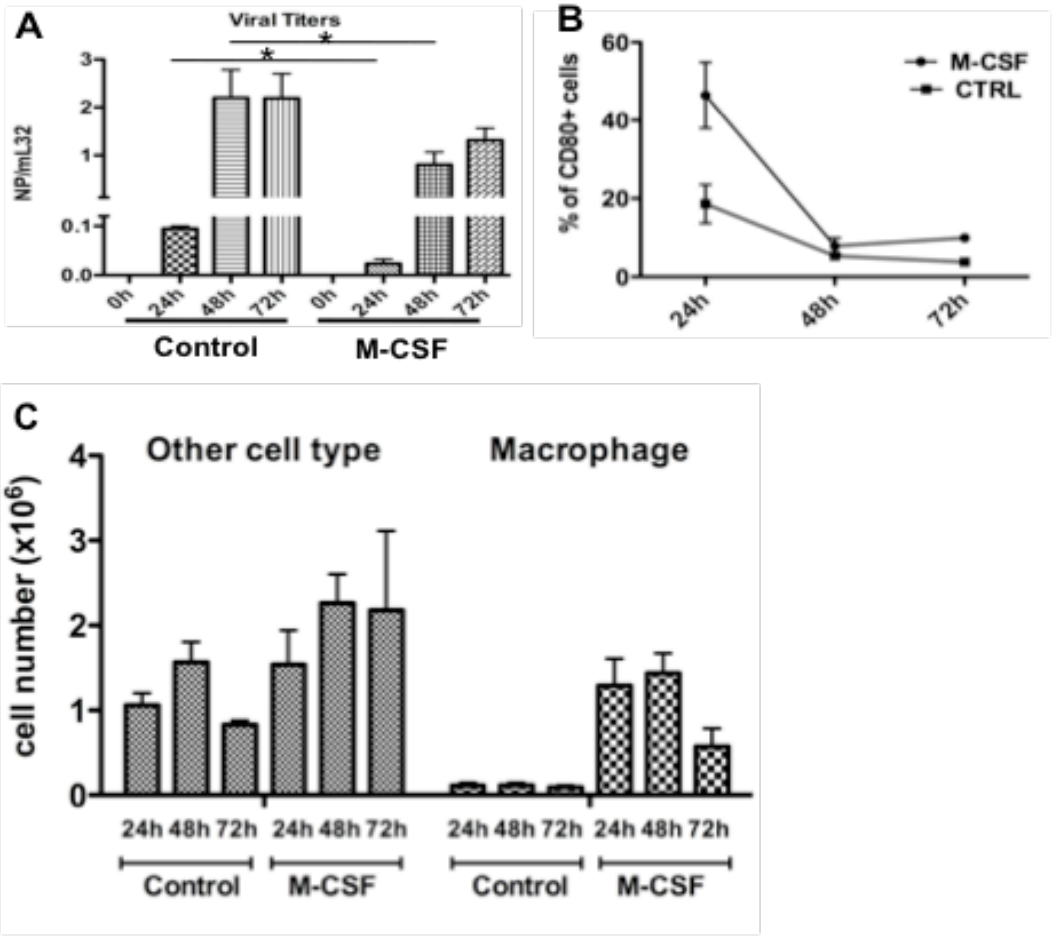

Figure 18. Influenza A virus infection in M-CSF treated mice.
Humanized mice were hydrodynamically injected with pcDNA vectors expressing M-CSF and untreated humanized mice from the same batch were used as controls. 7 days post-injection, M-CSF treated mice and untreated mice were infected intratracheally with influenza A virus. Mice were killed 0 h, 24 h, 48 h and 72 h post infection, and single cell suspensions from the left lobe of lung were prepared. (A) Quantitative RT-PCR analysis of viral nuclear protein (NP) gene expression in lung tissue from untreated humanized mice and M-CSF treated mice 0 h, 24 h, 48 h and 72 h post-infection. Expression was normalized to mouse L32. Shown are the mean values of three mice per group. Error bars indicate SEM. Data are representative of two independent experiments. (B) The frequency of human $CD80^+$ cells in $CD45^+CD14^+CD11b^+$ cells in lung was determined in untreated humanized mice and M-CSF treated mice. Shown are the mean values of three mice per group. Error bars indicate SEM. Data are representative of two independent experiments. (C) Single cell suspensions from left lobe of lung were prepared from untreated humanized mice and M-CSF treated mice 0 h, 24 h, 48 h and 72 h post-infection. The frequency of macrophages were determined by flow cytometry analysis of CD14 in $CD45^+CD45.1^-$ population. The absolute number of each cell lineage was counted by total cell counts x human cell percentage x corresponding cell percentage. Shown are the mean values of three mice per group. Error bars indicate SEM. Data are representative of two independent experiments.

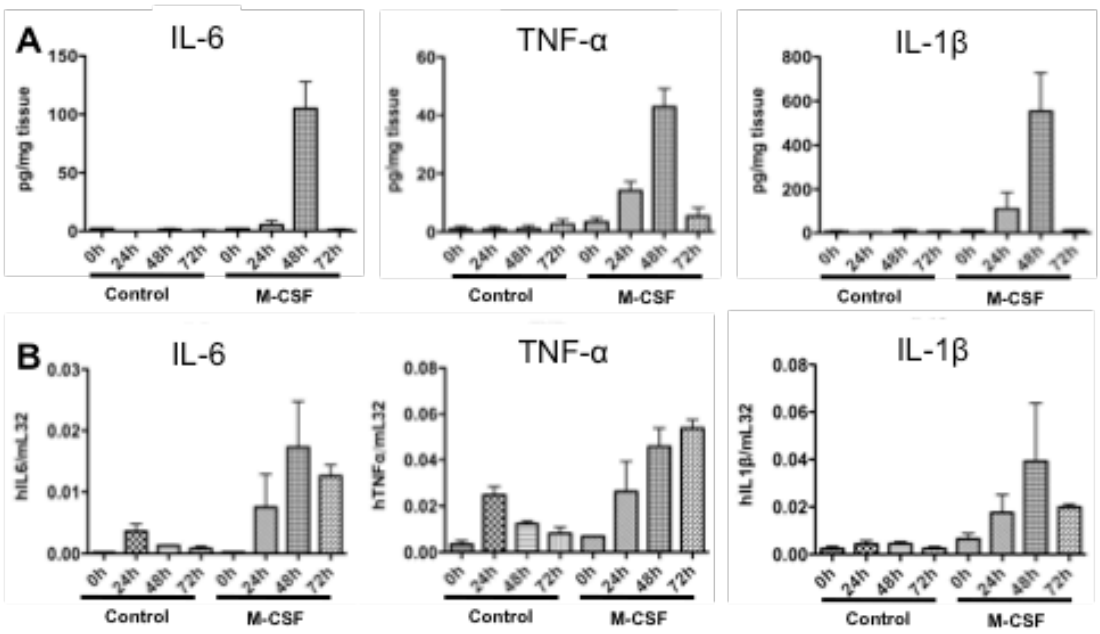

Figure 19. Enhanced human inflammatory responses in M-CSF treated mice after influenza infection.

(A) CBA assay of human IL-6, TNF-α and IL-1β protein were performed on lung homogenates from untreated humanized mice and M-CSF treated mice at 0 h, 24 h, 48 h, and 72 h post-infection. Untreated humanized mice from the same batch were used as controls. Shown are the mean values of triplicate samples. Error bars indicate SEM. Data are representative of two independent experiments. (B) Quantitative RT-PCR analysis of human IL-6, TNF-α and IL-1β expression in lung tissue from untreated humanized mice and M-CSF treated mice 0 h, 24 h, 48 h and 72 h post-infection. Expression was normalized to mouse L32. Shown are the mean values of three mice per group. Error bars indicate SEM. Data are representative of two independent experiments.

Other interesting results were observed in influenza infected M-CSF treated mice. Unlike LPS stimulated human macrophages that secreted IL-10 into serum, human IL-10 protein was not detected both in lung homogenate and serum samples of M-CSF treated and untreated mice after influenza infection (data not shown). However, mRNA expression of human IL-10 showed a higher level and an increasing trend in M-CSF treated mice compared to untreated mice (Figure 20A). Activated antigen specific T cells and tissue resident macrophages are the main producers of IL-10 during influenza virus infection to suppress the inflammation and prevent injury to tissue. Since the specific role of IL-10 in the acute influenza infection is still unclear, M-CSF treated mice may offer a tool to study the regulation and function of this cytokine. Furthermore, since alveolar macrophages are the main type-I IFN producer cells after infection with pulmonary viruses, we also measured the IFN-β mRNA in lung homogenate. M-CSF treated mice had a steadily increasing trend of human IFN-β mRNA expression during the course of infection compared to untreated mice (Figure 20B) (Kumagai et al., 2007). During influenza A virus infection, macrophages function also as accessory cells by producing chemokines and cytokines to promote T cell responses to clear the viruses. In M-CSF treated mice, an increasing trend of human IL-12 mRNA expression correlated with the increased production of IFN-γ mRNA most likely because macrophages secrete IL-12 to stimulate the production of IFN-γ by NK cells and cytotoxic T cells (Desmedt et al., 1998; Murray and Wynn, 2011) (Figure 20C). In untreated mice, both IL-12 and IFN-γ production decreased from 24 h post-infection.

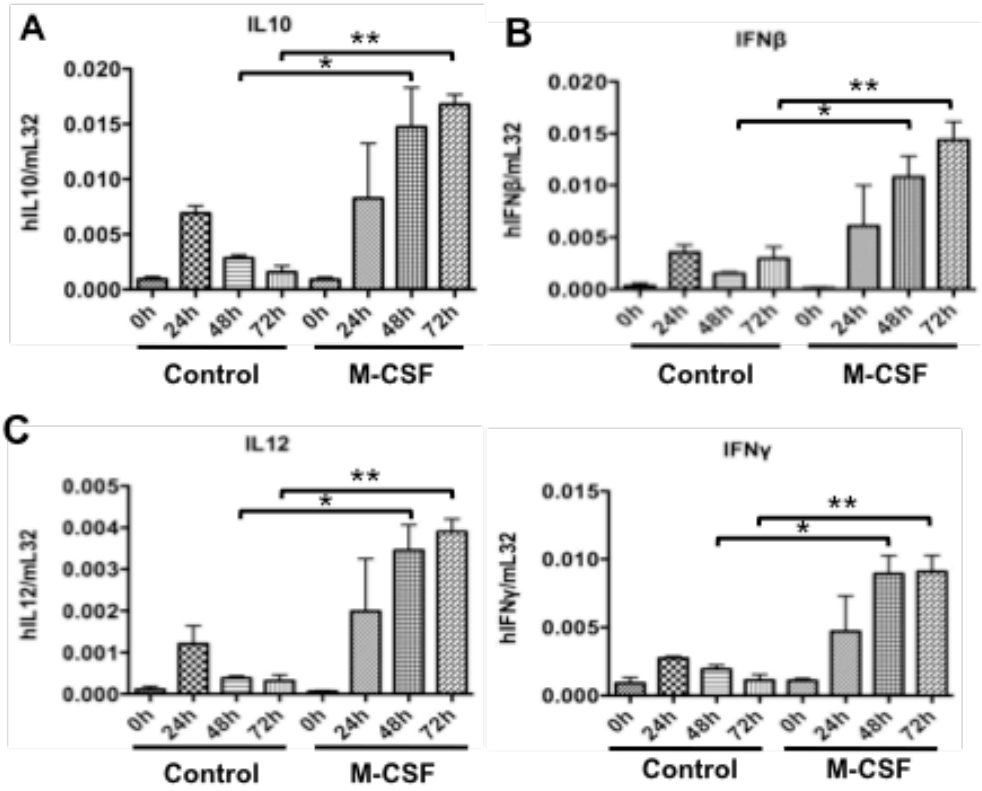

Figure 20. Q-PCR analysis of cytokine production after influenza A virus infection.

Quantitative RT-PCR analysis of human IL-10, IFN-β, IL-12 and IFN-γ expression were performed on RNA extracted from lung tissue of untreated humanized mice and M-CSF treated mice 0 h, 24 h, 48 h and 72 h post infection. Untreated humanized mice from the same batch were used as controls. Expression was normalized to mouse L32. Shown are the mean values of three mice per group. Error bars indicate SEM. Data are representative of two independent experiments.

4.2.7 Human peritoneal macrophages are recruited during Mycobacterium infection in M-CSF treated mice

Macrophages are critical components of the innate and adaptive immune responses to bacterial pathogens. Infection of mice with the intracellular bacterium BCG via the i.p. route has served as a classic system for the study of macrophage activation (Hamerman and Aderem, 2001). We have demonstrated the systemic responses of monocytes and macrophages to LPS challenge, and the local response of tissue resident macrophages to influenza A virus infection. Here, we took the advantage of BCG infection as a model to test the ability of monocytes to migrate to inflammatory sites and to differentiate into activated macrophages. 5 million CFUs of BCG were injected into mice. Mice were sacrificed at 24 h and 72 h post-infection. Peritoneal lavage and spleen homogenates were collected for BCG colony assays. Significantly lower numbers of BCG colony counts found in the spleen and peritoneal lavage samples of M-CSF treated mice (Figure 21A). Moreover, human $CD14^{+}CD11b^{+}$ macrophages were present only in M-CSF treated mice at 24 h post-infection (Figure 21B). These macrophages may be derived from activated monocytes recruited from the peripheral blood in response to BCG stimulation. However, the majority of peritoneal cells were still of mouse origin (>80%) during the course of infection. We also extracted RNA from peritoneal cells and examined the expression of human inflammatory cytokines (Figure 21C). Only M-CSF treated mice showed increased expression of IL-6 and IL-1 β at 24 h post-infection. Taken together, a proportion of human monocytes in M-CSF treated mice can be recruited to inflammatory sites to differentiate into macrophages to control the growth of BCG.

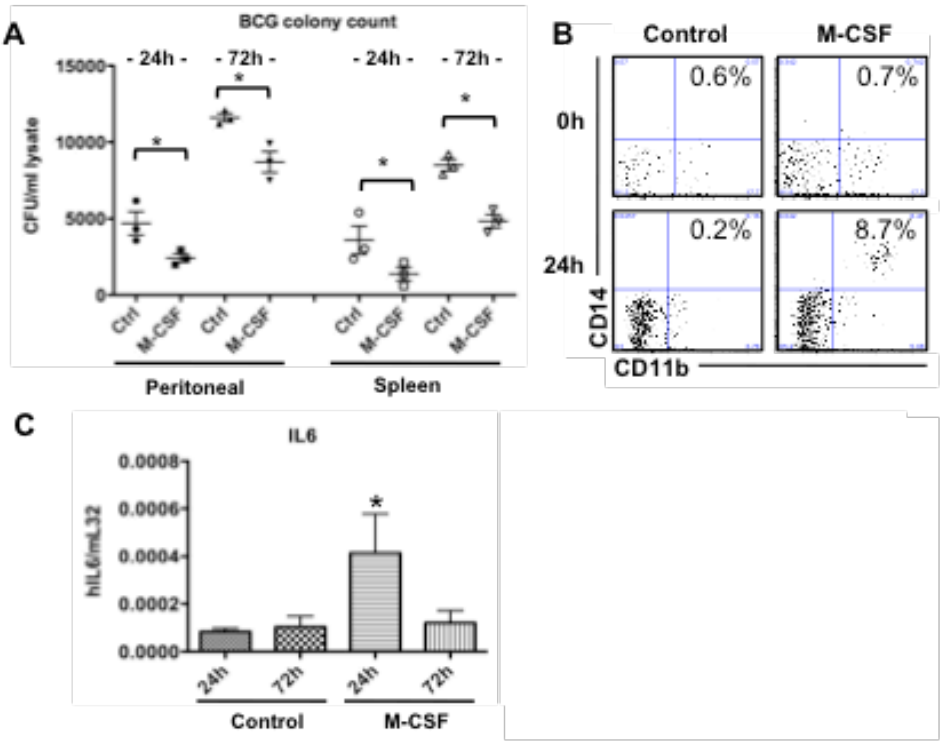

Figure 21. BCG infection in M-CSF treated mice.
Humanized mice were hydrodynamically injected with pcDNA vectors expressing M-CSF and untreated humanized mice from the same batch were used as controls. 7 days post-injection, M-CSF treated mice and untreated mice were infected intraperitoneally with BCG. Peritoneal lavage and spleen was collected at 0 h, 24 h, and 72 h post-infection. (A) BCG colony count from untreated humanized mice and M-CSF mice at 24 h and 72 h post-infection. Each symbol represents an individual mouse; horizontal bars indicated the mean values. Error bars indicate SEM. Data are representative of two independent experiments. (B) Peritoneal cells from untreated humanized mice and M-CSF treated mice 0 h and 24 h after infection were stained for CD45, CD45.1, CD14 and CD11b human antibodies. Cells that are human $CD45^{+}CD45.1^{-}$ were pregated and discriminated based on CD14 versus CD11b expression. (C) Quantitative RT-PCR analysis of human IL-6 expression in total peritoneal cells from untreated humanized mice and M-CSF treated mice 24 h and 72 h post-infection. Expression was normalized to mouse L32. Shown are the mean values of four mice per group. Error bars indicate SEM. Data are representative of two independent experiments

4.3 Discussion

The ability to study human hematopoiesis with in vivo settings is one of the advantages of humanized mice. Given that cytokines are important for human monocyte/macrophage development (e.g. M-CSF, GM-CSF and IL-34), and are species-specific (Manz, 2007; Wei et al., 2010), humanized mice may be regarded as a 'human knockout model' to study the impact of these cytokines. GM-CSF induces proliferation and differentiation of intermediate myeloid precursors, while M-CSF induces not only the proliferation and differentiation of late myeloid precursors but also the maturation of monocytes into macrophages (Ogawa, 1993). IL-34 increases monocytes viability, induces macrophage proliferation, and synergized with other cytokines to generate macrophages and osteoclasts from cultured progenitors. Based on the functions of these three cytokines, human myelogenesis would stop at CFU-GM or CFU-M stages. However, as described in our results, the characterization of humanized mice revealed that there was a unique human cell population in BM, and human monocyte/macrophage development was blocked mainly at the promonocyte stage. Hence, it would be necessary to reevaluate our knowledge obtained from mouse experiments and in vitro data, and human monocyte/macrophage progenitors might exploit other cytokines other than GM-CSF and M-CSF for differentiation to promonocytes in vivo.

Studies on op/op mice, which are deficient for mouse M-CSF, reveal that the hematopoietic system has the capacity to use alternative mechanisms to compensate for the absence of M-CSF probably by the effect of GM-CSF and IL-34 (Begg et al., 1993; Nilsson and Bertoncello, 1994; Wei et al., 2010). Hence, at least one of three

cytokines has to be presented for monocyte/macrophage development. With supplementation of human GM-CSF or M-CSF by hydrodynamic injection, the blocked development could be restored and monocytes/macrophages could be efficiently generated. Human monocytes/macrophages induced by M-CSF are known to express high CD14, whilst GM-CSF or IL-4 differentiated monocytes down-regulate the expression of CD14 and exhibit accessory cell functions like DCs (Ruppert et al., 1991; Shalhoub et al., 2011). The same phenomenon was observed in our cytokine treated humanized mice. Absence of both M-CSF and GM-CSF would hinder human monocyte/macrophage development in later stages rather than early stages of hematopoiesis, and the ratio of expression of these 2 cytokines affects the homeostasis of monocytes, macrophages and DCs.

In agreement with the indispensable role of M-CSF in generation of mouse tissue resident macrophages in organs from studies on op/op mice (Cecchini et al., 1994), various human tissue macrophages were significantly induced after M-CSF treatment. Previous efforts to improve human monocyte/macrophages, such as human GM-CSF/IL-3 or M-CSF knock-in Balb/c $Rag2^{-/-}\gamma c^{-/-}$ mice, have increased levels of alveolar macrophages and circulating monocytes compared to control mice, respectively (Rathinam et al., 2011; Willinger et al., 2011). However, tissue macrophages other than alveolar macrophages, and circulating monocytes in GM-CSF/IL-3 knock-in mice were not significantly improved. Although high percentages of circulating monocytes were achieved in M-CSF knock-in mice, the highest level of tissue monocyte/macrophage reconstitution was still under 5% of human $CD45^{+}$ cells. There could be several reasons for the limited improvements seen in these knock-in mice. First, these mice were created on Balb/c $Rag2^{-/-}\gamma c^{-/-}$ mice, which do not have the SIRP-α polymorphism like NSG mice. The functional mouse macrophage would

prevent high levels of reconstitution of human cells in tissues. Hence, the developing tissue macrophages and monocytes could be phagocytosed by mouse macrophages in M-CSF and GM-CSF knock-in mice. Second, M-CSF is not only produced by endothelial cells and fibroblasts, but also by monocytes, granulocytes. Moreover, mouse cells also consume human M-CSF. Poor tissue macrophage reconstitution in M-CSF knock-in mice may be the result of competition between human and mouse macrophages for the less than optimal amount of human M-CSF produced from mouse nonhematopoietic cells. Hence, a useful control to compare the human M-CSF levels in serum of knock-in mice would be the endogenous concentration of mouse M-CSF in serum of wild type mice rather than immunodeficient mice. As we used hydrodynamic injection to deliver cytokines into NSG mice, we circumvented the above problems confronted by knock-in mice. Although hydrodynamic injection provides short-term and variable levels of expression in vivo, the levels of expression are much higher in hydrodynamic condition compared to the levels in knock-in condition. This excessive amount of cytokines guarantees that both human and mouse monocyte and macrophages could be efficiently generated. Hence, both high levels of circulating monocytes and different types of tissue macrophages were induced after a single hydrodynamic injection of M-CSF plasmids. This method renders researchers a rapid approach to test the effect of one or several secreted proteins in mice. Furthermore, the level and duration of the expression of gene expression can be optimized in hydrodynamic injection by the amount of plasmids, choice of the promoters, and number of injections. Therefore, hydrodynamic injection of M-CSF encoding plasmids into NSG mice leads to high levels of human tissue resident macrophage reconstitution, and the tissue macrophage in NSG mice offers a unique and powerful tool to study the development of human monocyte/macrophage

development and compare the human macrophage with their cognate mouse model systems. This is an area with great potential, as the chasm between understanding human and mouse macrophages is wide.

Along with the improved reconstitution of human monocytes and tissue macrophages, M-CSF treated humanized mice also exhibit significantly stronger proinflammatory response to LPS and enhanced protection against influenza virus and BCG infection. The levels of proinflammatory cytokine induced in M-CSF treated mice are ~46 fold and ~34 fold higher than untreated mice for IL-6 and TNF-α at 2 hour post-injection, respectively. The LPS stimulation in current knock-in mice results in only 2-3 fold higher level of IL-6 than control humanized mice (Rathinam et al., 2011; Willinger et al., 2011). In addition, we first reported the enhanced immune response against influenza virus and BCG infection in humanized mice. No difference of viral load in the course of infection was seen between GM-CSF/IL-3 knock-in mice and control mice, whereas significant lower level of viral load was seen between M-CSF treated mice and untreated mice. In BCG infection, the numbers of BCG colonies were significantly lower in spleen and peritoneal of M-CSF treated mice than untreated control during the period measured. These improvements should be attributed to higher engraftment and better function of monocytes and tissue macrophages.

Despite the presence of tissue resident macrophages and enhanced responses against infections, the virus burden and bacteria were still significant in M-CSF treated mice over the course of infection. This could imply that resident macrophages alone are insufficient to control the infection, and that subsequent T and B cell responses are needed to clear the infection (McGill et al., 2009). In M-CSF treated mice, more human T cells could be found in lung after 3 days of infection (data not shown). This

suggests functional macrophages may secrete chemokines and cytokines to attract more T cells and induce their differentiation into effector cells. To prove these hypothesis, *in vitro* experiments, such as mixed leukocyte reaction, need to be conducted to test the function of human T cells and macrophages from M-CSF treated mice in the future. *In vivo* experiments, such as immunization of tetanus toxoid and infection of influenza virus, also need to be carried out to test human B cell and T cell response in M-CSF treated mice. With information from these experiments, not only the functionality of M-CSF induced macrophages could be proved, the puzzle around human T cell activation in humanized mice may also be answered. If human macrophages in M-CSF treated mice were capable of stimulating human T cells *in vitro* but not *in vivo*, it would be necessary to investigate the impact of missing lymph nodes in humanized mice on efficient human immune responses. In addition, most cells in the peritoneal lavage after BCG infection were of mouse origin and mouse tissue resident macrophages were coexisting with human ones by histological analysis. Further improvement of this model by temporary depleting mouse macrophages before hydrodynamic injection may remove the undesired mouse macrophages and make humanized mice a promising tool to model human tuberculosis.

Another limitation of this approach to improve human tissue macrophage in humanized mice is that several types of macrophages are M-CSF independent. The results have shown that macrophages in brain and kidney of humanized mice increased to a much less extent than macrophages in other organs. This suggests M-CSF do not have a critical impact on the development or proliferation of these types of macrophages. However, this limitation could be easily overcome by injecting other cytokines or combining other cytokines with M-CSF. GM-CSF and IL-34 might be

interesting candidates to test. A more challenging and significant study would be improving human macrophage populations, which originate from non-hematopoietic progenitors, such as embryonic progenitors. Current humanized mice permit high levels of engraftment of human hematopoietic cells but the engraftment of cells derived from human tissues or embryos are generally poor. To improve the engraftment of macrophages, such as Langerhans cells, a subpopulation of microglia and Kuffer cells, which develop in the embryo before the appearance of definitive HSCs, humanized mice have to be further improved to achieve stable and high levels engraftment of nonhematopoietic cells of human origin (Ginhoux et al., 2010; Ginhoux and Merad, 2010; Hoeffel et al., 2012; Schulz et al., 2012).

In summary, the findings in this study may answer a long-lasting question of monocyte/macrophage development in humanized mice. Hydrodynamic injection of human M-CSF encoding plasmids into NSG mice greatly improved the reconstitution of tissue resident macrophages and the related innate immune responses against selected human pathogens. Apart from being a model for human hematopoiesis and immune responses to human pathogens, the M-CSF treated mice may facilitate studies on the role of human macrophages in the course of tumorigenesis, autoimmune diseases and tissue homeostasis.

CHAPTER 5

EFFORTS TO IMPROVE

ANTIGEN-SPECIFIC ANTIBODY RESPONSES

IN HUMANIZED MICE

This work is still in progress. LI Yan designed and performed experiments, analyzed and interpreted data.

To improve antibody responses in humanized mice has been an intense topic of research on humanized mice for many years. A humanized mouse with a proper humoral immune response holds promise for developing effective vaccines or even generating neutralizing antibodies against human pathogens for which we do not have good prophylaxis and therapeutics. Since *de novo* human T cell development and stable reconstitution were achieved in NSG mice, it was expected that human antibody response would also be greatly improved. However, the human Ig detected in sera of humanized mice is still mainly IgM, while very low levels of antigen-specific IgG could be generated. As reviewed in the Introduction, the cause of this problem is attributed to insufficient human T cell help. Jackson Laboratory tries to modify the NSG mice to express one specific human HLA-DR so that human CD4 helper T cells will be selected on human HLA-DR, and be properly activated when stimulated with antigen. This effort is still undergoing. Even if this approach was successful, the difficulty to find matched HSC would still limit the application of humanized mice. Hence, we tried to overcome this problem via two approaches detailed below:

5.1 Expression of human CD40 ligand (CD40L) on human T cells to promote the maturation and proliferation of antigen-experienced B cells.

The problems caused by the T cell activation may be partially remedied by expressing CD40L on human CD4 T cells. Human CD40 is constitutively expressed on B cells and other antigen-presenting cells. The CD40-CD40L interaction is essential for humoral responses. Upon the recognition of antigen by B cell receptor, the CD40-CD40L interaction provides the second signal to induce B cell activation and promote

B cell proliferation, germinal center formation, and Ig Isotype-switching (Grewal and Flavell, 1998).

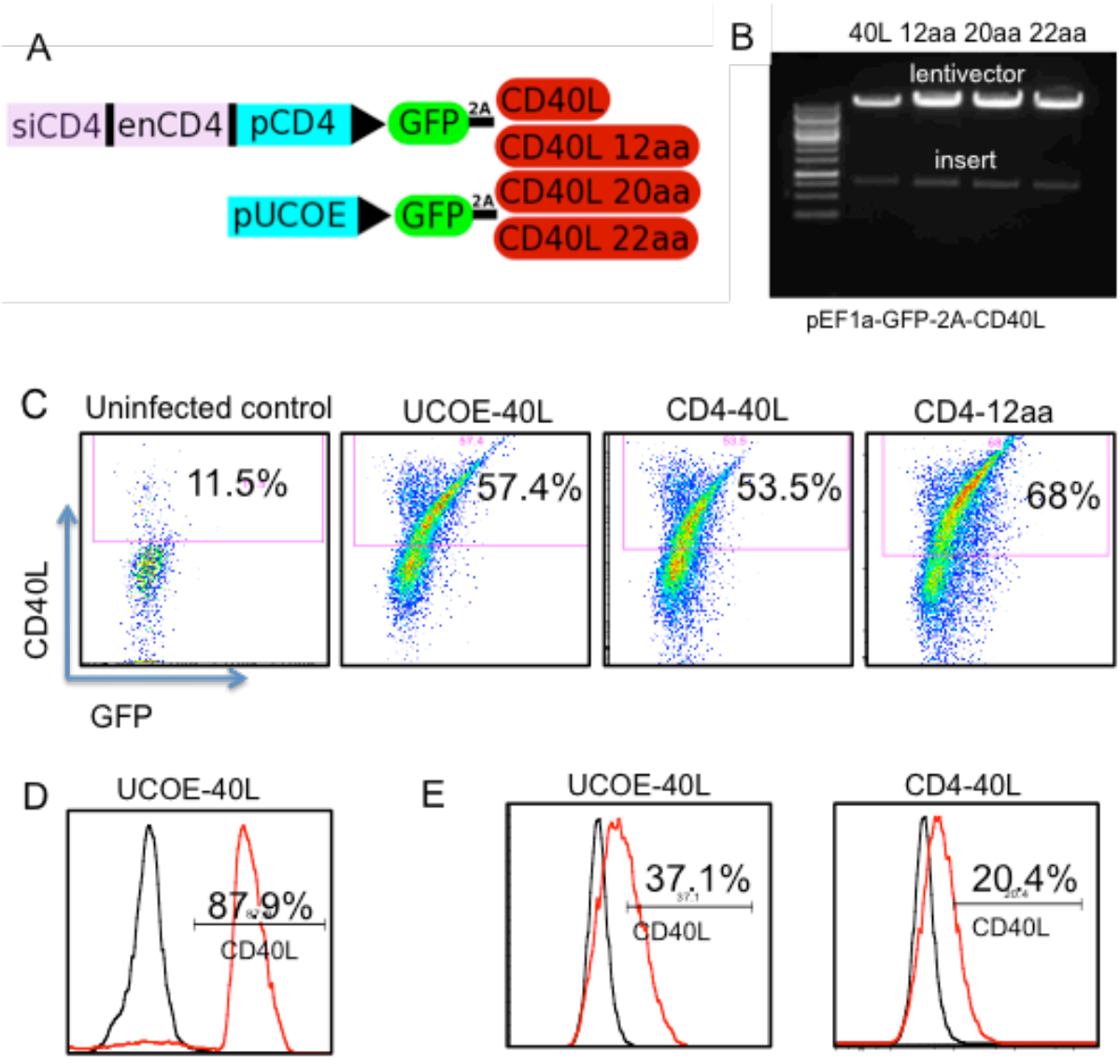

Figure 22. Expression of human CD40L on human cells

(A) Schematic diagram for lentivector encoding a human CD40L or its truncated forms. The expression of human CD40L is controlled either by lineage specific human CD4 promoter or by unspecific UCOE promoter. (B) The digestion of lentivector containing full length, 12aa truncated, 20aa truncated and 22aa truncated CD40L shown in 1% agarose gel. EcoRI and NotI were used to excise the insert. (C-E) The infection of lentivirus in Jurkat cell line (C), primary $CD3^+$ T cells isolated from cord blood (D) and Raji cell line (E). Cells are stained for human CD40L, and analyzed by flow cytometry.

However, CD40L expression is tightly controlled in T cells. It is only transiently expressed for less than 24 hours on the surface of activated T cells (Castle et al., 1993; Grewal and Flavell, 1998). Studies have shown that CD40L on activated helper T cells is internalized upon encountering CD40-expressing B cells (Yellin et al., 1994). Since human T cells are educated on mouse MHC and are difficult to be activated by human DCs, genetic engineering of human T cells in humanized mice to express CD40L may circumvent the need of T cell activation. Hence, antigen-experienced B cells could be directly transformed into antibody-secreting cells with the help of CD40L expressing T cells.

In order to express CD40L on human T cells, we constructed a lentiviral vector with human CD40L under the human CD4 promoter and regulatory sequences (Figure 22A, B). We plan to infect HSCs with this lentivirus and inject infected HSCs into NSG mice. When mice are reconstituted with human cells, the human helper T cells would express CD40L to enhance the antibody production. With a proper immunization protocol, we could amplify the antigen-specific antibody producing B cells for further isolation of neutralizing antibody. Because murine CD40L, similar to human CD40L, has shown that the removal of the intracellular domain increases the time of surface expression of CD40L (Higham et al., 2010), we also constructed several truncations to remove the first 13-22 amino acids of the intracellular domain of human CD40L. These constructs successfully expressed human CD40L both on a cultured human T cell line (Figure 22C) and on primary human T cells isolated by CD3 selection from cord blood (Figure 22D). However, two issues prevented this project from moving forward. First, the CD4 promoter and regulatory elements did not restrict the expression of CD40L on CD4 T cells (Figure 22E). During *in vitro* culture, lentiviral-infected 293FT cells and human B cell line (Raji cell line) also

weakly expressed CD40L. We attempted to add more regulatory elements and redesign the promoter, but the results were not satisfactory. Second, the expression of CD40L was turned off after reconstitution of lentiviral-infected HSCs into NSG mice (data not shown). After 2 to 3 months reconstitution with CD40L-expressing HSCs infected with lentivirus, we could detect human cells but without the expression of CD40L. The antibody isotype in these mice was still mainly IgM and low levels of antigen specific IgG to TT were detected after immunization. The reason for the apparent silencing of CD40L expression is unknown, but it may be due to the poor engraftment property of CD40L-expressing HSCs or the tight transcriptional regulation of CD40L expression in cells. To solve this problem, it is critical to obtain a promoter to restrict the expression of CD40L to a specific cell type or to use an inducible promoter to control the timing of CD40L expression.

The limitation of this approach is the unspecific activation of B cells. Given that the CD40L would be forced to express on human T cells, all antigen-experienced human B cells could transform into antibody-producing cells. With an inducible promoter to control the expression of CD40L along with an immunization protocol, the targeted antigen-specific B cells might be able to proliferate and outgrow those unspecific activated B cells.

5.2 Expression of NSG mouse MHC class II (IA-g7) on reconstituted human cells for proper T cell activation and antibody response

The most obvious approach to correct the problem of human T cell selection in NSG mice is to replace NSG murine MHC class II gene with human HLA-DR gene. The human cells will thus be positively selected on mouse thymic epithelial cells expressing HLA-DR. However, this approach requires gene knock-in on the NSG background, and HLA-DR matched human HSCs to avoid graft-versus host response. The embryonic stem cell line for the creation of knock-in NSG mice is currently under development at the Jackson Laboratory. The requirement of HLA-DR matched HSCs also limit a wider application of these mice. An alternative approach is to express NSG mouse MHC class II, termed as IA-g7, on reconstituted human cells by lentiviral transduction of the IA-g7 gene into HSCs. In this approach, the human T cells are still educated on mouse MHC content. However, since human antigen-presenting cells utilize the NSG murine MHC molecule IA-g7 to present antigen, human T cells would have no difficulty to recognize the signal for activation. In addition, no HLA mapping and matching is necessary before each injection of HSCs into NSG mice, and expression of the mouse IA-g7 may increase the engraftment of human HSCs in NSG mice due to the MHC compatibility.

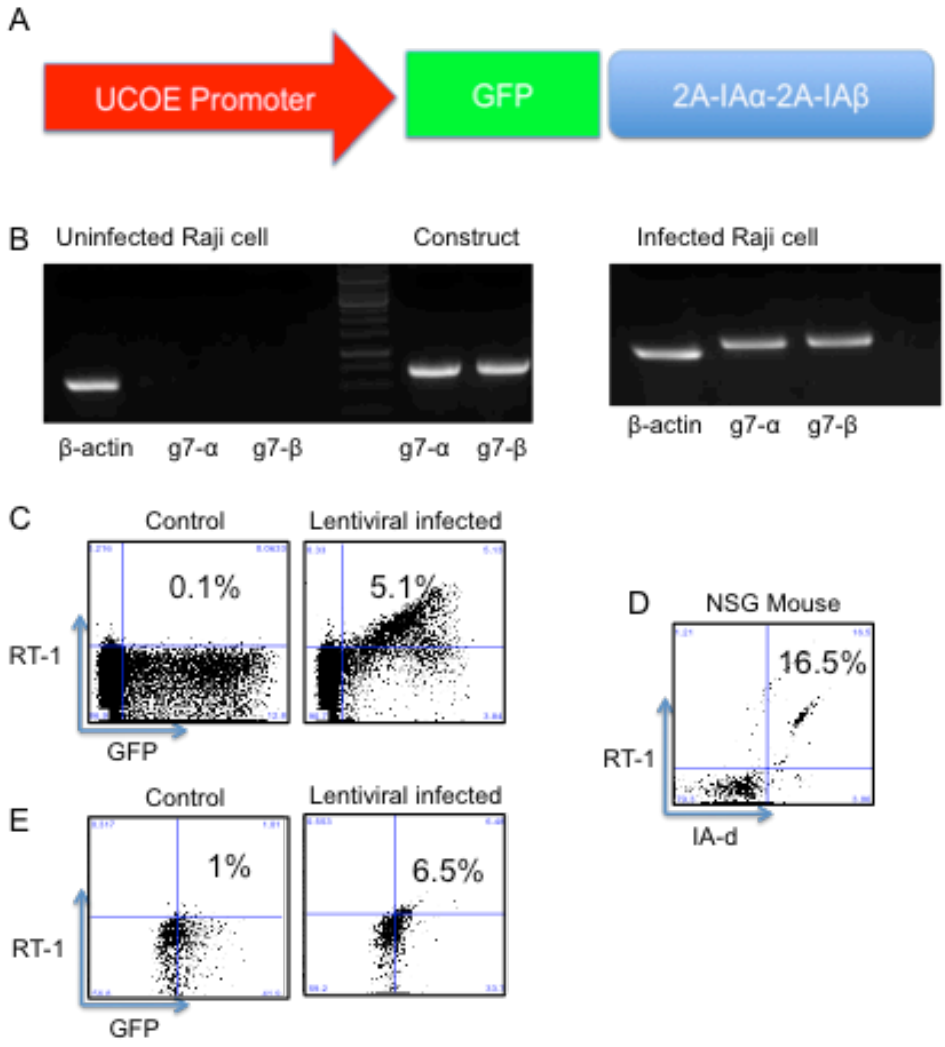

Figure 23. IA-g7 lentival vector and its expression on human cells
(A) schematic diagram for lentivector encoding NSG mouse MHC class II IA-g7. GFP, IA-g7 alpha chain and beta chain are connected by 2A peptides and expressed under the UCOE promoter. (B) RT-PCR from cDNA of Raji cells and lentiviral infected Raji cells with primers specific for IA-g7 alpha, beta chain and beta actin. IA-g7 lentivirus and lentivirus made from GFP expressing lentivector were use (C, E) to infect Raji cell line (C) and human HSCs (E). Cells are stained with RT-1 antibody to detect IA-g7 expression on flow cytometry. (D) RT-1 antibody and IA-d antibody staining on blood leukocytes from NSG mice.

We constructed a lentiviral vector to express IA-g7 under the ubiquitous chromatin opening element (UCOE) promoter (Figure 23A). This promoter drives stable and reproducible expression of IA-g7 alpha and beta chains. We infected the human Raji cell line with this lentiviral construct to verify the expression of IA-g7 on human B cells *in vitro* by RT-PCR (Figure 23B). Initially, we failed to detect the expression of IA-g7 by an antibody specific for IA-d, which shares the same alpha chain as IA-g7 and detects IA-g7 on NSG mouse cells. Since there was no specific antibody produced against IA-g7 (Rinderknecht et al., 2010), we suspected that IA-d antibody might not recognize IA-g7 molecule expressed by human cells. Hence, we tested other antibodies specific for mouse and rat MHC. Finally, we identified a clone (RT-1, targeted for rat MHC class I) that could recognize IA-g7 both on NSG mouse cells and the lentiviral-infected human B cell line (Figure 23C, D). However, this project encountered another problem. First, the expression of IA-g7 was very weak on HSCs after infection (Figure 23E). HSCs usually do not express MHC class II molecules, and associated accessory proteins, and this could explain the low expression of IA-g7 on the surface of human HSCs. Hence, we proceeded to reconstitute infected HSCs into NSG mice. 2 to 3 months later, we could not detect any human cells expressing IA-g7 in these mice (data not shown). To solve this problem, we will have to verify the expression of IA-g7 in infected HSCs by RT-PCR. If the IA-g7 is inserted into the human genome but the expression is still not detected in reconstituted human cells, it may be the possible that the RT-1 antibody fails to recognize the IA-g7 expressed on primary human cells, or the expression of IA-g7 is switched off. Currently, a new batch of mice with IA-g7 infected HSCs is being generated for this experiment.

Although these two projects are experiencing difficulties, efforts to find solutions to those problems will continue. Besides these two approaches, there are also attempts to

increase the antibody response by boosting the DCs with human GM-CSF and IL-4 in NSG mice (manuscript in preparation). This approach may enhance the antigen delivery to human T cells and B cells. However, as GM-CSF and IL-4 induced DCs are proinflammatory DCs, the massive production of proinflammatory cytokines and unspecific activation of human T cells may also pose potential problems.

CHAPTER 6

SUMMARY AND FUTURE PERSPECTIVES

6.1 Summary of thesis

Humanized mice offer a great platform to study human diseases *in vivo* and to test new therapies. Although much progress has been made on the generation of new mouse strains permissive for high human cell engraftment, many critical issues still remain to be solved in order to bring the application of humanized mice to clinical and preclinical use.

Focused on the widely used immunodeficient NSG mice, we addressed three urgent problems as a priority: 1) Low HSC numbers in a single unit of cord blood caused sample-to-sample variations, and restricted the application of humanized mice to large-scale preclinical studies. 2) Imbalanced human blood lineage reconstitution, especially monocytes/macrophages, caused poor immune responses against pathogens and reduced the predictive value of the human immune responses observed in humanized mice. 3) Human T cell selection in the mouse thymus environment rendered antigen-specific T cell activation difficult, and consequently poor B cell response and antibody production. In this thesis, we targeted these three problems, and found partial solutions to the first two.

For the first issue of limited HSCs in a single unit of cord blood, we developed an improved culture system that combines MSCs engineered to express Angptl5 with other growth factors recently identified to support HSC expansion. Using this method, $CD34^+$ $CD133^+$ cord blood cells were expanded 60 and 150 fold in 11 and 14-day cultures, respectively. The expanded $CD34^+$ $CD133^+$ cells efficiently reconstituted secondary recipients. Furthermore, the expanded cells gave rise to multilineage reconstitution of human blood cells in NSG mice, and a significantly higher level of T

cell reconstitution. These results demonstrate that expanded cells possess both short-term and long-term HSC activity, and the improved approach should provide a more efficient method for expanding human HSCs for both clinical and preclinical applications.

For the second problem of poor reconstitution and function of human monocytes/macrophages, we analyzed various organs of humanized mice to understand the root of the problem, and discovered that human monocyte/macrophage progenitors accumulated in the promonocyte stage. To restore the normal monocyte/macrophage development *in vivo*, we used hydrodynamic injection to express human cytokines in humanized mice. Mice with human M-CSF or GM-CSF expression significantly improved engraftment of monocytes/macrophages. In particular, M-CSF treated humanized mice supported the development of tissue resident macrophages in various organs, such as liver and lung. Moreover, M-CSF treated humanized mice had enhanced responses against influenza virus and BCG infection. Hence, the humanized mice with human tissue resident macrophages offer a approach for the study of human macrophages and their roles in disease progression in a small animal model.

Although these two improvements in humanized NSG mice partially solved the above problems in principle, it is acknowledged that several key issues still exist. For instance, expansion of HSCs eliminates the variation among cord blood samples, but *in vitro* culture is still expensive because of the relatively high concentration and cost for IGFBP2. Nonetheless, if the method developed here is widely applied and is marketed, mass production may reduce the cost. Furthermore, the improvements of human tissue macrophage reconstitution do not change the fact that many other

human myeloid compartments are still poorly reconstituted in humanized mice, such as neutrophils, erythrocytes and megakaryocytes.

In this thesis, I also made two attempts at improving human IgG antibody response in humanized mice. In the first attempt, several lentivectors were constructed to express human CD40L on reconstituted human CD4 T cells. This approach planned to utilize the costimulatory ability of CD40L to activate antigen experienced human B cells to secret antibodies. However, the expression of CD40L was not restricted on T cells despite that the CD40L gene expression was driven by a CD4 promoter. Moreover, the expression of CD40L was switched off after 2-3 months reconstitution. In another attempt, several lentivectors were cloned to express mouse MHC class II molecule IA-g7 on reconstituted human cells. This approach targeted the problems of human T cell selection on murine MHC content and hoped that matched MHC-TCR interaction would enhance the human B cell activation in humanized mice. However, this approach encountered the similar problem of target gene silencing like CD40L project. These problems are currently under investigations.

Despite room for further improvement on these problems in NSG mice, the current achievements on HSC expansion and macrophage reconstitution have already broadened the application of humanized mice to many new research areas. The bigger group of humanized mice created from a single donor's HSCs would make humanized mice applicable to large-scale studies or preclinical test without concerns about the variations within the group. The tissue macrophages in NSG mice offer a unique and powerful tool to study the development of human monocyte/macrophage development, and to compare the human macrophages with their cognate mouse model systems. This is an area with great potential, as the chasm between our understanding human and murine macrophages is wide.

6.2 Future perspectives

This thesis focused on three problems in current humanized mice, and partially solved two of them. For future investigations, work should continue to solve the antigen-specific antibody response. As discussed in Chapter 5, the expression of CD40L and IA-g7 was successful *in vitro*, but could not detected in the human cells in humanized mice reconstituted with infected HSCs. We need to isolate human cells from these mice, and examine if CD40L or IA-g7 is still within the human genome after reconstitution. This result will guide the direction for the next steps. Once these technical issues are solved, and CD40L or IA-g7 strategies indeed improve the antigen-specific antibody response, this model may have potential for generating monoclonal antibodies against various exogenous substances, such as viral or bacterial target proteins. Such monoclonal antibodies may act as therapeutic agents for prevention of infections or allergies.

Another direction to improve humanized mice is to increase the reconstitution of granulocytes and erythrocytes. Unlike macrophages and NK cells, the problem of granulocyte and erythrocyte engraftment may not be simply the result of lacking growth factors. Since granulocytes can only be found in the BM of humanized mice after cytokine treatment (Billerbeck et al., 2011; Rongvaux et al., 2011), they probably need serial sets of cytokines to help them further mature and migrate into the periphery. Moreover, transient depletion of mouse granulocytes by antibody or chemical treatment before adding human cytokines may provide space for human granulocytes to grow. For erythrocyte development, more than one month is required to detect the population of human erythrocytes in peripheral blood after EPO/IL-3 treatment (Chen et al., 2009). This result indicates that human erythrocyte

development may be blocked at very early stages. If human EPO/IL-3 knock-in mice could be developed, the human erythrocyte reconstitution may be improved, and it will facilitate the study of malaria in humanized mice. Currently, human erythrocytes from human blood must be successively injected into the mice *i.p.* in order to obtain persistent circulating human erythrocytes in blood for Plasmodium parasites to infect.

Recently, progress in the field of regenerative medicine has drawn the attention of the humanized mouse research community. Following the establishment of human embryonic stem cells, inducible pluripotent stem cells, and various identified adult tissue stem cells, artificial human organs or HSCs developed from embryonic stem cells or inducible pluripotent stem cells may be available. Although these techniques have not yet impacted the field of humanized mice, the future focus on improvements in humanized mice could shift from purely hematopoietic cell engraftment to artificial human organs and HSC-integrated systems.

After all, a mouse is not “human”. No matter how many improvements we make, the results obtained from humanized mice still need to be verified by clinical studies. However, this does not mean our efforts are meaningless. On the contrary, the more alike humanized mice and humans are, the fewer resources will be wasted on failed drug in clinical trials, and increasing knowledge will be gained from the direct study of human cells *in vivo*.

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