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Récepteur Wnt Non-Canonique Frizzled-6 Régule Hématopoïèse Induite Par Les Stress

Non-canonical Wnt Receptor Frizzled-6 Regulates Stress-Induced Hematopoiesis

by

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Robert Heinlein said, “Love is that condition in which the happiness of another person is essential to your own.” With this ideal in mind, **I dedicate my thesis to *my rock*, *my wonderful mother***, for her endless love, measureless support, encouragement and understanding, **and to *the memory of my father*** for his constant love and support from the Heaven. Without them, this journey would not have been possible.

RÉSUMÉ

L'homéostasie du sang est maintenue par l'équilibre entre l'auto-renouvellement et la différenciation des cellules souches/progénitrices hématopoïétiques (HSPC). Bien que les cellules souches hématopoïétiques (HSC) soient principalement maintenues dans un état de dormance, elles peuvent entrer rapidement dans le cycle cellulaire et se différencier en précurseurs lymphoïdes et myéloïdes pour reconstituer les cellules immunitaires suite à une myelosuppression ou à des infections systémiques. La signalisation de Wnt a été suggérée pour maintenir l'équilibre dynamique du pool de HSPC en régulant leur division et les signaux provenant du microenvironnement de la cellule souche. Récemment, notre groupe a montré que la protéine Wnt4 augmente l'expansion des HSPC par une voie de la polarité planaire cellulaire (PCP). Cependant, l'impact physiologique de la voie PCP sur l'hématopoïèse reste à être identifié. Pour aborder cette question, nous avons examiné le rôle d'un récepteur central de la PCP, Frizzled-6 (*Fzd6*), dans la spécification de la lignée et la fonction des HSPC pendant l'homéostasie et la réponse au stress. À l'état de base, nous n'avons observé aucune différence dans le nombre de HSC phénotypiquement enrichis dans la moelle osseuse (MO) *Fzd6*$^{-/-}$. Cependant, nos expériences de transplantations compétitives ont démontré que les HSPC des souris *Fzd6*$^{-/-}$ n'ont pas réussi à reconstituer une hématopoïèse à court et long terme chez les souris receveuses en raison d'une forte activation de la caspase-3.

Pour tester si les HSC *Fzd6*$^{-/-}$ répondent à une hématopoïèse d'urgence induite par une inflammation, nous avons injecté les souris *Fzd6*$^{-/-}$ avec une dose sous-létale de lipopolysaccharides bactériens (LPS) et de parasites *Leishmania donovani* qui établissent une infection chronique dans la MO et la rate. Les HSPC *Fzd6*$^{-/-}$ se sont peu multipliées et ont produit moins de progéniteurs myéloïdes dans les deux modèles. Cette diminution est accompagnée d'une production réduite de monocytes inflammatoires Ly6C^{hi} dans la MO et d'une diminution de l'accumulation de cellules myéloïdes dans la rate. Par conséquent, les souris *Fzd6*$^{-/-}$ sont plus sensibles à l'inflammation aiguë induite par l'endotoxine, mais présentent un faible taux de parasites dû à un nombre moins élevé de cellules monocytes/macrophages. Nos résultats établissent un lien mécanique entre la signalisation de *Fzd6* et la régulation de la réponse des HSC pendant le stress. Nous pensons que la signalisation de *Fzd6* est une

cible thérapeutique prometteuse pour moduler l'activation des HSC pour de nouveaux concepts de traitement afin de surmonter les limites de la transplantation de MO. En outre, nos observations reliant *Fzd6* à la myélopoïèse peuvent également avoir des implications pour les infections aiguës et chroniques.

Mots clés: cellules souches hématopoïétiques, signalisation Wnt/Frizzled, auto-renouvellement, myélopoïèse, inflammation, infection chronique

ABSTRACT

Blood homeostasis is maintained by the fine balance between self-renewal and differentiation of hematopoietic stem/progenitor cells (HSPCs). Although hematopoietic stem cells (HSCs) are maintained in a predominantly quiescent state, they can rapidly enter the cell cycle and differentiate into committed lymphoid and myeloid precursors to replenish immune cells in response to hematopoietic injury or systemic infections. Wnt signaling has been suggested to maintain the dynamic balance of HSPC pool by regulating HSPC divisions and the signals derived from the stem cell microenvironment. Recently our group has shown that non-canonical Wnt4 signaling increases HSPC expansion through a planar cell polarity (PCP)-like pathway. However, the wider physiological impact of PCP signaling on hematopoiesis is yet to be identified.

To address this question, we examined the role of a core PCP receptor, Frizzled-6 (*Fzd6*) in the lineage specification and the function of HSPCs during homeostatic maintenance and the stress response. At steady-state, we did not observe any differences in the numbers of phenotypically enriched HSCs in *Fzd6*$^{-/-}$ bone marrow (BM). However, our competitive transplantation experiments demonstrated that HSPCs from *Fzd6*$^{-/-}$ mice failed to reconstitute short and long term hematopoiesis in recipient mice due to a strong activation of caspase-3 and defective engraftment.

To test whether *Fzd6*$^{-/-}$ HSCs respond to inflammation-induced emergency hematopoiesis, we challenged *Fzd6*$^{-/-}$ mice with a sub-lethal dose of bacterial endotoxin lipopolysaccharide (LPS) and *Leishmania donovani* parasites that establish a chronic infection in the BM and spleen. *Fzd6*$^{-/-}$ HSPCs expanded poorly and produced significantly fewer myeloid progenitors in both models. This decrease was accompanied by a reduced production of Ly6C^{hi} inflammatory monocytes in BM and decreased accumulation of myeloid cells in spleen. *L. donovani* specifically induced the production of BM-derived factors that promote myeloid differentiation in both *Fzd6*$^{-/-}$ and wild-type BM. However, more pronounced elevations in inflammatory cytokines in *Fzd6*$^{-/-}$ BM resulted in decreased HSPC expansion and myeloid differentiation both in culture and *in vivo*. As a result, *Fzd6*$^{-/-}$ mice were more susceptible to endotoxin-induced acute inflammation but presented with lower parasite burden due to a corresponding decrease in monocyte/macrophage lineage cells.

Our results establish a mechanistic link between *Fzd6* signaling and demand adapted regulation of HSC response during stress. We anticipate that *Fzd6* signaling is a promising therapeutic target to modulate HSC activation for new treatment concepts to overcome the limitations of BM transplantation. Furthermore, our observations linking *Fzd6* to myelopoiesis may also have implications for acute and chronic infections.

Key words: Hematopoietic stem cell, Wnt/Frizzled signaling, self-renewal, myelopoiesis, inflammation, chronic infection

SYNOPSIS

L'hématopoïèse chez l'adulte consiste en une production de cellules sanguines suite à un processus d'engagement et de différenciation des cellules souches et progénitrices. Une cellule souche hématopoïétique (*hematopoietic stem cell*; HSC) est une cellule unique qui peut, à elle seule, reconstituer le système hématopoïétique d'un hôte suite à une irradiation létale (Osawa *et al.*, 1996). Pour que la reconstitution se produise, la cellule souche doit se doter de trois caractéristiques importantes. Premièrement, elle doit pouvoir s'auto-renouveler afin de produire une copie identique à la cellule d'origine et de maintenir son état indifférencié. Deuxièmement, elle doit posséder un potentiel de prolifération extensive pour repeupler le système sanguin. Finalement, elle doit aussi avoir la capacité de différenciation pour donner naissance à tous les éléments sanguins, autant lymphoïdes, myéloïdes qu'érythroïdes (Seita *et al.*, 2010).

Au sommet de la hiérarchie du système hématopoïétique, les HSC à long terme (*long-term* HSC; LT-HSC) possèdent le plus haut degré d'auto-renouvellement et la capacité de repeupler *in vivo*. Elles fournissent une reconstitution hématopoïétique à vie. Les LT-HSC ne se divisent qu'environ cinq fois au cours de la vie d'une souris adulte en l'absence de facteurs de stress externes (Wilson et al., 2008). Cette quiescence profonde protège les HSC de l'épuisement fonctionnel pour soutenir la production sanguine et prévient le développement de tumeurs malignes (Nakamura-Ishizu *et al.*, 2014). En aval des LT-HSC, les HSC à court terme (*short-term* HSC; ST-HSC) maintiennent l'équilibre hématopoïétique en produisant des progéniteurs multipotents (MPP). Ces trois populations cellulaires sont dans la fraction Lin^- $Sca\text{-}1^+$ $c\text{-}KIT^+$ (LSK) de la moelle osseuse (MO). Contrairement aux LT-HSC, leur progéniture engagée prolifère activement pour préserver l'intégrité de l'ensemble du tissu hématopoïétique (Wilson et al., 2008). Les cellules lymphoïdes et myéloïdes sont produites par des progéniteurs engagés qui comprennent les progéniteurs lymphoïdes communs (CLP), les progéniteurs myéloïdes communs (CMP), les progéniteurs lymphoïdes multipotents (LMPP), les progéniteurs de granulocytes et macrophages (GMP) et les progéniteurs de mégacaryocytes et érythrocytes (MEP). Ces progéniteurs sont générés par des MPP et ont une capacité de différenciation limitée (Adolfsson *et al.*, 2001, Morrison, 2002, Muller-Sieburg *et al.*, 2012).

Les populations de cellules souches/progénitrices hématopoïétiques (HSPC) résident dans un microenvironnement spécialisé (niche) entourées de cellules stromales dans la MO (Boulais *et al.*, 2015). Normalement, l'équilibre entre la division et la différenciation des HSC est contrôlé par différentes voies de signalisation intrinsèques à la cellule et des signaux de la niche provenant des cellules voisines dans la MO (Cheung *et al.*, 2013, Ema *et al.*, 2012, Nakamura-Ishizu *et al.*, 2014). Cependant, un certain nombre de conditions de stress telles les radiations et les infections peuvent renverser l'état de dormance des LT-HSC et les faire entrer en cycle cellulaire pour remplacer les cellules perdues (Allakhverdi *et al.*, 2009, MacNamara *et al.*, 2011a, Matatall *et al.*, 2016, Wright *et al.*, 2002). La manière dont les HSPC coordonnent des mécanismes opposés comme l'auto-renouvellement et la différenciation en conditions de stress reste à être clarifiée.

Il est bien établi que la signalisation de Wnt regroupe un large éventail de processus physiologiques et développementaux, comprenant la croissance, la division et la différenciation cellulaire (Cadigan *et al.*, 1997, Eisenmann, 2005). Différentes études ont rapporté que le dérèglement de cette cascade de signalisation joue un rôle central dans le développement des maladies hématologiques malignes et des leucémies (Johnson *et al.*, 2006, Lento *et al.*, 2013). Les protéines Wnt sont sécrétées par divers types de cellules stromales et agissent sur de courtes distances comme molécules de signalisation. Elles agissent également sur de longues distances dans la MO en suivant un gradient. Les protéines Wnt transmettent le signal via des récepteurs transmembranaires Frizzled (*Fzd*) pour activer au moins deux types de voies de signalisation intracellulaire. La voie canonique de Wnt contrôle la transcription des gènes cibles et est dépendante de la β-caténine pour induire une réponse cellulaire. Les voies de signalisation non canoniques sont β-caténine indépendantes et incluent les voies Wnt-Ca^{2+} et polarité planaire cellulaire (PCP). L'activation de la voie Wnt/Ca^{2+} conduit à la mobilisation de calcium (Ca) intracellulaire et à l'activation de la protéine kinase II dépendante à la calmoduline. La voie de la polarité cellulaire planaire (PCP) passe par la Jun N-terminal kinase (JNK) pour organiser le cytosquelette de manière asymétrique et polariser les cellules dans le tissu (Kikuchi *et al.*, 2007, Komiya *et al.*, 2008).

La majorité des études suggère que les voies de signalisation non canonique de Wnt jouent un rôle essentiel pour le maintien à long terme d'un pool de cellules HSC dans la M(Nemeth et al., 2007, Povinelli et al., 2014, Sugimura et al., 2012).

Récemment, notre laboratoire a démontré que la signalisation de Wnt4 augmente l'expansion des HSPC via une voie de type PCP (Heinonen et al., 2011b, Louis et al., 2008). Le récepteur de la voie PCP *Fzd6* est au moins partiellement requis pour l'expansion de HSPC médiée par Wnt4. Cependant, le rôle fonctionnel de la signalisation *Fzd6* dans les cellules engagées dans la lignée hématopoïétique est très peu connu. Dans le système hématopoïétique, il a été démontré que les HSPC et les cellules formant le sang mature chez l'humain et la souris expriment *Fzd6* avec un niveau d'expression plus élevé chez les cellules les plus immatures (Wagner et al., 2004, Yokota et al., 2008). Par conséquent, nous avons proposé que la signalisation de *Fzd6* puisse réguler l'hématopoïèse chez l'adulte à l'état d'équilibre et sous des conditions de stress.

Pour étudier le rôle de *Fzd6* dans l'hématopoïèse, nous avons croisé les souris *Fzd6*$^{+/-}$ avec des souris C57Bl/6 pendant 10 générations, ce qui a permis de minimiser la variabilité génétique dans nos expériences. Nous avons effectué une analyse détaillée de l'hématopoïèse *in vivo* chez les souris *Fzd6*$^{-/-}$ pour caractériser le phénotype hématopoïétique des souris *Fzd6*$^{-/-}$ pendant l'hématopoïèse chez l'adulte.

À l'état de base, la proportion de LSK CD150^{+} qui contiennent des LT- et ST-HSC est normal dans la MO *Fzd6*$^{-/-}$. Cependant, nous avons observé une augmentation de MPP amorcé vers la lignée lymphoïde chez les souris *Fzd6*$^{-/-}$. À l'inverse, nous n'avons détecté aucune différence dans le développement lymphoïde par rapport aux souris de type sauvage (*wild-type*; WT). Pour examiner la repopulation et le potentiel de différenciation des HSC *Fzd6*$^{-/-}$ *in vivo*, nous avons effectué des tests de repeuplement compétitifs et suivi la reconstitution dans le sang périphérique et la MO en comparant la greffe de cellules de MO *Fzd6*$^{-/-}$ avec des cellules témoins WT. Nous avons montré que le manque de *Fzd6* entraîne une perte progressive des HSPC du donneur en raison d'une forte activation de la caspase-3 dès 7 à 8 jours après la transplantation. Cela indique que la greffe, le repeuplement et l'auto-renouvellement des HSPC *Fzd6*$^{-/-}$ sont inefficaces.

Afin de déterminer si les HSC *Fzd6*$^{-/-}$ répondent à d'autres types de stress dans leur microenvironnement, nous avons ensuite soumis les souris *Fzd6*$^{-/-}$ à une dose sous-létale de lipopolysaccharide (LPS), une endotoxine bactérienne. Des études antérieures ont montré que l'hématopoïèse d'urgence induite par LPS « réveille » les HSC en dormance et les fait entrer en cycle cellulaire avec un changement dans la différenciation favorisant la production de cellules myéloïdes (Boettcher *et al.*, 2012,

Scumpia *et al.*, 2010). Nous avons montré qu'un traitement systémique de LPS entraîne une diminution rapide de la cellularité de la MO qui s'accompagne d'une augmentation de 10 à 15 fois du nombre de HSC. Malgré la grande proportion de HSC (~65-70%) qui résident dans la phase G0 du cycle cellulaire pendant l'homéostasie, plus de la moitié de ces HSC en dormance ont été retrouvés dans les phases G1 ou S-G2-M du cycle cellulaire suite à l'injection de LPS. Nous n'avons observé aucune différence dans le comportement cyclique entre les HSC *Fzd6*$^{-/-}$ et WT lorsqu'analysé avec la coloration Ki67/Hoechst. Cependant, les souris *Fzd6*$^{-/-}$ ne parviennent pas à obtenir l'expansion prévue de la population HSPC. Ces résultats suggèrent que *Fzd6* est nécessaire pour une expansion efficace des HSPC de la MO et leur auto-renouvellement, non seulement après la transplantation, mais aussi suite à un stress prolifératif comme une inflammatoire aiguë.

L'inflammation aiguë se caractérise par une génération rapide et une mobilisation de cellules effectrices myéloïdes de la MO conduisant à une leucocytose inflammatoire. La capacité des HSPC à répondre aux infections en augmentant le nombre de progéniteurs et en produisant des cellules myéloïdes capables de détruire les agents pathogènes microbiens tout en préservant un pool de cellules souches intactes est une caractéristique critique de la défense de l'hôte (Scumpia *et al*., 2010, Ueda *et al*., 2005). Lors d'une administration sous-létale de LPS, la moelle des souris injectées démontre une réduction du nombre absolu de cellules lymphoïdes et de granulocytes. Nous avons aussi observé une augmentation relative de l'accumulation de HSC et de cellules myéloïdes effectrices (granulocytes et monocytes) dans la rate. Nous avons observé une augmentation significative des GMP Sca-1^{+}, également nommés GMP d'urgence (*emergency GMP*; eGMP) car ils n'apparaissent que pendant la myélopoïèse d'urgence. Même si des nombres similaires de HSC ont été observés dans les rates des souris *Fzd6*$^{-/-}$ et WT, la rate *Fzd6*$^{-/-}$ contient beaucoup moins d'eGMP. Cette diminution est accompagnée d'une production réduite de monocytes inflammatoires Ly6C^{hi} dans la MO et une diminution de l'accumulation de cellules myéloïdes effectrices dans la rate. Des essais réciproques de greffe de MO ont révélé que des facteurs dépendants du stroma et intrinsèques aux cellules jouent un rôle dans la différenciation myéloïde inefficace des HSPC *Fzd6*$^{-/-}$. Collectivement, nos données suggèrent que *Fzd6* joue un rôle essentiel dans la détermination du sort des HSPC vers les lignées myéloïdes lors de l'inflammation aiguë induite par des endotoxines. Les mécanismes sous-jacents la défectuosité de la réponse myéloïde des HSPC doivent

encore être identifiés. Pour répondre à cette question, nous avons poursuivi en étudiant l'importance de *Fzd6* dans l'activation des HSPC dans une infection chronique en utilisant un modèle de souris de leishmaniose viscérale.

La leishmaniose viscérale est une maladie transmissible par un vecteur et causée par le protozoaire intracellulaire *Leishmania donovani*. Des études antérieures ont montré que le parasite *L. donovani* n'infecte pas directement les HSC ou leurs progéniteurs en aval. Il établit plutôt une infection persistante dans les macrophages de la MO, ce qui corrèle avec une amélioration de la production des cellules myéloïdes par la MO et la rate (Cotterell *et al.*, 2000a, Cotterell et al., 2000b). Cependant, la contribution de l'amélioration de la myélopoïèse au cours de l'infection n'est pas bien comprise. Étant donné que la déficience en *Fzd6* réduit l'activation des HSC en réponse à une inflammation induite par l'endotoxine, nous avons analysé le compartiment hématopoïétique de souris *Fzd6*$^{-/-}$ et WT au cours de l'infection. De 14 à 28 jours après l'infection (*post-infection*; pi), l'expansion du parasite dans la MO et la rate a entraîné une augmentation graduelle de la fréquence et du nombre de LT- et ST-HSC dans la MO, suivi de l'accumulation de HSPC dans la rate. 21 jours après l'infection (21 pi), la majorité des HSC se sont divisés (>75-80%). La progression des HSC dans le cycle cellulaire et leur expansion sont accompagnées d'une augmentation de l'activation de la β-caténine au cours de l'infection, ce qui suggère que la signalisation Wnt/Frizzled (*Fzd*) joue un rôle dans l'activation des HSPC dans la leishmaniose.

Parallèlement à la charge parasitaire, la proportion et le nombre de eGMP Sca-1^{+} ont également augmenté, atteignant un plateau entre les jours 21 et 28, selon la force de l'infection. La progéniture myéloïde de ces eGMP consiste principalement en des monocytes Ly6C^{hi} avec un phénotype régulateur de type suppresseur de cellules. Étonnamment, nous avons observé que les parasites *L. donovani* n'infectent pas seulement les macrophages de la MO, mais également les précurseurs de monocytes et les monocytes matures, confirmés par une coloration Giemsa de la MO provenant des souris infectées au jour 28 pi. Pour savoir si les cytokines de l'environnement dans la MO influencent la différenciation des HSPC et augmentent la charge parasitaire dans la MO, nous avons analysé le surnageant de la totalité des cellules de la MO en comparant des souris non infectées à des souris infectées par *L. donovani* à différents jours après l'infection. Nous avons détecté une augmentation significative dans la MO du niveau d'expression de différentes cytokines et chimiokines qui pourraient contribuer à l'expansion et à la différenciation myéloïde des HSPC dans la phase chronique de

l'infection. Pour tester si les monocytes générés dans la MO infectée sont fonctionnellement différents des monocytes normaux différenciés *in vitro*, nous avons exposés les cultures aux amastigotes fluorescents de *L. donovani*. Les surnageants provenant de la MO infectée ont augmenté non seulement la proportion de monocytes infectés par des parasites, mais aussi la réplication du parasite dans ces cellules avec le temps. Ces résultats suggèrent que l'expansion du parasite *L. donovani* dans la MO active efficacement les HSC et les pousse à produire un grand nombre de cellules myéloïdes, qui sont parmi les cibles préférées du parasite, et deviennent encore plus sensibles à l'infection. À l'inverse, les HSPC *Fzd6*$^{-/-}$ ont généré moins de progéniteurs myéloïdes et, par conséquent, moins de monocytes Ly6C^{hi} et une réduction de l'accumulation de cellules myéloïdes dans la rate *Fzd6*$^{-/-}$ par rapport aux témoins WT. Cette diminution dans la différenciation myéloïde corrèle avec la diminution de l'expansion du parasite, la charge parasitaire ayant été mesurée dans la MO et la rate. Cela établissant un lien entre la signalisation *Fzd6* et la réponse aux infections des HSPC. Par conséquent, nos résultats démontrent un rôle important pour l'activation des HSPC dépendante de Wnt/*Fzd* dans la régulation de l'infection chronique par les parasites.

Compte tenu du potentiel thérapeutique des HSC, il est nécessaire de mieux comprendre les mécanismes impliqués dans le contrôle des HSPC. Nous prévoyons que la signalisation non canonique de *Fzd6* est une cible thérapeutique prometteuse pour de nouveaux traitements pour moduler l'activation des HSC et le développement des cellules myéloïdes et surmonter les limites de la transplantation de moelle osseuse et le traitement des maladies inflammatoires aiguës et chroniques.

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TABLE OF CONTENTS

RÉSUMÉ i

ABSTRACT iii

SYNOPSIS v

ACKNOWLEDGEMENT xi

TABLE OF CONTENTS xii

LIST OF FIGURES xv

LIST OF TABLES xvii

LIST OF ABREVIATIONS xviii

CHAPTER 1 INTRODUCTION 1

1. Matter of life: Blood 2

2. Ontogeny of hematopoietic stem cells 4

3. Transition towards an adult phenotype 6

4. Steady-state hematopoiesis 9

4.1. Maintenance of hematopoietic stem cell quiescence 14

4.1.1. Intrinsic regulation of the cell-cycle 14

4.1.2. Regulation of quiescence by hematopoietic stem cell niches 16

4.2. Hematopoietic progenitor cells 22

4.3. The role of classical cytokines in the hematopoietic diversity 26

5. Stress response of hematopoietic stem cells 30

5.1. Homing and cell kinetics of transplanted hematopoietic stem cells 31

5.2. Inflammation- and pathogen-induced emergency hematopoiesis 35

5.2.1. Direct recognition of pathogens by hematopoietic stem cells 41

5.2.2. Effect of pro-inflammatory signals on emergency hematopoiesis 44

6. Regulation of hematopoiesis by Wnt/Frizzled signaling 50

6.1. Wnt proteins acts as morphogens....50

6.2. Wnt recognition by Frizzled receptors....51

6.3. Wnt signaling pathways....53

6.4. Multi-faced role of Wnt ligands in hematopoiesis....57

6.5. Frizzled receptors in hematopoiesis....64

6.6. The role of Wnt signaling in inflammation....68

7. OBJECTIVES....70

CHAPTER 2 PUBLICATION NO.1....72

FRIZZLED-6 REGULATES HEMATOPOIETIC STEM/PROGENITOR CELL SURVIVAL AND SELF-RENEWAL....73

1. SUMMARY....75

2. INTRODUCTION....76

3. RESULTS....78

3.1. Fzd6 deficiency does not affect intracellular β-catenin levels in HSPCs....78

3.2. *Fzd6* has an age-dependent effect on hematopoietic progenitor cell maintenance....80

3.3. *Fzd6* negatively regulates Cdc42/JNK signaling in HSPCs....82

3.4. *Fzd6* is essential for competitive repopulating capacity of HSCs and long-term granulocytic reconstitution....84

3.5. Long-term reconstitution defects of *Fzd6* deficient HSPCs are not due to altered homing but rather to defective survival and expansion in the recipient bone marrow....87

3.6. *Fzd6* deficient HSPCs expand poorly in response to emergency signals....90

4. DISCUSSION....92

5. MATERIAL AND METHODS....95

CHAPTER 3 PUBLICATION NO.2....99

INFECTION-ADAPTED EMERGENCY HEMATOPOIESIS PROMOTES VISCERAL LEISHMANISIS....100

1. ABSTRACT....102

2. AUTHOR SUMMARY 103

3. INTRODUCTION 104

4. RESULTS 106

4.1. L. donovani induces the expansion of HSC-like cells in the bone marrow and spleen 106

4.2. Induction of myelopoiesis during *L. donovani* infection results in the generation of altered progeny with a regulatory phenotype 109

4.3. *Fzd6* promotes bone marrow response to *L. donovani* 115

4.4. Enhanced myelopoiesis correlates with increased parasite burden 121

4.5. Decreased parasite expansion in *Fzd6*$^{-/-}$ mice is not due to enhanced T lymphocyte activity 129

4.6. Bone marrow cytokine environment promotes the generation of permissive monocytes 132

5. DISCUSSION 137

6. EXPERIMENTAL PROCEDURES 140

CHAPTER 4 GENERAL DISCUSSION 145

1. *Fzd6* is indispensable for the self-renewal and repopulation of hematopoietic stem cells 146

2. *Fzd6* regulates endotoxin-induced expansion and myeloid differentiation of hematopoietic stem/progenitor cells 151

3. *Fzd6* signaling accelerates the progression of *Leishmania donovani* parasite infection in mice 156

4. Conclusion: a new role for *Fzd6* signaling in homeostatic and stress-induced hematopoiesis 159

CHAPTER 5 REFERENCES 161

ANNEX-I The role of *Fzd6* in endotoxin-Induced inflammation 195

ANNEX-I List of communications 215

LIST OF FIGURES

CHAPTER 1: INTRODUCTION

Figure 1.Establishment of primitive and definitive hematopoiesis in mice 5

Figure 2.HSC transition from fetal and adult properties 8

Figure 3. Self-renewal and differentiation of hematopoietic stem cells.....10

Figure 4.Cycling activity of hematopoietic stem and progenitor cells 13

Figure 5.Cell-intrinsic regulation of hematopoietic stem cell quiescence.....15

Figure 6. Maintenance of quiescent hematopoietic stem cells in the bone marrow.....18

Figure 7. Crosstalk signaling between hematopoietic stem cells and osteoblasts in the niche.....20

Figure 8. The current scheme of hematopoietic hierarchy 24

Figure 9. Cytokine regulation of hematopoietic differentiation in semisolid medium.....27

Figure 10. Hematopoietic stem cell homing following bone marrow transplantation.....33

Figure 11. Initial stages of inflammation-induced emergency hematopoiesis.....36

Figure 12. Direct and indirect recognition of danger signals by hematopoietic stem cells.....43

Figure 13. G-CSF-mediated mobilization of hematopoietic stem/progenitor cells 46

Figure 14. The structure of Frizzled receptors 51

Figure 15. Canonical Wnt/β catenin signaling pathway.....54

Figure 16. Non-canonical Wnt signaling pathways 56

Figure 17. Frizzled receptor expression on hematopoietic/progenitor cells.....64

CHAPTER 2: PUBLICATION NO.1

Figure 1. Non-canonical *Fzd6* is expressed on $CD150^+$ HSCs and influences the ratio of $Flt3^+$ vs $CD150^+$ progenitors.....79

Figure 2. *Fzd6* is a negative regulator of Cdc42/Jnk signaling 83

Figure 3. *Fzd6*$^{-/-}$ HSPCs display defective long-term engraftment and self-renewal *in vivo*.....85

Figure 4. *Fzd6*$^{-/-}$ HSPCs display defective long-term engraftment and self-renewal *in vivo*.....86

Figure 5. Defective long-term reconstitution of *Fzd6*$^{-/-}$ HSPCs is not due to altered homing 88

Figure 6. *Fzd6*$^{-/-}$ HSPCs cannot expand and die by apoptosis in the first week after transplant. 89

Figure 7. *Fzd6*$^{-/-}$ HSPCs exhibit poor emergency hematopoiesis. 91

CHAPTER 3: PUBLICATION NO.2

Figure 1. Parasite expansion coincides with proliferation and accumulation of bone marrow hematopoietic stem/progenitor cells.....107

Figure 2. Bone marrow HSCs switch their differentiation towards non-classical myeloid progenitors 110

Figure 3. *Leishmania* parasite expansion promotes myeloid output in the bone marrow 113

Figure 4. Frizzled-6 is required for parasite-induced expansion and myeloid differentiation of HSPCs 117

Figure 5. Diminished myeloid output in *Fzd6*$^{-/-}$ mice correlates with a reduced parasite burden during the chronic phase of infection.....122

Figure 6. Decreased accumulation of myeloid cells is accompanied with reduced parasite burden in *Fzd6*$^{-/-}$ spleen 125

Figure 7. *Fzd6*$^{-/-}$ T lymphocytes are functionally indistinguishable from their *Fzd6*$^{+/+}$ counterparts 130

Figure 8. *Fzd6*$^{-/-}$ bone marrow microenvironment is enriched in pro-inflammatory cytokines and chemokines.....133

Figure 9. Infected bone marrow microenvironment directly promotes HSPC expansion and the generation of permissive monocytes.....136

LIST OF TABLES

Table 1.Antigenic proteins and glycoproteins used to purify hematopoietic stem cells...11

Table 2. Cell surface phenotype of identified hematopoietic stem /progenitor cells.....25

Table 3. Cytokines and hormones regulating hematopoietic cells.....29

Table 4. Pathogen specific response of hematopoietic stem/progenitor cells.....40

Table 5. Expression of Wnt ligands and Frizzled receptors in the bone marrow 57

Table 6. Wnt ligands and their function in hematopoietic stem cell regulation.....63

Table 7. Known function of Frizzled receptors in hematopoiesis.....67

LIST OF ABREVIATIONS

5-FU: 5- Fluorouracil

ABC: ATP-binding cassette

ACD: Asymmetric cell division

AGM: Aorta-gonad-mesonephros region

Ang-1: Angiopoietin-1

APC: Adenomatous polyposis coli

Axin: The axis inhibition protein-1

BaP: Basophil progenitor

BM: Bone marrow

BMDM: Bone marrow-derived macrophages

BMCP: Basophil-mast cell progenitor

BMP: Bone morphogenic protein

$Ca2^{+}$: Calcium

CAR: CXCL12-abundant perivascular cell

CDK: Cyclin dependent kinase

CDP: Common-dendritic cell progenitor

CFU: The colony-forming unit

CFU-E: Colony-forming-unit-erythroid

CFU-G: Colony-forming unit-granulocytes

CFU-GEMM: Colony-forming unit-granulocyte-erythrocyte-monocyte- megakaryocyte

CFU-GM: Colony-forming unit-granulocyte-monocyte

CFU-M: Colony-forming unit-monocyte

CK1α: Casein kinase 1α

CLP: Common lymphoid progenitor

cMOP: Common monocyte precursors

CMP: Common myeloid progenitor

CRD: Cysteine-rich domain

DAMP: Danger-associated molecular pattern

Dkk1: The Dickkopf protein -1

Dsh: Dishevelled (in Drosophila)

Dvl: Dishevelled (in vertebrates)

ECM: Extracellular matrix

EGF: Epidermal growth factor

ELP: Early-lymphoid progenitor

EOP: Eosinophil progenitor

EP: Erythroid progenitor

EPO: erythropoietin

ETP: Early thymic progenitor

FACS: Fluorescence activated cell sorting

FGF: Fibroblast-growth factor

FL: Fetal liver

Fz: Frizzled in Drosophila

***Fzd*:** Frizzled ortholog in mammals

G-CSF: Granulocyte colony stimulating factor

GM-CSF: Granulocyte-monocyte colony stimulating factor

GM-CSFR: Granulocyte-macrophage colony-stimulating factor receptor

GMP: Granulocyte-monocyte progenitor

GPCR: G-protein-coupled receptor

GSK-3β: Glycogen synthase kinase-3β

HGF: Hepatocyte growth factor

HSC: Hematopoietic stem cell

HSPC: Hematopoietic stem/progenitor cell

ICAM-1: Intercellular adhesion molecule-1

IFN: Interferon

IFNAR: Interferon-α/β receptor

IGF-1: insulin-like growth factor-1

IL: Interleukin

IRF: Interferon regulatory transcription factor

Jnk: c-Jun N-terminal kinase

LCMV: Lymphocytic choriomeningitis virus

LEF: Lymphoid enhancer binding factor

LFA-1: Lymphocyte-function antigen-1

LIF: Leukemia Inhibitory Factor

Lin: Lineage

LMPP: Lymphoid-primed multipotent progenitor

LRP: Low density lipoprotein *receptor*-related protein

LPS: Lipopolysaccharide

LT-HSC: Long-term hematopoietic stem cell

Madcam1: Mucosal vascular addressin cell adhesion molecule-1

MCP: Mast cell progenitor

M-CSF: Macrophage colony-stimulating factor

M-CSFR: Macrophage colony-stimulating factor receptor

MDP: Macrophage-dendritic cell progenitor

MEP: Megakaryocyte-erythrocyte progenitor

Mk: Megakaryocyte

MkP: Megakaryocyte progenitor

MkRP: Megakaryocyte progenitor with repopulating ability

MMP: Matrix metallopeptidase

MPP: Multipotent progenitor

MSC: Mesenchymal stem cell

MyRP: Myeloid progenitor with repopulating ability

NK: Natural killer

NP: Neutrophil-progenitor

OB: Osteoblast

PAMP: Pathogen-associated molecular pattern

PCP: Planar cell polarity

PDE: Phosphodiesterase

PDGF-BB: Megakaryocyte-secreted platelet-derived growth factor

PKC: Protein kinase C

PLC: Phospholipase C

PRR: Pattern recognition receptor

PSGL-1: P-selectin glycoprotein-ligand-1

PTH: Parathyroid hormone

Rb: Retinoblastoma

ROS: Reactive oxygen species

SCD: Symmetric cell division

SCF: Stem cell factor

SDD: Symmetric differentiation division

SFRP: Secreted Frizzled-related protein

SLAM: Signaling lymphocyte activation molecule

ST-HSCs: Short-term hematopoietic stem cell

TCF: T-cell factor

TGF-β: Transforming growth factor-β

Th: T-helper

TLR: Toll-like receptor

TNF: Tumor necrosis factor

TNFR: Tumor necrosis factor receptor

TPO: Thrombopoietin

VCAM-1: Vascular-adhesion protein-1

VEGF: Vascular endothelial growth factor

VLA-4: Very-late antigen-4/ Integrin α4β1

Wg: Wingless

WIF: Wnt Inhibitory Factor

YS: Yolk sac

CHAPTER 1

INTRODUCTION

1. Matter of life: Blood

Over centuries, blood has been considered as a magical symbol, which is often linked to courage, power and immortality in many faiths and religions. Due to its mysterious healing properties, blood was described as 'the centre of life itself' (Learoyd, 2006, Meletis *et al.*, 2010). *Hematopoiesis*—the Greek *haima*, meaning "blood" and *poiesis*, meaning "formation"— refers to the process of mature and functional blood cell generation (Damjanov, 2013).

Our understanding of hematopoiesis is the combination of ongoing research and extensive effort that have been carried out for several decades. The concept of hematopoiesis was introduced in 19th century by the establishment of bone marrow (BM) as the source of blood cells (Cooper, 2011). The existence of a "mother cell" for all blood elements was first proposed in Maximov's theory in 1909, while its potential participation in radiation damage was postulated by Sabin in 1932 (Friedenstein, 1989, Sabin *et al.*, 1932). The threat of continued atomic warfare in the period of mid-1940s and 1960s, marked a new era in the hematopoietic research, which is mainly based on studying the effects of irradiation and BM transplantation. Jacobson and his colleagues found that mice could survive an otherwise lethal irradiation exposure if a hematopoietic organ is protected by lead foil (Jacobson *et al.*, 1949). Soon afterwards, it became apparent that adult BM cells transplanted into syngeneic recipients were capable of rescuing lethally irradiated mice from hematopoietic failure (Lorenz *et al.*, 1952). But, it was not until early 1960s that first *in vivo* evidence provided by Till and McCulloch that radioprotection was due to the transplanted multipotent stem cells (Till *et al.*, 1961). The ability of BM cells to reconstitute the entire hematopoietic system in recipients provided an *in vivo* assay for the identification of hematopoietic stem cells (HSCs), thus setting the basis towards further evaluation (Becker *et al.*, 1963, Siminovitch, 1964).

While it was initially thought that HSCs are a homogenous population, the development of *in vitro* clonal assays progressively revealed the heterogeneity of hematopoietic stem/progenitor cells (HSPCs) in various aspects (Bradley *et al.*, 1966, Pluznik *et al.*, 1965). Consequently, the description of methods to characterize HSCs and their progeny did not occur another decade or more (Metcalf, 1980, Spangrude, 1991, Spangrude *et al.*, 1988). Today, we can enrich adult HSCs using the combination of monoclonal antibodies and high-speed fluorescence activated cell sorting (FACS)

technique based on the expression of different cell surface markers (Adolfsson *et al.*, 2001, Ikuta *et al.*, 1992, Oguro *et al.*, 2013). However, there is not any unique marker to identify true HSCs, thus more fastidious approaches are required for the functional characterization. Since the discovery of transplantable multipotent HSCs in adult BM to the present day, BM transplantation assays are considered “the gold standard” to demonstrate HSC function. The term ‘HSC’ refers to a single cell which is sufficient to rescue a host from otherwise lethal irradiation exposure and completely establish its entire hematopoietic system (Osawa *et al.*, 1996). This reconstitution ability requires: (i) *self-renewal*; to make an identical copy of itself while preserving the undifferentiated state, (ii) *extensive proliferation potential*; to repopulate, and (iii) *potency*; to differentiate and give rise to all blood elements.

2. Ontogeny of hematopoietic stem cells

During embryogenesis and fetal development, hematopoiesis occurs in different hematopoietic sites (niches) including the extraembryonic yolk sac (YS), the intraembryonic aorta-gonad-mesonephros region (AGM), fetal liver (FL), spleen and BM (Figure 1). Primitive hematopoiesis is initiated in the YS of the developing mouse embryo at around embryonic day 7.5 (E7.5). During the primitive streak stage, development of a putative YS, also known as the hemangioblast, marks the onset of hematopoiesis (Baron, 2003, Kennedy *et al.*, 2007). First hematopoietic cells produced in the YS are primitive red blood cells, termed erythroblasts, which contain embryonic hemoglobin. These large nucleated erythroblasts are surrounded by a layer of endothelial cells, which form blood islands. Macrophages and megakaryocytes are also generated at this stage (Kennedy *et al.*, 2007, Sasine *et al.*, 2016). At a slightly later stage, lymphoid precursors are also found in YS (Godin *et al.*, 1995). However, the first adult-type HSC, which can reconstitute the hematopoietic system are generated in AGM at ~E10.5 (Sasine *et al.*, 2016). Emergence of HSC from AGM marks the establishment of definitive hematopoiesis. Definitive HSCs circulate via umbilical vessels and migrate to YS and placenta. These sites provide potent hematopoietic niches for the maturation of immature “pre-HSCs” into functional HSCs (Gekas *et al.*, 2010, Swiers *et al.*, 2010). At E11.5, HSCs migrate to the FL in which they will be directed towards differentiation and give rise to definitive hematopoietic progenitors (Gekas *et al.*, 2010, Sasine *et al.*, 2016). Thereafter, hematopoietic activity stops in AGM, but still detected in YS and placenta (de Bruijn *et al.*, 2000, Sasine *et al.*, 2016). HSCs daily double their numbers in FL to seed the newly forming hematopoietic niches in the spleen and BM. Before the birth, the primitive HSCs migrate and colonize spaces filled out with embryonic bone and cartilage, establishing a close connection with BM microenvironment in early life (Taichman *et al.*, 1998). During transition from FL to BM, the frequency of HSCs dramatically declines. By the time of birth, the BM remains the primary hematopoietic site in which all HSC activity is retained during the adult life (Calvi *et al.*, 2003, Kunisaki *et al.*, 2013).

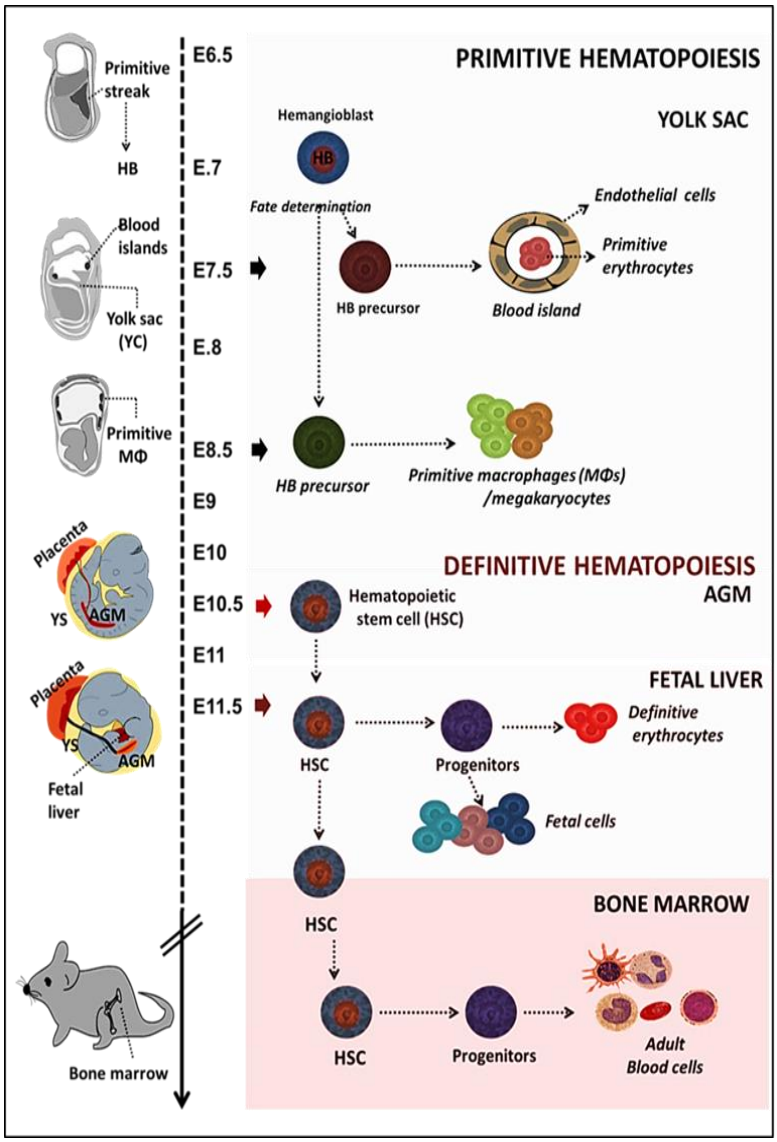

Figure 1.Establishment of primitive and definitive hematopoiesis in mice

Primitive hematopoiesis occurs between embryonic day E7.5 and E10. Main purpose of the primitive hematopoiesis is to provide the oxygenation of the developing embryo. Both endothelial cells and hematopoietic cells are generated from a common progenitor, the hemangioblast. The extraembryonic yolk sac serves as the source of primitive erythrocytes. The extraembryonic yolk sac blood islands, a structure formed by endothelial cells surrounds primitive erythrocytes. Primitive macrophages and megakaryocytes are also found in the yolk sac during this stage. Emergence of HSCs from the intraembryonic AGM around day E10.5, marks the beginning of definitive hematopoiesis which comprises: (i) expansion of 'pre-HSC' and maturation to functional HSC in fetal liver, yolk sac and placenta and (ii) differentiation of HSC into multipotent hematopoietic progenitors and generation of definitive hematopoietic cells in the fetal liver which is the major hematopoietic site during mid-gestation. Before the time of birth, fetal HSCs migrate to hematopoietic niches in BM in which all hematopoietic activity is retained in late-gestation and during adult life. *AGM: aorta-gonad-mesonephros region, HSC: hematopoietic stem cell.* Adapted from "Baron et al., 2012; Boisset et al., 2012; Ginhoux et al., 2013; Medvinsky et al., 2011".

3. Transition towards an adult phenotype

During the development and throughout aging, HSCs undergo phenotypic and functional changes. Mouse fetal HSCs actively cycle in order to expand the stem cell pool and seed newly forming hematopoietic organs in the developing embryo. The post-natal switch in HSC turnover occurs around 3 to 4 weeks of age at which HSCs become predominantly quiescent with only 5 to 10% remain cycling (Makio Ogawa, 1993, Pietras *et al*., 2011). While both adult BM and FL stem cells show an extensive capacity for self-renewal, FL has a greater hematopoietic reconstitution potential in transplant settings when compared to adult (Christensen *et al*., 2004, Morrison *et al*., 1995). Throughout the ontogeny, HSCs acquire different differentiation potentials. FL HSCs have a strong bias towards myeloid differentiation when compared to adult HSCs, which give a balanced lymphoid-myeloid lineage output (Bowie *et al.*, 2007). HSPC populations generate distinct types of hematopoietic cells at different stages of the development. Lymphoid and myeloid cell subtypes differ in phenotype and function (Ghosn *et al.*, 2012, Mold *et al.*, 2010), suggesting that HSCs generate distinct hematopoietic cell types at different stages of the development.

These developmental changes are linked to the differences in the gene expression profiles, transcriptional programs and cell cycle regulation of fetal and adult HSCs. Some of the cell-autonomous mechanisms, which selectively regulate fetal and adult HSCs, are depicted in Figure 2. The generation of HSCs from hemogenic endothelium requires Notch signaling *(Kumano et al., 2003).* Notch signaling occurs through the interaction of transmembrane receptors (Notch1-4 in mice) and Notch ligands (Jagged and Delta) on the signal receiving cells, which leads to the transcriptional regulation of Notch target genes (Kim et al., 2014). Notch1 and Jagged-1 display specific expression patterns within the arterial endothelium in the developing mouse embryo (Robert-Moreno et al., 2005). While Notch1 signaling plays an essential role in the generation of HSCs within the AGM *region (Kumano et al., 2003),* it is dispensable for primitive or definitive hematopoiesis in the YS *(Hadland et al., 2004, Robert-Moreno et al., 2008).* Jagged-1 coupling with Notch1 initiates direct transcriptional activation of GATA2 in the hemogenic endothelium *(Robert-Moreno et al., 2005).* A transcriptional network, which include GATA2, Ets and Scl contributes to the early hematopoietic expression of Runx1 (also known as Aml1), which is essential for the generation of definitive HSCs in mice (Nottingham et al., 2007). Thus, mice that are

deficient in either GATA2 or Aml/Runx1 are embryonically lethal due to the lack of definitive hematopoietic cells (Okuda et al., 1996, Tsai et al., 1994). It is demonstrated that Aml/Runx1 and Notch1 are not required for the maintenance of adult HSCs, while GATA2 plays a key role also in adulthood (Ichikawa et al., 2004, Mancini et al., 2005, Rodrigues et al., 2005).

After HSCs are formed, fetal specific genes such as the polycomb-group gene Ezh2, which regulates Lin28/let7 pathway plays an essential role in the acquisition of a fetal gene signature (Oshima et al., 2016). The Lin28/let7 pathway is critically required for the higher self-renewal activity of fetal HSCs and production of fetal-type lymphocytes (Copley et al., 2013). Conversely, the transcription factor, *Sox17* acts in the downstream of *Aml-1/Runx1* and *Gata-2* and maintains fetal HSCs prior to the acquisition of an adult phenotype (Kim et al., 2007). Germline deletion of *Sox17* reduces the expression of *Dickoff-1 (DKK-1)* which negatively regulates canonical Wnt pathway (Kim *et al.*, 2007). Canonical Wnt signaling is transmitted by binding of Wnt secreted glycoproteins to the Frizzled (Fz) family of cell surface receptors, which leads to the formation of a larger surface complex with the co-receptors, the low-density lipoprotein-related receptor (LRP) 5 and 6 (Malhotra et al., 2009). Activation of canonical Wnt pathway regulates the amount of a multifunctional adaptor protein β-catenin and directs transcriptional activation of intracellular signaling cascades (Komiya et al., 2008, Schubert et al., 2013). In the developing embryo, Wnt/β-catenin signaling regulates HSC emergence in a dose-dependent paracrine fashion. At around E10.5, Wnt/β activity is restricted to endothelial non-hematopoietic cells localized at the base of hematopoietic clusters in the AGM. Inactivation of Wnt/β-catenin signaling at this stage not only decreases the numbers of newly generated HSCs but also alters the function of these cells as shown in transplantation settings (Ruiz-Herguido *et al*., 2012). Interestingly, already formed HSCs contained in the E11.5 AGM appear to be β-catenin independent, suggesting a transient activation of Wnt/β-catenin signaling in HSC emergence (Ruiz-Herguido *et al*., 2012). Although Wnt/β-catenin activity is dispensable for the stem cell maintenance after E11.5 in fetal life, it is involved in the regulation of adult HSPC pool in a dose-dependent manner (Luis et al., 2011, Ruiz-Herguido et al., 2012). Likewise, transcriptional regulators including the proto-oncogene Bmi-1 and the transcriptional repressor Gfi-1 play an essential role in for the generation and maintenance of adult HSCs, while they are not required for the emergence and maintenance of fetal HSCs (Hock et al., 2004, Park et al., 2003). Interestingly, the changes in the cycling status of

HSCs, which occur by 3 weeks of age in mice also appears to be a cell-intrinsic developmental event. Evidence shows that the transcription factor C/EBPα which negatively regulates fetal HSCs self-renewal is at least partially required for the acquisition and maintenance of adult HSCs and for *in vivo* switch in HSC cycling activity in mice (Ye et al., 2013). These observations suggest that intrinsically determined molecular signals mediated by different developmental regulators collectively control properties and appropriate numbers of HSCs during fetal and adult life.

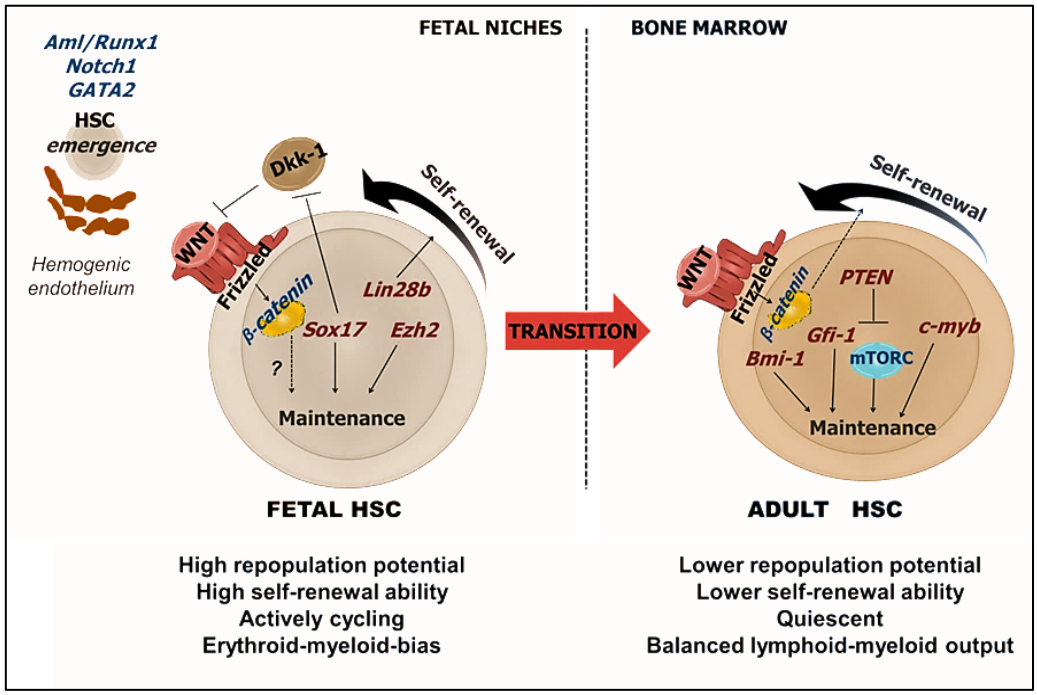

Figure 2. HSC transition from fetal and adult properties

A molecular switch during the transition from fetal to adult phenotype, results in decreased HSC expansion, cycling, self-renewal ability and myeloid differentiation potential. Aml/Runx-1, Notch1 and GATA2 are essential for the HSCs in the developing embryo, but dispensable for adult HSC maintenance. When HSCs are formed, transcription factor Sox17 regulates the acquisition of an adult phenotype via promoting Wnt/β-Catenin signaling. Ezh2 and its downstream Lin28b are crucial for the maintenance of fetal HSCs while Bmi-1 is required for HSC maintenance/function only in adult. PTEN and the transcription factors Gfi-1 and c-myb are dispensable for the primitive stages of hematopoiesis but essential for the definitive hematopoiesis while Wnt/β-Catenin activity regulates HSC maintenance/function transiently in a dose-dependent manner throughout developmental stages and adult life. Compiled from "Copley et al., 2013; Dejana et al., 2017; Ichikawa et al., 2004; Jang et al., 2007; Luis et al., 2011; Magee et al., 2012; Mancini et al., 2005; Oshima et al., 2016; Rodrigues et al., 2005; Ruiz-Herguido et al., 2012".

4. Steady-state hematopoiesis

Adult hematopoietic system consists of stem and progenitor cells, which continuously feed blood cell production through the commitment and differentiation processes. HSC *self-renewal* occurs through symmetric and asymmetric cell divisions. Symmetric divisions can yield either two identical stem cell daughters for stem cell expansion (SCD), or two committed daughter cells, termed symmetric differentiation division (SDD). Asymmetric divisions (ACD) generate one daughter that remains in a quiescent state as a reservoir for the stem cell pool and a committed daughter, which proliferates and differentiates to sustain hematopoiesis (Ito *et al*., 2012, Ting *et al*., 2012, Yamamoto *et al*., 2013). The progenitor cells are a subset between HSCs and mature blood cells. The restricted-differentiation potential and lack of self-renewal capacity distinguish committed-progenitors from HSCs (Seita *et al*., 2010).

Evidence from a growing number of single-cell transplantation and lineage tracing studies indicates to a significant heterogeneity in HSC subtypes with differential *repopulation* kinetics (Dykstra *et al.*, 2007, Sieburg *et al.*, 2006), *self-renewal* ability (Yamamoto *et al.*, 2013), *cell cycle* status (Mallaney et al., 2014, Wilson et al., 2008) and *multi-lineage differentiation* output (Dykstra *et al.*, 2007, Ema *et al.*, 2014). Accordingly, short-lived HSCs (ST-HSCs) provide radioprotection and the initial regeneration of hematopoiesis for up to 3-4 months post-transplant while their ancestors, long-term repopulating HSCs (LT-HSC), retain their repopulation and multi-lineage reconstitution activity for the life time of a recipient mouse (Harrison *et al*., 1993, Yamamoto *et al*., 2013).

During the commitment, *self-renewal* capacity and *multi-lineage differentiation* potential gradually lost (Figure 3), whereby a developmental switch occurs in genetic, metabolic and transcriptional programs associated with cell fate. Multipotent progenitors (MPPs) generate all mature blood lineages but possess transient reconstitution ability due to the lack of self-renewal capacity (Adolfsson *et al*., 2001, Ikuta *et al*., 1992, Morrison *et al*., 1997, Osawa *et al*., 1996, Weissman *et al*., 2001). Notably, steady-state hematopoiesis is largely sustained by ST-HSCs and progenitor cells rather than LT-HSCs. Thus, the frequency of LT-HSCs is considerably lower than any other progenitor population within BM (Busch *et al*., 2015).

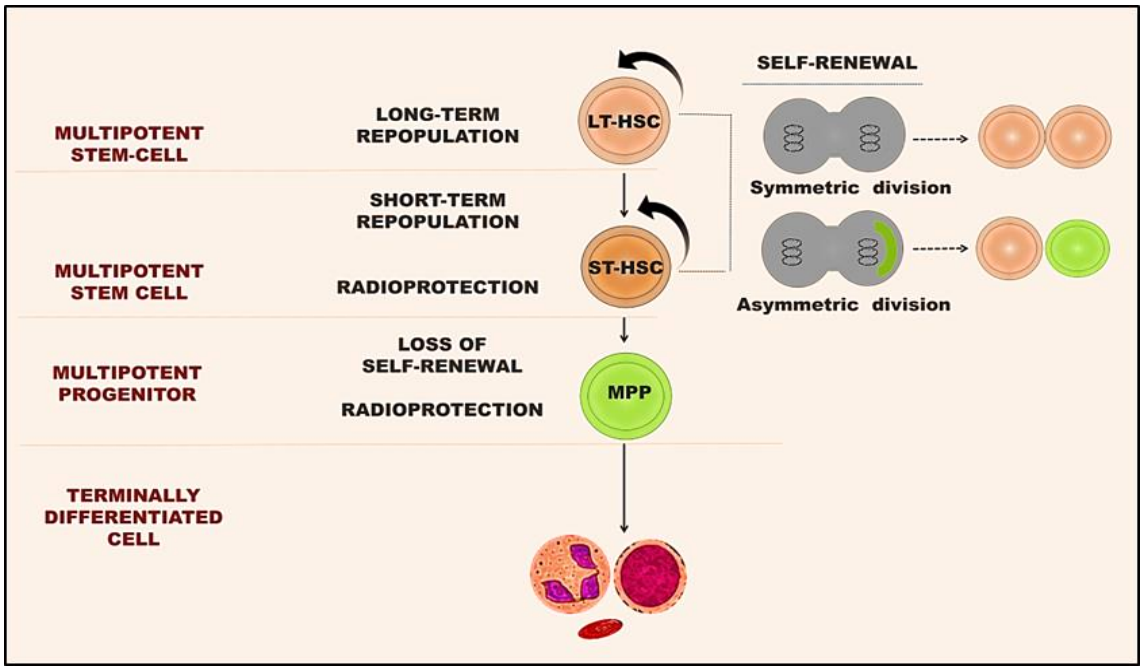

Figure 3. Self-renewal and differentiation of hematopoietic stem cells

Hematopoietic stem cells (HSCs) are a rare slow-dividing fraction of the bone marrow (~0.01% of all marrow). HSCs are identified with their unique *self-renewal* ability to reserve their pool and *multi-lineage differentiation* capacity to give rise to the committed progenitors, which supply mature blood lineages. HSC *self-renewal* occurs through symmetric and asymmetric cell divisions. At the top of hierarchy, LT-HSCs have the highest *self-renewal* and *in vivo repopulation ability* and provide a life-time hematopoietic reconstitution. When LT-HSCs divide, the *self-renewal* ability and function are gradually lost. When transplanted, ST-HSCs provide radioprotection and initial reconstitution of recipient's hematopoietic system for a limited time. MPPs lack self-renewal ability but have multi-lineage potential. *LT-HSC: long-term hematopoietic stem cell, ST-HSC: short-term hematopoietic stem cell, MPP: multipotent progenitor cell. Figure adapted from "Passegué et al., 2003; Suda et al., 2011; Wilson et al., 2006".*

FACS is commonly used for the identification of HSCs. Isolation of HSCs with this technique includes a negative selection using lineage (Lin) markers and a positive selection (Morrison, 2002). Lin selection cocktail, typically includes; CD3, CD4 and CD8 to exclude T lymphocytes, CD19 and B220 for B lymphocytes, CD11b and Gr-1 to remove myeloid cells and glycophorin-A or Ter119 to eliminate erythroid cells. After the removal of Lin^- cells, HSCs are further characterized based on the presence of stem-cell specific antigens on their surface. Some of the cell surface molecules expressed on mouse HSPCs are summarized in Table 1.

Table 1.Antigenic proteins and glycoproteins used to purify mouse hematopoietic stem cells

Antigen	Known function	Reference
CD34	a type I transmembrane phosphoglycoprotein which regulates cell cycle activity of HSCs and mediates HSC adhesion within hematopoietic niche	(Krause *et al.*, 1996)
CD117 (c-Kit)	the receptor tyrosine kinase which supports the maintenance and survival of HSCs via binding its ligand stem cell factor	(Broudy, 1997)
Sca-1	stem cell antigen-1; facilitates HSC maintenance and lineage specification	(Bradfute *et al.*, 2005)
Thy-1 (CD90)	a heavily N-glycosylated glycophosphatidylinositol-linked surface protein which is postulated to participate in lymphocyte-cell interactions and adhesion	(Barda-Saad *et al.*, 1999)
CD150	a member of the signaling lymphocytic activation molecule (SLAM) family receptors expressed on megakaryocytes and HSPCs	(Oguro et al., 2013)
CD48	a member of SLAM family receptors required for the initiation of T-cell activation and proliferation; absent on HSCs; upregulated on MPPs during inflammation	(González-Cabrero et al., 1999) (Boles et al., 2011)
CD244	a ligand for CD48 regulates the activation and cytotoxicity of T and NK cell functions during infections; not expressed on HSCs	(Waggoner et al., 2012)
IL-7Rα (CD127)	a receptor for interleukin 7 (IL-7) which plays a critical role in lymphocyte development; not expressed on HSCs	(Barata, 2013)
Flt3 (Flk2 or CD135)	a fms-related tyrosine kinase which contributes to the proliferation and differentiation of BM hematopoietic cells	(Gilliland *et al.*, 2002)
CD41	mainly expressed on megakaryocytes and platelets but transiently expressed on HSPCs at different stages of development and in response to stress	(Gekas *et al.*, 2013) (Pietras et al., 2016)

The most primitive HSC/HPC population remains within $Lin^{-}Sca\text{-}1^{+}c\text{-}KIT^{+}$ (LSK) fraction of the mouse BM (Ikuta *et al*., 1992, Minetaro Ogawa *et al*., 1991, Gerald J Spangrude *et al*., 1988). LSK fraction consists of LT-HSCs, ST-HSCs and MPPs (Figure-4).These populations are distinguished based on the expression of Thy1.1, CD34 and a HSC-specific fms like tyrosine kinase, CD135/Flt3. LT-HSCs are identified as IL-$7R\alpha^{-}Flt3^{lo/-}Thy1^{-}CD34^{-}$ LSK fraction, which give rise to ST-HSCs (IL-$7R\alpha^{-}Flt3^{lo/-}Thy1^{-}CD34^{+}$ LSKs) whose further downstream is MPPs (IL-$7R\alpha^{-}Flt3^{lo/hi}Thy1^{-}CD34^{+}$ LSKs) (Adolfsson *et al.*, 2001, Ikuta *et al.*, 1992, Morrison *et al.*, 1997, Osawa *et al.*, 1996, Weissman *et al.*, 2001). Differential expression of SLAM (the signaling lymphocytic activation molecule) markers, CD150, CD48 and CD244 enable further separation of distinct HSC and MPP subpopulations. While ST-HSCs express CD48 and its ligand CD244 on their surface, LT-HSCs lack these antigens. Lack of CD150 expression distinguishes MPPs from LT and ST-HSCs (Morita *et al.*, 2010, Oguro *et al.*, 2013).

Similar to mouse HSPCs, human HSPC populations can be isolated using a combination of monoclonal antibodies and FACS cell-sorting technology. Lin surface markers, which are used for the negative selection of human HSPCs include CD2 and CD3 for T lineage, CD19 for B cells, CD16 for NK cell lineage, Glycophorin A for red blood cells and CD14 and CD15 for myeloid cells. Following negative selection, HSCs are further enriched into Thy-1^{+}, Thy-$1^{+}CD38^{-lo}$, or $CD133^{+}$ fractions within $CD34^{+}$ cells (Reitsma et al., 2002). In contrast to mouse HSPCs, the SLAM receptor CD150 does not appear to be differentially expressed on human HSCs compared to more differentiated progenitors. Conversely, CD48 expression is found not only on committed human hematopoietic progenitor cells but also non-hematopoietic cell populations including endothelial cells. Interestingly, CD244, a ligand for CD48 is specifically expressed on human HSCs (Sintes et al., 2008, Zaiss et al., 2003).These observations suggest differential expression pattern of surface cell markers that differs on mouse and human HSCs.

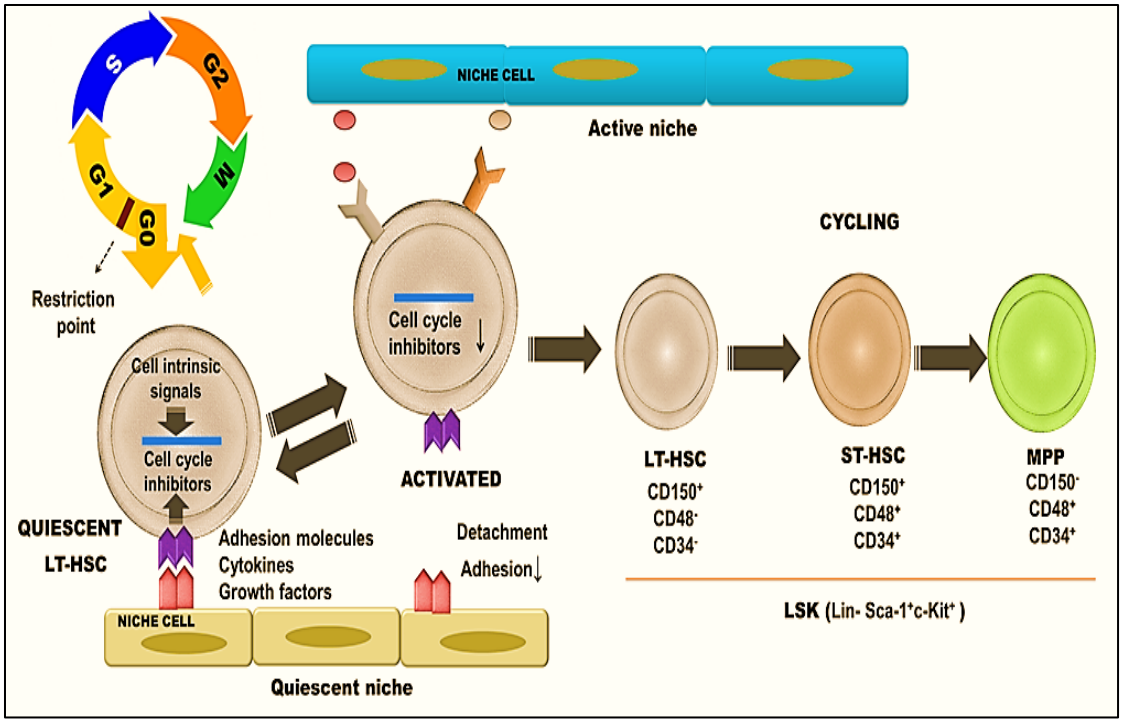

Figure 4. Cycling activity of hematopoietic stem and progenitor cells in mice

The most primate HSCs are found in the LSK ($Lin^-Sca\text{-}1^+c\text{-}Kit^+$) fraction within the bone marrow. LSK cell subset is composed of LT-HSC, ST-HSC and MPPs. Under steady-state conditions, the majority of LT-HSCs are found in G0 of the cell cycle phase while only >2% remain cycling (S-G2-M). Quiescence is maintained by cell-intrinsic signals and cell-niche interactions. Conversely, only 39% of ST-HSCs are found in G0 whereas MPPs are actively proliferating (only 16% in G0) (Wilson et al., 2008). *HSC: hematopoietic stem cell, LT-HSC: long-term hematopoietic stem cells, MPP: multi-potent progenitor cell, ST-HSC: short-term hematopoietic stem cell. Figure adapted from "Arai et al., 2008; Nakamura-Ishizu et al., 2014; Raaijmakers, 2010".*

The cell cycle is divided into four phases: G1, S, G2 and M phases (Figure-4). Two key checkpoints control the cell cycle and interrupt its progression when DNA damage occurs or the cells have failed to satisfy the requirement due to growth factor /nutrition deprivation. As a consequence, cells permanently exit the cell cycle and enter into an inactive state (G0) before apoptosis. The G0 phase is associated with the loss of cycling potential, senescence and death (Pietras *et al.*, 2011). In contrast to short-lived somatic cells, the majority of HSCs remain resting in the G0 phase. As they divide and differentiate into MPPs, which possess restricted lineage potential, the majority of the committed progeny become active in the S, G2, and M phases to maintain blood cell production (Steinman, 2002).

4.1. Maintenance of hematopoietic stem cell quiescence

Quiescence is crucial to protect HSCs from oxidative stress, DNA damage and the accumulation of replication-associated mutations. Stress resistance in quiescent HSCs is regulated by distinct programs. For instance, HSCs have specific ATP-binding cassette (ABC) transporter gene expression, which provides high drug efflux ability. The ABC transporters protect stem cells against toxic compound accumulation by pumping them across their cell membranes (Tang *et al.*, 2010). Dormant HSCs also display distinct apoptotic programs (Kosan *et al*., 2015), which probably facilitates their resistance to cytotoxic agents including ultraviolet light, ionizing radiation, and chemicals (Blanpain *et al*., 2011). The proper balance between HSC quiescence and expansion is critical to maintain homeostatic hematopoiesis and prevent hematopoietic failure or excessive expansion of stem pool that can lead to exhaustion or cancer formation. During steady-state hematopoiesis, HSC quiescence is tightly controlled by cell-intrinsic mechanisms, which are modulated by the niche signals in the BM.

4.1.1. Intrinsic regulation of the cell-cycle

The cell cycle regulation plays an important role in longevity and functional potential of HSCs. Yet, increased cell cycle activation in HSCs can lead to premature depletion and eventually exhaustion of the stem cell pool (Thompson *et al.*, 2008). The cell cycle activity of HSCs is dynamically controlled by cyclins and cyclin dependent kinases (CDKs) as depicted in Figure 5. During quiescence, the retinoblastoma (Rb) proteins prevent cell cycle progression by a complex mechanism, which includes the inactivation of transcription factors such as the E2F family. Progression through G1 and S phase requires the phosphorylation and suppression of the Rb protein by D cyclin/Cdk4/Cdk6 complex. Inactivation of the Rb protein activates the E2F transcription factors, which in turn induces HSC entry into the S phase (Steinman, 2002, Walkley *et al*., 2007).

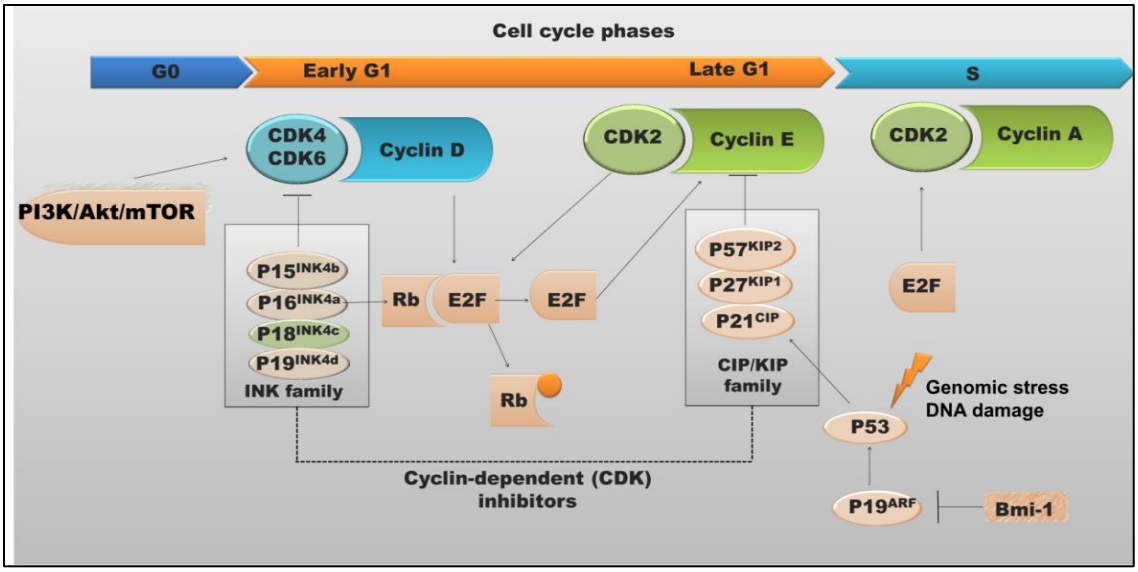

Figure 5.Cell-intrinsic regulation of hematopoietic stem cell quiescence

The cell cycle activity of HSCs is regulated by cyclins and CDKs. Cell-autonomous and external signals activate Cyclin D/CDK4-6 complex. The cell cycle is promoted by the inactivation of Rb/E2F complex in early G1. Late G1 progression is controlled by cyclin E-CDK2 complex. Cyclin/CDK activity leads to the phosphorylation of the Rb protein. The phosphorylation of the Rb protein results in the separation of Rb and E2F and the activation of genes encoding proteins required for the S phase. INK family and CIP/KIP family of CDK inhibitors are able to inhibit cyclins and CDKs that can arrest cell cycle progression. HSC cell cycle activity is also subject to the regulation via p53, either in response to cellular damage or p19ARF activity. The p53 activation in HSCs promotes the upregulation of pro-apoptotic genes (i.e., bax, nova,and puma) and induces DNA repair pathways. *CDK: cyclin-dependent kinase, Rb: retinoblastoma protein. Adapted from "Hao et al., 2016; Pietras et al., 2011".*

All three members of D cyclin family (Ccnd1, Ccnd2 and Ccnd3) are expressed in HSCs (Passegué *et al.*, 2005). While mice deficient for a single D-cyclin show no apparent hematopoietic defects (Fantl *et al.*, 1995), triple knockout mice for all three D cyclins die before the birth due to hematopoietic failure (Kozar *et al.*, 2004). Likewise, mice lacking CDK4/6 are also embryonically lethal due to impaired fetal hematopoiesis. In contrast, CDK6 deficient embryos are viable and display slightly less severe defects in hematopoiesis (Malumbres *et al.*, 2004). Interestingly, LT-HSCs lack CDK6 expression that leads to a delay in their cell cycle entry by 5-6 hours. Conversely, ST-HSCs express moderate levels of CDK6 that permits relatively rapid cell cycle entry in response to stress (Kosan *et al.*, 2015).

The members of the INK4 (p15, p16, p18, and p19) and CIP/KIP family (p21, p27, and p57) of CDK cell cycle inhibitors restrain the ability of HSCs to expand in response to various types of stress (Viatour *et al*., 2008). INK4 family exclusively binds

and inhibits the D cyclins while the CIP/KIP family interacts with all CDKs (Roussel, 1999). The genes of the INK4 locus, $p16^{INK4a}$ and $p19^{ARF}$, are actively repressed by the polycomb protein Bmi-1, which is preferentially expressed in HSCs. Lack of Bmi-1 completely blocks the proliferation/self-renewal in HSCs and increases p53-dependent cell death, resulting in lethal hematopoietic failure (Lessard *et al.*, 2003, Park *et al.*, 2003). The p53 tumor suppressor gene regulates many aspects of HSC behavior including proliferation, differentiation, stress response, apoptosis, and aging (Asai *et al.*, 2011). LT-HSCs express high levels of p53 (Dumble *et al.*, 2007), which promotes enhanced quiescence in these cells (Liu *et al.*, 2009). P53 appear to mediate its effects on HSCs at least partly through binding the Gfi-1 proto-oncogene and activating its expression (Liu *et al.*, 2009). Transcription factor Gfi-1 has been shown to restrict the proliferation of HSCs. As a consequence, the deletion of Gfi-1 leads to excessive cycling in HSCs that is accompanied by impaired repopulation potential (Kubota *et al*., 2012). Interestingly, caspase-3 upregulation is also observed in p53 deficient HSC (Liu *et al.*, 2009). The caspase 3, a downstream target of the PTEN/Akt pathway is generally recognized as the major effector of apoptosis. *In vivo* transplantation studies suggest that deletion of caspase-3 augments the proliferation of HSCs while negatively affecting their differentiation with a delay at specific stages of hematopoiesis, therefore indicating a role in HSC regulation (Janzen *et al.*, 2008). Yet, limited activation of the PTEN/Akt pathway has shown to preserve HSC quiescence, presumably minimizing reactive oxygen species (ROS) production, which limits cell cycle progression in HSCs (Pietras *et al.*, 2011). Taken together, these studies demonstrate that quiescence is tightly regulated by a complex cell-intrinsic network, which directly controls HSC expansion and self-renewal activity.

4.1.2. Regulation of quiescence by hematopoietic stem cell niches

As ‘everyday language is a part of the human organism’ (McDonough, 1986), the signaling networks and the crosstalk between stem cells and neighboring cells, are the language, which provides necessary information for the quiescence and activation of HSCs within the BM microenvironment. Niche signals modulate cell-divisions of HSCs, which are regulated by cell-intrinsic machinery. While some of these external cues suppress the cell cycle entry, others enhance cell cycle progression under stress

conditions. These regulatory networks include cytokines, chemokines, transcription factors, adhesion molecules and proteolytic enzymes (Guerrouahen *et al*., 2011).

Within the marrow, the location of HSCs distinguishes them from their progenitors. It is assumed that stem cell quiescence is partially enforced by the lack of oxygen in the local microenvironment (Suda *et al.*, 2011). Indeed, the hallmark of HSC niches is low levels of oxygenation, termed 'hypoxic niche'. Hypoxia slows the metabolic state of HSCs while promoting anaerobic glycolysis. This metabolic state protects stem cells from toxic and mutagenic effects of oxygen-generated free radicals (Parmar *et al.*, 2007, Suda *et al.*, 2011). The endosteal region is distant from the vasculature and defined with the deepest hypoxia due to the limited perfusion and low partial pressure of the oxygen (Eliasson *et al.*, 2010). LT-HSCs are found tightly adherent to the endosteum in the proximity of the osteoblasts (OBs) or closely associated with small arterioles (Wilson *et al*., 2008). HSCs, which are located in the endosteal niche are extremely quiescent with an estimated rate division of five times during lifetime of an adult mouse and have the highest functional potential (Wilson *et al*., 2008). This data suggests that LT-HSCs do not only tolerate low oxygen levels, but also it is a requisition for their longevity and function (Eliasson *et al*., 2010).

The endosteum is enriched in micro vessels, which carry oxygenated blood to the BM and connect the hematopoietic niche to the blood circulation (Bianco, 2011). A quiescence pool of LT-HSCs is also maintained around these small arterioles, which are surrounded by surrounded by sympathetic nerves, layers of smooth muscle cells and matrix components. In the arteriolar niches, HSCs are surrounded by quiescent $Nestin^{-}$ mesenchymal stem cells (MSCs) and $NG2^{+}LepR^{-}$ pericytes in association with endothelial cells (Kunisaki *et al*., 2013). Once activated, quiescent LT-HSCs detach from the endosteum and migrate towards the central parts of the bone and to the vascular areas in which they differentiate and are directed for the mobilization in order to re-establish hematopoiesis (Silberstein *et al.*, 2013). It has been shown that sympathetic nervous system might also regulate trafficking of HSCs into the bloodstream and could serve to maintain hematopoietic homeostasis. Indeed, the release of HSCs from bone marrow into the circulation is tightly regulated through the circadian release of adrenergic signals, which are delivered by nerves in the BM, transmitted to stromal niche cells (Méndez-Ferrer et al., 2008).

Cellular components of the BM hematopoietic niches comprise bone-forming OBs, bone-resorbing osteoclasts, endothelial cells, adipocytes, megakaryocytes and stromal cells including MSCs and reticular cells (Figure 6).

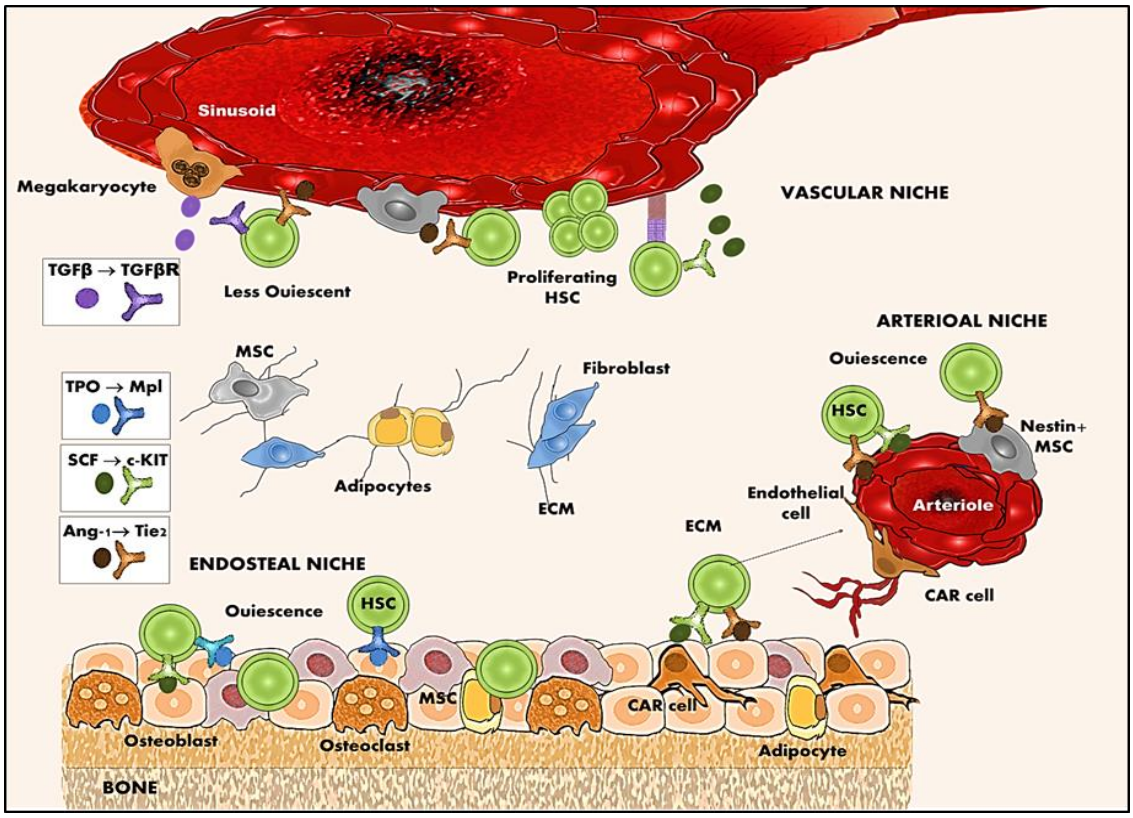

Figure 6. Maintenance of quiescent hematopoietic stem cells in the bone marrow

The quiescent LT-HSCs are found in the bone lining of the endosteum and are associated with small arterioles. Less quiescent or activated HSCs reside in vascular regions and are likely mature to hematopoietic progenitors. Osteoblasts and stromal cells maintain a quiescent HSC pool through secretion of TPO, SCF and Ang-1. Endothelial cells stimulate HSC expansion through adhesion molecules. Megakaryocytes, which are localized in perivascular areas, prevent HSC expansion via TGF-β signaling, while FGF4 expression induces HSC proliferation. ***Ang-1: angiopoietin-1, ECM: extracellular matrix, FGF-4: fibroblast-growth factor-4, SCF: stem cell factor, MSC: mesenchymal stem cell, TGF-β: transforming-growth factor-β, TPO: thrombopoietin. Figure is compiled from "Blank et al., 2015; Boulais et al., 2015; Grassel et al., 2007; Kopp et al., 2005; Mendelson et al., 2014; Reagan et al., 2015".***

Osteoblastic lineage cells: OBs are the main players of the endosteal niche, which maintain a quiescent stem cell pool via crosstalk signaling with HSCs and other stromal niche components. OBs evolve for the purpose of : (i) forming the bone by secreting bone matrix proteins, (ii) regulating bone-resorbing osteoclast activity and (iii) facilitating to the regulation of the BM microenvironment by secreting cytokines and growth factors (Dominici *et al.*, 2004). Genetic studies suggest that the physical contact with OBs plays an important role in the maintenance and function of HSCs. Indeed, an increase in the numbers of OBs is accompanied by a parallel increase in LT-HSC numbers with minimal effect on their downstream progenitors (Scadden, 2006, Zhang *et al*., 2003). OBs secret cytokines, growth factors and chemokines such as the stem cell factor (SCF), thrombopoietin (TPO), angiopoietin-1 (Ang-1), CXCL12, Jagged-1 and Wnt proteins, which positively regulates HSC quiescence and maintenance in the endosteum (Figure 7). Osteoblasts extensively inhibit HSC proliferation by producing hematopoietic inhibitory factors such as leukemia inhibitory factor (Rulifson *et al.*), osteopontin and transforming growth factor-β (TGF-β) to control HSC pool size. As a consequence, depletion of osteoblastic lineage cells results in severely impaired HSC function, and eventually exhaustion of HSC pool (Coşkun *et al.*, 2014).

Osteoclasts: The hematopoietic niche is a dynamic structure, which can be remodeled in response to exogenous stimuli and stress. Because stem cells are closely associated with the bone surface, the modifications or alterations in the bone composition directly affect HSC behaviour. Mature osteoclasts not only contributes to the bone resorption and remodeling of the bone, but also regulates bone-forming of OBs. Hence, osteoclast activity directly and indirectly controls the mobilization and retention of HSCs (Martin *et al*., 2005). Osteoclastic differentiation requires M-CSF and the interaction between the stromal protein activator of NF-κB ligand-RANKL and its receptor RANK on OB progenitors. RANKL induces osteoclast formation and activates mature osteoclasts (Boyce *et al.*, 2008). RANKL stimulated osteoclasts reduces the hematopoietic niche components, CXCL12, SCF and osteopontin along the endosteum, thereby increasing the mobilization of HSPCs from the niche (Kollet *et al.*, 2006). The loss of osteoclast activity results in disruption of the niche through a decrease in OB pool that in turn inhibits HSC colonization in the BM (Mansour *et al.*, 2012). These studies suggest that osteoclast activity might not be required for HSC expansion and maintenance, but plays a key role in their mobilization to the blood stream.

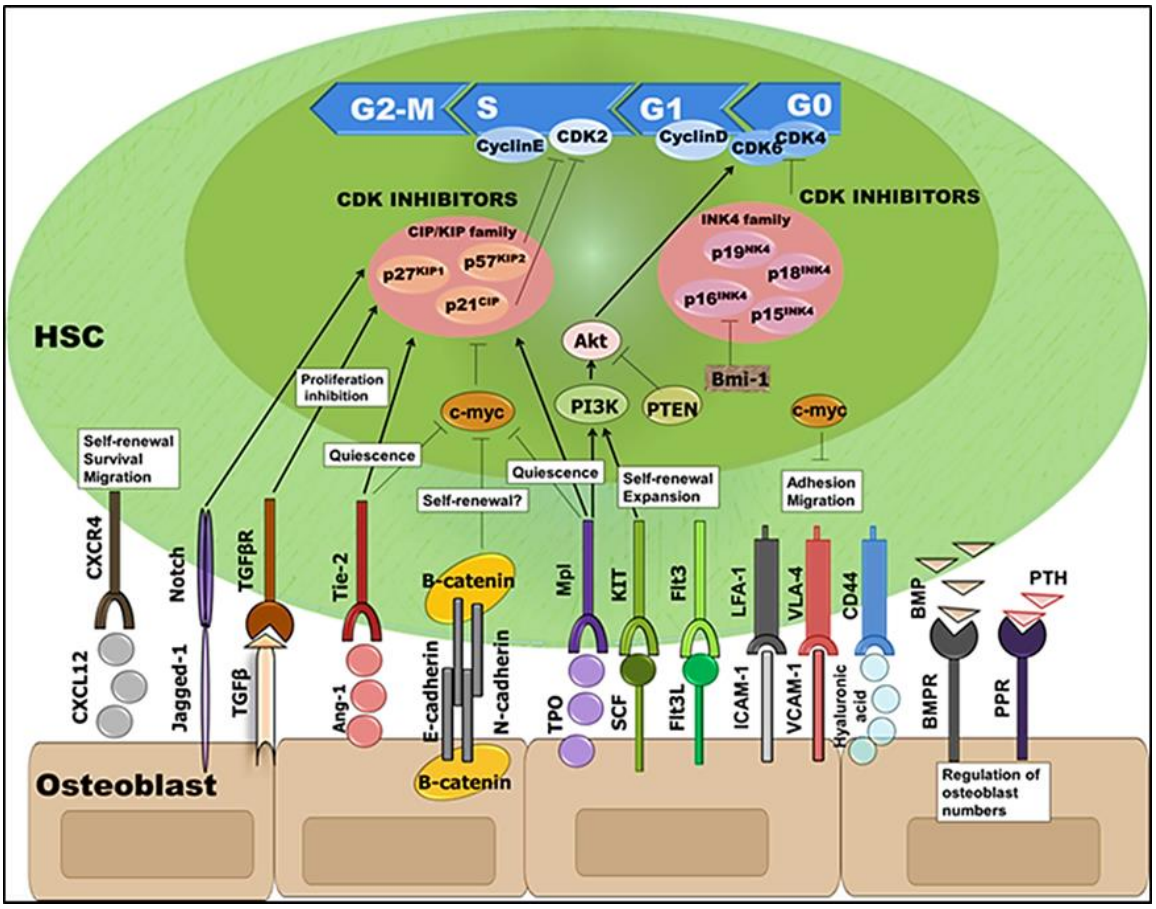

Figure 7. Crosstalk signaling between hematopoietic stem cells and osteoblasts in the niche

Within the niche, HSC fate is balanced by the integration of cell-autonomous factors and external cues derived from the niche components. Osteoblasts secrete cytokines and growth factors, which maintain HSCs in a quiescent state. The cell adhesion molecules, E- cadherin/N cadherins and integrins (VLA4, LFA-1), increases HSC adhesion to the bone surface. Ang-1 and TPO promotes quiescence whereas TGF-β limits expansion of HSCs. SCF and Flt3L play a crucial role in HSC self-renewal and expansion. CXCL12 signaling does not only maintain HSC quiescence but also facilitates to the survival and mobilization of HSPCs. The c-myc regulates the adhesion and cell cycle entry of HSCs. Osteoblastic niche size is controlled by BMP and PTH signaling pathways. *Ang-1: angiopoietin-1, BMP: bone morphogenic protein, LFA-1: lymphocyte function-associated antigen-1, PTH: parathyroid hormone, SCF: stem cell factor, TGF- β: transforming growth-factor, TPO: thrombopoietin, VLA-4: very late antigen-4/Integrin α4β1. Figure is adapted from "Forsberg et al., 2009; Pietras et al., 2011; Rizo et al., 2006; Taichman, 2005; Wilson et al., 2006".*

MSCs: MSCs are the key players of the BM microenvironment. They give rise to many of the niche elements including OBs, adipocytes, fibroblasts, and chondrocytes. Nestin^{-} Osx^{+} immature MSCs are mainly located on the endosteum and express high levels of matrix proteins (e.g., proteoglycan, osteopontin) and CXCL12, which are implicated HSC regulation. While early MSCs regulate HSC quiescence, more mature Nestin expressing

MSCs, which are located in the perivascular niche, control the proliferation and mobilization of HSCs (Hsu *et al.*, 2012). Nestin^{+} MSCs express relatively higher levels of soluble factors CXCL12, Ang-1 and vascular-adhesion protein-1 (VCAM-1) when compared to those secreted by OBs (Ehninger *et al*., 2011). It is suggested that perivascular cells are originated from a subgroup of MSCs which express the putative marker STRO-1. Because, these MSCs are able to express specific pericyte markers such as α-smooth muscle actin and CD146 (Shi *et al.*, 2003). Perivascular MSCs are located in close proximity with arterioles and directly touch sympathetic nerves and Schwann cells, which play a crucial role in the maintenance of steady state HSC homeostasis HSC retention under regenerative conditions (Katayama et al., 2006, Kunisaki et al., 2013, Méndez-Ferrer et al., 2010). MSCs localized near adrenergic nerve fibers express high levels of HSC maintenance genes, which mediate osteoblastic differentiation and enhanced maintenance of HSCs (Méndez-Ferrer et al., 2010).

Endothelial cells: It is well established that both endothelial cells and primitive HSCs arise from specialized vascular endothelial cells in the YS (Sasine *et al.*, 2016). Adult HSCs are closely localized to sinusoidal endothelial cells and perivascular cells in the trabecular regions of long bones (Kiel et al., 2005). Endothelial cells express adhesion molecules such as VCAM-1 and E-selectin, which facilitate HSC trafficking by modulating HSC-niche interaction (Schweitzer *et al.*, 1996). Similar to Nestin^{+} MSCs, BM endothelial cells support hematopoiesis through the expression of CXCL12, Ang-1, SCF and growth factors including fibroblast growth factor (FGF) and epidermal growth factor (EGF) (Guerrouahen *et al.*, 2011, Mendelson *et al.*, 2014).

Megakaryocytes (MKs): During homeostasis, MKs, a terminally differentiated cell type derived from HSCs, reside in close proximity to the BM sinusoidal vessels (Battinelli *et al.*, 2007). Intriguingly, MKs share similar common surface receptors, lineage-specific transcription factors, and specialized signaling pathways with adult HSCs. Similar to HSCs, MKs express Mpl, CXCR4, CD150, c-Kit, CD34 and Tie-2. These similarities might be due the developmental origin, since they arise from the same endothelial progenitors in early development (Huang *et al.*, 2009). MKs maintains HSC quiescence by producing factors, which limit HSC proliferation such as TGFβ and CXCL4 (Bruns *et al.*, 2014, Zhao *et al.*, 2014b). They also express Ang-1, FGF and CXCL12, which

regulate HSC expansion (Day *et al.*, 2014). Selective depletion of MKs within the niche, alters quiescence and induces HSCs into the cell cycle (Bruns *et al.*, 2014).

Other cell types are also implicated in the regulation of HSC fate. For instance, a subpopulation of monocytes/macrophages have been implicated in the regulation of steady-state and stress-induced hematopoiesis (Chow *et al.*, 2013). These macrophages are able to stimulate HSC retention by acting on CXCL12 expressing Nestin+ MSCs (Chow *et al.*, 2011). In addition to cellular components of the BM microenvironment, extracellular matrix (ECM) molecules including collagens, proteoglycans and glycoproteins, are also involved in the regulation of HSC behavior through cell-cell adhesion, which includes integrins, adaptors and signaling proteins. ECM presents various cytokines and growth factors, which regulate HSC maintenance and expansion. Examples of these factors are FGF, hepatocyte growth factor (HGF), vascular endothelial growth-factor (VEGF), BMPs and TGF-β (Gattazzo *et al.*, 2014, Klein, 1995).

4.2. Hematopoietic progenitor cells

The hematopoietic compartment displays a specific arrangement of blood cells in the BM. These sites are where HSPCs travel and receive necessary lineage-specific signals from specialized BM stromal cells for their differentiation (Wintrobe *et al.*, 2009). MPPs, which are generated from HSCs are localized in the niches, closely associated with RANKL-producing OBs and CXCL12 expressing $VCAM1^+$ reticular cells. MPP gives rise to myeloid, lymphoid and megakaryocyte/erythroid progenitors (Melchers, 2015). Granulocytes, monocytes and their precursors, collectively termed myeloid cells, play a key role in the development, tissue regeneration and humoral response. Lymphocytes or lymphoid cells including T cells, B cells, natural killer (NK) cells and certain type of dendritic cells, participate in the immune response (Janeway *et al.*, 1997).

During HSC differentiation towards a particular lineage, the genes associated with extensive self-renewal are downregulated, while a defined subset of lineage-specific genes is upregulated at the progenitor level. For instance, *Tie2* expression is only enriched in HSCs but not downstream committed progenitors (Terskikh *et al*., 2003). Likewise, the transcription factors *Meis1, Hoxa9*, and *Prdm16*, which are involved in the maintenance of undifferentiated state are progressively methylated and transcriptionally silenced during differentiation. In contrast, transcriptionally inactive genes are found to

be methylated. For instance, myeloid lineage specification is accompanied by the transcriptional upregulation of *Mpo*, which encodes myeloperoxidase enzyme required for neutrophil function (Kosan *et al.*, 2015). Therefore, hematopoietic lineage specification occurs as a gradual process, which involves many transcriptional and functional intermediate states. Different MPP subsets give rise to oligopotent common-lymphoid progenitor (CLP) and common-myeloid progenitors (CMP). Flt3 expression marks a subset within MPPs, termed lymphoid-primed multipotent progenitors (LMPPs), with a robust *in vivo* lymphoid reconstitution potential (Adolfsson *et al*., 2001, Kondo *et al*., 1997). Indeed, mice lacking Flt3 have reduced numbers of lymphocytes (McKenna *et al*., 2000). MPPs can be further fractionized based on VCAM-1 expression whereas PU.1 transcription factor marks different subsets of CMPs (Lai *et al.*, 2006, Lai *et al.*, 2005, Nutt *et al.*, 2005). MPPs co-express lymphoid and myeloid genes, whereas CMPs co-express myeloid and erythroid genes but not lymphoid genes (Akashi *et al*., 2003). CMP and CLPs further give rise to bi-potent progenitors, which generate uni-potent precursors and terminally differentiated blood cells.

According to myeloid bypass model, LT-HSCs can give rise to the lineage-restricted progenitors, which possess long-term repopulating ability. These progenitors can generate terminally differentiated hematopoietic cells without passing through the MPP stage. Accordingly, myeloid progenitor with repopulating ability (MyRPs), which arise directly from LT-HSCs, are the major suppliers of myeloid cells during stress hematopoiesis (Yamamoto *et al*., 2013). More recently, four different MPP subsets are identified based on SLAM markers and Flt3. It is proposed that the MPP compartment is composed of at least two distinct subsets of myeloid-biased MPPs, which work together with lymphoid-primed MPPs to regulate blood production (Pietras *et al*., 2015). Figure 8 depicts a working model of these findings. Cell surface phenotypes of distinct HSPC subsets are listed in Table 2.

Differentiation and maturation of blood lineages are regulated by transcription factors, genetic programs and cytokines in a stepwise fashion under steady-state conditions. It must be noted that demanding hematopoietic stress conditions can promote changes in cell surface markers of HSPCs in addition to differential cell kinetics.

Yet it is frequent, mutant HSCs can fail to reconstitute hematopoiesis upon transplantation due to LT-HSC defects while they sustain normal hematopoiesis at steady-state, which is mainly governed by ST-HSCs (Scheicher *et al*., 2011).

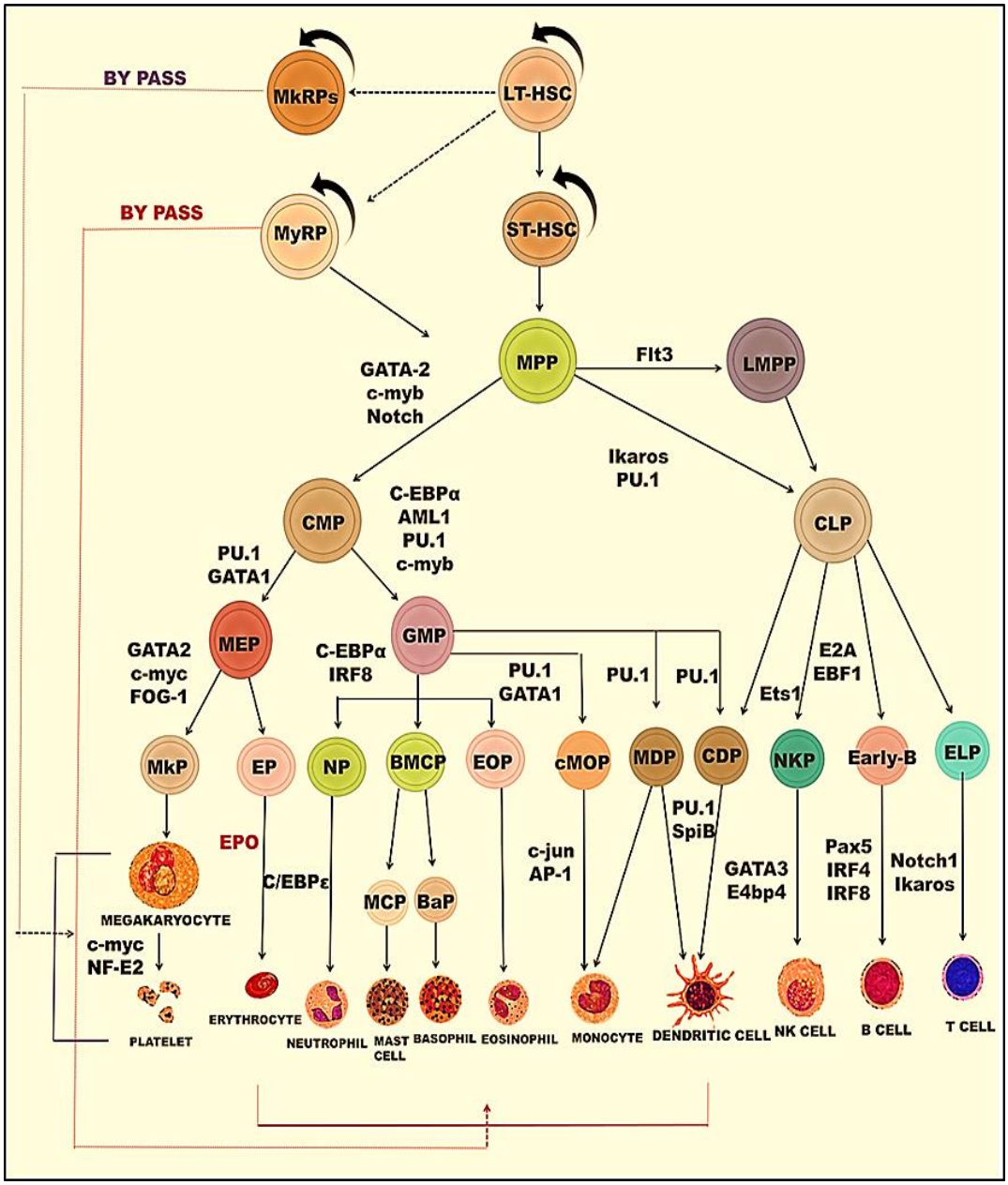

Figure 8. The current scheme of hematopoietic hierarchy

The figure displays HSC subsets that have been identified by *in vivo* transplantation assays or more complex assays. *MyRP; myeloid-progenitor with repopulating ability, MkRP; megakaryocyte progenitor with repopulating ability, MPP; multipotent progenitor, LMPP; lymphoid-primed multipotent progenitor, CMP; common-myeloid progenitor, CLP; common-lymphoid progenitor, GMP; granulocyte-monocyte progenitor, MEP; myeloid-erythroid progenitor, MkP: megakaryocyte progenitor, EP; erythroid-progenitor, NP; neutrophil-progenitor, EOP; eosinophil progenitor, BMCP; basophil-mast cell progenitor, MCP; mast cell progenitor, BaP; basophil progenitor, cMOP; common-monocyte progenitor, MDP; macrophage-dendritic cell progenitor, CDP; common-dendritic cell progenitor, ELP; early-tlymphoid progenitor.* The transcription factors that control lineage fate at each step are also illustrated. Figure is adapted from "Lichtman et al., 2006; Nimmo et al., 2015; Woolthuis et al., 2016".

Table 2. Cell surface phenotype of identified hematopoietic stem /progenitor cells

CELL TYPE	CELL SURFACE PHENOTYPE	Reference
LT-HSC	IL-7Rα$^-$Flt3$^{lo/-}$Thy1$^-$CD34$^-$ LSK CD34$^{lo/-}$Flt3$^-$CD150$^+$CD48$^-$ LSK CD150$^+$CD48$^-$CD41$^-$ CD244$^-$LSK	(Yokota *et al.*, 2012) (Morita *et al.*, 2010) (Chen *et al.*, 2016a)
ST-HSC	IL-7Rα$^-$Flt3$^{lo/-}$Thy1$^-$CD34$^+$ CD34$^+$Flt3$^-$CD150$^+$LSK CD150$^+$CD48$^+$CD41$^-$CD244$^+$LSK	(Yokota *et al.*, 2012) (Challen *et al.*, 2009) (Chen *et al.*, 2016a) (Oguro *et al.*, 2013)
Classical MPP	Lin$^-$Sca-1$^+$Kit$^+$CD34$^+$IL-7Rα$^-$Flt3$^{lo/-}$VCAM-1$^+$	(Lai *et al.*, 2005) (Lai *et al.*, 2006)
LMPP	Lin$^-$Sca-1$^+$Kit$^+$CD34$^+$IL-7Rα$^-$Flt3^{hi}V-CAM1$^-$	(Adolfsson *et al.*,
Myeloid-biased MPPs	MPP2; Lin$^-$ Sca-1$^+$Kit$^+$CD34$^+$Flt3$^-$CD150$^+$CD48$^+$ MPP3; Lin$^-$ Sca-1$^+$Kit$^+$CD34$^+$Flt3$^-$CD150$^-$CD48$^+$	(Pietras *et al.*, 2015)
Lymphoid-biased MPPs	MPP4; Lin$^-$ Sca-1$^+$Kit$^+$CD34$^+$Flt3$^+$CD150$^-$CD48$^+$	(Pietras *et al.*, 2015)
CMP	Lin$^-$Sca1$^-$Kit$^+$CD34$^+$FcRII/III$^-$	(Akashi *et al.*, 2000)
CLP	Lin$^-$IL-7Rα$^+$Sca-1^{lo}Kit^{lo}	(Akashi *et al.*, 2000)
GMP	Lin$^-$Sca1$^-$Kit$^+$CD34$^+$CD16/32$^+$	(Challen *et al.*, 2009)
MEP	Lin$^-$Sca1$^-$Kit$^+$CD34$^-$CD16/32$^{lo/-}$	(Challen *et al.*, 2009)
MDP CDP	Lin$^-$Sca-1$^-$Kit^{int}IL-7Rα$^-$Flt3$^+$CX3CR1$^+$	(Auffray *et al.*, 2009) (Fogg *et al.*, 2006) (Onai *et al.*, 2007)
cMOP	Lin$^-$Sca-1$^-$Kit^{int}IL-7Rα$^-$Flt3$^-$CX3CR1$^+$Ly6C$^+$	(Hettinger *et al.*, 2013)
BMCP	Lin$^-$ Sca-1$^-$Kit$^{int/hi}$CD34$^+$ CD16/32^{hi}B7^{hi}	(Arinobu *et al.*, 2005)

LMPP: lymphoid-primed multipotent progenitor, CMP: common myeloid progenitor, CLP: common lymphoid progenitor, GMP: granulocyte-monocyte progenitor, MEP: megakaryocyte-erythrocyte progenitor, MDP: macrophage-dendritic cell progenitor, CDP: common-dendritic cell progenitor, cMOP: common-monocyte progenitor, BMCP: basophil-mast cell progenitor

4.3. The role of classical cytokines in the hematopoietic diversity

Numerous attempts have been made to identify and purify cytokines with their abilities to support *in vitro* colony formation of hematopoietic progenitors. Methylcellulose cultures are commonly used to analyze the frequency of lineage progenitors in response to different cytokines. Traditionally, a single suspension is seeded on a solid media (methylcellulose or agar) supplemented with colony-stimulating factors and after an incubation period, generated colonies are counted and classified based on the morphology. Since the semisolid agar prevents cellular migration, mature cells, which are generated in each individual colony are derived from a single progenitor, termed a colony-forming unit (CFU). Although lymphoid differentiation media is commercially available, CFU assays mainly measure myeloid-erythroid potential of HSPCs (Pereira *et al.*, 2007). CFUs are composed of lineage-committed colonies as depicted in Figure 9.

Many of the cytokines have been investigated in the differentiation of HSPCs. Accordingly, the commitment of erythroid progenitors is regulated by erythropoietin or EPO (Ogawa, 1993); SCF and granulocyte-colony-stimulating factor (G-CSF) promote neutrophil maturation; macrophage-colony-stimulating factor (M-CSF) is specific for monocyte/macrophage differentiation (Metcalf, 1980); and IL-7 stimulates B lymphocyte maturation *in vitro (Kondo et al., 1997),* whereas GM progenitors require combination of IL-3, granulocyte-macrophage-colony-stimulating factor (GM-CSF) and SCF for their multi-lineage differentiation (Metcalf, 1980). The mutant mouse models suggest that humoral regulation might be required for the proliferation/survival of hematopoietic progenitors and the terminal differentiation of mature cells rather than hematopoietic commitment. Indeed, lack of G-CSF does not result in complete absence of mature neutrophils. Mice deficient for both G-CSF and M-CSF have macrophages and neutrophils, respectively (Lieschke *et al*., 1994). Even in mice lacking GM-CSF, G-CSF and M-CSF, myeloid cells are still present. However, these mice fail to respond to inflammatory insults (Friedman, 2007, Hibbs *et al*., 2007), suggesting that cytokine regulation is required for the proper function of mature myeloid cells and their precursors. It is thus possible that interruption in one molecular pathway can result in the invocation of compensatory pathways. Yet, two or more pathways can synergically affect the lineage-restriction. For instance, Epo deficiency is lethal due to the failure in definitive erythropoiesis (Wu et al., 1995). However, evidence from in vitro culture systems indicates that Epo does not directly affect commitment but rather regulates

apoptosis protection of late erythroid progenitors (Dolznig et al., 2002, Koury et al., 1990). The anti-apoptotic protein Bcl-xL has been proposed to mediate EPO-dependent survival during erythroid differentiation (Dolznig et al., 2002, Silva et al., 1996). Bcl-xL is known to be upregulated at the terminal stages of erythroblast differentiation (Aerbajinai et al., 2003, Gregoli et al., 1997). Mice deficient for Bcl-xL is embryonic lethal due to massive cell death of immature hematopoietic cells, which results in defective definitive hematopoiesis (Motoyama et al., 1995). In the absence of Epo, cultured murine hematopoietic progenitor cells undergo apoptotic cell death, presumably due to the downregulation of anti-apoptotic proteins Bcl-2 and Bcl-XL. Indeed, infection of these progenitors with a retroviral vector encoding human Bcl-XL or Bcl-2 increases the survival in the absence of Epo (Silva et al., 1996). Induction of Bcl-xL has been also shown to induce in vivo-like terminal differentiation into enucleated erythrocytes in serum-free cultures (Dolznig et al., 2002).

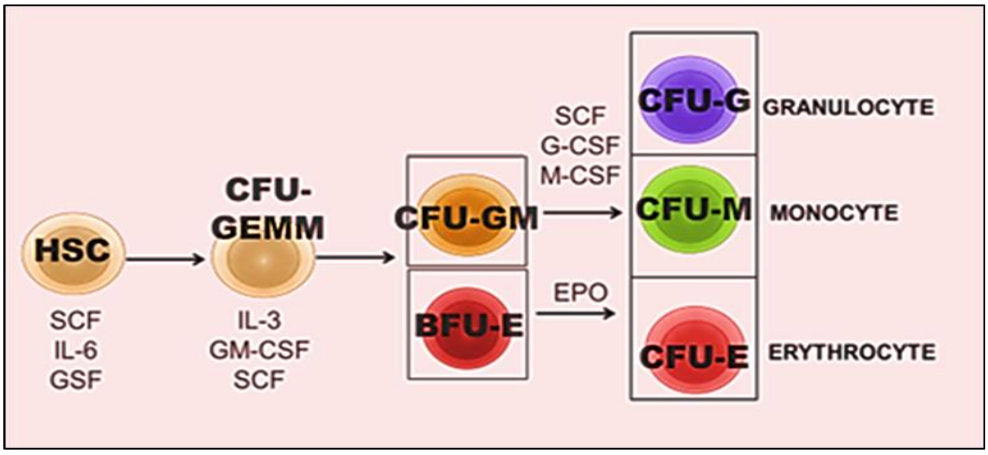

Figure 9. Cytokine regulation of hematopoietic differentiation in semisolid medium

The colony-forming unit (CFU) assay identifies hematopoietic progenitors by evaluating their ability to form hematopoietic colonies in response to cytokines in semisolid medium (agar or methylcellulose). Erythroid colonies are consisted of *colony-forming-unit-erythroid (CFU-E)*, which generates small erythroblast colonies and *erythroid-restricted burst-forming unit (BFU-E)*, which is more immature than CFU-E and produce larger colonies. Myeloid-restricted progenitors include *colony-forming unit-granulocytes (CFU-G)* and *colony-forming unit-monocyte (CFU-M)*. Multipotent progenitors consist of *colony-forming unit-granulocyte-monocyte (CFU-GM)* and *colony-forming unit-granulocyte-erythrocyte-monocyte- megakaryocyte (CFU-GEMM).* Cytokines which regulates *in vitro* differentiation are also depicted. *SCF; stem cell factor, GM-CSF; granulocyte-monocyte colony stimulating factor, G-CSF; granulocyte-colony stimulating factor, M-CSF; monocyte/macrophage colony stimulating factor, EPO; erythropoietin, IL-3/6; interleukin 3/6. Figure is adapted from "Eaves, 2015; Quesenberry et al., 2014".*

Consistent with these results, the location of hematopoietic cells within the BM niches suggests that distinct cell subsets have specific needs for humoral regulation and stromal support for their development, maturation and function. For instance, essential need for IL-7 in lymphopoiesis has been demonstrated by several studies (Milne *et al.*, 2006). Within the niche early-B cell precursors are found in close contact with CXCL12-expressing reticular cells surrounded by sinusoidal endothelial cells in the niche while late-B cells are located near the IL-7 expressing reticular cells, which secrete low levels of CXCL12. The decreased production of IL-7 leads to impaired B lymphopoiesis with a specific block in the transition from early to late B cell precursors (Panaroni *et al.*, 2013). Yet, immature B cells generated in BM as well as mature B cells that return back to BM after differentiation in peripheral lymphoid tissues, are found in the perivascular niche associated with high levels of CXCL12 and IL-7 (LeBien *et al*., 2008, Tobón *et al*., 2013, (Panaroni *et al.*, 2013). Taken together, the establishment of hematopoietic diversity is regulated by orchestrated interplay between cell-intrinsic transcription networks in HSPCs and cellular components of the niche, which provides stromal support and appropriate cytokines. Pivotal cytokines and their action in hematopoiesis are summarized in Table 3.

Table 3. Cytokines and hormones regulating hematopoietic cells

CYTOKINE	PRIMARY ACTION	REFERENCE
G-CSF	Acts on the survival of granulocyte progenitor and terminal maturation stages of granulopoiesis	(Liu *et al.*, 1996) (Zhan *et al.*, 1998)
M-CSF	Promotes proliferation of monocyte/macrophage progenitors	(Mossadegh-Keller *et al.*, 2013)
GM-CSF	Stimulates survival, proliferation and differentiation of granulocyte/monocyte progenitors	(Zhan *et al.*, 1998)
EPO	Affects proliferation of erythroid progenitors EPO protects against apoptosis through induction of Bcl-XL	(Ogawa, 1993) (Dolznig *et al.*, 2002)
TPO	Acts on HSC maintenance and megakaryopoiesis	(Yoshihara *et al.*, 2007) (Yu *et al.*, 2012)
IL-1	Induces other colony-stimulating factors in the proliferation of primitive HSPCs	(Fibbe *et al.*, 1991)
IL-3	Stimulates the growth of multiple myeloid cell lineages	(Evans *et al.*, 1999)
IL-4	Regulates B cell maturation and T cell function	(Wurster *et al.*, 2002) (Barner *et al.*, 1998)
IL-5	Primary regulator of eosinophil, basophil and mast cell growth	(Stone *et al.*, 2010)
IL-6	Acts on the proliferation and differentiation of granulocyte, monocyte, megakaryocyte, and erythroid progenitors	(Bernad *et al.*, 1994)
IL-7	Stimulates lymphocyte maturation	(Kondo *et al.*, 1997)
IL-15	Facilitates in the lymphoid cell development	(Colpitts *et al.*, 2013)
IL-16	Promotes proliferation of primitive HSPCs and *in vitro* dendritic cell differentiation	(Rofani *et al.*, 2009)
IL-17	Facilitates in the regulation of HSPCs through stimulating G-CSF, IL-6, and EPO	(Mojsilović *et al.*, 2015)

G-CSF: granulocyte-colony stimulating factor, M-CSF: macrophage-colony stimulating factor, GM-CSF: granulocyte-monocyte colony stimulating factor, EPO: erythropoietin, TPO: trombopoietin, IL: interleukin.

5. Stress response of hematopoietic stem cells

Given that stem cell therapies and BM transplantation are commonly used in clinics to treat hematopoietic diseases, experimental HSC research is currently focused on understanding the mechanisms, which regulate stem cell behavior in response to hematopoietic stress. Under homeostatic conditions, the generation of hematopoietic cells in the BM and the egress into circulation occur as a continuously balanced process (Busch *et al*., 2015). However, hematopoietic stress can induce quiescent HSCs into the cell cycle followed by their proliferation and differentiation. Increased numbers of LSKs and/or HSCs in the BM is extensively reported in response to hematopoietic challenges, e.g., transplantation (Wilson et al., 2008), physical exercise (De Lisio *et al.*, 2012, Kroepfl *et al.*, 2012), psychosocial stress (Heidt *et al.*, 2014), administration of cytotoxic drugs (Wright *et al*., 2002), inflammation (Allakhverdi *et al.*, 2009, Haas *et al.*, 2015a), and infection (Matatall *et al.*, 2016). Many of these studies have showed that HSPCs quickly respond to depletion of mature blood cells in situations of emergencies. It should be noted that some of these responses associated with HSC activation are dose-dependent. For instance, the stress of moderate bleeding results in partial increase in HSC self-renewal divisions (Cheshier *et al*., 2007). In contrast, severe myelosuppression caused by a chemotherapeutic agent fluorouracil (5-FU) awakens almost all of the quiescent LT-HSCs, stimulating their proliferation and mobilization into the bloodstream. Because 5-FU incorporates with G1/S phases of the actively cycling cells, its administration does not directly affect LT-HSCs, which are retained in G0. Hence, HSC activation following 5-FU administration occurs as a tissue injury response due to massive loss of hematopoietic cells and the alterations in the BM microenvironment (Radley *et al*., 1979, Xian *et al*., 2004). Intriguingly, HSCs return back to their normal quiescent state when the lost cells have been replaced and homeostatic hematopoiesis is restored (Wilson et al., 2008).

During steady state conditions, HSCs are strongly retained in BM niches (Figure 6 and Figure 7). The transition from resting to a proliferation state associates with changes in the genetic and metabolic programs of HSCs that allows the detachment from the niche followed by a shift in the BM location, proliferation and active trafficking

into the circulation. Quiescent LT-HSCs have slow metabolism due to their relatively low energy demand that prevents production of ROS, a natural by-product of active mitochondrial metabolism (Kohli *et al*., 2014). The endosteal niche provides a hypoxic niche in which HSCs depend on anaerobic glycolysis for ATP synthesis (Ito *et al*., 2014). Hematopoietic stress enhances mitochondrial respiration and NADPH oxidase activity that elevates ROS levels in HSC (Pang, 2011, Walter *et al*., 2015), thus facilitating detachment from the endosteum and shift their location to more oxygenic vascular niche for further differentiation and mobilization (Jang *et al*., 2007). Notably, repetitive cell cycle divisions can permanently change the epigenetic landscape and gene-expression programs in HSCs. Strikingly, the genes involved in the niche communication are downregulated, when HSCs exit dormancy and undergo serial self-divisions. When these cells return to dormancy, they are located in a more active niche and probably they would enter the cell cycle more quickly due to their new genetic program (Qiu *et al*., 2014). Increased DNA damage can lead to elevated ROS levels that increase p38MAPK activation, which is associated with upregulation of p16 (Shao, 2011).

5.1. Homing and cell kinetics of transplanted hematopoietic stem cells

Most of our knowledge on murine HSCs and the molecular mechanisms, which regulate their function, comes from *in vivo* competitive repopulation assays, which measure the repopulating ability of transplanted mutant-test HSC with unknown functional properties against an adequate number of genetically distinct wild-type HSCs in irradiated recipient mice (Yuan *et al*., 2005). Radiation exposure induces profound perturbations of the hematopoietic niches in which HSCs will engraft following transplantation. Induction of free radical production not only leads to apoptosis of hematopoietic cells, but also results in severe damage and destruction to BM blood vessels, the vascular niche and the bone structure (Cao *et al.*, 2011). Recovery of BM microenvironment from ionizing irradiation occurs through the expansion of radio-resistant habitant niche components (mesenchymal stem cells and mature megakaryocytes) and the repopulation of donor HSCs (Dominici *et al.*, 2009, Nicolay *et al.*, 2015). Upon transplantation, efficient repopulation requires a series of balanced interactions between donor HSCs and its supporting niche, which promotes: (i) HSC recruitment to the BM, termed *homing*, (ii) adhesion within the hematopoietic niche,

known as engraftment and (iii) the expansion and multi-lineage differentiation of HSPCs for the reconstitution of the hematopoietic system (Figure 10).

After transplantation, the initial step is the survival of HSCs that is initiated by (i) tethering, (ii) rolling along the endothelial blood vessels and (iii) the strong attachment to the marrow endothelium. While initial tethering and rolling is mediated by P-selectin, E-selectin is required for slow rolling and activation of adhesion to the endothelial cells. The p-selectin glycoprotein-ligand-1 (PSGL-1) binds to P- and E-selectins and CD44 only binds E-selectin. The enforced CD44 expression increases E-selectin adherence and enhances HSC homing (Merzaban *et al.*, 2011, Winkler *et al.*, 2012). When CD44 is blocked with antibodies, HSPCs are trapped in the periphery (Vermeulen *et al.*, 1998). The next step is the firm adherence of HSCs to the endothelial cells that occurs through the interactions between integrins and endothelial VCAM-1, intercellular adhesion molecule 1 (ICAM-1) and mucosal vascular addressin cell adhesion molecule-1(Madcam1). The integrin family members expressed on HSCs including very-late antigen-4 (VLA-4), α4β7, and lymphocyte-function antigen-1 (LFA-1), facilitate attachment to the endothelium. This strong adhesion supports HSCs to overcome the shear stress of blood flow and promotes trans-endothelial migration (Guerrouahen *et al.*, 2011, Koni *et al.*, 2001, Lapidot *et al.*, 2005, Sahin *et al.*, 2012). The stimulatory class of Gα subgroup of G-protein coupled-receptors (GPCR) are also involved in HSC trafficking and promote homing to the BM. For instance, the inhibition of CXCR4 that is thought to signal through Gα proteins, markedly decreases HSC homing. Furthermore, Gα deficiency alters homing efficiency of transplanted HSCs (Méndez-Ferrer *et al.*, 2009).

Under steady state conditions, murine HSCs are maintained in the diaphysis of the long bones. Radiation injury induces the expansion of host OBs located in the epiphysis and metaphysis to form transient endosteal niches for HSC lodgment. This activity reaches near-maximal at 24 hours, therefore delaying the transplantation of BM cells, increases HSC engraftment compared to immediate transplantation (Marino *et al.*, 2013). Proliferating OBs secrete increased amounts of CXCL12 that diffuses in the marrow space and promotes circulating HSCs migrate across the sinusoidal endothelial cells (Dominici *et al*., 2009, Lapidot *et al*., 2005). Approximately 5 hours after transplantation, HSCs are detected in close association with vascular structures. Recruitment of HSCs to the BM is completed during the first 12 to 24 hours following injection (Lewandowski *et al.*, 2010).

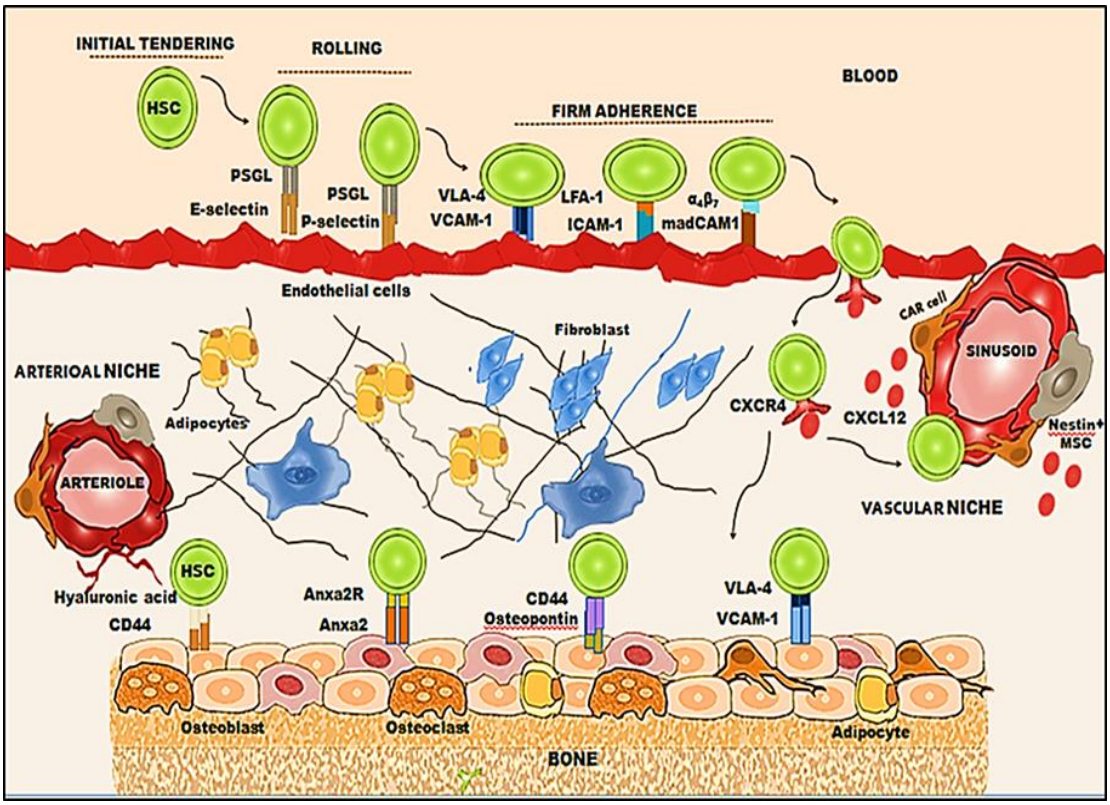

Figure 10. Hematopoietic stem cell homing following bone marrow transplantation

Initial tendering and rolling of HSCs are mediated by E- and P-selectins, which interact with PSGL-1 on stem cells. Initial recognition proceeds by firm adherence to endothelium to overcome the shear flow conditions. HSCs strongly attach to endothelial cells through binding of integrins (VLA-4, α4β7, and LFA-1) on endothelial VCAM-1, ICAM-1 and madCAM-1. Thereafter, HSCs transmigrate through the endothelial layer in CXCL12 mediated manner and transiently lodge in parasinusoidal niche. Proliferating OBs secrete molecules including Anxa2, CXCL12, osteopontin and VCAM-1 which promote migration to BM microenvironment and adhesion to the endosteum, thereby enhancing HSC engraftment within the niche. Anxa2: annexin-II, *ICAM-1: intercellular adhesion molecule-1, LFA-1: lymphocyte-function antigen-1, Madcam-1: mucosal vascular addressin cell adhesion molecule-1, PSGL-1: p-selectin glycoprotein-ligand-1, VCAM-1: vascular-adhesion protein-1, VLA-4: very-late antigen-4.Figure is adapted from "Hidalgo, 2008; Mazo et al., 2011; Sahin et al., 2012".*

Massive loss of hematopoietic cells in the BM promotes the rapid expansion of HSC pool upon trafficking and lodgment. Shortly after transplantation, more than half of the engrafted HSCs are found in the active phases of cell cycle (Nilsson *et al.*, 1997). HSCs proliferation and expansion occurs on the second or third day following transplantation (Lewandowski *et al.*, 2010). HSCs bind to surviving host megakaryocytes in the parasinusoidal niche and induce their migration to the endosteal surface in the epiphysis and metaphysis. 2 days after transplantation, donor HSCs are found in close

contact with N-cadherin+ OBs (Dominici *et al.*, 2009). The shift in the location of MKs is mainly governed by TPO/Mpl signaling. MKs enhance their close contact with OBs by CD41 mediated adhesion (Olson *et al.*, 2013). Thereafter, osteoblastic expansion is induced by MKs through a pathway in which TPO/Mpl signaling leads to an increase in MK-secreted platelet-derived growth factor (PDGF-BB) and insulin-like growth factor-1 (IGF-1) (Olson *et al.*, 2010, Olson *et al.*, 2013). The crosstalk between MKs and OBs as well as soluble factors produced by expanding pool of osteoblastic cells including annexin-II, VCAM-1 and ICAM-1, enhance HSC adhesion within the niche (Jung *et al.*, 2007, Sahin *et al.*, 2012). By day 5 to 7, almost all HSCs undergo self-renewal division to multiple their numbers (Lewandowski *et al.*, 2010). At 10 days after transplantation, OBs form a single layer, which contributes to the restoration of the endosteal niches. Afterwards, MKs are relocated in the center of marrow and vascular areas (Dominici *et al.*, 2009), whereas HSCs can be detected in additional hematopoietic sites in the BM (Cao, 2004).

Together, these observations suggest that hematopoietic engraftment is predominantly determined by the efficiency of homing and cell-intrinsic factors. HSCs proliferate and temporarily lodge in their initial site of lodgment and multiply their numbers to engraft in additional locations during the later stages of reconstitution (Cao, 2004, Dominici, 2009, Lewandowski, 2010). It is thus possible that distinct hematopoietic sites in which HSCs engraft differ in their ability to support the proliferation and differentiation of HSPCs.

Although the mechanisms affecting self-renewal divisions of HSCs are not clearly understood, it is established that self-renewal capacity and repopulation ability of HSCs following transplantation are at least partly sustained by intrinsic levels of cell cycle regulators. It has been shown that reduced levels of $p21^{CIP1}$, the $p27^{KIP1}$ and the $p16^{INK4a}$ yield defects in the self-renewal and repopulating ability of HSCs, whereas lack of the $p18^{INK4C}$ induce active cycling, leading to premature senescence (Cheng *et al.*, 2000a, Cheng *et al.*, 2000b, Yu *et al.*, 2006). Moreover, the absence of $p21^{CIP1/WAF1}$ and $p18^{INK4C}$ accelerates hematopoietic exhaustion after transplantation (Yu *et al*., 2006). For efficient repopulation, HSCs not only expand by numbers but also prevent differentiation and apoptosis. For instance, overexpression of anti-apoptotic member BCL-2 prevents apoptosis but facilitates in spontaneous differentiation of HSCs *in vitro* (Fairbairn *et al.*, 1993). When overexpressed *in vivo*, BCL-2 protects HSCs against apoptosis but results in a moderate increase in their expansion (Domen *et al.*, 2000). These results

demonstrates that apoptosis is an important factor in the regulation of HSCs. However, prevention of apoptosis is not sufficient for considerable expansion of HSCs *in vivo*. It is likely that a tight regulation of signaling networks (e.g., Wnt, BMP, TGF-β and Notch ligands), transcription factors, transcriptional regulators, cell-cycle regulators and epigenetic modifiers is required to balance HSC fate for efficient repopulation during regeneration (Kosan *et al.*, 2015, Krause, 2002, Trompouki *et al.*, 2011).

5.2. Inflammation- and pathogen-induced emergency hematopoiesis

The innate immune system, which consists of epithelial barriers, phagocytic leukocytes—monocyte/macrophages and neutrophils, dendritic cells, natural killer (NK) cells—, and circulating plasma proteins provides a first line of defense against pathogenic assaults. The innate immune system not only plays a crucial role in the initiation and direction of adaptive immune response but also participates in the clearance of pathogenic agents and worn-out cells. The adaptive immune system consists of: (i) humoral response; mediated by antibody production of B lymphocytes and (ii) cell-mediated response;, which includes T lymphocyte activation/response and the secretion of various cytokines (Alberts *et al.*, 2002, Medzhitov *et al.*, 1997).

The initial reaction of the immune system to invading pathogens and hematopoietic injury is the immediate inflammatory response, termed inflammation. During inflammation, leukocytes recognize pathogens via chemokine receptors and pathogen recognition receptors on their surface and migrate to the infection sites. Neutrophils are the first to be recruited to the inflammation site to eliminate the pathogens by phagocytosis, production of toxic molecules, release of enzymes, and formation of extracellular traps (Lacy, 2015). Because of the short-life of neutrophils with a half-life up to 12.5 hours, they are quickly eliminated in the spleen, liver and BM (Furze *et al.*, 2008, Suratt *et al.*, 2001). Dramatic depletion of neutrophils during an infection generates signals to the BM that promotes emergency hematopoiesis. The hallmark of this demand-driven hematopoietic state is the expansion of primitive HSCs followed by increased production of myeloid cells, which are shunted to the periphery to combat pathogens (Takizawa *et al.*, 2012). Figure 11 illustrates the initial stages of hematopoietic response during acute inflammation.

Acute microbial infections elicit profound alterations in hematopoietic compartment of the BM. For example, infection with *Escherichia coli*, a gram negative bacteria found in intestines, induces neutrophil mobilization from the BM to the blood or interstitial fluid (Kwak *et al.*, 2015). Following *E.coli* infection, decreased BM cellularity and the alterations in BM microenvironment induce proliferation of BM LSKs (Zhang *et al.*, 2008) and adjacent myeloid progenitors (Kwak *et al.*, 2015), resulting in increased neutrophil generation. Likewise, *Staphylococcus aureus* infection or polymicrobial sepsis induced by bacterial endotoxin lipopolysaccharide (LPS) are marked by the expansion of LT- and ST-HSCs in the BM, followed by enhanced delivery of HSPCs and neutrophils into peripheral circulation (Scumpia *et al.*, 2010).

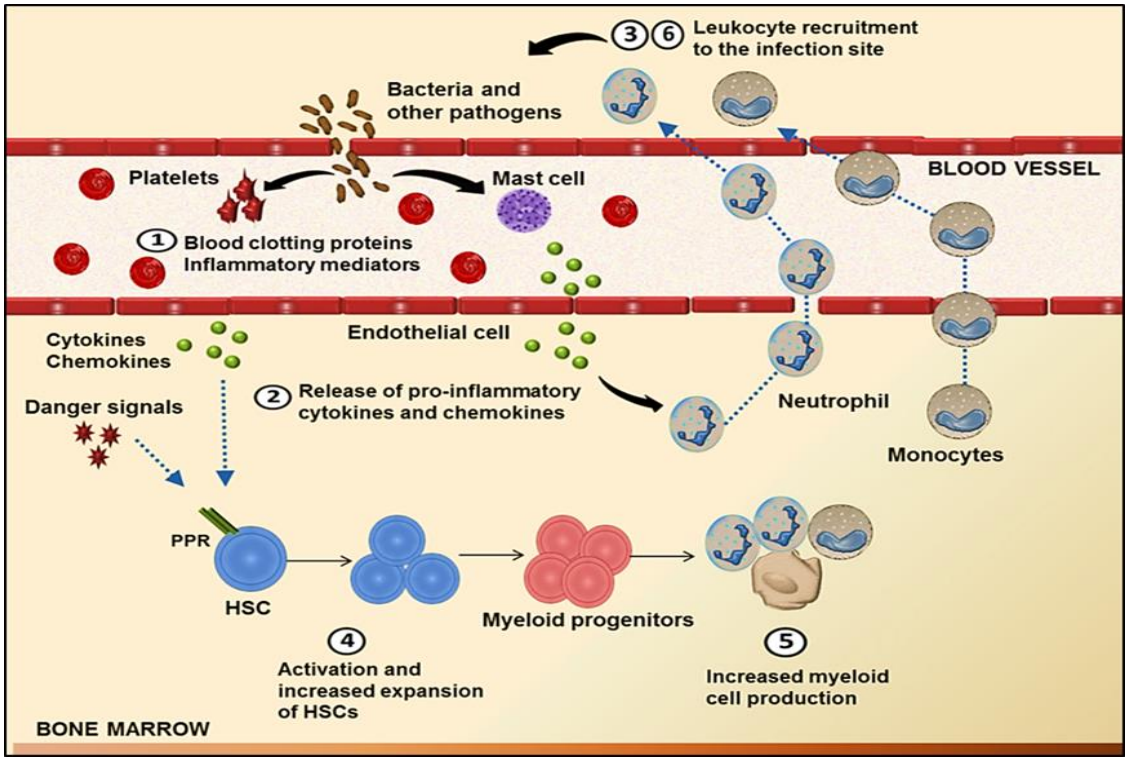

Figure 11. Initial stages of inflammation-induced emergency hematopoiesis

Systemic infections induce profound alterations in numerous hematopoietic and non-hematopoietic cell populations in the BM. Upon infection and during other inflammatory insults, leukocytes are mobilized from the BM to the infected tissues to combat microbial pathogens. Dramatic depletion of immune cells place demands on HSPCs to increase hematopoietic cell production. This demand-adapted state of hematopoiesis, also referred to emergency hematopoiesis, is characterized by (1) recognition of invading pathogens PRRs on the immune and non-immune cells (etc., endothelial cells, stromal cells), (2) release of inflammatory mediators which modify leukocyte and endothelial responses, (3) release and migration of leukocytes from the BM to the inflamed tissue through bloodstream, (4) activation of quiescent HSCs via direct (recognition of PAMPs, through cell surface PRRs) or indirect (in response to pro-inflammatory cytokines produced by other immune- or niche cells), (5) increased myelomonocytic

cell production which facilitates increased mobilization of these cells (6) to the circulation until the inflammation is resolved. ***PAMP: pathogen-associated molecular patterns, PRR: Pattern recognition receptor. Figure is adapted from Takizawa et al., 2012.***

Remarkably, experimental pneumonia (Shi *et al.*, 2011c) and acute malaria (Belyaev *et al.*, 2010) studies suggest that parasitic organisms, which are not found in the BM can also lead to a significant increase in LSK and HSC numbers while shifting hematopoietic differentiation in favor of myeloid cells. Although many retroviruses can directly infect HSPCs (Banerjee *et al.*, 2010), intracellular infection is not the only mechanism, which is involved in inflammation-induced HSC activation. Increasing evidence suggest that HSPCs respond to infections or exposure to microbial products through multiple mechanisms. Indeed, neither HSCs nor myeloid progenitors transmit infectious bacteria which typically require internalization mechanisms for their establishment (Baldridge *et al.*, 2010a, Kolb-Mäurer *et al.*, 2002, MacNamara *et al.*, 2011b). HSPCs can directly recognize pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) through toll-like receptors (TLRs) on their surface (Nagai *et al*., 2006, Yáñez *et al.,* 2011). However, the observation that the lack of TLR adaptor signaling molecules does not affect HSPC expansion during *Staphylococcus aureus* infection or polymicrobial sepsis (Scumpia *et al.*, 2010) points to a crosstalk between the peripheral immune response and hematopoietic compartment in the BM. It is well appreciated that infections and tissue damage trigger production of pro-inflammatory cytokines and paracrine signals by hematopoietic compartment as well as non-hematopoietic cells such as fibroblasts and endothelial cells, which counteract with the initial infection (Turner *et al.*, 2014). Indeed, tissue resident macrophages are known to produce growth factors that promote emergency hematopoiesis upon recognition of bacterial by products through their surface pattern-recognition receptors (PRRs) (Nishizawa *et al.*, 1990). Monocytes might also serve critical function selective production of BM myeloid cells through secretion of growth factors and cytokines in response to infection (de Waal Malefyt *et al.*, 1991, Vellenga *et al.*, 1988). The data from stringent *in vivo* experiments suggest that pathogens also induce endothelial cells to produce granulopoietic growth factors, which lead to the initiation of emergency hematopoiesis (Boettcher *et al.*, 2014). Because, LT-HSCs and their down-stream progeny express several classes of cytokine and chemokine receptors that participate in infection response (Zheng *et al*., 2011), these inflammatory signals do not only serve to amplify the inflammatory response but also participate in the mobilization, proliferation

and differentiation of HSPCs during emergency myelopoiesis (Baldridge *et al.*, 2011, King *et al.*, 2011). Recent evidence indicates that inflammatory conditions can also stimulate HSPCs to directly produce cytokines including IL-6, tumor necrosis factor (TNF)-α, IL-1β and GM-CSF which play a critical role in mediating rapid myeloid cell recovery (Zhao *et al.*, 2014a). This body of work illustrates that HSPCs play an active role in the first-line host defense to replenish depleted immune cells. Furthermore, HSPCs also contribute to the humoral immunity by acting as potent Th2 cytokine producers in response to inflammatory signals. Ex vivo stimulation of circulating human HSPC obtained from cord blood or adult peripheral blood with IL-3, IL-4, SCF, interferon (IFN)-γ, and TNF induce the secretion of IL-5, IL-13 and IL-6, in addition to GM-CSF and other chemokines (Allakhverdi *et al.*, 2009). Although how HSCs influence T-helper (Th)-2 effector cell function under physiological conditions remains to be defined, there is evidence that mouse Lin- MPPs mobilize to the gut-associated lymphoid tissue where they promote Th2 cytokine responses in a model of intestinal helminth infection (Saenz *et al.*, 2010).

It is evident that distinct pathogens elicit differential alterations in HSPC behaviour and function (Table 4). Inflammation and infection alters phenotype and function of HSPCs in a context dependent manner, which reflects the severity of different host-pathogen interactions. Altered expressions of some markers which are used to define primitive HSCs have been reported in response to inflammatory signals. For example, Sca-1 expression is upregulated following cytokine stimulation such as TNF and IFN, (Malek *et al*., 1989, Zhao *et al*., 2010) and in response to bacterial infection (Shi *et al*., 2013). Other work has shown that LT-HSCs express reduced levels of c-Kit when challenged with 5-FU administration which stimulates HSC response similar those in systemic infection (Randall *et al*., 1997). It is thus possible to speculate that these markers may play a functional role in HSC behavior. Particularly, the observation of severe reduction in BM myeloid cells in Sca-1 deficient mice following bacteremia (Shi *et al*., 2013) suggests that Sca-1 directly affect inflammation-induced HSC response. It will be important to determine how the other surface molecules facilitate the integration of multiple signals in HSC response.

Despite profound changes in BM cells in response to inflammation, hematopoietic compartment restore to a normal homeostatic state when the inflammation is resolved (Baldridge *et al.,* 2010, Essers *et al.,* 2009). While acute exposure to pro-inflammatory cytokines such as IFN-α does not affect HSC activity

(Essers *et al*., 2009), chronic stimulation of TLR signaling (Zhao *et al.,* 2013) or prolonged inflammation compromise HSC functionality as shown in the repopulating experiments (Matatall *et al.,* 2016).

Table 4. Pathogen specific response of hematopoietic stem/progenitor cells

Pathogen	Effect on bone marrow HSPCs	Reference
Staphylococcus aureus	TLR2 dependent expansion of LSKs	(Granick *et al.*, 2013)
Candida albicans	Increased *in vitro* proliferation and differentiation of LSKs via MyD88 signaling	(Yáñez *et al.*, 2009)
Chronic low dose-LPS	Increased HSC cycling, loss of self-renewal and myeloid differentiation ability	(Esplin *et al.*, 2011)
Short-term high dose-LPS	Increased HSC multi-lineage repopulating ability.	(Takizawa et al., 2011)
Pseudomonas aeruginosa	Expansion of LSKs, reduced repopulating capacity	(Rodriguez *et al.*, 2009)
Ehrlichia muris	IFN dependent expansion, reduced engraftment ability and a reduced bias towards myeloid lineage	(MacNamara *et al.*, 2011a)
Salmonella enteritidis	Reduction in the numbers of LT-HSCs accompanied by an increase in ST-HSC pool	(Kobayashi *et al.*, 2015)
LCMV	Altered maintenance and impaired proliferation by stromal cells	(de Bruin et al., 2013)
Mycobacterium aviumn	IFN-γ dependent dysfunctional LSK expansion	(Baldridge *et al.*, 2010a)
Vaccinia virus	MyD88-dependent expansion of LSKs	(Singh *et al.*, 2008)
Plasmodium chabaudi	IFN-γ dependent expansion of LSKs	(Belyaev *et al.*, 2010)
Leishmania donovani	Increased *in vitro* myeloid colony-forming ability of HSPCs which are obtained from infected mice	(Cotterell *et al.*, 2000a)
Helmint infection	IL-25 induced accumulation of MMPs in inflamed gut tissue where they promoted TH2 cytokine responses	(Saenz *et al.*, 2010)
Blood-stage malaria	IFN-γ dependent expansion of LSKs followed by increased myeloid differentiation at the expense of neutrophils	(Furusawa *et al.*, 2016)

LPS: lipopolysaccharide, LCMV: the lymphocytic choriomeningitis virus

5.2.1. Direct recognition of pathogens by hematopoietic stem cells

Innate immune cells as well HSPCs recognize molecular signatures of pathogens arising from PAMPS including LPS from gram-negative bacteria, bacterial/viral nucleic acid and peptidoglycans through cell surface-localized TLRs, a family of transmembrane PRRs (Yáñez *et al.*, 2013). Ten human and thirteen mouse TLR genes have been described (Mahla *et al.*, 2013). TLRs which are localized on the plasma membrane (TLR1, 2, 4, 5 and 6) recognize bacterial wall components or flagellin, whereas intracellular TLRs (TRL3, 7, and 9) which are found in endosomes are associated with nucleic acid recognition (O'Neill *et al.*, 2007). Specifically, TLR2 recognize a variety of PAMPs from gram-positive bacteria, including bacterial lipoproteins, lipoteichoic acids and lipoarabinomannan, and from fungus including zymosan and β-glucan. TLR3 is required for the detection of virus-derived double-stranded RNA. TLR4 is specific for the recognition of LPS whereas TLR7 and TLR9 is implicated for single-stranded RNA (ssRNA) (Kawai *et al.*, 2011).

The role of TLRs in differentiated myeloid cells is well established. Neutrophils express many members of TLRs with the exclusion of intracellular TLR-3, and 7 (Sabroe *et al.*, 2005). Similar expression levels of TLR-2, 4 and 9 are observed in monocytes when compared to neutrophils whereas neutrophils express relatively lower levels of TLR5 compared to monocytes (O'Mahony *et al.*, 2008). Activation of TLR signaling induces the production of inflammatory cytokines and expression of cell-specific receptors in these cells which is accompanied by enhanced phagocytic activity (Kawai *et al.*, 2010). Experimental evidence indicates that murine HSPCs and GMPs also express TLR2, TLR4, TLR7 and TLR9 on their cell surface as depicted in Figure 12. TLR2 and TLR4 are highly expressed on HSCs and GMPs whereas expression levels of TLR7 and TLR9 appear to be higher on CLPs when compared to other progenitor populations in the BM (Takizawa *et al.*, 2012). It is proposed that TLR signaling plays an active role not only in HSPC emergence and throughout embryonic hematopoiesis but also in functional response to pathogen induced stimulation during emergency hematopoiesis (Clapes *et al.*, 2016). For example, *In vitro* stimulation of TLR2 on HSPCs with a synthetic triacylated lipopeptide (Pam3CSK4, recognized by TLR1/TLR2 heterodimers) results in rapid acquisition of myeloid markers and increased monocyte/macrophage cell output even in the absence of exogenous growth and differentiation factors (Nagai *et al.*, 2006). Similar to *in vitro* stimulation, TLR2-dependent activation of HSCs during infections results in the expansion of not only LSK population, but also GMPs in a chronic murine

tuberculosis model (Choi *et al.*, 2011). TLR2-dependent expansion of LSKs is also observed during *Candida albicans* infection which promotes differentiation of HSCs towards myeloid lineage (Yáñez *et al.*, 2011). In addition to expansion of HSPCs, the promotion of hematopoietic commitment toward myeloid lineage development maximizes myeloid cell production which is a crucial element of host response to systemic infection. Activation of TLR4 by LPS is characterized by rapid cell cycle entry of HSPCs followed by increased expansion and differentiation of myeloid progenitors which favors myelopoiesis over lymphopoiesis (Ueda et al., 2005, Zhang et al., 2016). TLR2 or TRL4-mediated signaling modulates HSPCs via causing alterations in cytokine receptor and transcription factor expressions which are consistent with monocyte and/or macrophage differentiation. While M-CSFR is increased upon stimulation with TLR ligands, key transcription factors associated with lineage fate decisions such as SCL and GATA2 transcripts are depleted (Nagai *et al*., 2006). Transient downregulation of PU.1 and C/EBPα have also been reported following TLR stimulation by systemic bacterial infection (Zhang *et al.*, 2016). Indeed, myeloid progenitors, which are responsible for the production of myelomonocytic cells, represent the most TLR responsive population within the BM (Nagai *et al.*, 2006). Together these results suggest that TLR activation in HSCs not only induce activation and expansion of HSCs but also modulate differentiation by regulating cell-intrinsic transcriptional factors.

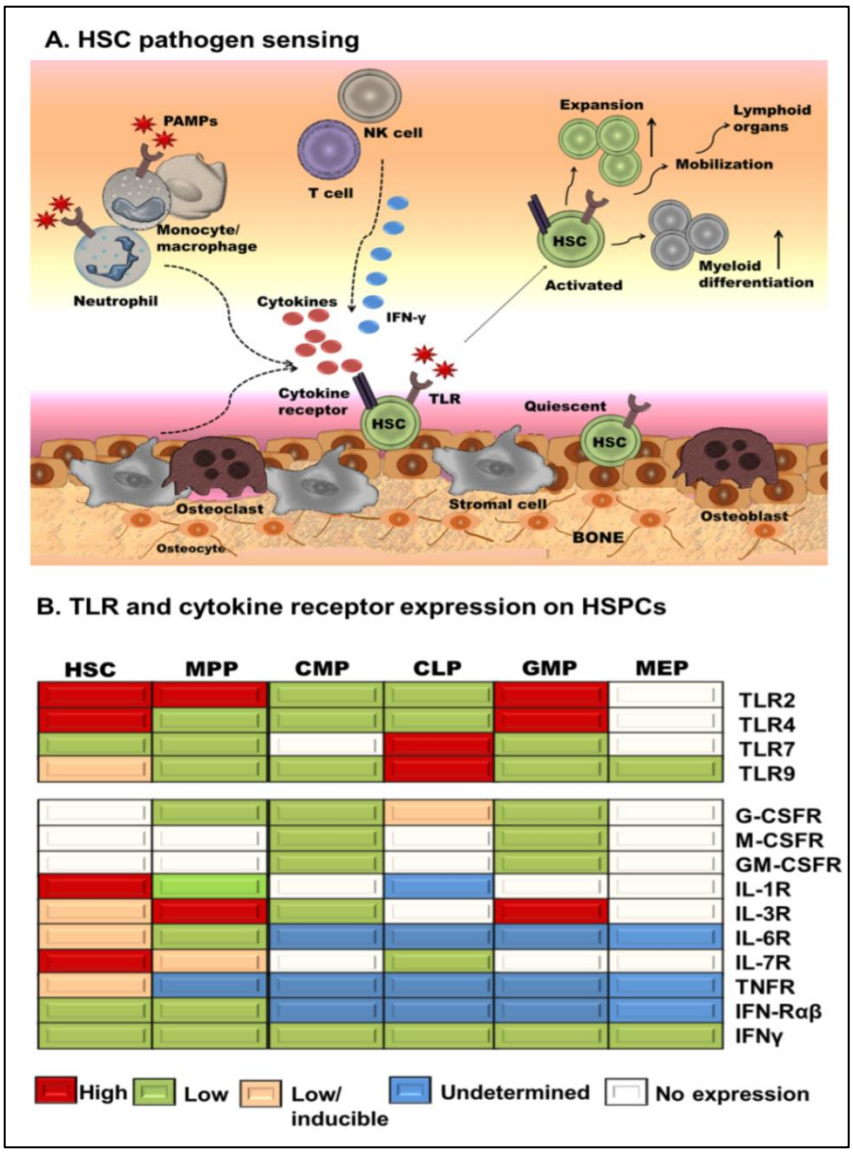

Figure 12. Direct and indirect recognition of danger signals by hematopoietic stem cells

(A) When the immune system encounters pathogens, microbial products are recognized by immune cells via cell-surface PPRs which triggers release of numerous immune cell attractants (chemokines) as well as pro-inflammatory signals (cytokines). Although HSCs remain quiescent under steady-state conditions, infection-induced danger signals promote HSCs into the cell cycle. During infection, HSCs can be activated through either direct recognition of PAMPs by TLR signaling or indirectly via stimulation by growth factors, cytokines and chemokines expressed by PAMP sensing- and/or infected immune cells (adapted from Zaretsky et al., 2014). (B) Expression of various TLRs and cytokine receptors on HSPCs that have been reported. Heat map summarizes differential expression levels of selected inflammatory response receptors on steady-state HSCs and their progenitors (adapted from Takizawa *et al*., 2012). *CLP: common lymphoid progenitor, CMP: common-myeloid progenitor, GMP: granulocyte-monocyte progenitor, HSC: hematopoietic stem cell, MEP: megakaryocyte-erythroid progenitor, MPP: multi-potent progenitor, TLR: Toll-like receptor, PAMP: pathogen-associated molecular patterns, PRR: pattern-recognition receptor.*

5.2.2. Effect of pro-inflammatory signals on emergency hematopoiesis

During inflammation and myelosuppression, increased levels of hematopoietic colony-stimulating factors and pro-inflammatory cytokines induce proliferation and mobilization of HSPCs which results in increased myeloid output (Zaretsky *et al.*, 2014). Some of these inflammatory signals in infection-induced HSC response are summarized below.

Hematopoietic growth factors: Despite differences in kinetics and magnitude of the responses, the expansion of HSC compartment is frequently accompanied by the development of extramedullary hematopoiesis in the spleen and liver (Burberry *et al*., 2014, MacNamara *et al.,* 2011, Matatall *et al*., 2016), which provide alternative cellular niches for further expansion and differentiation of HSPCs. G-CSF is the best studied myeloid cytokine in neutrophil and HSC recruitment to the peripheral blood due to its common use in the clinic settings (Bender, 1992, Sheridan, 1992). Although G-CSF levels are considerably low at steady-state, a rapid increase in its production by endothelial cells, macrophages, epithelial cells and fibroblasts is observed in response to inflammatory mediators such as LPS, TNF-α, interferon IFN-β, vascular endothelial growth factor (VEGF), IL-17 and IL-1 (Bendall, 2014). Increased systemic G-CSF levels often correlates with increased infiltration of phagocytic cells to the inflammation site. The experimental infection studies demonstrated that G-CSF treatment reduces the mortality rates in infected mice due to increased phagocytic activity (Dunne *et al.*, 1996, Kullberg *et al.*, 1998). G-CSF mediates its effect by binding to its receptor G-CSFR on neutrophils and HSPCs. Mice lacking G-CSFR demonstrate defects in neutrophil migration in lymphoid organs in response to infections (Bendall, 2014). In contrast, HSC activation in response to G-CSF appears to be cell-extrinsic, since the absence G-CSFR does not affect HSPC mobilization following G-CSF administration (Greenbaum *et al*., 2011). Elevated G-CSF levels alters BM microenvironment by supressing CXCL12 production in OBs (Semerad *et al*., 2005), endothelial cells (Eash *et al.*, 2010) and CAR cells (Day *et al.*, 2015). CXCL12 act as an inhibitor of the cycling status of HSCs to maintain their pool (Cashman *et al*., 2002). Hence, down-regulation of CXCL12 at protein and mRNA levels enables local gradients towards blood and the mobilization of HSPCs. Increased levels of other cytokines such as SCF and Flt3-L are also reported to induce such down-regulation in BM CXCL12 levels (Christopher *et al.*, 2009). Blocking

CXCL12 or its receptor CXCR4 reduces the mobilization of HSCs following G-CSF administration, suggesting a critical role for CXCL12/CXCR4 axis in the mobilization of stem/progenitor cells (Petit *et al.*, 2002). Disruption of adhesive interactions between HSCs and surrounding BM stromal niche plays an important role in the stem cell retention. G-CSF increases ROS production of BM neutrophils that results in elevated secretion of neutrophilic enzymes and serine proteases, which are capable of cleaving adhesion molecule VCAM-1 (Singh *et al.*, 2012). Administration of anti-VLA-4, the ligand for VCAM-1 which is present on stromal cells, has been shown to induce HSC mobilization (Papayannopoulou *et al.*, 1995), which is accomplished through cooperative c-Kit/SCF signaling (Papayannopoulou *et al.*, 1998). Inhibition of other adhesion molecules such as CD44 which interacts with ECM components also enhances HSPC retention (Vermeulen *et al.*, 1998).

There is emerging evidence that monocyte/macrophages also play a critical role in the regulation of HSPC trafficking in response to G-CSF. G-CSF treatment of transgenic mice in which G-CSFR expression is restricted to $CD68^+$ monocytes, enables HSPC mobilization by supressing osteoblastic activity (Heidt *et al.*, 2014). Further analysis of monocytic cell population in BM revealed that the ablation of $CD169^+$ macrophages leads to a profound decrease in CXCL12, SCF, and Ang-1 from Nestin-perivascular cells which is accompanied by increased HSPC mobilization from BM (Chow *et al.*, 2011). These studies together demonstrate that subtypes of monocyte/macrophage populations facilitate in G-CSF induced HSPC recruitment from BM to the peripheral tissues through the regulation of HSC-niche interactions. A model for G-CSF mediated mobilization and egress of HSPCs is depicted in Figure 13.

M-CSF (or CSF-1) is another important myeloid cytokine that is released during infection and inflammation. It is shown that M-CSF treatment of freshly transplanted recipient mice prior to infection with *Pseudomonas aeruginosa* and the fungus *Aspergillus fumigatus* increases the survival. Because M-CSF treatment results in increased production of granulocyte-monocyte lineage cells, increased systemic levels of this cytokine can more efficiently protect mice in response to these opportunistic pathogens when compared to G-CSF treatment (Kandalla *et al.*, 2016). The myelo-proliferative effect of M-CSF in infection-induced HSPC response has been further confirmed by time-lapse single cell imaging and single cell gene expression. It appears that M-CSF but not G-CSF directly induces endogenous PU.1 protein and instructs

myeloid-differentiation fate in HSCs which serve as an adaptation mechanisms combat pathogenic challenge (Mossadegh-Keller *et al.*, 2013).

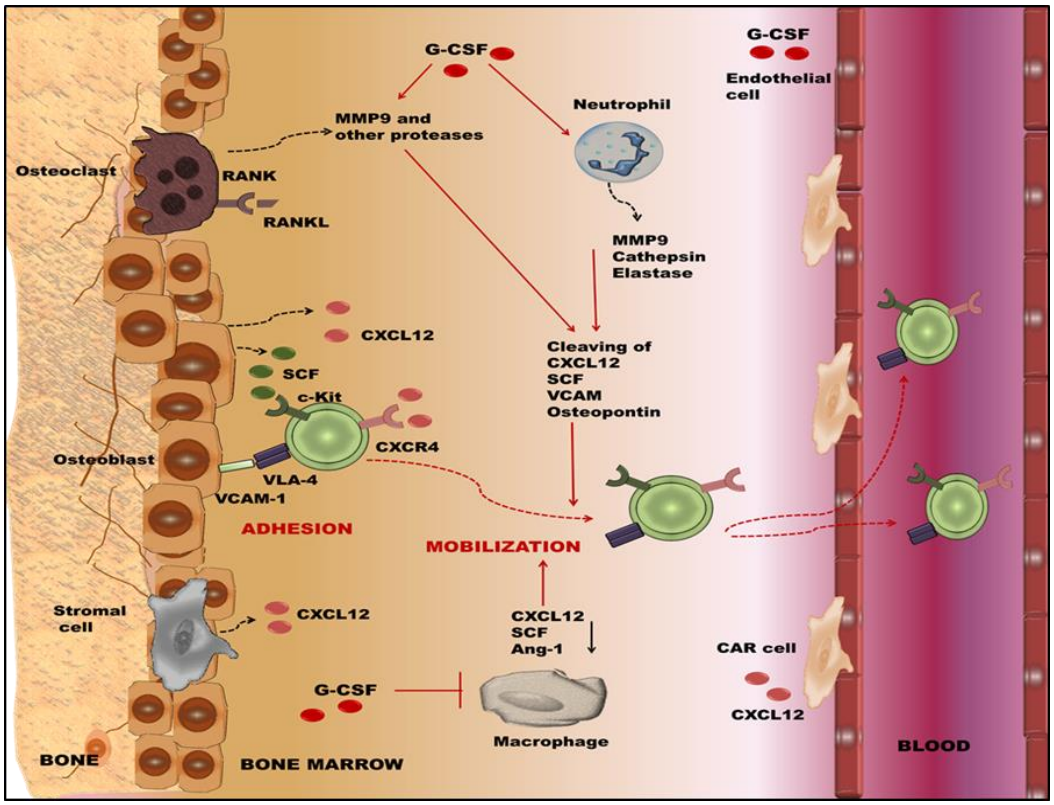

Figure 13. G-CSF-mediated mobilization of hematopoietic stem/progenitor cells

During steady-state hematopoiesis, high levels of CXCL12 expressions from multiple niche components maintain HSCs in a quiescent state. Systemic infection increases G-CSF levels in the BM which in turn results in reduced CXCL12 expression from OBs, mesenchymal stromal cells and perivascular cells. This enables the detachment of stem cells from the OBic niche and facilitates their migration to the vascular niches for further differentiation and mobilization. Figure is adapted from ``Ehninger and Trumpp 2011; Tall, Yvan-Charvet et al. 2012; Hosoba and Waller 2014; Boulais and Frenette 2015``.

Besides G-CSF and M-CSF, there are also reports that have intended to delineate the role of GM-CSF in infection-induced myelopoiesis. GM-CSF is commonly used in clinics to promote the mobilization of neutrophils and their progenitors after completion of chemotherapy or radiation (Iii *et al*., 1988). The neutralization of GM-CSF markedly decreases the accumulation of GMPs in inflammation sites as shown in experimental colitis (Sainathan *et al*., 2008). Increased GM-CSF levels in response to infection not only lead to an increase in myeloid cell production but also prime myeloid cells for efficient microbial killing ability. *In vitro* culture of macrophages with GM-CSF

promotes the production of the pro-inflammatory cytokines TNF, IL-6, IL-12p70 and IL-23 following LPS stimulation (Fleetwood *et al*., 2007). Likewise, increased production of G-CSF in the BM and spleen of infected mice has been reported in a mouse of visceral *Leishmania*sis (Cotterell *et al*., 2000a). Visceral *Leishmania*sis is caused by *Leishmania donovani* parasites which primarily infect stromal macrophages. It is proposed that infection of macrophages by these parasites increases the capacity of macrophages to further support emergency myelopoiesis via the selective induction of GM-CSF as well as TNF-α in the BM (Cotterell *et al*., 2000b). The increased levels of GM-CSF was accompanied by increased hematopoietic activity, which was shown by increased *in vitro* myeloid colony-forming ability of HSPCs obtained from infected mice (Cotterell *et al.*, 2000a). In agreement with these observations, the mice lacking GM-CSF show increased susceptibility to infections with pathogenic organisms including Listeria monocytogenes (Zhan *et al*., 1998), pulmonary streptococcus (LeVine *et al*., 1999) and blood-stage malaria (Riopel *et al*., 2001). These data indicates that hematopoietic colony-stimulating factors not only support steady-state myelopoiesis but also exert crucial function in immune response by steering HSPCs towards granulocyte and monocyte differentiation and priming myeloid cells for efficient pathogen clearance.

IL family cytokines: It has been well documented that increased levels of interleukins play a key role in inflammatory response in infectious conditions and inflammatory diseases, and is associated with increased myeloid cell production (Beuscher *et al.*, 1992, Couper *et al.*, 2008, Gee *et al.*, 2009, Van der Poll *et al.*, 1999). Recently, Pietras *et al*. reported the role of IL-1 pro-inflammatory signaling on HSC fate in a study using single-cell tracking technology (Pietras et al., 2016). This study showed that the chronic exposure to IL-1 accelerate cell division kinetics of HSCs priming them towards a myeloid differentiation via activation of PU-1 myeloid program. Like IL-1, IL-27 has been shown to directly regulate expansion, mobilization and myeloid differentiation fate of HSCs in mouse model of malaria (Furusawa *et al.*). During infection, IL production can also be induced by other pro-inflammatory cytokines. For instance, IFN-γ secretion during acute the lymphocytic choriomeningitis virus (LCMV) infection enhances IL-6 production of MSCs, thus increasing the numbers of MPPs and myeloid precursors in the BM through transient down-regulation of Runx-1 and Cebpα in HSPCs (Schürch *et al.*, 2014).

IFN family cytokines: IFNs are a group of cytokines produced by immune cells in response to viruses, bacteria, and parasites and tumor cells. IFN signaling plays a key role in macrophage activation, immunoglobulin switch in B cells, altered T helper response, inhibition of cell growth and induction of apoptosis. Type I IFNs (IFN-α, IFN-β) are secreted by hematopoietic (lymphocytes, dendritic cells, macrophages) and non-hematopoietic (fibroblasts, endothelial cells, and OBs) cells and bind to the IFN α/β receptor (IFNAR) on target cells. Type II IFN (IFN-γ) is produced by T-cells and NK-cells and uses the IFN-γ receptor (IFNγR) for target cell recognition (Le Page *et al.*, 1999). Despite the wide clinical use of recombinant IFNs in the treatment of viral infections and autoimmune diseases (Meyer, 2009), cellular targets of these cytokines are still largely unknown. Recent studies demonstrated that IFNs could affect HSPC compartment in infectious settings. Essers *et al*. have shown that *in vivo* stimulation of mice with IFN-α induce expansion of LSK population (Duchosal *et al.*, 2009). These results were further confirmed in a study by Sato *et al*. who showed that the lack of IRF2, a transcriptional repressor of IFN signaling leads to enhanced HSC cycling but a functional decline in repopulating ability of HSCs. Furthermore, they demonstrated that HSC function was partially restored when type I IFN signaling was disabled in this cell population (Sato *et al.*, 2009). Although short-time (three doses) exposure does not appear to affect repopulating ability of HSCs, chronic exposure IFN-α stimulation (eight doses over 2 weeks) markedly reduce HSC function in competitive transplantation assays (Duchosal *et al.*, 2009). Paradoxically, IFNs does not seem to stimulate proliferation of HSCs *in vitro* (de Bruin *et al*., 2013). However, acute exposure to type-I IFNs alters the distribution of HSCs in the BM niches (Kunisaki *et al.*, 2013), facilitating HSC activation. In contrast, chronic *in vivo* exposure to IFN-I decreases the expression of Foxo3a, p53, p27 and p57 and downregulates Notch and TGF pathways which in turn enforcing acutely proliferating HSCs to a quiescent state. However, enforcement of IFN-I exposed HSCs to enter cell cycle (i.e., by transplantation) trigger direct cell death in proliferating HSCs via activation of p53-dependent pro-apoptotic gene program (Pietras et al., 2014). Furthermore, recent work has shown that proliferating IFN-I-exposed HSCs move away from quiescence-associated periarteriolar niches (Kunisaki *et al*, 2013), suggesting a regulatory link between the BM niche and HSCs during IFN-I-induced proliferation.

Increased proliferation and impaired long-term repopulating capacity of HSC have also been reported in response to IFN-γ in mice infected with Ehrlichia muris

(MacNamara *et al.*, 2011a) and Mycobaterium avium (Baldridge *et al.*, 2010a). IFN- γ signaling appears to enhance HSPC expansion at least partly via STAT1 activation (Zhao *et al.*, 2010). Others showed that IFN-γ reduces TPO-mediated phosphorylation of signal transducer and activator of transcription STAT-5, via SOCS-1 which in turn impairs maintenance and self-renewal of HSPCs (de Bruin et al., 2013). Collectively, these studies suggest that both type I and II IFNs directly stimulate HSC response and function upon infection. While short-term exposure induce cell-cycle entry in HSCs, prolonged stimulation results in the exhaustion of the stem cell pool.

TNF : TNF family of pro-inflammatory cytokines were originally identified as necrotic factors which stimulates apoptosis in tumor cells. Today, it is now established that TNF-α which is mainly produced by activated monocyte/macrophages, lymphocytes and endothelial cells, is involved in host defense and protective immune response when the immune system is exposed to infectious pathogens (Pfeffer, 2003). Two distinct TNF receptors have been identified: TNFR1, which induce apoptosis via its intracellular death domain and TNFR2, which is shown to promote cellular proliferation (Aggarwal, 2003). Although several *in vitro* studies have shown that TNF-α supresses HSPCs in culture (Broxmeyer *et al.*, 1986, Bryder *et al.*, 2001, Dybedal *et al.*, 2001), *in vivo* functional relevance of these reports is more controversial. For instance, it was shown that TNF-α produced by $CD8^+$ cells enhance *in vitro* myeloid colony-forming ability and *in vivo* engraftment capacity of HSPCs by supressing apoptosis (Rezzoug *et al.*, 2008). Likewise, a following study reported that the absence of TNF receptors impair HSC function at steady-state and under transplantation stress (Rebel *et al.*, 1999), thus implicating a stimulatory role for TNF in HSC maintenance and stress response. However, observations coming from a recent study were in apparent discrepancy with previous studies. Although steady-state hematopoiesis was not altered in the absence of TNFR1 or TNFR2, serial transplantation experiments revealed enhanced the long-term repopulating capability of $TNFR1^{-/-}$ or $TNFR2^{-/-}$ HSCs, thereby suggesting a suppressive role for TNF in *in vivo* HSC activity (Pronk *et al.*, 2011). The different results coming from these studies could be explained by the dose, age-dependent differences and exposure time of TNF signaling as well as the environmental factors that could differentially affect HSC response.

6. Regulation of hematopoiesis by Wnt/Frizzled signaling

As stressed in previous sections, several recent studies have highlighted the importance of the balanced self-renewal cell divisions and quiescence of stem cells in normal and demand-adapted hematopoiesis. However, the precise mechanisms that regulate HSC maintenance and regeneration remain incompletely understood. The best candidate factors are the ones that modulate stem cell fate by acting in an autocrine or paracrine fashion. Those include members of the BMP, Hedgehog, Notch and Wnt signaling pathways (Kim *et al.*, 2014). Among them, the components of the Wnt pathway stand out because of their critical roles not only in embryonic development, but also governing the behavior of many different stem cells in tissue homeostasis (Nusse *et al.*, 2008). As a result, deregulation of Wnt signaling pathways play a causal role in the promotion of malignant transformation and the pathogenesis of many autoimmune diseases (Johnson *et al.*, 2006).

Wnt signaling pathway is evolutionary conserved from nematodes to human. Wnt signals are transmitted by binding of Wnt proteins to specific cell surface receptors, which trigger intracellular signalling cascades (Komiya *et al.*, 2008, Schubert *et al.*, 2013). Members of the Frizzled (*Fzd*) family serve as receptors to transduce signals from the Wnt proteins that can function alone or with co-receptors such as the low-density lipoprotein-related receptor (LRP) 5 and 6 (Malhotra *et al.*, 2009).

6.1. Wnt proteins acts as morphogens

The members of Wnt gene family are identified based on the full-length sequences that show identity to their original members Wnt-1 (initially named Int-1) in the mouse and wingless in Drosophila melanogaster (Cadigan *et al.*, 1997). There are five Wnt genes in Caenorhabditis elegans (Eisenmann, 2005), eight in Drosophila (Graba *et al.*, 2013), and nineteen in the human and murine genome (Schubert *et al.*, 2013). The current list of known vertebrate Wnt genes can be found on the Wnt gene homepage (Nusse, 2013). The degree of sequence identity in Wnt genes ranges from 20% to 85% across species (Nusse, 2013).

The Drosophila Wnt gene, wg induces the correct differentiation and distribution of epithelial cells which generate the wing structures of the flies (Swarup *et al.*, 2012). As

a result, mutant larvae develop into a wingless phenotype (Sharma *et al.*, 1976). In mammals, the directional information provided by Wnt signaling gradient play an important role in cell fate determination and the specification of cell identity along the anterior-posterior axis during the development of specific body parts and organs (Yamaguchi, 2001).

Wnt genes encode secreted glycoproteins that range from 350 to 400 amino acids in length (Nusse, 2013). Wnt proteins are secreted by a diversity of cells subsets and act as short-range signaling molecules and long-range morphogens in a graded fashion throughout tissues. In contrast to freely diffusible morphogens, Wnts are highly hydrophobic and mostly attached to cell-membranes and ECM, therefore their secretion and movement in extracellular space is tightly controlled (Mikels *et al.*, 2006). Wnt proteins have at least one N-linked glycosylation site for their targeting to the secretory pathway and 22 to 24 conserved cysteine residues, which are involved in Wnt folding and multimerization (Takada *et al.*, 2006). One of the common features of Wnt proteins is the palmitate modification following by their synthesis that facilitates the movement to target particular domains of the membrane and mediates intracellular protein trafficking in Wnt responding cells (Mikels *et al*., 2006). Palmitate modification of Wnt proteins not only influence membrane localization of Wnts but also required for the recognition by the extracellular domain receptors (Cong *et al.*, 2004).

6.2. Wnt recognition by Frizzled receptors

The *Fzd* proteins are a family of 10 seven-pass transmembrane domain-spanning cell surface receptors which play a key role in the initiation of nearly all Wnt pathways (Dijksterhuis *et al.*, 2014). Historically, the frizzled (*fz*) gene was first identified in Drosophila in which fz is cell autonomously required for the correct coordination of epidermal cells during wing development. In normal wing, the orientation of hair cells points distally, while in fz mutants, the hair cells points in random directions and form swirling patterns (Gubb *et al.*, 1982, Vinson *et al.*, 1987). Today the members of *Fzd* receptor family are defined by homology to the encoded protein of the original member Fz locus in Drosophila (Vinson *et al.*, 1989). In Drosophila, there are five *Fzd* receptors including the most studied members *Fz*, and *Fz* homologs *Dfz*2, and *Dfz*3 (Wu *et al.*, 2002). In mammals, ten *Fzd* genes have been identified that are grouped into four

clusters: *Fzd*1, *Fzd*2, and *Fzd*7 that have approximately 75% amino acid identity; *Fzd*5 and *Fzd*8 that share 70% identity to each other; *Fzd*4, *Fzd*9 and *Fzd*10 that have 65% identity; *Fzd*3 and *Fzd6* that have 50% identity in amino acid sequence (Huang *et al.*, 2004). *Fzd* receptors contain an extracellular N-terminus and an intracellular C-terminus (Figure 14).

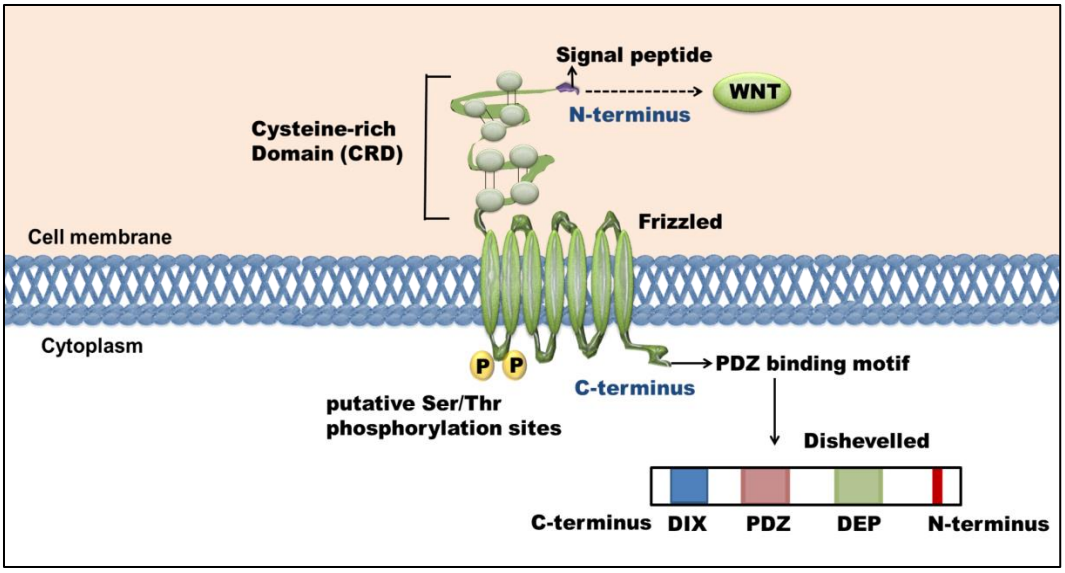

Figure 14.The structure of Frizzled receptors

Adapted from "Lee et al., 2015 and Schulte, 2010".

The extracellular N terminus contains a cysteine-rich domain (CRD) that consists of 120-125 residues with ten conserved cysteines, each forming disulphide bonds. There is also a hydrophilic linker region of 40-100 amino-acids in the N-terminus (Huang *et al*., 2004). Due to the presence of hydrophobic domains, *Fzd*s are classified as unconventional G-protein-coupled receptor (GPCR) (Huang *et al.*, 2004). Indeed, *Fzd* receptors are able to signal through heterotrimeric G proteins (Chen *et al*., 2008). The CRD domain is required for binding the palmitate group of Wnt ligands (Huang *et al*., 2004). The internal motif of *Fzd* does not contain a free C-terminus and bind to the PDZ domain of adapter protein Dishevelled (Dsh in Drosophila; Dvl in vertebrates) which later interacts with the conserved KTxxxW motif in the cytoplasmic tail to initiate intracellular Wnt signaling downstream of *Fzd*s (Chen *et al*., 2008). Notably, C-terminal sequences are not well conserved in *Fzd* receptors that affect their binding affinity to Dvl proteins (Umbhauer *et al.*, 2000). Notably, eight of ten *Fzd*s differ in the length and similarity in the carboxy-terminus sequence while *Fzd*3 and *Fzd6* lack a carboxy-terminal PDZ

domain-binding motif (Romero *et al.*, 2011). These differences suggest that *Fzd* receptors might also differ in their activation and pattern of triggered downstream signalling.

6.3. Wnt signaling pathways

The interaction of Wnts with their corresponding *Fzd* receptors triggers at least three different signal transduction cascades; (i) canonical Wnt/β-catenin signaling, non-canonical (ii) planar cell polarity (PCP) pathway and (iii) Wnt/calcium [Ca2+] (Malhotra *et al*., 2009, Schubert *et al*., 2013). The transcription factor β-catenin plays a central role in the canonical Wnt signaling (Figure 15). β-catenin is involved in the cadherin-catenin complex. In this inactive form, the stabilization and cytosolic pool of β-catenin is regulated by a destruction complex which is composed of casein kinase 1α (CK1α), glycogen synthase kinase-3β (GSK-3β), the axis inhibition protein 1 (AXIN1) and adenomatous polyposis coli (APC). When included in the destruction complex, β-catenin is constitutively phosphorylated by CK1α and GSK-3β at several residues in the N-terminus. Phosphorylated β-catenin is then ubiquitinated and subsequently degraded by the proteosome. The interaction of Wnts with *Fzd* receptors in association with LR5/6 induces phosphorylation of Dvl that leads the inhibition of the destruction complex, promoting accumulation β-catenin in the cytosol and its translocation to the nucleus. In the nucleus, β-catenin binds to lymphoid enhancer binding factor (LEF)/ T-cell factor (TCF) family to induce transcription of Wnt target genes including cyclin D1, c-myc, COX-2 and inducible NOS (Dijksterhuis *et al*., 2014, Kikuchi *et al*., 2007). Wnt-1, Wnt-2b, Wnt-3a, Wnt-8a, and Wnt-8b appear to activate canonical signaling pathway in several cell types (Schubert *et al*., 2013).

The canonical Wnt signaling cascade facilitates proliferation and fate specification of stem and progenitor cells. Aberrant activation in canonical Wnt activity is frequently reported as an autocrine feedback in leukemia as well as other cancers (Staal *et al.*, 2016a). Hence, the fine-tuning of this pathway is required for stem cell response. Numerous extracellular modulator molecules can bind either Wnt ligand or *Fzd* receptors to inhibit Wnt/β catenin signaling. Among those, The Dickkopf protein 1 (Dkk1) inhibit Wnt response by competitively binding to the Wnt co-receptors LRP5/6. The other

antagonists, secreted Frizzled-related proteins (SFRPs) and Wnt Inhibitory Factors (WIFs) directly bind Wnt proteins and inhibit Wnt/*Fzd* coupling.

In contrast to canonical pathway, Wnt/$Ca2^{+}$ and Wnt/PCP pathways require *Fzd* and the intracellular transduction component Dvl, but not LRP or β-catenin Figure 16. In the Wnt/Ca2+ pathway, activation of G-proteins, phospholipase C (PLC) and phosphodiesterase (PDE) results in increased levels of intracellular $Ca2^{+}$ levels which in turn activates enzymes such as CaMKII and protein kinase C (PKC) (Kühl *et al*., 2000).

The Wnt/PCP signaling forms the most extensively studied Wnt signaling pathway among others. The term “planar cell polarity” refers to the asymmetrical distribution of cells within a tissue plane that is observed in a range variety of tissues (Huang *et al.*, 2004). PCP signaling components are highly conserved from Drosophila to vertebrates. In PCP signaling, upstream PCP components, Four-jointed, Dachsous, Fat and Atrophin generate signals for the global direction of polarity across the tissue. These directional signals are interpreted by the core PCP components, *Fzd*, Dvl, Celsr, Vangl, and Prickle, which establish planar polarity within individual cells along the axis. The downstream PCP signaling is mediated by Daam1, Rho, Rac, Rho kinase, JNK, and Profilin, which are involved in rearrangement of cytoskeletal elements, oriented cell division, differential adhesion across cells, regulation of cell extensions and cell movement. Wnt/PCP signaling not only generates the distinct planar polarity phenotypes but also required for proper cell/tissue function (Wang, 2009, Yang *et al*., 2015).

Non-canonical Wnt pathways are implicated in tissue morphogenesis due to their regulatory role in cell migration and polarity (Dijksterhuis *et al*., 2014). Wnt4, Wnt5a, Wnt-11 and Wnt16 have shown to signal through the non-canonical pathways in different cell types (Clements *et al*., 2010, Geetha-Loganathan *et al*., 2014, Heinonen *et al*., 2011, Qian *et al.,* 2007, Wu *et al*., 2013). The non-canonical Wnt proteins are also able to bind a number of unconventional Wnt receptors including Ror, Ryk and Ptk7 (Berger *et al.*, 2017, Hendrickx *et al.*, 2008) as depicted in Figure 16.

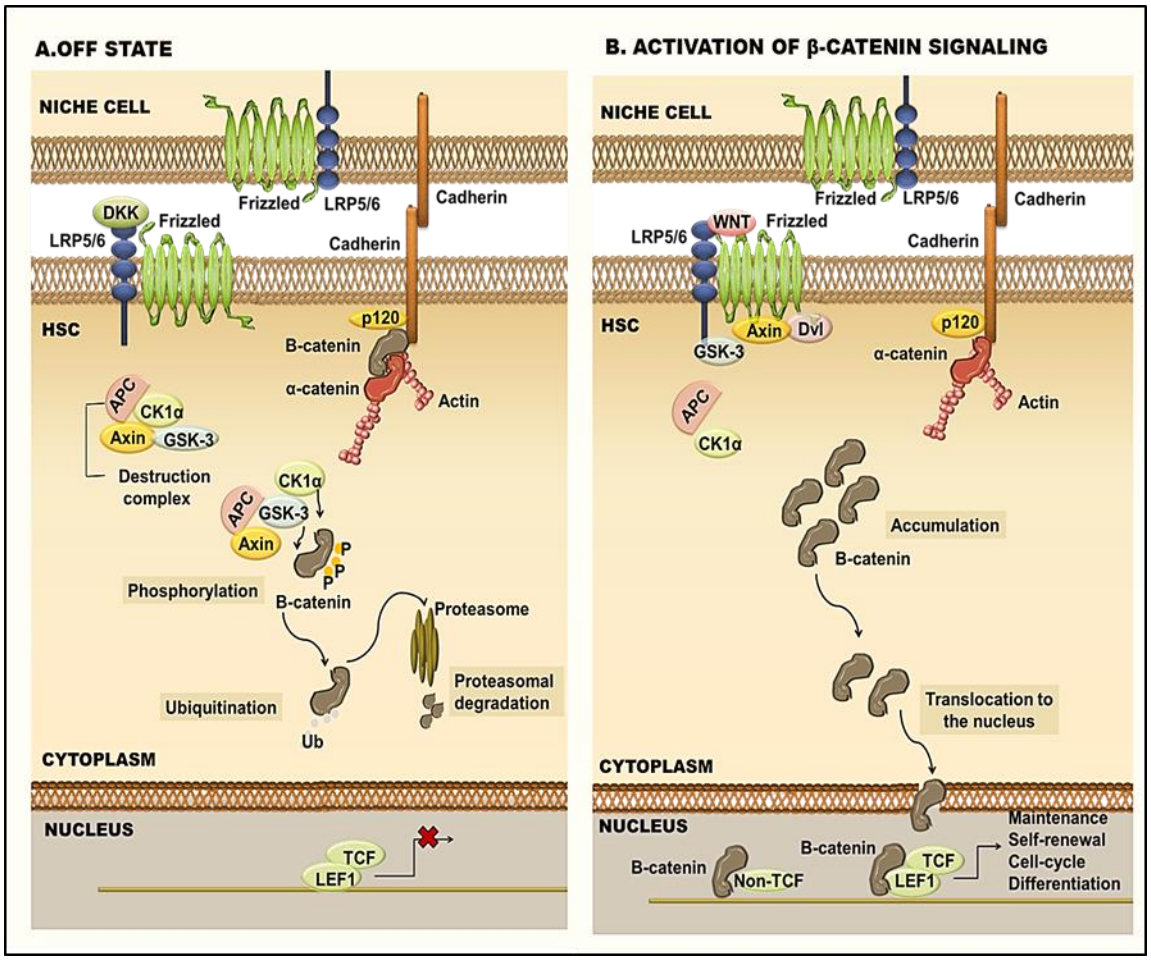

Figure 15. Canonical Wnt/β catenin signaling pathway

(A) In the inactivated state, β-catenin is associated with the cell membrane where it interacts with cadherin molecules (E- and N cadherin) and α-catenin to form the cytoskeleton and mediate cell-cell adhesion via homophilic interactions. β-catenin is constitutively phosphorylated by CK1α and GSK-3β. Phosphorylated β-catenin in the destruction complex (CKIα, GSK3β, APC, Axin) is subsequently ubiquitinated and degraded by the proteasome. (B) Binding of Wnt ligand to the Frizzled/LRP-5/6 receptor complex enables β-catenin to escape from the degradation and results in its accumulation in cytoplasm. Hypophosphorylated β-catenin is further translocated in the nucleus, where it binds TCF/LEF proteins to activate transcription of Wnt target genes. *Dsh: Dishevelled, GSK-3β: glycogen synthase kinase-3β, CK1α: casein kinase 1α, Axin: the axis inhibition protein 1, APC: adenomatous polyposis coli, LEF: lymphoid enhancer binding factor, TCF: T-cell factor. Adapted from "Li et al., 2012; Piersma et al., 2015;Vinyoles et al., 2014".*

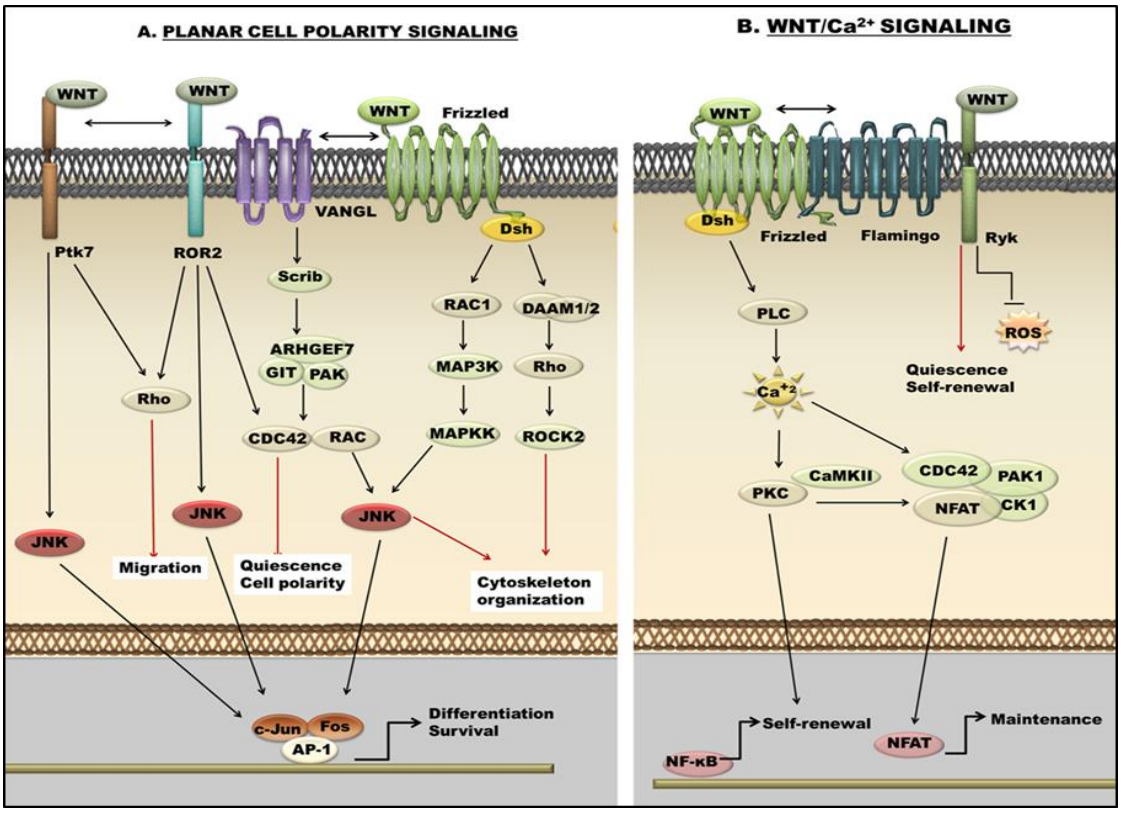

Figure 16. Non-canonical Wnt signaling pathways

(A) The planar cell polarity (PCP) components are asymmetrically distributed in the cells and directly or indirectly interact with Wnt ligands. Localization of Dsh in these interactions is a key determinant in activation or inhibition of this pathway. Upon *Fzd* activation, Dsh forms a complex with Daam, which activates Rho and ROCK that are involved in cytoskeleton organisation. Dsh can also directly activate Rac1 which regulates JNK. Ror2 and Ptk7 can interact to initiate PCP signaling via JNK. In another pathway, Fz and Vang can physically interact to initiate signaling through Scrib which regulates CDC42 and JNK. (B) In the Wnt/$Ca2^{+}$ pathway, activation of *Fzd* receptors recruits heteromeric G-proteins that activate phospholipase C (PLC) which results free intracellular calcium and activation of CAMKII. In a second pathway, physical interaction between *Fzd*, Fmi and Ryk results in inhibition of intracellular ROS levels. *Dsh: Dishevelled, Daam-1: Dishevelled associated activator of morphogenesis-1, JNK: c-Jun terminal kinase, ROCK: Rho-associated kinase, PLC: phospholipase C, PKC: protein kinase C, CaMKII: Ca2+/calmodulin-dependent protein kinases II. Adapted from "Fedon et al., 2012; Grishina, 2014; Katoh et al., 2007;Pataki et al., 2015".*

6.4. Multi-faced role of Wnt ligands in hematopoiesis

The expression of Wnt genes in hematopoietic system has been studied both in mice and human. Accordingly, Wnt5a and Wnt10b were initially detected in mouse FL progenitor cells (Austin *et al*., 1997) while Wnt5a, 10b and 2b were shown to be expressed in primitive human HSCs (Van Den Berg *et al*., 1998). During early hematopoiesis, a biphasic expression pattern of Wnt ligands is observed in the hematopoietic tissue. While some of the Wnt proteins (Wnt 3, 5a and 8a) are highly expressed in primitive hematopoietic sites, the others (Wnt3a, 6, 7b, 10b, and 16) are upregulated in definitive hematopoietic niches at later stages (Corrigan *et al.*, 2009). This data suggests that Wnt ligands differentially regulate hematopoiesis in a stage-specific fashion. In the adult BM, Wnt ligands are extensively produced by stromal cells surrounding HSCs (Table 5).

Table 5. Expression of Wnt ligands and Frizzled receptors in the bone marrow

Cell type	Wnt ligands	Frizzled receptors	Reference
Osteoblasts	Wnt1, Wnt2, Wnt2b, Wnt3a, Wnt4, Wnt5a, Wnt5b, Wnt6, Wnt10a, Wnt11, Wnt16	*Fzd*3, *Fzd*4, *Fzd*5, *Fzd6*	(Spencer *et al.*, 2006) (Sugimura et al., 2012)
Osteoclast precursors	Wnt2b, Wnt4, Wnt6, Wnt7b	*Fzd*1, *Fzd*4, *Fzd*5, *Fzd6*, *Fzd*7, *Fzd*8, *Fzd*9,	(Qiang *et al.*, 2010)
Mesenchymal stem cells	Wnt2, Wnt4, Wnt5a, Wnt11, Wnt16	*Fzd*2, *Fzd*3, Fdz4, *Fzd*5, Fdz6	(Etheridge *et al.*, 2004)
Endothelial cells	Wnt2, Wnt2b, Wnt3, Wnt5A, Wnt7A, Wnt 11	*Fzd*3, *Fzd*4, *Fzd6*	(Goodwin *et al.*, 2006) (Planutiene *et al.*, 2011)

Wnt production by mononuclear hematopoietic cells has also been reported. For instance, mature B cells produce Wnt-1 (Laine *et al*., 2013) and Wnt5a (Liang *et al*., 2003), whereas Wnt10b is secreted by immature B cells and erythroid cells (Congdon *et*

al., 2008). The expression analysis of Wnt genes indicates a limited and reduced expression in HSCs while increasing and continuous expression is observed in more differentiated progeny (Hardiman et al., 1996). There is also evidence suggesting the involvement of Wnt ligands in the crosstalk between mature blood cells and bone marrow niche components including osteoblasts. PTH, which is known as a canonical Wnt agonist has been shown to increase β catenin levels in osteoblastic cells by modulating the protein kinase A and protein kinase C pathways (Kulkarni et al., 2005, Tobimatsu et al., 2006) .PTH does not only stimulate bone formation (Kroll, 2000), but also regulates HSC numbers by modulating the niche size (Calvi et al., 2003). In addition to osteoblastic lineage, T lymphocytes also respond to PTH, sensitize the stromal niche elements of BM to PTH (Gao et al., 2008) and stimulate osteoblastic differentiation (Pacifici, 2013). Importantly, PTH stimulation of $CD8^+$ T cells induces *in vitro* Wnt10b production (Terauchi *et al*., 2009), which has been shown to exert a coordinated control over inhibition of adipogenesis and stimulation of osteoblastogenesis (Bennett et al., 2005).

The first evidence for the regulatory role of Wnt proteins in hematopoiesis was reported by *in vitro* culture studies. Stromal cells transduced with Wnt ligands Wnt1, Wnt5a, or Wnt10b stimulated a seven to eleven fold increase in the expansion of FL cells (Austin *et al.*, 1997). Intriguingly, *in vitro* expansion rate of human progenitors in response to Wnt1, Wnt5a, or Wnt10b were found to be similar to those observed with recombinant human SCF (Van Den Berg *et al.*, 1998). Subsequent genetic studies demonstrated the autocrine and paracrine mode action of Wnt signaling in HSCs using gain- and loss-of-function approaches.

Canonical Wnt/β signaling is the most studied pathway in hematopoiesis. Deficiency of Wnt3a, a prototypical canonical Wnt ligand is embryonically lethal in mice. FL HSCs from these mice have irreversibly impaired self-renewal and differentiation capacity due to complete depletion of canonical Wnt signaling in the HSC compartment (Luis *et al.*, 2009). Importantly, Wnt3a deficiency does not influence the expression of the other Wnt genes (Luis *et al.*, 2010). When Wnt3a activity is prevented by ectopic expression of a Fz ligand-binding domain or Axin in adult HSCs, a severe decline in their expansion and self-renewal capacity is observed (Reya *et al.*, 2003). In contrast, Wnt3a treatment prior to BM transplantation enables the maintenance of an immature phenotype in HSCs accompanied by increased expansion and self-renewal in recipient

mice (Willert *et al.*, 2003). Since Wnt3a is not secreted by HSPCs, these results suggest that Wnt3a acts in a paracrine fashion to regulate hematopoiesis. To confirm this hypothesis, Fleming and colleagues investigated the role of canonical Wnt signaling in the context of BM niche by using an osteoblast-specific promoter driving expression of Dkk-1(Fleming *et al.*, 2008). Dkk-1 is known to compete with ligands by binding the Wnt co-receptor LRP5/6 (Ahn *et al.*, 2011). In the study of Fleming, increased osteoblast-specific Dkk-1 expression resulted in markedly inhibited Wnt signaling in HSCs (Fleming *et al.*, 2008). Unexpectedly, decreased Wnt activity resulted in increased cell cycling and enhanced short-term repopulation activity as shown by transplantation assays. However, HSC function gradually declined reflecting a cell-autonomous defect in self-renewal and long-term function (Fleming *et al.*, 2008). These results indicate a requirement for the niche-dependent Wnt signaling in HSC maintenance and longevity.

Other studies focus on the β-catenin, the nuclear effector of canonical Wnt signaling. β-catenin deficiency is embryonic lethal due the lack of mesoderm organization. Because HSCs are believed to derive from the embryonic mesoderm, β-catenin has been a great candidate in HSC research (Staal *et al.*, 2005). As previously mentioned, β-catenin is involved in the cadherin-catenin complex. During cell-cell contact, the cadherin family of cell-cell adhesion proteins forms adhesive bonds at the site of contact which is mediated by the binding of cadherin extracellular regions of the opposing cells. Classical cadherins are known to be Ca^{2+}-dependent transmembrane proteins, thus, the rigidity of the extracellular region is maintained by binding of a network of Ca^{2+} ions to the linker region between adjacent extracellular regions. The formation of cell-cell adhesion by cadherins occurs via directly through adhesion rigidity and indirectly via modulating mechanical tension and transmitting signals to the actomyosin cytoskeleton of apposed cells (Maître et al., 2013). In the cadherin-catenin complex, β-catenin is associated with the cell membrane where it interacts with cadherin molecules (E- and N-cadherin). Binding of β-catenin by the cytoplasmic domain of these classical cadherins forms a complex with in turn binds α-catenin. The interaction of cadherins with catenins allows their physical connection to the cytoskeleton and mediate cell-cell adhesion via homophilic interactions (Alberts et al., 2002, Daugherty et al., 2007). The cleavage of cadherins by proteases leads to the release of β-catenin, which in turn results in the up-regulation of β-catenin signaling. Likewise, stabilization of cell-adhesion promotes the degradation of β-catenin and decreases β-catenin mediated

transcription (Brembeck *et al.*, 2006). LT-HSCs mainly adhere to the N cadherin^{+} osteoblasts. Asymmetric localization of two junction molecules, N-cadherin and β-catenin between stem cells and these osteoblasts shown to support HSC maintenance by controlling the niche size (Zhang *et al*., 2003). Constitutive expression of activated β-catenin has shown to induce expansion of HSCs in *in vitro* cultures (Baba *et al.*, 2006, Reya *et al.*, 2003). Vav-Cre-mediated conditional deletion of β-catenin in hematopoietic cells also leads to impaired proliferation in HSCs (Zhao *et al.*, 2007). Likewise, conditional expression of a stable form of β-catenin results in a multi-lineage differentiation block and impaired HSC maintenance, eventually exhaustion of the stem cell pool (Kirstetter et al., 2006). While these observations suggest a role for β-catenin in HSC regulation, other researchers did not observe any hematopoietic phenotype when they studied Cre-Lox-P mediated inactivation of β-catenin (Cobas *et al.*, 2004) or β- and γ-catenin in BM progenitor cells (Jeannet *et al.*, 2008, Koch *et al.*, 2008). It is possible that in these studies, Wnt signaling was not completely depleted (Jeannet *et al*., 2008). This notion was further supported by Luis *et al*., who studied a gradient of five different levels of *in vivo* canonical Wnt signaling activation using different Apc-mutant mouse strains (Luis et al., 2011). Their results indicate that while very low levels of canonical Wnt activity is sufficient to sustain adult hematopoiesis, intermediate and higher levels, however, lead to a downregulation in HSC self-renewal genes which can result in reduced repopulation capacity (Luis et al., 2011). Collectively, these results suggest a dose-dependent requirement for canonical Wnt/ β-catenin pathway in HSC function.

Unlike canonical Wnt signaling pathway, the role of non-canonical Wnt signaling is not well defined in hematopoiesis. Wnt5a is the best studied Wnt in this pathway. In addition to results obtained from *in vitro* studies, the expression on Wnt5a on primitive HSCs further support its role in hematopoiesis (Van Den Berg *et al.*, 1998). Murdoch and colleagues have shown that Wnt5a treatment of mice prior to transplantation, significantly increases the engraftment potential of human cord blood cells (Murdoch et al., 2003). Likewise, culture-treated LSKs with recombinant murine Wnt5a increased the repopulation ability of these cells in transplant settings. Notably, Wnt5a enhanced HSC function by maintaining a quiescent state (Nemeth et al., 2007). The effect of Wnt5a on HSC quiescence can be partly explained by its suppressive effect on ROS levels in HSC (Povinelli et al., 2014). Although Wnt5a does not induce β-catenin activation, it can downregulate Wnt3a-mediated gene expressions associated with HSC differentiation,

such myc (Nemeth et al., 2007). Thus, Wnt5a act as a canonical Wnt signaling antagonist in HSC maintenance and function. Indeed, the opposing impact of Wnt5a and Wnt3a is also observed in lineage differentiation of HSPCs. Wnt3a overexpression favors lymphopoiesis whereas Wnt5a increases myelopoiesis in the BM (Famili *et al.*, 2015). A recent study reported a role for Wnt5a in context HSC aging (Florian *et al.*, 2013a). HSCs from 24-month old mice show a dysfunctional expansion with impaired repopulation potential and augmented myeloid output over lymphopoiesis (Geiger *et al.*, 2013). In their study, Florian *et al*. observed a shift from canonical to non-canonical Wnt signaling as a result of elevated Wnt5a expression. Consistent with this finding, treatment of young mice with Wnt5a resulted in acquisition of an aged phenotype with diminished repopulation capacity and differentiation switch in favor of myeloid cells via activation of the small Rho GTPase Cdc42 (Florian *et al.*, 2013a). Cdc42 is known to act as a key regulator of cell polarity, modulating actin and tubulin orientation, cell-cell and cell-extracellular matrix adhesion during asymmetric cell division (Cau *et al.*, 2005). In young LT-HSC, Cdc42 and tubulin are asymmetrically distributed at the same location whereas Cdc42 and tubulin are randomly distributed throughout the body of aged LT-HSCs in an unpolarized fashion (Florian *et al.*, 2012a, Florian *et al.*, 2013a). These results suggest that stem-cell-intrinsic reduction of non-canonical signaling affects the mode of the cell division and fate, resulting in functional exhaustion.

Recently, our laboratory demonstrated that Wnt4 regulates HSC numbers in a non-canonical fashion (Heinonen et al., 2011b, Louis et al., 2008). In mice, Wnt4 is strongly expressed in thymus, FL and neonatal BM (Heinonen et al., 2011a, Heinonen et al., 2011b, Louis et al., 2008). Consequently, $Wnt4^{-/-}$ and $Wnt4^{+/-}$ neonates are present with a smaller thymic size and a reduction in the numbers of LSKs (Louis et al., 2008). In their study, they used a retroviral vector to enforce Wnt4 expression in FL cells prior to transplantation. Enforced expression of Wnt4a in FL cells sufficiently increased the thymic size and also led to a modest increase in BM LSKs in recipient mice. Moreover, similar to those seen in Wnt5a treatment, Wnt4 also increased the numbers of LSK cells in G0, suggesting an acquisition of an immature phenotype (Louis et al., 2008). Since deletion of β-catenin did not affect Wnt4-induced HSPC expansion, it is likely that Wnt4 signal through non-canonical pathway. Indeed, overexpression of Wnt4 increased levels of phosphorylated JNK which is involved in the downstream of non-canonical Wnt signaling. They also performed competitive transplant assays where $Jnk2^{-/-}$ and $Jnk2^{+/+}$

cells were transduced with a retroviral vector MSCV-Wnt4-IRES-GFP prior to transplantation. Jnk2$^{+/+}$ Wnt4 expressing myeloid cells were in a higher frequency than competing cells 12 weeks post-transplant, thus suggesting that the effect of Wnt4 was cell autonomous and dependent of Jnk2. It is possible that Wnt4 may act through a mechanism that resembles the PCP pathway. Indeed the expressions of seven polarity complex genes were upregulated in Wnt4$^{-/-}$ FL liver cells. They also showed that the PCP receptor, *Fzd6* is at least partly required for Wnt4-mediated expansion of the stem cell pool (Heinonen *et al.*, 2011b). Collectively these results suggest a link between *FZD6*, WNT4, JNK, and expression of PCP pathway target genes in the regulation of hematopoiesis.

Other studies indicate that non-canonical Wnt ligands may also play an important role in the hematopoietic development. (Vijayaragavan *et al.*, 2009) and niche modulation (Spencer *et al.*, 2006, Yavropoulou *et al.*, 2007). Wnt ligands and striking findings on their role in HSPC regulation are summarized in Table 6.

Table 6. Wnt ligands and their function in hematopoietic stem cell regulation

Ligand	Effect on hematopoietic stem cells	Reference
Wnt1	*In vivo* expansion of fetal liver HSPCs Ex vivo expansion of human HSPCs	(Austin *et al.*, 1997, Chotinantakul *et al.*, 2013, Laine *et al.*, 2013)
Wnt2	Hemangioblast differentiation from primitive mesoderm Increased erythroid colony formation of human HSPCs in culture	(Lento *et al.*, 2013, Van Den Berg *et al.*, 1998, Wang *et al.*, 2007)
Wnt3	*In vivo* and *in vitro* expansion Activates dormant HSCs Enhances self-renewal and differentiation of fetal and adult HSPCs	(Lento *et al.*, 2013, Luis *et al.*, 2010, Reya *et al.*, 2003, Reya *et al.*, 2000, Trowbridge *et al.*, 2010)
Wnt4	Required for *in vivo* T and B lymphopoiesis *In vitro* expansion of HSPCs -Enforced expression increases the repopulation ability of fetal liver HSC in transplant assays	(Chang et al., 2007, Heinonen et al., 2011b, Louis et al., 2008, Yu et al., 2014)
Wnt5a	Inhibits caonical Wnt signaling in HSPCs, regulates maintenance, self-renewal and repopulation capacity of HSPCs	(Nemeth et al., 2007, Pereira et al., 2008, Trowbridge et al., 2010)
Wnt10b	Enhance *in vivo* expansion of hematopoietic progenitor cells in response to GSF treatment	(Austin *et al.*, 1997, Cho *et al.*, 2014, Congdon *et al.*, 2008, Lento *et al.*, 2013)
Wnt11	Stimulates hemogenic and hematopoietic differentiation of human embryonic stem cells in culture	(Lento *et al.*, 2013, Vijayaragavan *et al.*, 2009)
Wnt16	Embryonic HSC specification in zebrafish	(Clements *et al.*, 2011)

6.5. Frizzled receptors in hematopoiesis

The *Fzd* family receptors are expressed on primitive and adult HSPCs both in human (Austin *et al.*, 1997, Van Den Berg *et al.*, 1998) and mice (Heinonen *et al.*, 2011b, Van Den Berg *et al.*, 1998, Yokota *et al.*, 2007). In the adult mouse, the expressions of *Fzd* receptors appear to decline during myeloid/lymphoid commitment with the exception of Fzd1, *Fzd*2 and *Fzd6*. Fzd2 expression is not detectable on HSPCs while *Fzd*1 is highly expressed on CMPs. *Fzd6* expression, however, can be detected in HSCs and downstream progenitors (Yokota et al., 2008). High expression levels of *Fzd* receptors on both HSCs (Figure 17), and non-hematopoietic niche components (Table 5), suggest that they can play an active role in HSCs regulation.

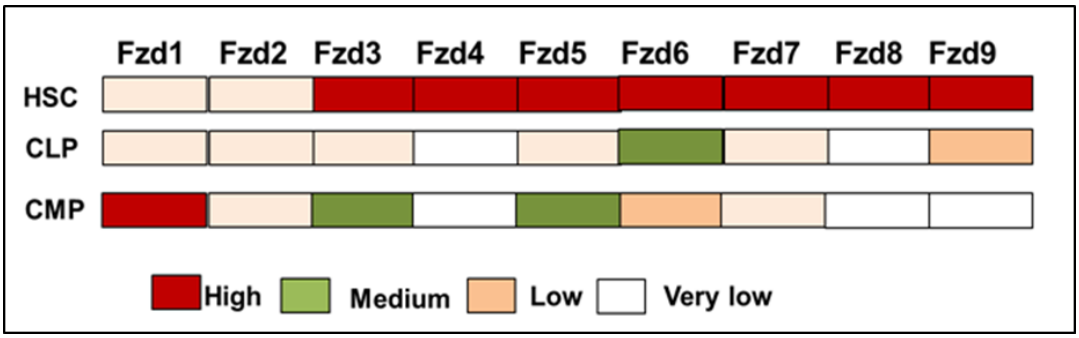

Figure 17. Frizzled receptor expression on hematopoietic/progenitor cells

HSCs express 7 out of 9 Frizzled receptors, whose expression decline as they give rise to committed myeloid and lymphoid progenitors. *Fzd*1 is not detectable on HSCs but highly expressed on CMPs. *Fzd6* remain detectable in all progenitors with relatively higher expression levels in HSCs. Figure adapted from the results obtained by (Yokota et al., 2008).

Despite the fact that *Fzd*s are predominantly expressed in HSCs, their role in hematopoiesis is understudied. From a pharmacological view, the ligand selectivity and signaling events which they trigger have not been fully understood in the other cell types (Dijksterhuis *et al.*, 2014), that also hold true for hematopoietic system. Because of the complexity of Wnt signaling, the relationship between ligands and receptors are hardly defined. First, there are ten *Fzd* receptors for nineteen Wnt ligands. Second, Wnt/*Fzd* coupling occurs as a context dependent manner. Best example of this notion can be found in Wnt5a-mediated non-canonical signaling. In a study, Wnt5a was shown to

regulate *in vitro* cytokine production of human myeloid cells via *Fzd*5 (Blumenthal *et al.*, 2006). Others showed that Wnt5a-mediated *in vitro* proliferation of endothelial cells can be blocked by *Fzd*4 extracellular domain (Masckauchán *et al.*, 2006). Recently, Sugimura and colleagues demonstrated that Wnt5a can exert its effect through *Fzd*8 to maintain a quiescent HSC pool in the niche (Sugimura et al., 2012). Following this study, other researchers have shown that the interaction between Wnt5a and an alternative Wnt receptor Ryk is required to induce HSC quiescence and enhance ST- and LT-hematopoietic repopulation (Povinelli et al., 2014), thus adding more complexity to the non-canonical Wnt signaling. Third, *Fzd* receptors can bind to more than one Wnt ligand to activate different signaling pathways. For instance, the binding of Wnt5a to *Fzd*2 activates the Wnt/Ca2+ pathway in human melanoma cells (Bazhin *et al*., 2010, Ma *et al*., 2007), while stimulation of Fzd2 with Wnt3a has shown to activate β-catenin-dependent Wnt signaling in different cell types (Verkaar *et al*., 2009). Finally, *Fzd* receptors are able to initiate intracellular Wnt signaling even in the absence of a CRD domain or Wnt ligand. In model organism Drosophila, *fz* is able to recruit *dsh* to the cell membrane and initiate PCP signaling independent of Wnt ligand (Adler, 2002). In this case, the direct protein-protein interactions between *Fzd* and *flamingo/vangl* complex on adjacent cells (Wu and Herman 2006; Wu and Mlodzik 2008) may account for the acquisition of polarity. The example of these types of interactions was shown between *Fmi/Celsr1* and *Fzd*8 in HSC regulation (Sugimura et al., 2012). *Fmi* is an atypical cadherin-type receptor which activates PCP signaling cascade and interferes with canonical Wnt signaling in other model systems (Morgan *et al.*, 2004). Both Fmi and *Fzd*8 predominantly expressed on primitive HSCs. Intriguingly, *Fzd*8 expression is relatively higher in quiescent HSCs than in active HSCs (Akashi *et al.*, 2003). It is well demonstrated that Fmi mediates a homophilic interaction between N-cadherin^{+} OBs and HSCs via regulating *Fzd*8 distribution at the interface of these cells to maintain HSCs in a quiescent state (Sugimura et al., 2012). This effect is partially through downregulation of IFN-γ expression, and partially via repression of canonical Wnt signaling (Sugimura et al., 2012). It is possible that Ryk might be also involved in the complex interaction of Fmi/*Fzd*8 in the BM (Bazhin *et al*., 2010, Ma *et al*., 2007). These results suggest that *Fzd* receptors are the determinant factors for niche-dependent Wnt ligands to transmit necessary signals in HSC regulation.

Besides *Fzd*8, there are only two *Fzd* receptors, *Fzd6* and *Fzd*9, have been studied in the context of HSPC regulation (Table 7). Ranheim *et al*. demonstrated that *Fzd*9 is cell-intrinsically required for lymphopoiesis, particularly for B cell development (Ranheim *et al.*, 2005). Consistent with high expression levels of *Fzd*9 on CLPs and ELPs (Yokota *et al*., 2007), this study indicated a role for this receptor in committed progenitors rather than HSCs.

Historically, *Fzd6* is known as a core PCP receptor which has redundant roles in polarity establishment and cell-orientation. Absence of *Fzd6* signaling results in genetically mosaic animals characterized with polarity phenotypes (Chang et al., 2016, Guo et al., 2004, Wang et al., 2006). *Fzd6* deficiency in mice is generally associated with abnormal macroscopic hair whorls. In mice, the proper orientation of hair follicles depends on the equal distribution of PCP components, *Fzd6* and *Vangl2* in daughter cells upon progenitor division. During division, mitotic internalization of *Fmi/Celsr1* enables global alignment of polarity by temporarily blocking PCP signals (Devenport et al., 2011). Hence, loss of *Fzd6* alters polarity establishment resulting in random orientation of hair follicles, suggesting a crucial role for *Fzd6* in asymmetric/symmetric cell fate. This hypothesis is further supported by elevated levels of *Fzd6* expression in human cancers (Cantilena *et al*., 2011, Haider *et al*., 2006, Wissmann *et al*., 2003). In the hematopoietic system, *Fzd6* is also predominantly expressed in fetal (Heinonen *et al*., 2011b) and adult (Yokota *et al*., 2007) murine HSCs. Importantly, *Fzd6* is the only *Fzd* receptor which is abundantly upregulated in all three subsets (slow-dividing, fast-dividing and committed) of primitive human HSCs (Wagner *et al*., 2004). Enriched *Fzd6* expression in the slow-dividing fraction suggests a role for this receptor in the regulation of quiescence and self-renewal of HSPCs. Remarkably, human *Fzd6*, is 89% identical to mouse *Fzd6*. In mouse, *Fzd6* deficiency markedly delays the development of chronic lymphocytic leukemia (Wu *et al*., 2009). In parallel with these results *Fzd6* deficient mice have a reduction in the percentage of BM LSKs, HSCs and MPPs (Heinonen *et al.*, 2011b). Although Wnt4 appear to transmit signal through *Fzd6* receptor, the phenotypic differences in $Wnt4^{-/-}$ and $Fzd6^{-/-}$ mice suggest that other compensatory interactions might involve in Wnt-mediated signaling in association with *Fzd6*.

Table 7. Known function of Frizzled receptors in hematopoiesis

Receptor	Phenotype	WNT ligand	Known function in hematopoiesis	Reference
***Fzd*1**	Viable	Wnt1 Wn2 Wnt3* Wnt7b	Not expressed on fetal adult HSC but highly expressed on CMPs Elevated expression levels observed in activated macrophages during infection	(Dijksterhuis *et al.*, 2014, Heinonen *et al.*, 2011b, Neumann *et al.*, 2010, Yokota *et al.*, 2007)
***Fzd*2**	Viable	Wnt5a*	Not detected on fetal and adult HSPC surface Postulated to stimulate chemokine production in human neutrophils via binding Wnt5a.	(Heinonen *et al.*, 2011b, Jung *et al.*, 2013, Yokota *et al.*, 2007)
***Fzd*4**	Cerebellar dysfunction	Wnt2 Wnt2b Wn7b	Upregulated during primitive hematopoiesis and highly expressed on adult HSCs. Its knockdown inhibit CML progenitor growth	(Agarwal *et al.*, 2017, Corrigan *et al.*, 2009, Dijksterhuis *et al.*, 2014, Wang *et al.*, 2001)
***Fzd*5**	Defective yolk sac and placental angiogenesis	Wnt5a*	Upregulation is observed in activated macrophages in response to LPS and M. tuberculosis	(Blumenthal *et al.*, 2006, Ishikawa *et al.*, 2001, Yokota *et al.*, 2007)
Fzd6	Defects in hair patterning	Wnt4*	Loss of *Fzd6* alters the size of HSPC pool and myeloid development in mice Its dysregulation is implicated in a mouse model of B-cell leukemia.	(Heinonen *et al.*, 2011b, Wu *et al.*, 2009a)
***Fzd*8**	**Viable**	Wnt5a	Regulates HSPC pool size, self-renewal and engraftment in association with Flamingo co-receptor	(Sugimura et al., 2012)
***Fzd*9**	Defects in pre-B-cell stage	Wnt2	Required for B-cell development from progenitors in a cell-autonomous fashion	(Dijksterhuis *et al.*, 2014, Ranheim *et al.*, 2005)

***Wnt/*Fzd* ligand interactions implicated in hematopoiesis.**

Collectively these studies demonstrated a crucial role of Wnt signaling in homeostatic and deregulated hematopoiesis. While there still is much to be clarified regarding the role of Wnt signaling in hematopoiesis, based on the presented results, one can speculate that non-canonical Wnt signaling components supresses' the canonical Wnt activity to maintain a quiescent stem cell pool in the BM microenvironment. Any disruption of this balanced network results in the loss of HSC function and subsequent promotion of hematologic malignancies.

6.6. The role of Wnt signaling in inflammation

In addition to their role in normal hematopoiesis, Wnt signaling pathways were recently shown to contribute the resolution of inflammation and restoration of tissue homeostasis. For instance, canonical Wnt/β-catenin signaling not only contributes the development and lineage maturation of T and B cells, but also regulates the activation of regulatory T cells(Reya *et al.*, 2000, Staal *et al.*, 2008a, Staal *et al.*, 2008b). The activation of canonical Wnt signaling in inflammation triggers immune response by supressing Treg cell function, thus aberrant activation of this pathway can lead to the development of autoimmune responses (van Loosdregt *et al.*, 2013). Moreover, β-catenin activation directly participates in the generation of tolerogenic dendritic cells and regulation of inflammatory response (Suryawanshi *et al.*, 2016). Besides β-catenin, canonical Wnt ligand, Wnt3a promotes anti-inflammatory functions (Neumann *et al.*, 2010) and enhanced bacterial killing (Chen *et al.*, 2016b) in murine macrophages. Elevated expression of Fzd1 is also reported on macrophages in response to microbial infections. Though, Fzd1 inhibit Wnt3a induced Wnt/β-catenin signaling in mycobacteria-infected murine macrophages (Neumann *et al.*, 2010). It is noteworthy, elements of Wnt/β-catenin signaling are often upregulated in many cancer types and autoimmune diseases (Zimmerman *et al.*, 2012), which suggest that aberrant activation of this pathway may involve in sustained tissue inflammation.

Non-canonical Wnt signaling, particularly Wnt5a signaling has been also linked to the pathogenesis of inflammatory disorders such as rheumatoid arthritis (Sen *et al.*, 2000), sepsis (Schulte *et al.*, 2015) and atherosclerosis (Bhatt *et al.*, 2014). Consistently, Wnt5a has been reported to be upregulated in macrophages stimulated with IFN- γ and LPS, which is accompanied by enhanced expression of pro-inflammatory cytokines IL-6, IL-1β, IL-8, and macrophage inflammatory protein-1β (MIP-1β) (Pereira *et al.*, 2008). In

addition, Wnt5a and its receptor Fzd5 have shown to regulate IL-12 response in mycobacteria-induced human macrophages and IFN- γ production of mycobacterial antigen-activated T cells (Pereira *et al.*, 2008). While the activation of Wnt5a-Fzd5 signaling upregulates pro-inflammatory cytokines and augments bacterial phagocytosis in macrophages, it does not appear to enhance bacterial killing (Maiti *et al.*, 2012). In contrast, enhanced Wnt5a signaling has shown to inhibit *L. donovani* parasite infection in macrophages via activation of Rac-1 signaling (Chakraborty *et al.*, 2017).

These studies clearly imply that Wnt signaling regulates the host response to pathogenic insults by modulating cytokine production and immune cell functions. To date, however, Wnt signaling components have not been directly implicated in the activation and myeloid output of HSCs in response to inflammation and infection. A deeper understanding of Wnt signaling in the regulation of the BM microenvironment and its crosstalk with HSPC during inflammatory conditions is clearly needed. The knowledge on the role of Wnt signaling cascade in inflammation and infection may provide therapeutic benefits in the treatment of autoimmune diseases, and infectious diseases.

7. OBJECTIVES AND GENERAL HYPOTHESIS

Despite their silent state, HSCs can exit quiescence and expand their numbers in order to replenish blood cells that have been lost during stress conditions such as irradiation, injury and infections. This is of particular interest to understand the molecular mechanisms, which regulate HSC activation and lineage specification upon stress. Non-canonical Wnt signaling components have emerged as important regulators during these processes. Our laboratory has previously demonstrated that non-canonical (beta-catenin independent) Wnt4 signaling increases HSPC expansion through a planar cell polarity (PCP)-like pathway. PCP is known for its critical role in symmetric/asymmetric cell divisions in epithelial organs. The wider physiological impact of PCP signaling on hematopoiesis is yet to be identified. Our general hypothesis is that non-canonical Fzd6 receptor regulates the activation and the maintenance of HSPC during homeostasis and under hematopoietic stress conditions. With this end, this thesis project examines the role of non-canonical PCP receptor, Frizzled-6 (*Fzd6*) in normal and emergency hematopoiesis.

The first aim of the current study was to answer the question:

- Does *Fzd6* play a role in the regulation of HSPC function?

To answer this question, we characterized the hematopoietic phenotype of *Fzd6*$^{-/-}$ mice during steady state adult hematopoiesis and examined *in vivo* repopulation and multi-lineage differentiation potential *Fzd6*$^{-/-}$ HSCs through a series of transplantation experiments.

Inflammation and infections promotes expansion and differentiation of HSPCs, altering stem cell homeostasis. Wnt signaling components have been implicated in the modulation of inflammatory responses. However, the role of Wnt signaling in inflammation- and infection-induced HSC response had not been investigated. Our hypothesis is that Fzd6 regulates not only inflammation/infection-induced HSC activation but also lineage differentiation skewing of HSPCs toward the myeloid lineage under inflammatory conditions. Therefore, we pursued our study to further investigate the importance of *Fzd6* signaling in emergency hematopoiesis in response to natural stress conditions using mouse models of endotoxin-induced inflammation and chronic visceral leishmaniasis.

The second part of this thesis focused on the questions:

- How does *Fzd6* deficient HSPCs respond to endotoxin-induced acute inflammation?
- What is the impact of chronic *Leishmania donovani* infection on the BM HSPC compartment? How does the BM microenvironment contribute to enhanced myeloid output during *L. donovani* infection?
- How are the lineage commitment decisions from HSPCs initiated and regulated in the absence of *Fzd6* in response to endotoxin-toxin induced acute inflammation and chronic *L. donovani* infection?

CHAPTER 2

PUBLICATION NO.1

FRIZZLED-6 REGULATES HEMATOPOIETIC STEM/PROGENITOR CELL SURVIVAL AND SELF-RENEWAL

Frizzled6 regulates hematopoietic stem/progenitor cell survival and self-renewal[1]

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Contribution of the student

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1. SUMMARY

Adult hematopoietic stem/progenitor cell (HSPC) numbers remain stable in the absence of external stressors. After bone marrow transplant, HSPCs need to expand substantially in order to repopulate the bone marrow and to replenish the peripheral blood cell pool. Here we show that a non-canonical Wnt receptor, Frizzled-6 (*Fzd6*), regulates HSPC expansion and survival in a hematopoietic cell-intrinsic manner. *Fzd6* deficiency increased the ratio of $Flt3^{hi}$ multipotent progenitors to $CD150^{+}$ stem cells in the mouse bone marrow, suggesting defective stem cell maintenance. Competitive transplantation experiments demonstrated that $Fzd6^{-/-}$ HSPCs were able to home to the bone marrow but were severely impaired in their capacity to reconstitute a lethally irradiated host. Lack of *Fzd6* resulted in a strong activation of caspase-3 and a gradual loss of donor HSPCs and peripheral blood granulocytes. *Fzd6* was also necessary for the efficient HSPC expansion during emergency hematopoiesis. Mechanistically, *Fzd6* is a negative regulator of Cdc42 clustering in polarized cells. Furthermore, β-catenin-dependent signaling may be disinhibited in $Fzd6^{-/-}$ HSPCs. Collectively, our data reveal that *Fzd6* has an essential role in HSPC maintenance and survival. Non-canonical Wnt-*Fzd6* signaling pathway could thus present an interesting target for promoting HSPC expansion and multilineage hematopoietic recovery after transplant.

Keywords: hematopoietic stem cells; bone marrow transplantation; wnt signal transduction

2. INTRODUCTION

Adult hematopoietic stem/progenitor cells (HSPCs) are maintained in a specialized niche in the bone marrow (BM). Multiple signals from the niche regulate HSPC numbers and their ability to self-renew as well as to differentiate into all blood cell types (Martinez-Agosto *et al.*, 2007, Scadden, 2014). Micro-environmental cues also influence the cell cycle status of HSPCs and modulate stem cell polarity. Polarity has been suggested to maintain the dynamic balance of HSPC pool by regulating the outcome (self-renewal versus differentiation) of HSPC divisions (Florian *et al.*, 2012b, Florian *et al.*, 2010, Schroeder, 2007b, Wu *et al.*, 2007). The molecular mechanisms coordinating the cross-talk between niche-associated signals and HSPC-intrinsic polarity determinants remain unclear.

Wnt signaling is necessary for adult HSPC self-renewal in the BM, but it needs to be tightly regulated (Lane et al., 2011, Luis et al., 2011, Schaniel et al., 2011): mild increases in β-catenin-dependent signaling enhance HSPC function, while stronger signals would favor myeloid expansion to the detriment of stem cell self-renewal. Although non-canonical, β-catenin-independent, Wnt signaling pathways are known to modulate cell polarity, cell motility and tissue patterning (Angers et al., 2009, Devenport et al., 2008, Devenport et al., 2011, Gillies et al., 2011, Gray et al., 2011, Guo et al., 2004, Lapebie et al., 2011, Mirkovic et al., 2011), their role in hematopoietic cells is much less well established. Exogenous Wnt5a has been shown to activate β-catenin-independent signaling in $Lin^{-}Sca1^{+}cKit^{hi}$ (LSK) hematopoietic progenitors and to improve HSPC maintenance and function by promoting their quiescence (Buckley et al., 2011, Murdoch et al., 2003, Nemeth et al., 2007, Povinelli et al., 2014). By contrast, hematopoietic Wnt5a expression is augmented during HSPC aging and decreasing Wnt5a levels in aged HSPCs resulted in the restoration of their polarity and self-renewal (Florian et al., 2013). Wnt4 enhances the expansion of fetal liver LSKs, most notably $Flt3^{hi}$ LSKs, and improves thymic recovery after irradiation and hematopoietic cell transplant through mechanisms that depend on Jnk2 but do not require β-catenin or its binding partner Tcf1 (Heinonen et al., 2011b, Louis et al., 2008). Lastly, inhibition of canonical Wnt signaling through the activation of Frizzled (*Fzd*)-8 and the atypical cadherin Fmi maintained quiescent long-term hematopoietic stem cells in the adult mouse BM (Sugimura et al., 2012). Most polarity genes are expressed in fetal HSPCs

(Heinonen *et al.*, 2011b); however, the direct role of most core non-canonical Wnt signaling effectors and planar cell polarity (PCP) has not been addressed in hematopoietic cells.

We recently reported that the receptor *Fzd6* was at least partially required for the Wnt4-mediated expansion of fetal liver HSPCs in culture (Heinonen *et al.*, 2011b). Furthermore, we observed alterations in the frequency of BM HSPCs in *Fzd6*$^{-/-}$ mice on a mixed C57BL/6x129sv background. *Fzd6* expression has been demonstrated in HSPCs and mature blood-forming cells in human and mouse (Heng *et al.*, 2008) with the strongest expression levels corresponding to more immature cell types (Wagner et al., 2004, Yokota et al., 2008). *Fzd6* is generally associated with PCP signaling in epithelial cells (Angers et al., 2009, Gray et al., 2011, Guo et al., 2004, Lapebie et al., 2011) and it has been previously proposed to act as a negative regulator of the β-catenin-dependent canonical Wnt pathway (Golan *et al.*, 2004, Mirkovic *et al.*, 2011). Very little is known about the functional role of *Fzd6* signaling in the hematopoietic lineage, except for its being involved in the initiation and progression of chronic lymphocytic leukemia in the Eμ-TCL1 mouse model (Wu *et al.*, 2009b).

Here we have examined the impact of *Fzd6* in the regulation of HSPC function using mice on C57BL/6 background. We report a cell-intrinsic requirement for *Fzd6* in competitive short- and long-term hematopoietic reconstitution after irradiation and transplant. We show that *Fzd6* deficiency impaired the expansion and survival of HSPCs, resulting in the activation of caspase-3 and inefficient HSPC engraftment in the first week after BM transplant. We further show that *Fzd6* is necessary for efficient emergency responses during systemic inflammation. Overall we demonstrate a crucial role for *Fzd6* in HSPC self-renewal and negative regulation of Cdc42/Jnk signaling in the BM and suggest that *Fzd6* could be an interesting target for stimulating HSPC expansion in culture or *in situ* post-transplant.

3. RESULTS

3.1. Fzd6 deficiency does not affect intracellular β-catenin levels in HSPCs

Fzd6 mRNA is abundantly expressed by fetal liver (Heinonen *et al.*, 2011b) as well as BM HSPCs (Yokota et al., 2008). However, given the different methods of detection and the variable markers used for cell identification, we wanted to validate these results and directly compare *Fzd6* expression levels in fetal liver and adult mouse BM $CD150^+$ $Lineage^-Sca\text{-}1^+c\text{-}Kit^+$ (LSK) cells, which are enriched in hematopoietic stem cells (HSC) (Kiel et al., 2005), as well as different HSPC sub-populations in the BM. *Fzd6* mRNA levels were comparable between adult and fetal liver $CD150^+$ HSPCs as detected by quantitative RT-PCR on sorted cells (Fig. 1A). To evaluate *Fzd6* expression in different BM HSPC sub-populations, we took advantage of the *LacZ* construct inserted into the *Fzd6* mutant allele (Guo et al., 2004). Using fluorescein di-V-galactoside as the substrate for β-galactosidase, only $CD150^+$ HSPCs displayed detectable levels of *Fzd6* promoter activity (Fig. 1B). These results correlate well with previously published data (Yokota et al., 2008). as well as data found in the Immunological Genome project database (Heng *et al.*, 2008) (http://www.immgen.org/) and support the hypothesis that *Fzd6* play a key role in the regulation of fetal and adult hematopoiesis.

Fzd6 has been associated with non-canonical Wnt signaling (Gray et al., 2011, Guo et al., 2004) with the potential to inhibit β-catenin-dependent canonical Wnt pathway in non-hematopoietic cells (Golan *et al.*, 2004, Mirkovic *et al.*, 2011). In contrast, *Fzd6* expression correlated with increased levels of intracellular β-catenin in a mouse model of chronic B lymphocytic leukemia although there was no evidence of a direct functional relationship (Wu *et al.*, 2009b). To determine whether lack of *Fzd6* modulated β-catenin in normal BM HSPCs, we quantified intracellular active β-catenin levels by flow-cytometry using an antibody specific for the stable, non-phosphorylated form of the protein. We observed no difference in β-catenin staining between $Fzd6^{+/+}$ and $Fzd6^{-/-}$ $CD150^+$ HSPCs (Fig. 1C), indicating that *Fzd6* was not involved in the maintenance of intracellular β-catenin levels in HSPCs at steady-state and thus appeared to signal through a non-canonical pathway.

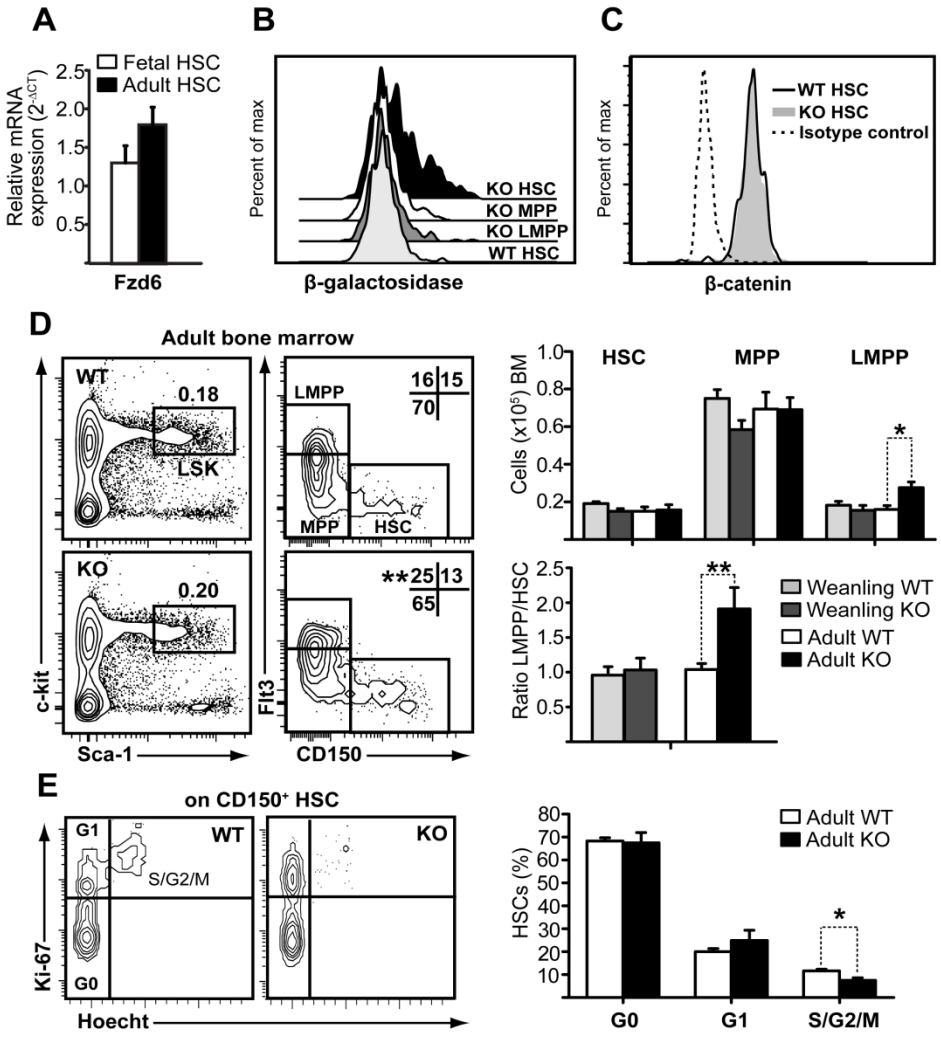

Figure 1. Non-canonical *Fzd6* is expressed on CD150$^+$ HSCs and influences the ratio of Flt3$^+$ vs CD150$^+$ progenitors

A) Analysis of *Fzd6* expression by quantitative RT-PCR in the E13.5 fetal liver and 6-week-old adult BM HSCs. Histogram represents mean +SEM from three independent experiments. B) β-galactosidase expression from the *Fzd6-nLacZ* allele was determined by its ability to cleave the fluorescent substrate FDG in *Fzd6*$^{-/-}$ (KO) and BM stem/progenitor cells. *Fzd6*$^{+/+}$ (WT) cells were used as negative control. Similar results were obtained from three independent experiments. C) Intracellular active β-catenin levels in BM CD150$^+$ *Fzd6*$^{-/-}$ and *Fzd6*$^{+/+}$ HSCs. Similar results were obtained from four independent experiments. D) Flow cytometry analysis of the proportion of CD150$^+$ CD135 (Flt3)$^-$ HSCs, CD150$^-$ CD135$^-$ MPPs and CD150$^-$ CD135$^{+/hi}$ LMPPs. Representative flow cytometry data are shown for 6-week-old *Fzd6*$^{-/-}$ and *Fzd6*$^{+/+}$ BM. Numbers within the flow cytometry panels represent the mean percentages of total LSKs as well as the different LSK subsets over total LSKs from eight animals per group. Histogram represents the absolute numbers of BM LSK subpopulations HSC, LMPP, MPP in 3-week (n=6) and 6-week-old (n=8) in *Fzd6*$^{-/-}$ and *Fzd6*$^{+/+}$ mice (mean + SEM). E) Cell cycle analysis of CD150$^+$ HSCs. Ki-67/Hoechst co-staining was used to distinguish the G0, G1 and S/G2/M cell cycle phases. Histogram represents mean + SEM from three animals per group. *P<0.05, **P<0.005 (two-tailed, unpaired student's t-test).

3.2. *Fzd6* has an age-dependent effect on hematopoietic progenitor cell maintenance

To determine whether *Fzd6* was required for resting hematopoiesis, we examined 3- and 6-week-old *Fzd6*$^{-/-}$ and sex-matched littermate *Fzd6*$^{+/+}$ mice. The frequency and the numbers of bone marrow LSKs, CD150^{-} multipotent progenitors (MPPs) and CD150^{+} CD135^{-} HSCs were not altered in *Fzd6*$^{-/-}$ weanling or young adult mice. Interestingly, 6-week-old adult *Fzd6*$^{-/-}$ BM presented a modest, approximately two-fold increase in the number of LSKs with high cell–surface Flt3 (CD135; lymphoid-primed MPPs, or LMPPs) while no significant differences were observed at three weeks (Fig. 1D). This was largely due to an overall increase in Flt3 staining intensity in *Fzd6*$^{-/-}$ BM, suggesting defective HSC maintenance. Given that a relative increase in more differentiated, Flt3^{+} subsets could correlate with HSC activation (Sugimura et al., 2012, Wilson et al., 2008, Wilson et al., 2004), our observation led us to speculate that *Fzd6* might be involved in the regulation of HSC turnover.

During resting hematopoiesis, the majority of long-term HSCs remain in a quiescent state with only a small fraction that are actively cycling. Adult HSC self-renewal has been frequently linked to HSC quiescence since disruption of quiescence leads to defects in HSC self-renewal (Nygren et al., 2006, Rossi et al., 2012, Sugimura et al., 2012, Wilson et al., 2008). More specifically, non-canonical Wnt5a signaling favors HSC maintenance via quiescence (Nemeth et al., 2007, Povinelli et al., 2014, Sugimura et al., 2012). To assess whether *Fzd6* regulated HSPC cycling, we stained *Fzd6*$^{+/+}$ and *Fzd6*$^{-/-}$ BM cells with the DNA dye Hoechst and an antibody against Ki-67. Somewhat unexpectedly, we found no significant difference in the proportion of resting (G0) HSPCs (Fig. 1E). However, we did observe a slight but reproducible decline in the proportion of dividing (S-G2-M phase) *Fzd6*$^{-/-}$ HSCs (Fig. 1E). These results suggested that *Fzd6* signaling might promote cell cycle progression and proliferation of HSCs.

To determine whether *Fzd6*$^{-/-}$ HSPCs were functionally biased toward lymphoid fates given their elevated levels of Flt3 expression (Sitnicka *et al.*, 2002), we assessed the frequency of early T lineage progenitors in the thymus and mature T and B lymphoid cell subsets in BM and spleen. Although we saw some evidence of an augmented lymphoid output as shown by an increase in *Fzd6*$^{-/-}$ thymic cellularity, flow cytometry analysis revealed no significant differences in the proportion of various lymphoid subsets

in the BM or lymphoid organs (spleen and thymus) between *Fzd6*$^{-/-}$ and *Fzd6*$^{+/+}$ mice (Supplementary Figure 1). Our data therefore indicate that *Fzd6* deficiency increases the ratio of Flt3^{hi} LMPPs to CD150^{+} HSCs within the LSK compartment (Fig. 1D) but the potential lymphoid bias appears compensated for during steady-state hematopoiesis.

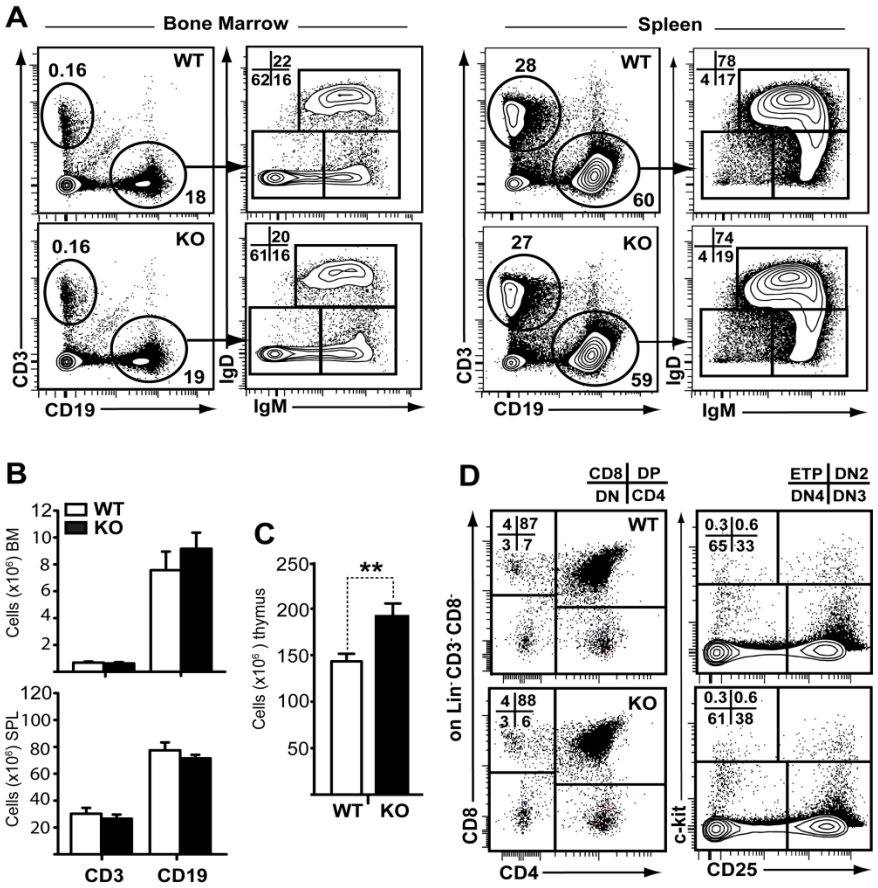

Figure S1. *Fzd6*$^{-/-}$ mice present grossly normal lymphoid development.

A) Flow cytometry analysis of CD3e+ T lymphocytes and CD19+ B lymphocyte populations in the bone marrow (left) and spleens (right) of adult *Fzd6*+/+ and *Fzd6-/-* mice. B lymphocyte subpopulations were gated as IgD+IgM+ mature/recirculating B cells, IgD-IgM+ immature B cells and IgD-IgM- pro- and pre-B cells within the CD19+ cells. Numbers shown in different quadrants indicate the mean percentage from four independent experiments. B) CD3e+ T lymphocyte and CD19+ B lymphocyte numbers in bone marrow (top) and spleen (bottom). Histograms represent the cell numbers (mean + SEM) from four independent experiments. C) Thymic cellularity for *Fzd6*+/+ and *Fzd6-/-* mice (mean ±SEM, n=5). D) Flow cytometry analysis of thymocytes from adult *Fzd6*+/+ and *Fzd6-/-* mice, showing CD4-CD8- double negative (DeKoter *et al.*) CD4+ or CD8+ single positive, and CD4+CD8+ double positive (DP) subsets on the left. T cell progenitors were first gated on Lin- (B220, CD3ε, CD11b, GR1, Ter119, CD8") and then separated according to c-Kit (CD117) and CD25 expression as follows: c-Kit+ CD25- early thymic progenitor (ETP), c-Kit+CD25+ DN2, and c-Kit-CD25+ DN3. Numbers shown in different quadrants indicate the mean percentage out of total thymocytes (for CD4/CD8) and Lin- cells (for T cell precursors) from four different experiments

3.3. *Fzd6* negatively regulates Cdc42/JNK signaling in HSPCs

Non-canonical Wnt signaling can modulate cell divisions (Gillies *et al.*, 2011, Lapebie *et al.*, 2011), HSPC quiescence (Nemeth et al., 2007, Povinelli et al., 2014, Sugimura et al., 2012), and polarity (Florian et al., 2013). In particular, an increase in Wnt5a/Cdc42-dependent signaling has been linked to the apolarization of HSPCs during aging (Florian et al., 2012, Florian et al., 2013), whereas *Fzd*8/Fmi-dependent suppression of HSPC activation is necessary for the maintenance of long-term HSCs (Sugimura et al., 2012). To evaluate the role of *Fzd6* in non-canonical signaling, we examined the expression of Celsr1, which has been shown to interact with *Fzd6* in other tissues (Devenport *et al.*, 2008), phosphorylated active JNK and Cdc42 in $Fzd6^{-/-}$ and $Fzd6^{+/+}$ HSPCs by flow cytometry. We did not observe any difference in Celsr1 expression (Fig. 2A), although we cannot conclude about its localization. However, both phospho-JNK and Cdc42 were present at higher levels in $Fzd6^{-/-}$ HSPCs (Fig. 2A), suggesting that *Fzd6* might negatively regulate Cdc42/JNK signaling.

The localization of Cdc42 appears essential for its role in HSPC polarity and aging (Florian et al., 2013). To visualize Cdc42 in $Fzd6^{-/-}$ and $Fzd6^{+/+}$ HSPCs, we resorted to imaging flow cytometry and evaluated Cdc42 polarization (asymmetrical distribution within the cell) and the staining intensity of Cdc42 clusters (bright detail intensity). There was no difference in the proportion of HSPCs with polarized Cdc42 between $Fzd6^{-/-}$ and $Fzd6^{+/+}$ BM cells (32% and 32%, respectively). However, $Fzd6^{-/-}$ HSPCs showed a greater proportion of bright Cdc42 clusters (Fig. 2B-C), with almost all polarized HSPCs falling under the Cdc42 bright category (96% vs 67% for $Fzd6^{+/+}$). Collectively these results suggest that *Fzd6* provides a regulatory function to restrict Cdc42 clustering and thus negatively regulates Cdc42/JNK signaling.

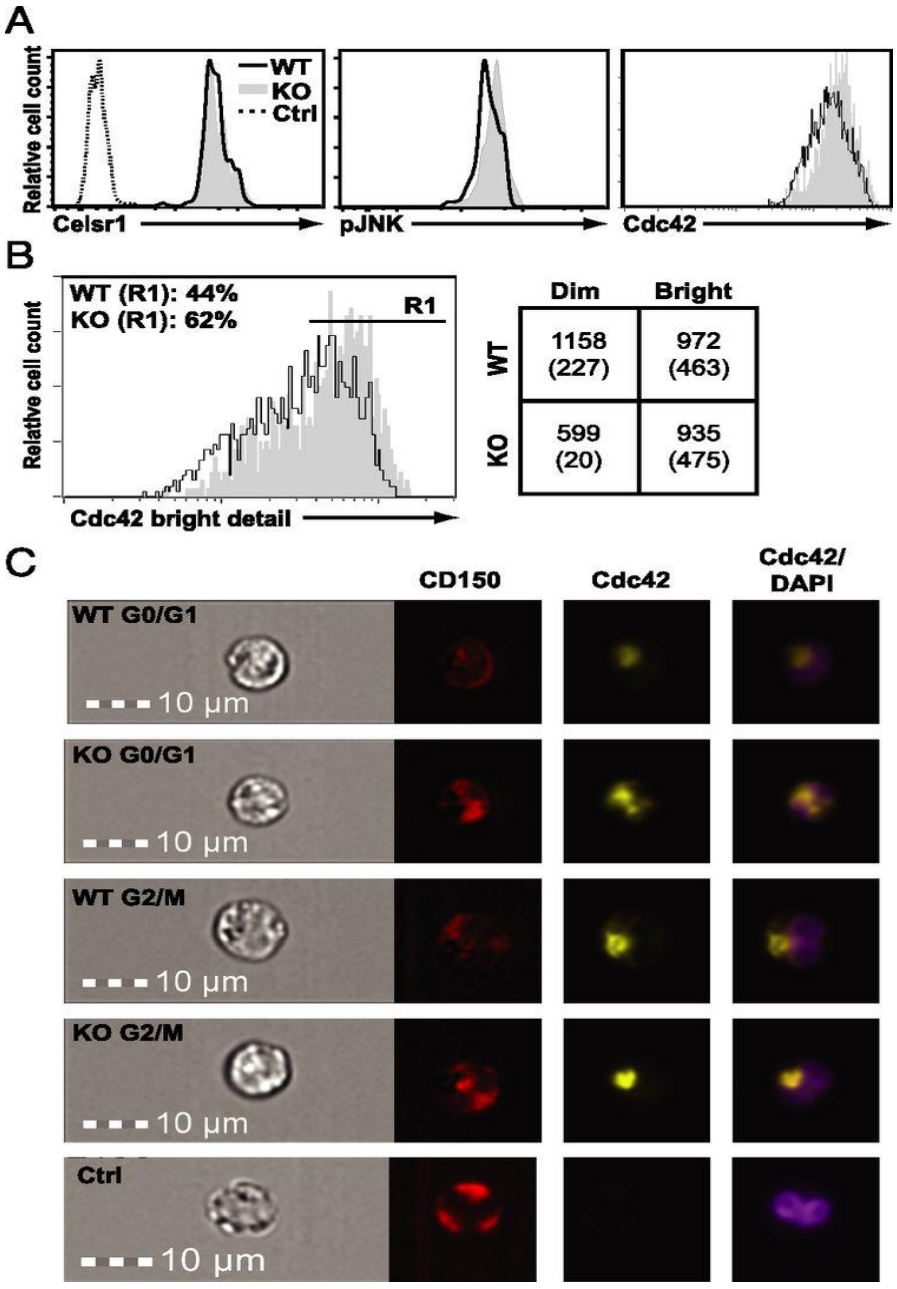

Figure 2. *Fzd6* is a negative regulator of Cdc42/Jnk signaling

A) Flow cytometry analysis of the expression of Celsr1, phospho-Jnk (T183/Y185) and Cdc42 in BM CD150^{+} *Fzd6*$^{-/-}$ and *Fzd6*$^{+/+}$ HSCs. Similar results were obtained from at least three independent experiments. B) Imaging flow cytometry analysis of Cdc42 staining in BM CD150^{+} Sca1^{+} cells. The data are pooled from two independent experiments with a total of four mice per genotype. The X-axis of the histogram represents the staining intensity of Cdc42 clusters for *Fzd6*$^{-/-}$ and *Fzd6*$^{+/+}$ cells. The table shows the numbers of cells within the Cdc42 dim gate (left side of the histogram) and the Cdc42 bright gate (right side of the histogram) according to genotype. The numbers in parentheses represent polarized events (polarized Cdc42 staining) in each group. *P*<0.0001 (Fisher's exact test). C) Representative images of *Fzd6*$^{-/-}$ and *Fzd6*$^{+/+}$ cells in G0/G1 and G2/M phases of the cell cycle. The bottom images show the staining control (secondary antibody only) for Cdc42.

3.4. *Fzd6* is essential for competitive repopulating capacity of HSCs and long-term granulocytic reconstitution

To directly determine whether *Fzd6* was required for cell-intrinsic regulation of HSPC function *in vivo*, we performed competitive transplantation assays. Fresh BM cells from *Fzd6*$^{-/-}$ and *Fzd6*$^{+/+}$ mice (CD45.2^{+}) were mixed with congenic competitor BM cells at a 1:1 ratio and transferred into lethally irradiated recipients (Fig. 3A). Donor chimerism was assessed by flow-cytometry analysis of peripheral blood leukocytes at 4, 8, 12 and 16 weeks post-transplant. Approximately half of the mice having received *Fzd6*$^{-/-}$ cells displayed poor reconstitution already at 4 weeks post-transplant (Fig. 3B). The defects in the repopulating ability of *Fzd6*$^{-/-}$ HSPCs became even more pronounced at 12 and 16 weeks (Fig. 3B) with a progressive loss of *Fzd6*$^{-/-}$ GR1^{hi} granulocytes, which suggested defective long-term HSC self-renewal (Fig. 3C). To further determine the cell-intrinsic role of *Fzd6* in myelo-lymphoid differentiation *in vivo*, the contribution to B cells, T cells and GR1^{hi} myeloid cells within CD45.2^{+} donor cells was evaluated by flow-cytometry analysis of peripheral blood at different time points. There was no difference in the distribution of granulocytes, monocytes and lymphocytes within *Fzd6*$^{-/-}$ donor cells when compared to *Fzd6*$^{+/+}$ donors at eight weeks after transplant. However, the proportion of granulocytes decreased significantly with time while the relative contribution of *Fzd6*$^{-/-}$ cells to the more long-lived lymphoid lineages and monocytes was not negatively affected (Fig. 3D). To obtain a more quantitative representation of lymphoid and myeloid lineages, we also analyzed the spleen at 16 weeks after transplant. The numbers of *Fzd6*$^{-/-}$ donor-derived cells were significantly decreased for all subsets (Fig. 3D), with an approximately 5-fold difference for B lymphocytes and monocytes and a nearly 10-fold decrease for T lymphocytes and granulocytes.

Flow-cytometry analysis of BM cells from the primary recipient mice confirmed the absence of *Fzd6*$^{-/-}$ HSCs in the majority of recipients at 16 weeks after transplantation (Fig. 4A). Consistent with the peripheral blood analysis, *in vitro* colony-forming ability of myeloid progenitor cells of *Fzd6*$^{-/-}$ origin was also significantly decreased (Fig. 4B). To further test the self-renewal activity of donor cells, we selected primary recipients of *Fzd6*$^{-/-}$ BM cells with detectable donor contribution at 16 weeks (11-32% total chimerism in peripheral blood) together with representative primary recipients of *Fzd6*$^{+/+}$ cells for secondary transplants. As expected from the frequency of phenotypic donor HSCs (Fig. 4A),

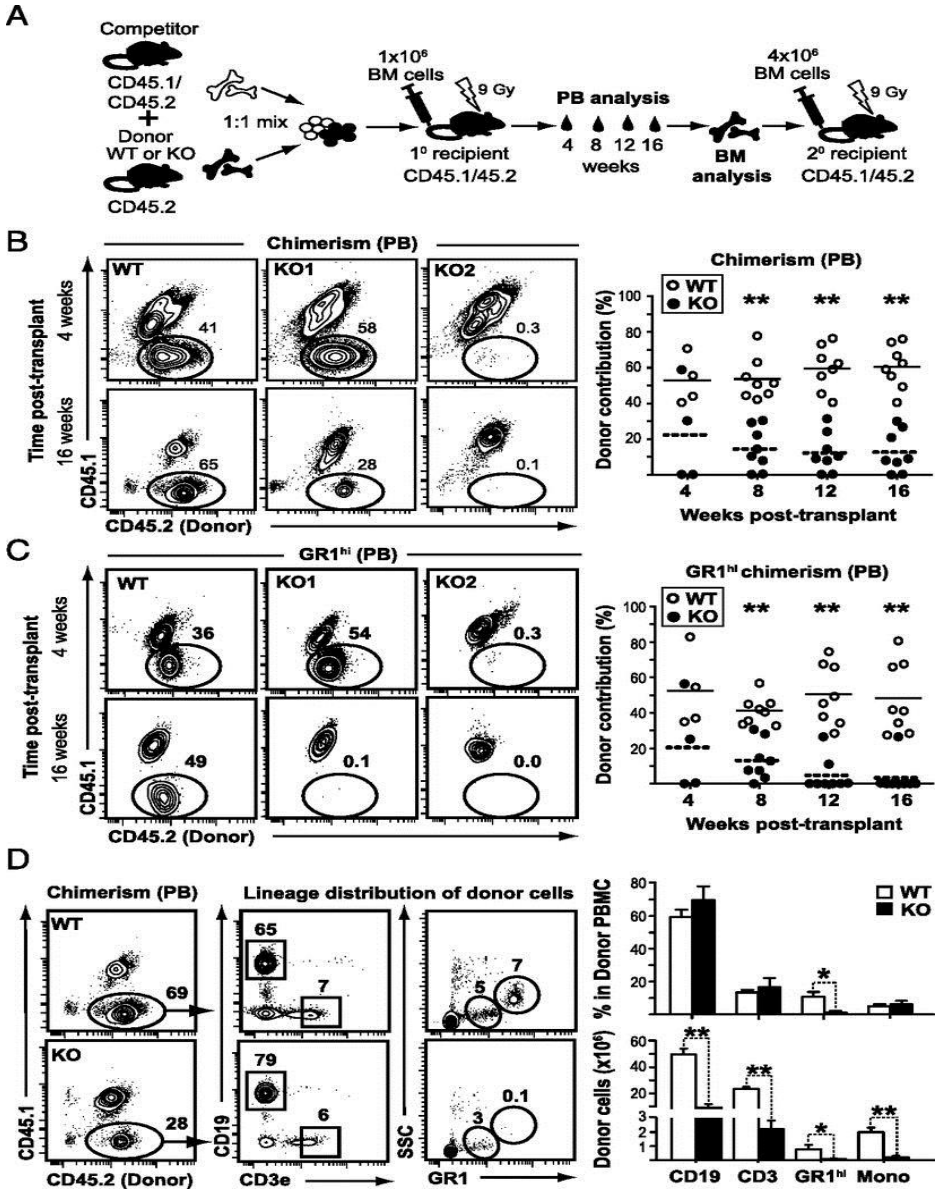

Figure 3. *Fzd6*$^{-/-}$ HSPCs display defective long-term engraftment and self-renewal *in vivo*.

(A) Experimental design of the competitive reconstitution assay. (B) Peripheral blood (PB) chimerism in primary recipient mice at 4, 8, 12, and 16 wk after transplant. Representative flow cytometry data at 4 and 16 wk are shown in the left panel. Pooled data from two independent groups of transplants are shown in the right panel. Dots represent individual mice (open circles for *Fzd6*$^{+/+}$ donors; black circles for *Fzd6*$^{-/-}$ donors), and horizontal lines represent the mean (solid line, *Fzd6*$^{+/+}$; dotted line, Fzd6$^{-/-}$). (C) Flow cytometry analysis of short and long-term *Fzd6*$^{+/+}$ and *Fzd6*$^{-/-}$ donor cell contribution to peripheral blood SSC^{hi}GR1^{hi} granulocytes. (D) Representative flow cytometry data and quantitative analysis of the relative distribution of CD19^{+} B lymphocytes, CD3ε^{+} T lymphocytes, CD11b^{+}GR1^{lo} monocytes, and GR1^{hi}SSC^{hi} granulocytes among donor-derived peripheral blood cells (top right panel) and donor-derived splenocytes (bottom right panel) in recipient mice. Histograms indicate mean + SEM from two independent experiments (n = 8).*p , 0.05, **p , 0.005 (two-tailed, unpaired Student t test).

Fzd6$^{-/-}$ donor cells were unable to establish multi-lineage hematopoietic reconstitution (>1% in all lineages) in secondary recipients, even at short-term (Fig. 4C). In comparison, *Fzd6*$^{+/+}$ donor cells were present in secondary recipients at a frequency that was at least equivalent to that detected in primary recipients (Fig. 4C-D). These data strongly suggest that *Fzd6* is crucial for *in vivo* repopulating activity and HSC self-renewal capacity, particularly under stress.

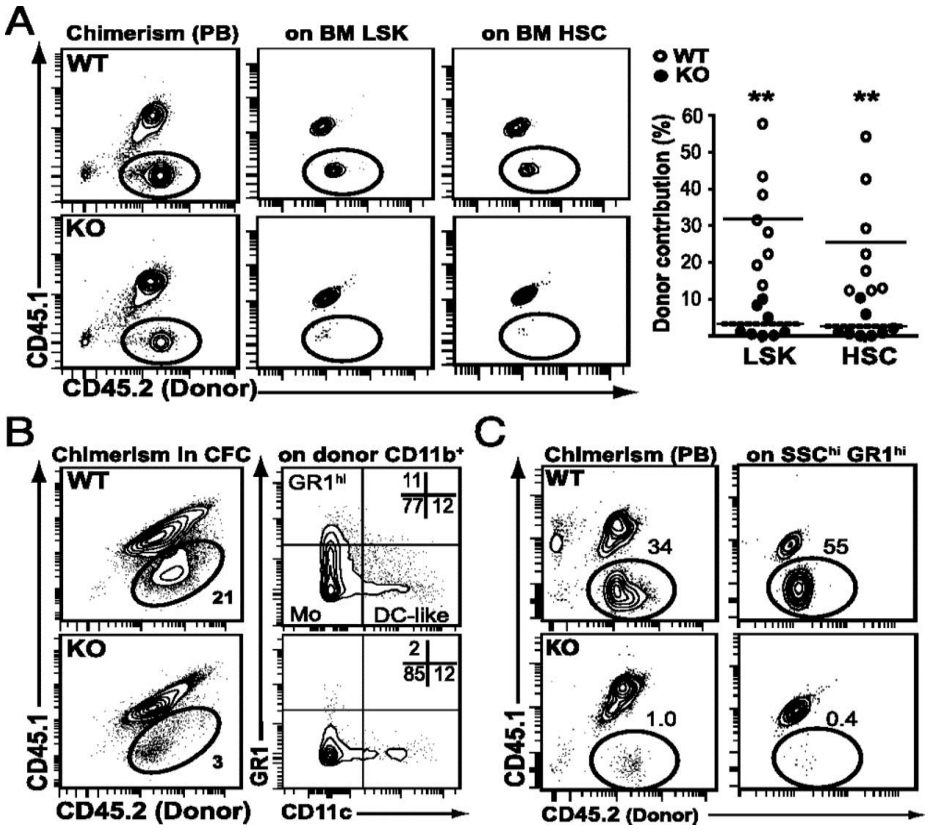

Figure 4. *Fzd6*$^{-/-}$ HSPCs display defective long-term engraftment and self-renewal *in vivo*

A) Representative flow cytometry data from peripheral blood (left) and BM 16 weeks after transplant. Pooled data from two independent groups of transplants are shown on the right. Dots represent individual mice (open circles for *Fzd6*$^{+/+}$ donors; black circles for *Fzd6*$^{-/-}$ donors) and horizontal lines represent the mean (solid line, *Fzd6*$^{+/+}$; dotted line, *Fzd6*$^{-/-}$). n=8 for both groups. B) Flow cytometry analysis of cells recovered from CFC assays. Left panel represents the percentage of donor-derived cells among all cells. Right panel shows the distribution GR1^{hi} granulocytes, GR1$^{lo/neg}$ monocytes and CD11c^{+} dendritic cells among donor-derived CD11b^{+} cells. Similar results were obtained from four independent experiments. C) Representative flow cytometry data from peripheral blood of secondary recipients. Left panel represents total chimerism and right panel shows the contribution of donor-derived cells to GR1^{hi} granulocytes. Numbers indicate the mean from one experiment (n=4 for both groups). Similar results were obtained from a second, independent transplant experiment. **P*<0.05, ***P*<0.005 (two-tailed, unpaired student's t-test).

3.5. Long-term reconstitution defects of *Fzd6* deficient HSPCs are not due to altered homing but rather to defective survival and expansion in the recipient bone marrow

To understand the mechanisms underlying the observed defects in the engraftment and reconstitution capacity of *Fzd6*$^{-/-}$ HSPCs, we first examined the role of *Fzd6* in BM homing. To assess the homing efficiency of HSPCs, *Fzd6*$^{-/-}$ or *Fzd6*$^{+/+}$ BM cells were transplanted into sub-lethally irradiated congenic recipient mice and the numbers of donor-derived HSPCs in the BM were determined by flow-cytometry at 16 h (Fig. 5A). Our results revealed no differences in the ability of *Fzd6*$^{-/-}$ cells to home to the BM when compared to *Fzd6*$^{+/+}$ controls (Fig. 5B). Furthermore, the percentage of donor-derived granulocytes generated in a colony-forming assay was similar for both *Fzd6*$^{+/+}$ and *Fzd6*$^{-/-}$ donors (Fig. 5C). Not surprisingly we saw no major differences in the expression levels of Cxcr4 or CD44 between *Fzd6*$^{+/+}$ and *Fzd6*$^{-/-}$ HSPCs at baseline (Fig. 5D). These results indicate that the presence of *Fzd6* is not necessary for migration to the BM. It might rather be required for HSPC expansion and maintenance.

Given the problems with myeloid reconstitution already present at four weeks post-transplant (Fig. 3C), we hypothesized that the loss of *Fzd6*$^{-/-}$ donor HSPCs would be an early event. We therefore decided to investigate the initial HSPC expansion to repopulate the recipient BM at four days and eight days post-transplant (Fig. 6A). *Fzd6*$^{-/-}$ HSPCs were present at comparable numbers on day four but failed to expand between days four and eight (Fig. 6B). Cell cycle analysis correlated with cell numbers, with the proportion of *Fzd6*$^{+/+}$ donor cells in the proliferating phase increasing between the two time points in contrast to *Fzd6*$^{-/-}$ HSPCs (Fig. 6D). *Fzd6*$^{-/-}$ cells showed similar behavior in both non-competitive (Fig. 6) and competitive settings (Fig. 3 and 4), suggesting a decrease in not only relative *Fzd6*$^{-/-}$ HSPC fitness but also in absolute terms. These results parallel the cell cycle analysis at steady-state (Fig. 1E), suggesting that either 1) *Fzd6* is directly implicated in cell cycle progression or that 2) dividing *Fzd6*$^{-/-}$ HSPCs are lost due to differentiation or cell death.

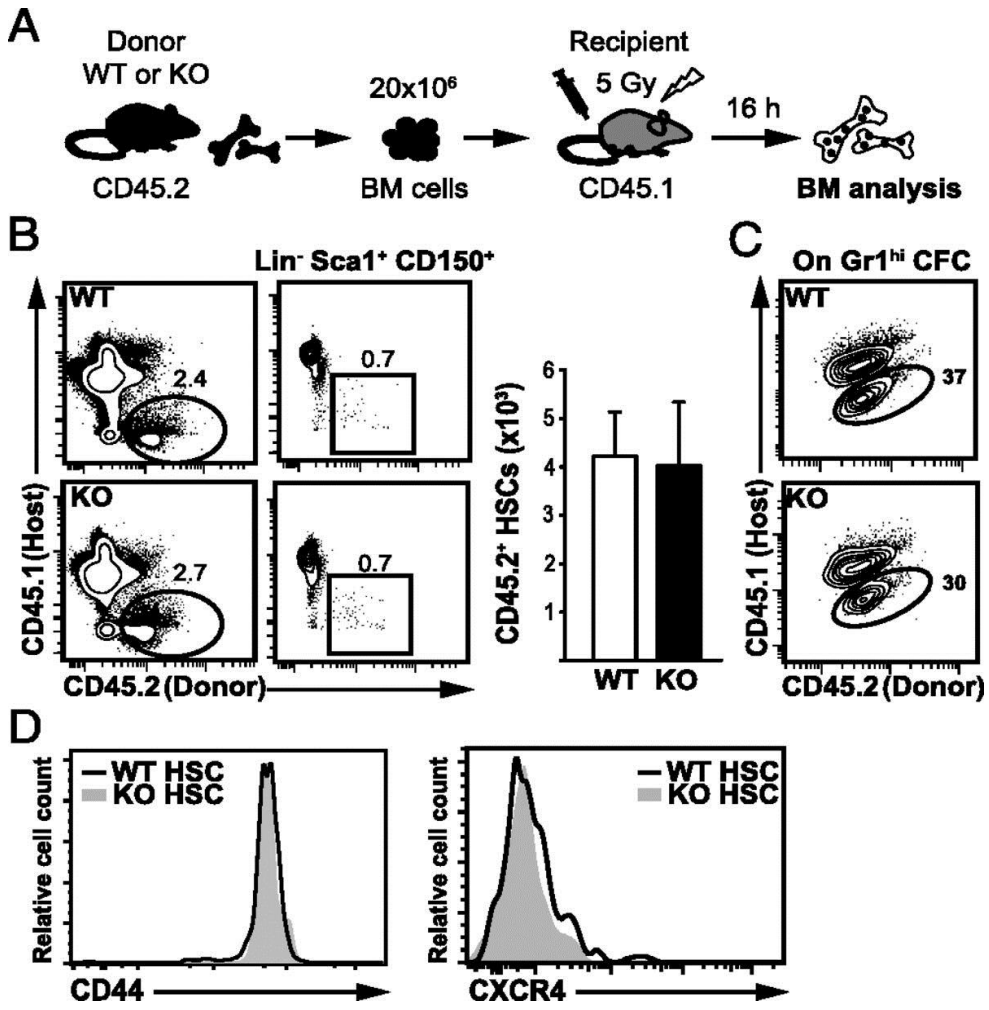

Figure 5. Defective long-term reconstitution of *Fzd6*$^{-/-}$ HSPCs is not due to altered homing

A) Experimental design for the homing assay. B) Representative flow cytometry data showing the percentage donor-derived cells and donor-derived CD150^{+} HSCs in recipient BM 16 h after transplant. Histogram represents the absolute number of donor-derived HSCs in BM. Similar results were obtained from two independent experiments with three mice per group in each experiment. C) Frequency of donor-derived granulocytes among cells recovered from mixed colony assays seeded with BM harvested 16h after transplant. Similar results were obtained from two independent experiments. D) Representative flow cytometry data depicting CD44 and Cxcr4 expression on *Fzd6*$^{+/+}$ (black line) and *Fzd6*$^{-/-}$ (solid gray) bone marrow HSPCs. Similar results were obtained from four pairs of mice.

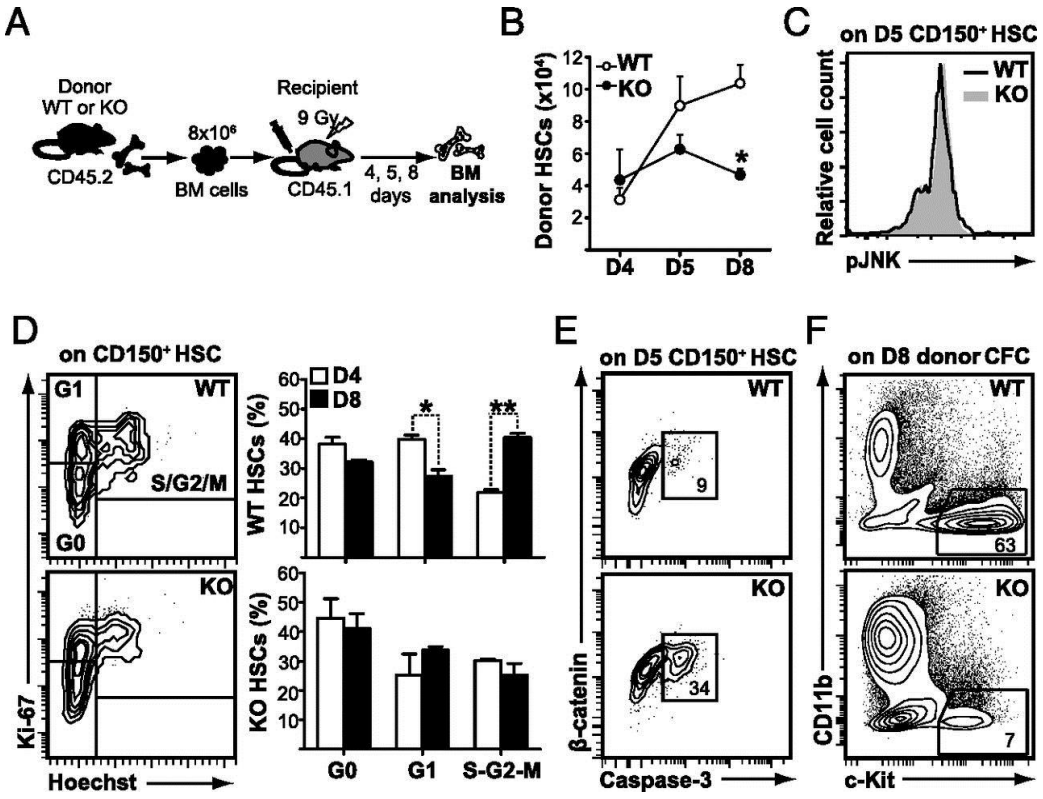

Figure 6. *Fzd6*$^{-/-}$ HSPCs cannot expand and die by apoptosis in the first week after transplant.

A) Experimental design for the short-term transplants. B) Numbers of donor-derived HSCs in BM on day 4 and day 8 after transplant. Graph represents pooled results from two independent experiments with three to five mice per group. C) Representative flow cytometry data depicting phospho-Jnk (T183/Y185) staining in *Fzd6*$^{+/+}$ and *Fzd6*$^{-/-}$ donor HSPCs. Similar results were obtained from four pairs of mice. D) Cell cycle analysis of donor-derived HSCs in the recipient bone marrow. Graphs represent pooled results from two independent experiments with two to five mice per group. E) Representative flow cytometry data depicting β-catenin and cleaved caspase-3 staining in *Fzd6*$^{+/+}$ and *Fzd6*$^{-/-}$ donor HSPCs. Similar results were obtained from four pairs of mice. F) Representative flow cytometry data depicting the proportion of c-Kit and CD11b staining within *Fzd6*$^{+/+}$ and *Fzd6*$^{-/-}$ donor-derived cells recovered from mixed colony assays. Similar results were obtained from four pairs of mice.

To investigate the second possibility, we stained post-transplant BM cells with an antibody against the active form of caspase-3 together with β-catenin. Sustained β-catenin expression has been shown to activate caspase-3 and result in loss of HSPCs shortly after transplant (Ming *et al.*, 2012). While *Fzd6*$^{+/+}$ donor HSPCs were mostly caspase-3 negative, 35-75% *Fzd6*$^{-/-}$ HSPCs stained positive for active caspase-3 (Fig. 6E). Caspase-3-positive cells also co-stained strongly for β-catenin (relative fluorescent intensity of 21-50 for *Fzd6*$^{-/-}$ cells, 8.5-11 for *Fzd6*$^{+/+}$). Furthermore, colonies generated by *Fzd6*$^{-/-}$ HSPCs contained a smaller proportion of cKit^{+} cells, which are indicative of colony-forming cells with high proliferative potential (Fig. 6F), suggesting that a larger proportion of the remaining *Fzd6*$^{-/-}$ HSPCs had initiated myeloid differentiation. In contrast to steady-state, we did not observe any difference in phospho-JNK activity (Fig. 6C). Collectively with the data presented in Figure 5 these results clearly demonstrate that *Fzd6*$^{-/-}$ HSPCs are fully able to reach the BM but once at destination, fail to expand due to a strong activation of caspase-3 and consequently decreased HSPC survival.

3.6. *Fzd6* deficient HSPCs expand poorly in response to emergency signals

To determine if *Fzd6*$^{-/-}$ HSPCs fail to expand also in response to other types of proliferative stress, we induced emergency myelopoiesis using a sublethal dose of LPS (Fig. 7A) (Boettcher *et al.*, 2012). Although both *Fzd6*$^{+/+}$ and *Fzd6*$^{-/-}$ HSPCs expanded in both proportion and number (Fig. 7B), BM from *Fzd6*$^{-/-}$ mice contained significantly fewer LSKs and CD150^{+} HSCs. This difference did not appear to stem from decreased cell cycle entry (Fig. 7C) but would be rather due to loss of cells either through cell death or differentiation. Similar numbers of HSCs were found in the spleen (on average, 5.0x10^{3} for *Fzd6*$^{+/+}$ vs 5.9x10^{3} for *Fzd6*$^{-/-}$), supporting the hypothesis of increased differentiation and BM exit. BM *Fzd6*$^{-/-}$ HSPCs also generated proportionately fewer cKit^{+} cells in a colony-forming assay (Fig. 7D), similar to what we saw shortly after transplant. Therefore, *Fzd6* is necessary for efficient BM HSPC expansion and self-renewal, not only after transplant but also under proliferative stress such as emergency myelopoiesis.

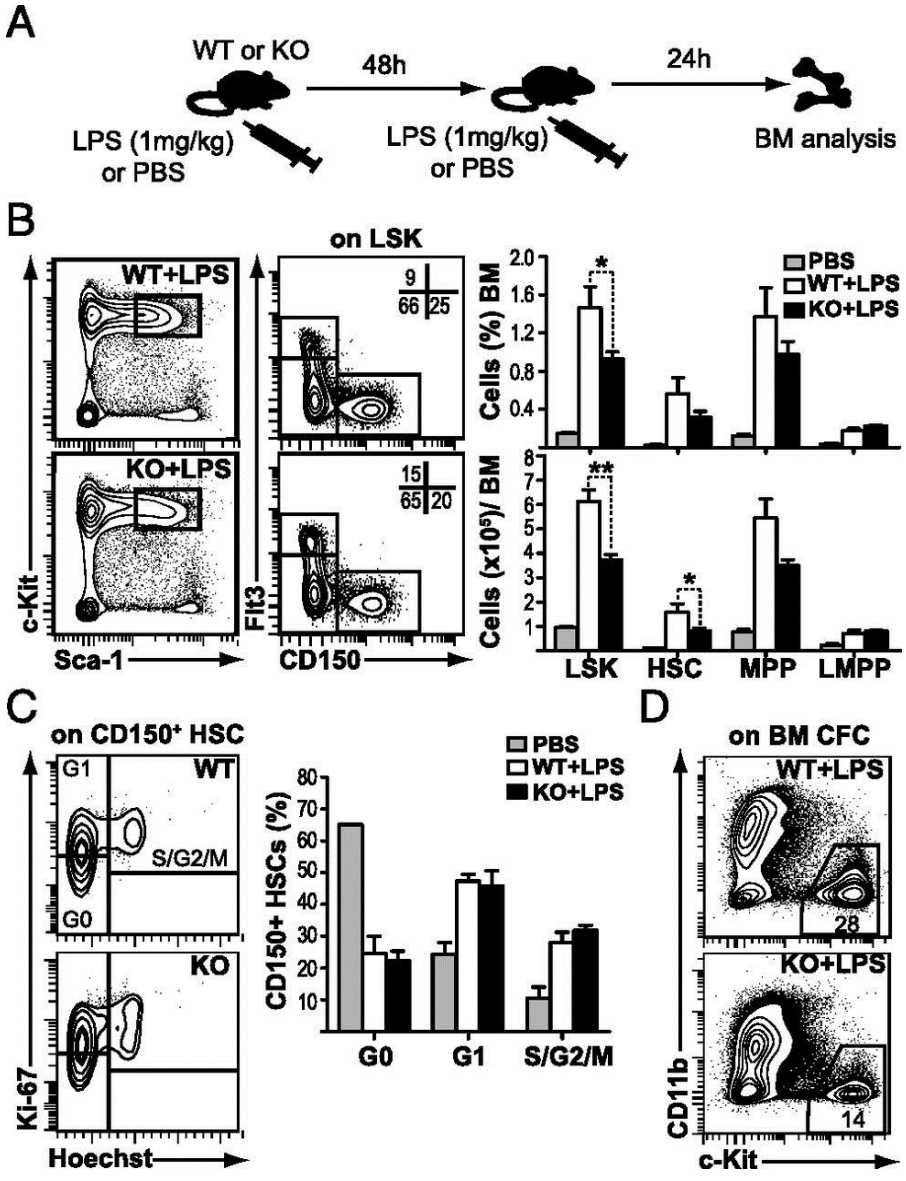

Figure 7. *Fzd6*$^{-/-}$ HSPCs exhibit poor emergency hematopoiesis.

A) Experimental design for the emergency response. B) Flow cytometry analysis of the BM HSPC compartment. Representative flow cytometry data are shown on the left. The numbers within flow cytometry plots show the percentage of HSPC subpopulations within LSKs. Histograms represent the proportion and absolute numbers of HSPCs per BM. Data are derived from three independent experiments with three to five mice per group. C) Cell cycle analysis of HSCs in the bone marrow. Histogram represents pooled results from three independent experiments with two to five mice per group. D) Representative flow cytometry data depicting the proportion of c-Kit and CD11b staining within *Fzd6*$^{+/+}$ and *Fzd6*$^{-/-}$ cells recovered from mixed colony assays. Similar results were obtained from four pairs of mice.

4. DISCUSSION

We have examined here the role of the Wnt signaling receptor *Fzd6* in the expansion and differentiation of mouse BM HSPCs at steady state and under replicative stress. At steady state, the proportion of $CD150^+$ HSCs was normal in the $Fzd6^{-/-}$ BM but $Fzd6^{-/-}$ HSPCs expressed a higher level of Flt3 than their $Fzd6^{+/+}$ counterparts. Furthermore, we show that although $Fzd6^{-/-}$ HSPCs were able to reach the recipient BM, they failed to reconstitute an irradiated host due to impaired expansion and survival. $Fzd6^{-/-}$ HSPCs also failed to expand in response to LPS. Thus, *Fzd6* was necessary for normal HSPC function in a hematopoietic cell-intrinsic manner.

Self-renewal is a crucial feature of HSCs, which allows them to replenish all hematopoietic lineages under conditions of BM stress e.g., ablative chemotherapy, irradiation, infections and injury (Baldridge et al., 2010b, Mendez-Ferrer et al., 2010, Wilson et al., 2008). Wnt signaling can play a critical role in HSPC self-renewal, although the extent and source of signals are still under debate and likely involve different pathways under different circumstances (Florian et al., 2013, Lento et al., 2014, Sugimura et al., 2012). Canonical Wnt signaling is induced by myeloablative stress (Sugimura et al., 2012) and reportedly required for hematopoietic recovery in response to irradiation (Lento *et al.*, 2014). However, in the absence of negative regulators, such as *Fzd6* (Golan *et al.*, 2004), the response is likely to remain unchecked. Although we did not detect differences in canonical Wnt signaling in $Fzd6^{-/-}$ HSPCs at steady-state, in contrast to what was reported for $Fmi^{-/-}$ and $Fzd8^{-/-}$ cells (Sugimura et al., 2012), we did observe stronger intracellular β-catenin staining after transplant. $Fzd6^{-/-}$ HSPCs were present at numbers comparable to controls at four days post-transplant but showed increased apoptosis and failed to expand over the following days. Sustained stabilization of β-catenin has been previously shown to be directly associated with HSPC exhaustion and apoptosis and could thus result in the loss of $Fzd6^{-/-}$ donor cells after transplant (Kirstetter et al., 2006, Ming et al., 2012, Scheller et al., 2006b).

Fmi and *Fzd8* loss-of-function studies suggested a non-canonical requirement for the maintenance of HSPC quiescence, as their absence altered the pool size, self-renewal and engraftment of HSPCs (Sugimura et al., 2012). Namely, *Fzd*8/Fmi signaling axis inhibited canonical Wnt signaling in HSCs and thus maintained HSC quiescence at

steady state. Label-retaining HSCs expressed much higher levels of *Fzd8* and *Fmi* than cycling HSCs, and both *Fmi* and *Fzd8* expression was decreased after activation of HSCs in response to 5-fluoro-uracil. Exogenous Wnt5a is also reported to maintain HSCs by enforcing their resting state (Nemeth et al., 2007, Povinelli et al., 2014). Although we did not detect significant differences in the fraction of quiescent (G0) HSCs in the *Fzd6*$^{-/-}$ BM, we cannot exclude possibility that *Fzd6* is also required for the quiescence of a specific subset of CD150^{+} HSCs. Loss of quiescence would ultimately result in decreased HSC survival and decreased long-term repopulation (Nygren et al., 2006, Pietras et al., 2014, Wilson et al., 2008), in agreement with our results after transplant. Further studies using, e.g., label-retention approaches will be required to conclusively clarify the issue.

Our results suggest that *Fzd6* is a negative regulator of both Cdc42/Jnk and β-catenin –dependent signaling in HSPCs. In contrast to *Fmi*$^{-/-}$ and *Fzd8*$^{-/-}$ cells, *Fzd6*$^{-/-}$ HSPCs displayed normal active β-catenin levels at steady state. There was also no difference in the expression of CD44, a classical Wnt target gene (Staal *et al.*, 2004). However, we observed enhanced Cdc42 and Jnk activity as indicated by the increased size of Cdc42 clusters and stronger staining with the phospho-specific Jnk antibody. Cdc42 activity can have opposing downstream results: activation of NFAT via the Cdc42/Ca^{2+} pathway and suppression of NFAT nuclear translocation through the Cdc42/Casein-kinase-1 pathway (Dejmek *et al.*, 2006). Fmi/*Fzd*8 signaling suppressed HSC activation through the inhibition of NFAT/IFN-γ axis and consequent downregulation of β-catenin activity (Sugimura et al., 2012), with *Fmi*$^{-/-}$ and *Fzd8*$^{-/-}$ HSPCs likely losing their self-renewal capacity due to excessive proliferation. Wnt5a/Cdc42 activity has been shown to inhibit canonical Wnt signaling also in culture (Nemeth *et al.*, 2007a) and during aging (Florian et al., 2013). It is thus possible that even though we do not detect altered active β-catenin levels in *Fzd6*$^{-/-}$ HSPCs, the enhanced Cdc42 activity could interfere with canonical Wnt signaling in our model and contribute to, e.g. decreased cell cycle progression. On the other hand, increased phosphorylation of β-catenin by Jnk could promote the translocation of stable β-catenin to the nucleus and thus enhance the potential for canonical Wnt signaling (Wu *et al.*, 2008).

Altered engraftment of HSPCs in the BM is frequently associated with impaired homing to the BM niche. This is certainly the case for HSPCs mutated for cell adhesion

related genes, such as Rho-family small GTPases Cdc42 and Rac1 (Gu *et al.*, 2003, Yang *et al.*, 2007). Although we cannot completely exclude defective interaction of *Fzd6*$^{-/-}$ HSPCs with the recipient stromal cells, we detected no impairment in their ability to home to the BM immediately after transplant. There was also no difference in the expression of CD44, a classical Wnt target gene (Staal *et al.*, 2004) that is involved in BM homing (Khaldoyanidi *et al.*, 1996). There may have been a subtle decrease in CXCR4 expression in *Fzd6*$^{-/-}$ HSPCs when compared to their *Fzd6*$^{+/+}$ counterparts and we certainly cannot exclude the possibility of defective CXCR4 signaling in *Fzd6*$^{-/-}$ cells. However, we saw no significant accumulation of *Fzd6*$^{-/-}$ HSPCs in the spleen, for example, demonstrating that *Fzd6*$^{-/-}$ donor cells failed to expand and generate differentiated progeny irrespective of their location.

Taken together, we have shown here that *Fzd6* plays an essential role in long-term maintenance and self-renewal of mouse HSPCs in a hematopoietic cell-autonomous manner that was likely independent of HSPC homing to the BM but rather related to their expansion, maintenance and survival. Mechanistically *Fzd6* is a negative regulator of both Cdc42/Jnk and β-catenin -dependent signaling. We propose that the activation of *Fzd6* signaling through pharmacologic means would constitute an interesting option for improving HSPC engraftment and the recovery of immune function after transplant.

5. MATERIAL AND METHODS

Experimental Animals

C57BL/6 (B6; CD45.2^{+}) and B6.SJL-PtprcaPep3b/BoyJ (Ly5a) (B6.SJL; CD45.1^{+}) mice were purchased from The Jackson laboratory (Bar Harbor, ME). Mice deficient in *Frizzled6* (*Fzd6*$^{-/-}$) are described elsewhere (Guo et al., 2004) and were originally a kind gift from J. Nathans (Johns Hopkins, Baltimore, MD). *Fzd6*$^{+/-}$ mice from mixed C57Bl/6x129sv background were backcrossed to C57Bl/6 for 10 generations and then maintained as *Fzd6*$^{+/-}$ to *Fzd6*$^{+/-}$ intercrosses. *Fzd6*$^{-/-}$ mice were compared to sex-matched *Fzd6*$^{+/+}$ littermates. For competitive transplant experiments, first generation progeny from B6.SJLx*Fzd6*$^{+/+}$ intercrosses were used as recipients and their littermates or B6.SJL mice were used as competing donors. For non-competitive short-term transplants and homing experiments the recipient mice were B6.SJL. All mice were bred and housed under specific pathogen-free conditions in sterile ventilated racks at the animal facility of INRS-Institute Armand-Frappier (Centre National de Biologie Expérimentale, CNBE). All procedures were in accordance with the Canadian Council on Animal Care guidelines.

Flow-cytometry Analysis and Cell-Sorting

BM was harvested by flushing tibias and femurs with PBS/0.1% BSA/0.5mM EDTA using a 25-gauge needle. To analyze BM HSPCs, the following antibodies were used: biotin-conjugated anti-lineage mAbs anti-CD3e (145-2C11), anti-CD11b (M1/70), anti-CD45/B220 (RA3-6B2), anti-GR1 (RB6-8C5), and anti-Ter119; streptavidin conjugated to BD Horizon™-V500 (BD Biosciences, Mississauga, ON) or FITC (BD Biosciences); PE anti-CD117 (c-kit, 2B8; BD Biosciences), PE-Cy7 anti-Sca-1 (Ly6A/E, D7; BD Biosciences), PerCP-eFluor® 710 anti-CD135 (Flt3, A2F10), and Alexa-Fluor® 647 anti-CD150 (TC15-12F12.2; BD Biosciences). PE-Cy7 anti-CD3, PE anti-CD19 (1D3), APC or FITC anti-CD45.1 (A20), eFluor® 450 or FITC anti-CD45.2 (104), and APC-Cy7 anti-GR1 (BD Biosciences) were used for the analysis of peripheral blood chimerism. For intracellular staining, surface-stained BM cells were fixed and permeabilized using the Foxp3 staining kit (eBioscience, San Diego, CA) and then

incubated with PE anti–β-catenin (15B8), Alexa-Fluor® 488 active Caspase-3 (Cell Signaling Technologies), PE-eFluor610® anti-Ki67 (SoIA15), PE anti-phospho JNK1/2 (pT183/pY185; BD Biosciences), unconjugated anti-active β-catenin (D13A1; Cell Signaling Technologies), unconjugated anti-Celsr1 (Abcam), or appropriate isotype controls. Unconjugated antibodies were detected with a PE-conjugated F(ab')2 fragment against rabbit IgG (Molecular Probes). All antibodies were purchased from eBioscience unless indicated otherwise. For cell-cycle analysis, BM cells were first incubated for 30 min at 37°C with Hoechst #33342 (Sigma Aldrich, Oakville, ON) in DMEM supplemented with 10% Premium FBS (Wisent Bioproducts, St-Bruno, QC) and 1 mM HEPES (Life Technologies, Burlington, ON), followed by staining with surface antibodies and intracellular anti-Ki67 as described above. The FluoReporter® *lacZ* Flow Cytometry kit (Life Technologies) was used for the detection of β-galactosidase expression from the *Fzd6* mutant allele. Samples were acquired with a four-laser LSR Fortessa flow cytometer (Beckton Dickinson, BD Biosciences, Mountain View, CA) and analyzed using BD FACS Diva software or FlowJo (for histogram overlays). For cell sorting, BM cells were stained as above. For fetal liver HSCs, CD11b was removed from the lineage panel and cells were further stained with Alexa Fluor® 647 anti-CD150, PE-Cy7 anti-Sca1, and APC-Alexa Fluor® 780 CD11b Samples were sorted for purity with a three laser FACS Aria-II (Beckton Dickinson).

Imaging Flow Cytometry (Amnis ImageStream)

BM cells were harvested as above and enriched for c-Kit$^+$ cells using biotinylated anti-mouse CD117 (BD Biosciences) and EasySep biotin selection kit (Stem Cell Technologies, Vancouver, BC). HSPCs were further identified by PE-Cy7 anti-Sca-1 and Alexa Fluor® anti-CD150. Flow cytometry analysis conducted in parallel confirmed that all Sca-1$^+$ CD150$^+$ cells were also c-Kit$^+$. Cells were washed in PBS, fixed and permeabilized at 4°C for 90 min, washed and blocked with 3% BSA for 30 min, followed by staining with anti-Cdc42 (EMD Millipore) for 1h. Staining was detected with a PE-conjugated F(ab')2 fragment of goat anti-rabbit IgG (Molecular Probes) in blocking buffer for 1h. Cells were counter-stained with DAPI (Life Technologies). Stained cells were washed in PBS and acquired with Amnis Imagestream Mark II imaging flow cytometer (EMD Millipore) and analyzed with IDEAS v6.1 software. A cell was considered

polarized when Cdc42 protein showed a distinctly asymmetrical distribution when a line was drawn across the middle of the cell.

Colony Assays

Single cell suspensions were prepared in IMDM containing 10% Premium FBS (Wisent Bioproducts), and cells were seeded into 35mm non-adherent petri dishes at a density of 10^4 cells/dish in methylcellulose medium containing stem cell factor, IL-3, IL-6, and erythropoietin (Methocult GF M3434; Stem Cell Technologies). The cultures were incubated at 37°C in 5% CO2 for 7–10 days and hematopoietic colonies were counted and identified based on morphology under an inverted microscope. Harvested colony forming cells were further stained with antibodies against CD11b, CD11c, CD117/c-Kit, GR1, CD45.1 and CD45.2. Stained cells were analyzed as described above.

Transplantation Assays

For competitive long-term reconstitution experiments, $5x10^5$ *Fzd6*$^{-/-}$ or *Fzd6*$^{+/+}$ BM cells (CD45.2$^+$) were mixed with $5x10^5$ competitor cells (CD45.1$^+$ or CD45.1$^+$/CD45.2$^+$) and injected into the lateral tail vein of lethally irradiated (two doses of 450 rads, 16 h apart) congenic recipient mice. For secondary transplants, an equal number ($2x10^6$) of total BM cells from two primary recipients were pooled and injected into lethally irradiated secondary recipients. For short-term analysis, $10x10^6$ donor cells were transferred without competition. To determine peripheral blood chimerism, blood samples were collected through the mandibular vein from recipient mice at 4, 8, 12 and 16 weeks and analyzed by flow cytometry. Sixteen weeks after transplantation, mice were euthanized and analyzed for reconstitution in BM and spleen.

Homing Assay

To determine the homing efficiency of hematopoietic cells, $20x10^6$ BM cells from *Fzd6*$^{-/-}$ or *Fzd6*$^{+/+}$ donors (CD45.2$^+$) were injected into sub-lethally irradiated (500 rads) CD45.1$^+$ recipient mice. Mice were euthanized and their BM harvested 16 h later for flow-cytometry and functional analysis of donor HSPCs.

Emergency hematopoiesis

To evaluate HSPC expansion during emergency hematopoiesis, *Fzd6*$^{-/-}$ and *Fzd6*$^{+/+}$ mice were given two i.p. injections of gamma-irradiated LPS (E.coli 0111:B4; Sigma-Aldrich) at a dose of 1mg/kg body weight, 48h apart (Boettcher *et al.*, 2012). Their BM was harvested 24h after the second injection for flow-cytometry and functional analysis.

Statistical analysis

Each value represents at least three independent experiments. Two-tailed student's t test was used to determine statistical significance unless otherwise noted. $P < 0.05$ was considered significant.

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CHAPTER 3

PUBLICATION NO.2

INFECTION-ADAPTED EMERGENCY HEMATOPOIESIS PROMOTES VISCERAL LEISHMANISIS

Infection-adapted emergency hematopoiesis promotes visceral *Leishmania*sis

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Contribution of the student

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1. ABSTRACT

Cells of the immune system are derived from hematopoietic stem cells (HSCs) residing in the bone marrow. HSCs become activated in response to stress, such as acute infections, which adapt the bone marrow output to the needs of the immune response. However, the impact of infection-adapted HSC activation and differentiation on the persistence of chronic infections is poorly understood. We have examined here the bone marrow outcome of chronic visceral *Leishmania*sis and show that the parasite *Leishmania donovani* induces HSC expansion and skews their differentiation towards non-classical myeloid progenitors with a regulatory phenotype. Our results further suggest that emergency hematopoiesis contributes to the pathogenesis of visceral *Leishmania*sis, as decreased HSC expansion results in a lower parasite burden. Conversely, monocytes derived in the presence of soluble factors from the infected bone marrow environment are more permissive to infection by *Leishmania*. Our results demonstrate that *L. donovani* is able to subvert host bone marrow emergency responses to facilitate parasite persistence, and put forward hematopoiesis as a novel therapeutic target in chronic infections.

2. AUTHOR SUMMARY

Hematopoietic stem cells (HSCs) are responsible for the generation of all blood cells and thus play an important but often underappreciated role in the host response to infections. HSCs are normally dormant, but they can become activated in response to stress, such as infections. This stress response is meant to generate more blood cells and help the body to eliminate the invading pathogen. We have studied here the activation of HSCs in a mouse model of chronic infection with the parasite *Leishmania donovani*. We found that the parasite efficiently activates HSCs and steers them to produce large numbers of specific blood cells that are among the preferred targets of the parasite and become even more susceptible to infection when produced within the diseased environment. Using a mouse strain in which HSC activation cannot be sustained, we found that diminished HSC activity correlated with decreased parasite numbers. We therefore propose that HSC activation by the parasite promotes the infection and could be used as a new target for treatment.

3. INTRODUCTION

Emergency hematopoiesis in response to severe bacterial infection is associated with an expansion of hematopoietic stem/progenitor cells (HSPCs) and their progeny, mediated by a combination of environmental cues, including inflammatory cytokines and microbial products (Baldridge *et al.,* 2010, Burberry *et al.,* 2014, Manz et al., 2014). Activated, proliferating HSPCs and myeloid progenitor cells (MPCs) leave the bone marrow and migrate to the spleen, liver, and other inflamed target organs where they can directly contribute to hematopoiesis (Burberry et al., 2014, Griseri et al., 2012, Haas et al., 2015b). In the case of acute bacterial infection, the resulting increase in myeloid differentiation promotes the immune response that is required for clearance of the pathogen (Baldridge *et al.*, 2010b, Manz *et al.*, 2014). Similarly, increased myelopoiesis in response to an acute viral infection can be beneficial for helping to mount the appropriate T cell responses to eliminate infected cells (Matatall *et al.*, 2014). Bone marrow homeostasis is usually restored after the infection is cleared. However, the potential impact of infection-adapted hematopoiesis on the ability of pathogens to persist and establish chronic infections has not been widely studied.

Persistent inflammatory conditions, including chronic parasitic infections such as visceral *Leishmania*sis may lead to an acquired bone marrow failure, where the HSPCs and bone marrow stroma are no longer able to support blood homeostasis (de Freitas *et al.*, 2016, Kaye *et al.*, 2011, Matnani *et al.*, 2016). The underlying mechanisms are poorly understood, but the pathology clearly suggests that the parasite infection profoundly changes normal hematopoiesis: visceral *Leishmania*sis is generally associated with hepatosplenomegaly, extramedullary hematopoiesis, pancytopenia, and immunosuppression. Previous studies using the Balb/c mouse model of infection with *Leishmania donovani* suggest that the parasite does not directly infect HSPCs or MPCs. Instead, *L. donovani* establishes a persistent infection in bone marrow macrophages, which correlates with an enhanced MPC output by bone marrow and spleen, and the production of myeloid growth factors (Cotterell *et al*., 2000a, Cotterell *et al*., 2000b, Kaye *et al.*, 2011). However, the contribution of enhanced myelopoiesis to the course of infection is not well understood.

HSPC activation corresponds to changes in Wnt signaling activity, with the various intracellular signaling pathways promoting either activation or quiescence (Abidin et al.,

2015, Staal et al., 2016b, Sugimura et al., 2012). We have previously shown that the absence of the Wnt signaling receptor Frizzled6 (*Fzd6*) results in defective stem cell self-renewal, completely abrogating their ability to reconstitute an irradiated host after transplant, and dampens HSPC expansion during LPS-induced emergency hematopoiesis (Abidin *et al*., 2015). Wnt signaling generally contributes to the establishment of T cell memory and regulates effector T cell responses (Gattinoni *et al.*, 2009, Zhou *et al.*, 2010) and leukocyte trafficking (Chae *et al.*, 2016), but its role in inflammation-induced myelopoiesis and the regulation of chronic infections is not known.

We show here that experimental *L. donovani* infection induces the expansion of hematopoietic stem cell (HSC)-like cells and $Sca1^{+}$ emergency MPCs in the bone marrow. The myeloid progeny of these emergency MPCs consists predominantly of $Ly6C^{hi}$ monocytes with a regulatory, suppressor cell-like phenotype. We further demonstrate that the expansion is functionally important, as monocytes generated in the presence of soluble factors extracted from the infected bone marrow are more permissive to infection, and a stunted emergency response such as seen in *Fzd6*$^{-/-}$ mice results in decreased parasite burden. Collectively our results support the hypothesis that *Leishmania* subverts the host bone marrow emergency response to promote its own proliferation and to allow for continued persistence of the infection.

4. RESULTS

4.1. L. donovani induces the expansion of HSC-like cells in the bone marrow and spleen

L. donovani infection results in enhanced myelopoiesis in the bone marrow and spleen of Balb/c mice (Cotterell *et al*., 2000a, Cotterell *et al*., 2000b); however, it is unclear which HSPCs the parasite targets and what functional consequences stem from the increased myeloid output. We initially followed the progression of *L. donovani* infection in the bone marrow of mice on C57Bl/6 background and observed a sharp increase in parasite burden beginning in the third week after infection (Fig. 1A). In parallel with parasite burden, the proportion and number of bone marrow $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ (LSK) cells and $CD150^{+}CD16/CD32^{-}$LSKs, which correspond to an HSC-like phenotype in uninfected mice, also increased, reaching a plateau between day 21 and day 28, depending on the strength of the infection (Fig. 1B, 1C, S1). A similar expansion was also observed in the spleen: both LSKs and HSC-like cells were virtually undetectable in the naïve spleen, but their numbers continued to augment in infected mice through day 35 (Fig. 1D, S1). These results indicate that the most immature HSPCs are indeed affected by infection with *L. donovani,* and that this effect persists in time.

Adult HSCs are usually dormant in the bone marrow, with more than two thirds of the cells residing in a quiescent state in the G0 phase of the cell cycle. However, they become readily activated under stress or in response to inflammatory cytokines (Baldridge et al., 2010b, Pietras et al., 2016, Wilson et al., 2008). Chronic infection with *L. donovani* induced HSC-like cells to enter cell cycle, resulting in a gradual loss of quiescent cells (Fig. 1E). This was accompanied by differentiation, as the proportion of $CD150^{+}$ HSC-like cells that had acquired CD48 and thus represented multipotent progenitors with myeloid bias (Kiel et al., 2005, Wilson et al., 2008) also increased from 25% to 75% (Fig. 1F, 1G); however, there was a significant expansion of the $CD48^{-}$ population as well. HSC activation and cell cycle entry have been shown to correlate with the induction of β-catenin-dependent Wnt signaling in non-infectious settings (Sugimura et al., 2012). We observed a significant increase in intracellular levels of active β-catenin that was specific for $CD150^{+}$ HSC-like cells (Fig. 1H), suggesting that Wnt signaling could contribute to regulating *L. donovani*-induced HSC expansion.

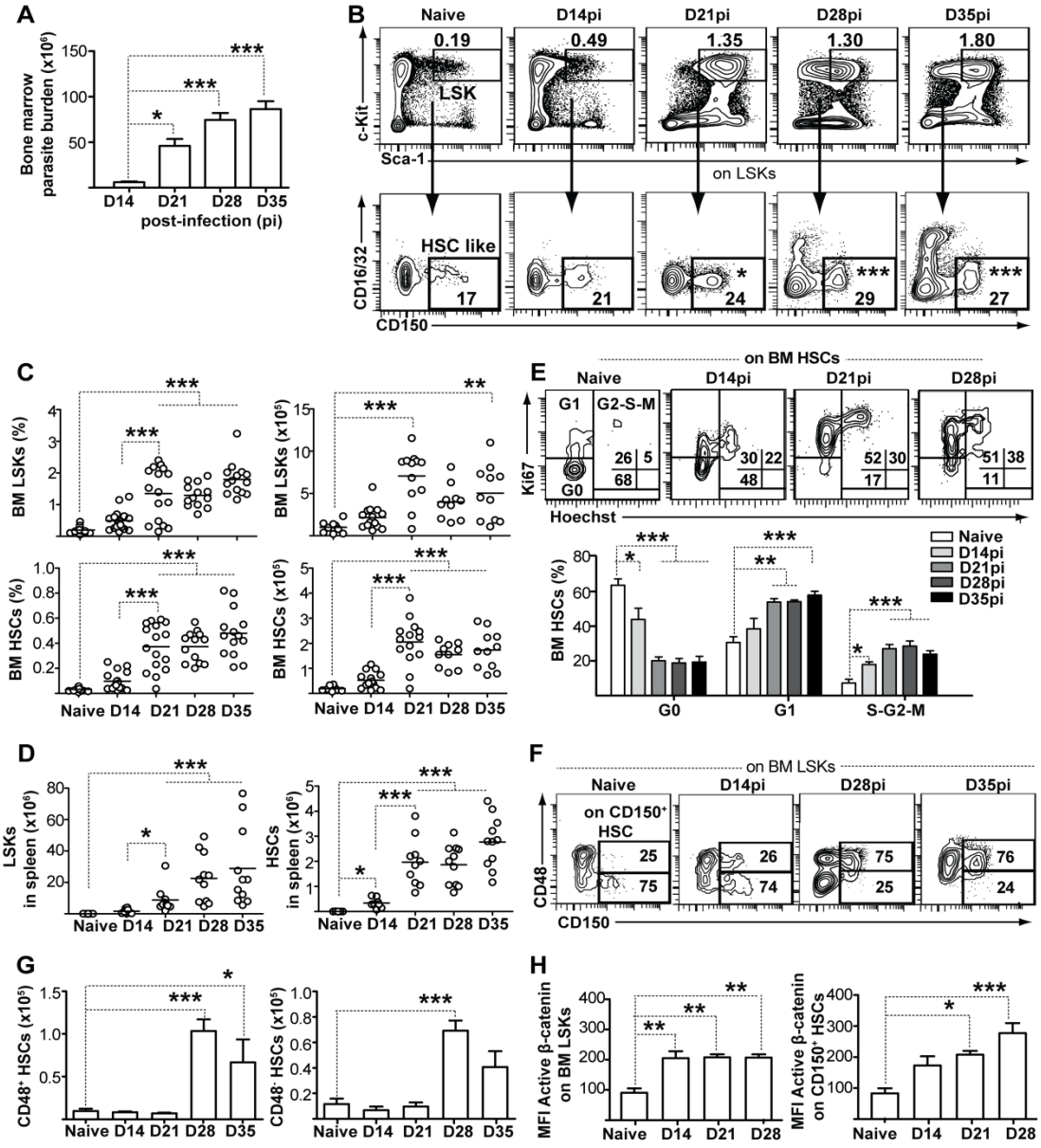

Figure 1. Parasite expansion coincides with proliferation and accumulation of bone marrow hematopoietic stem/progenitor cells

(A) Bone marrow parasite burden was assessed using the serial limiting dilution technique. Graph shows parasite burden per one femur and one tibia. (B) Representative flow cytometry data and gating strategy of BM HSPCs. BM cells were first gated on Lin$^-$ (B220$^-$CD3e$^-$CD11b$^-$GR1$^-$Ter119$^-$) and identified according to Sca1 and cKit (CD117) expression. HSCs were defined as CD150$^+$ CD16/CD32$^-$ within the Lin$^-$ cKit^{hi} Sca1$^+$ (LSK) population. Numbers within flow cytometry plots represent mean LSK percentage within total bone marrow and mean HSC percentage within LSKs. (C) Histograms show percentage and absolute numbers of LSKs and HSCs per two femora and two tibiae. Data are pooled from three independent experiments, with each individual dot representing one mouse. Horizontal lines represent the sample mean. (D) Absolute numbers of LSKs and CD150+ HSCs in spleens of infected mice. See also Fig. S1. (E) Ki-67/Hoechst co-staining was used to distinguish the G0, G1, and S/G2/M cell cycle phases of CD150+ HSC-like cells during infection. (F) Analysis of CD48 expression within BM CD150$^+$ HSC-like cells. Numbers within flow cytometry plots represent CD48+ and CD48- HSC subsets within CD150$^+$ HSC like cells. (G) Graphs depict the numbers of cells for the two subsets at various time points after

infection. (H) Intracellular active β-catenin levels (MFI) within CD150+ HSC-like cells. See also Fig. S1. All bar graphs represent mean + SEM with 4 mice per group coming from one single infection. Similar results were obtained in two additional independent experiments. *P<0.05; **P<0.01; ***P<0.001.

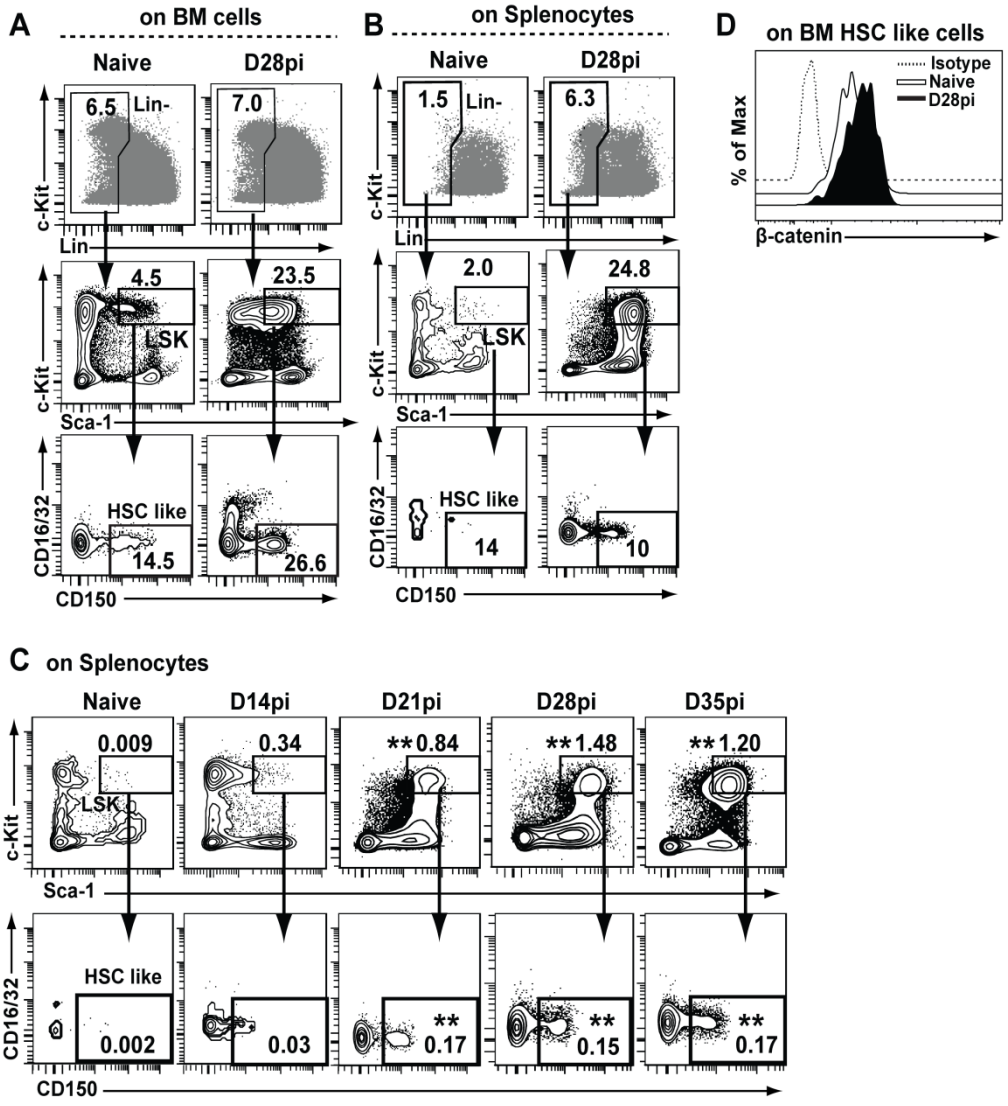

Figure S1. Analysis of hematopoietic stem/progenitor cells in the spleen (Related to Fig 1**).**

(A-B) Representative flow cytometry plots and gating strategy for LSKs and HSCs in the bone marrow and spleen. Numbers represent the percentage in each population for one individual mouse on day 28. (C) Representative flow cytometry plots and gating strategy for LSKs and HSCs in spleens of infected mice at various time points during infection. Mean percentage for each cell subset is indicated in histograms. (D) Intracellular β-catenin expression on bone marrow HSC cell like subsets in naïve and infected mice at D28pi.

4.2. Induction of myelopoiesis during *L. donovani* infection results in the generation of altered progeny with a regulatory phenotype

*Leishmania*sis is accompanied by an increase in circulating monocytes, and *L. donovani* induces the expansion and export to spleen of MPCs in Balb/c mice (Cotterell *et al.*, 2000b, Mirkovich *et al.*, 1986, Passos *et al.*, 2015). To better define the kinetics and the types of progenitors that were responding to the infection, we analyzed the bone marrow MPC compartment and observed that the number and proportion of granulocyte-monocyte progenitors (GMPs; CD16/CD32^{+}cKit^{+}CD41^{-}CD150^{-}Lin^{-}) remained stable over time (Fig. 2A, 2B, S2). There was no specific change in the proportion of actively cycling GMPs (Fig. 2C, S2), and in contrast to HSC-like cells, the increase in active β-catenin levels was very modest (Fig. 2F). However, there was a striking upregulation of Sca1 expression on GMPs (Fig. 2D, S2), which translated into a decline in the numbers of Sca1^{-}GMPs that are normally associated with steady-state hematopoiesis. The steady increase in the numbers of Sca1^{+} "emergency" GMPs (Fig. 2E; Buechler *et al*., 2013) together with the stable levels of total GMPs (Fig. 2A, 2B) suggested that the major change brought about by *L. donovani* did not impact as much the numbers of MPCs as the type of progeny they generated. B lymphopoiesis was nearly completely abrogated at later stages (Fig. S3), indicative of profound alterations to the bone marrow environment.

GMPs as a population give rise to both granulocytes (mostly neutrophils) and monocytes. During resting hematopoiesis, approximately half of the bone marrow myeloid cells are granulocytes (SSC^{hi}GR1^{hi}Ly6G^{+}), with the remaining half divided into Ly6C^{hi} monocytes ready to enter the circulation and a Ly6C$^{lo/-}$ population that comprises alternative monocytes and macrophages in addition to immature stages of both monocytes and granulocytes. Ly6C^{hi} monocytes represented the only myeloid cell type whose proportion steadily increased over time in the infected marrow (Fig. 3A). On day28, we observed infected cells with monocyte as well as myeloblast morphologies in bone marrow smears (Fig. 3B). All monocytes contained a single parasite, while the myeloblast-like cells, similar to mature macrophages, could be found to house several. These latter, productively infected cells could represent either immature myeloid cells or potentially an intermediate stage in monocyte maturation towards a more macrophage-like morphology.

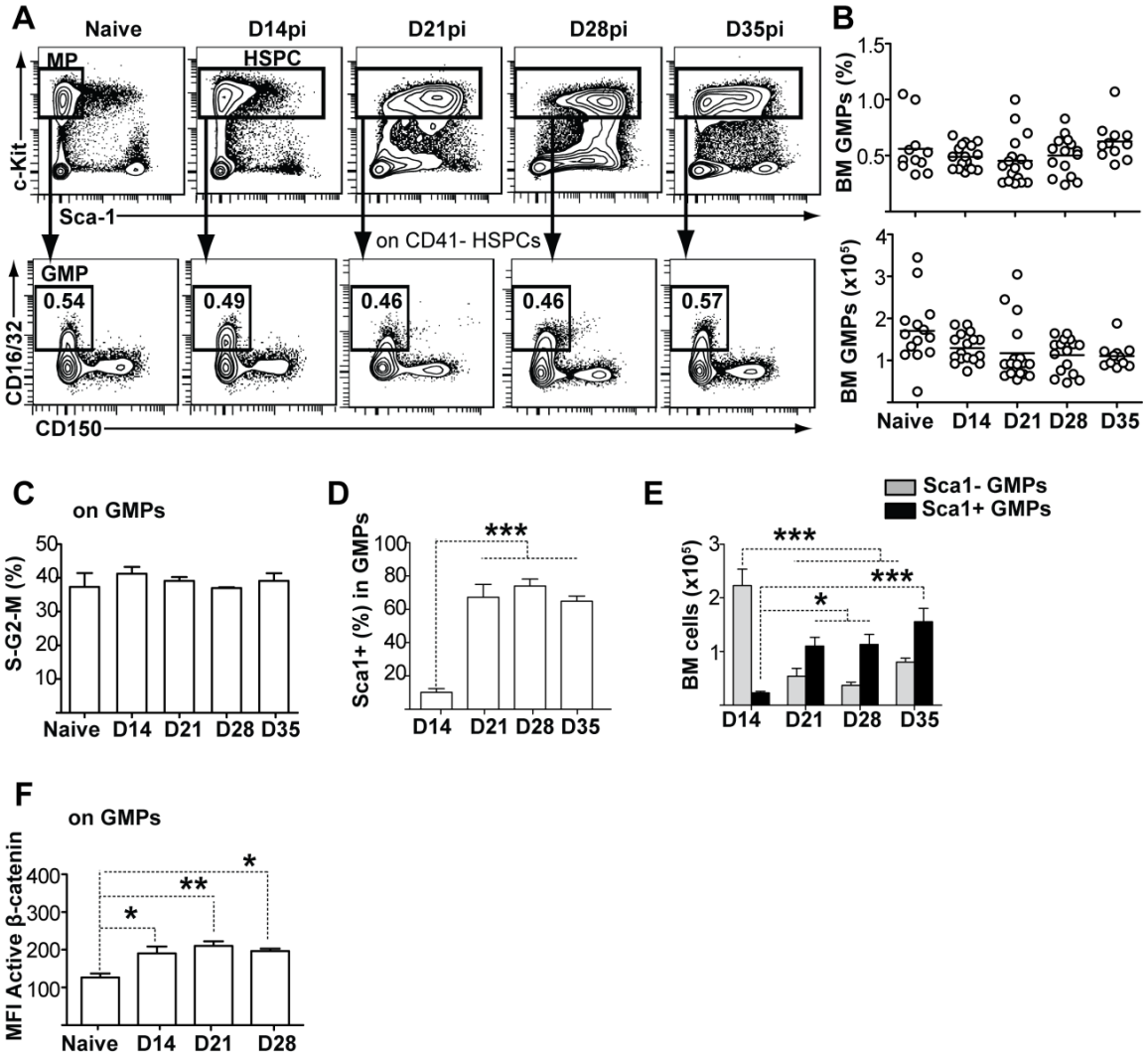

Figure 2. Bone marrow HSCs switch their differentiation towards non-classical myeloid progenitors

(A) Representative flow cytometry data and gating strategy of granulocyte-monocyte progenitors (GMPs) in the bone marrow. Steady state myeloid progenitor (MP) cells were first gated on Lin$^-$Sca1$^-$c-kit^{hi} and then subdivided according to the expression of CD41, CD150 and CD16/CD32. GMPs were identified as CD16/CD32$^+$ CD41$^-$ CD150$^-$. Due to the inflammation-induced shift in Sca1 expression, the total Lin$^-$ c-Kit^{hi} HSPC population was included for analysis during infection. See also Fig. S2. (B) Graphs show percentage and absolute numbers of GMPs at various time points. Data are pooled from three independent experiments, with each individual dot representing one mouse. Horizontal lines represent the sample mean. (C) Percentage of GMPs in S/G2/M phases of cell cycle. (D) Percentage of Sca-1$^+$ emergency GMPs within all GMPs. (E) Numbers of cells within Sca-1$^+$ and Sca-1$^-$ GMP subsets. (F) Intracellular active β-catenin levels (MFI) in GMPs.

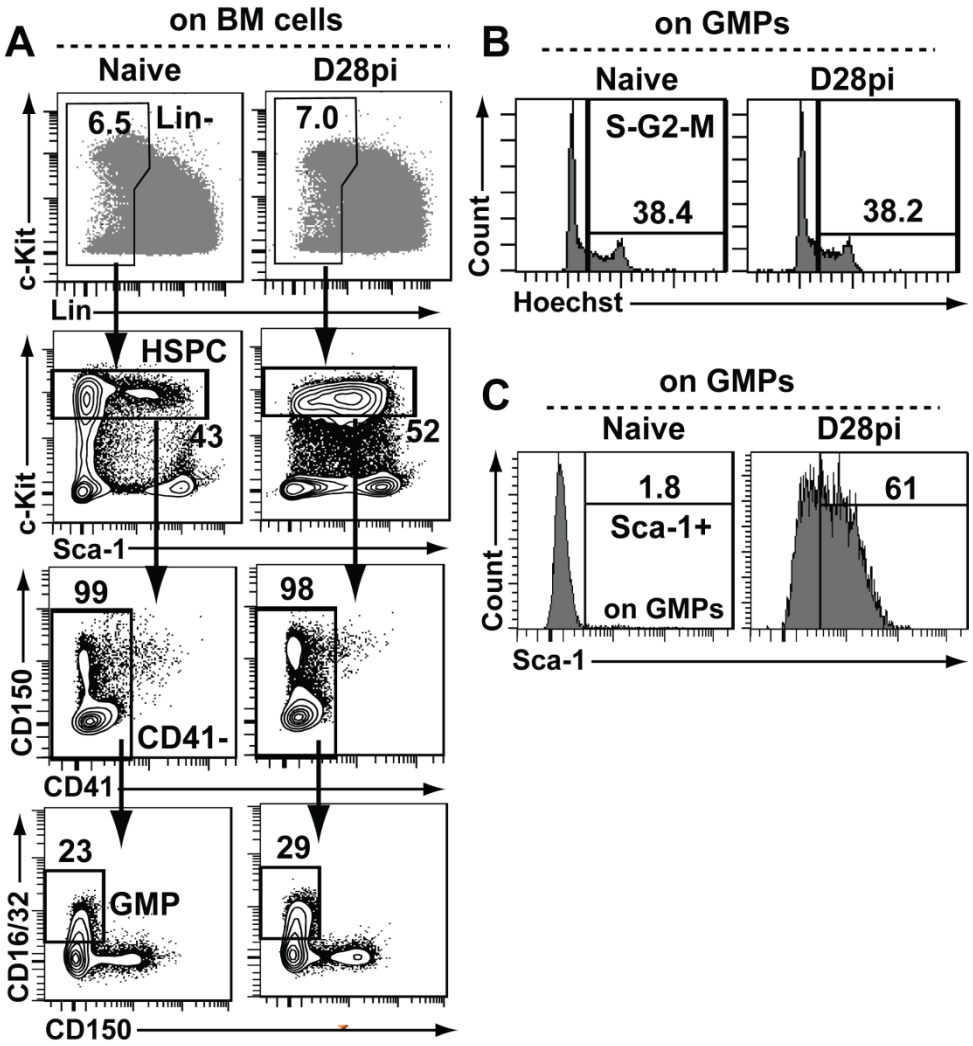

Figure S2. Analysis of myeloid progenitors in the bone marrow (Related to Figure 2)

(A) Representative flow cytometry plots and gating strategy for granulocyte-monocyte progenitors (GMPs) in the bone marrow of naïve and infected mice at D28pi. Numbers represent the percentage in each population for one individual mouse. (B) Representative histograms for cell-cycle analysis on bone marrow GMPs. (C) Representative flow cytometry data showing Sca-1 expression on GMPs in naïve and infected mice at D28pi.

To better evaluate monocyte differentiation and maturation, we sought to determine if the newly generated monocytes had acquired an altered, regulatory phenotype, similar to what has been reported in response to trypanosomal infection (Askenase *et al.*, 2015). Ly6C^{hi} monocytes rapidly acquired Sca1 expression, similar to the emergency GMPs (Fig. 3C, S3). In comparison, only a small percentage of Ly6C$^{lo/-}$ cells upregulated Sca1. Ly6C^{hi} monocytes also upregulated MHCII (Fig. 3C, S3) and down-regulated Ly6C expression (Fig. 3D),which suggested that they had been exposed to IFN-γ (Askenase *et al.*, 2015). To further support the hypothesis of monocyte developmental skewing, the Ly6C^{hi} monocytes generated in *L. donovani* –infected mice also upregulated Galectin-3 (Fig. 3D, S3), which is associated with alternative macrophage activation (MacKinnon *et al.*, 2008), IL-10 production (Chung *et al.*, 2013), and pro-fibrotic responses. These data collectively show that *L. donovani* subverts bone marrow hematopoiesis, enhancing the generation of emergency GMPs and the differentiation of monocytes that, due to their regulatory or immature phenotype, could represent safe targets and promote parasite expansion (Mirkovich *et al.*, 1986).

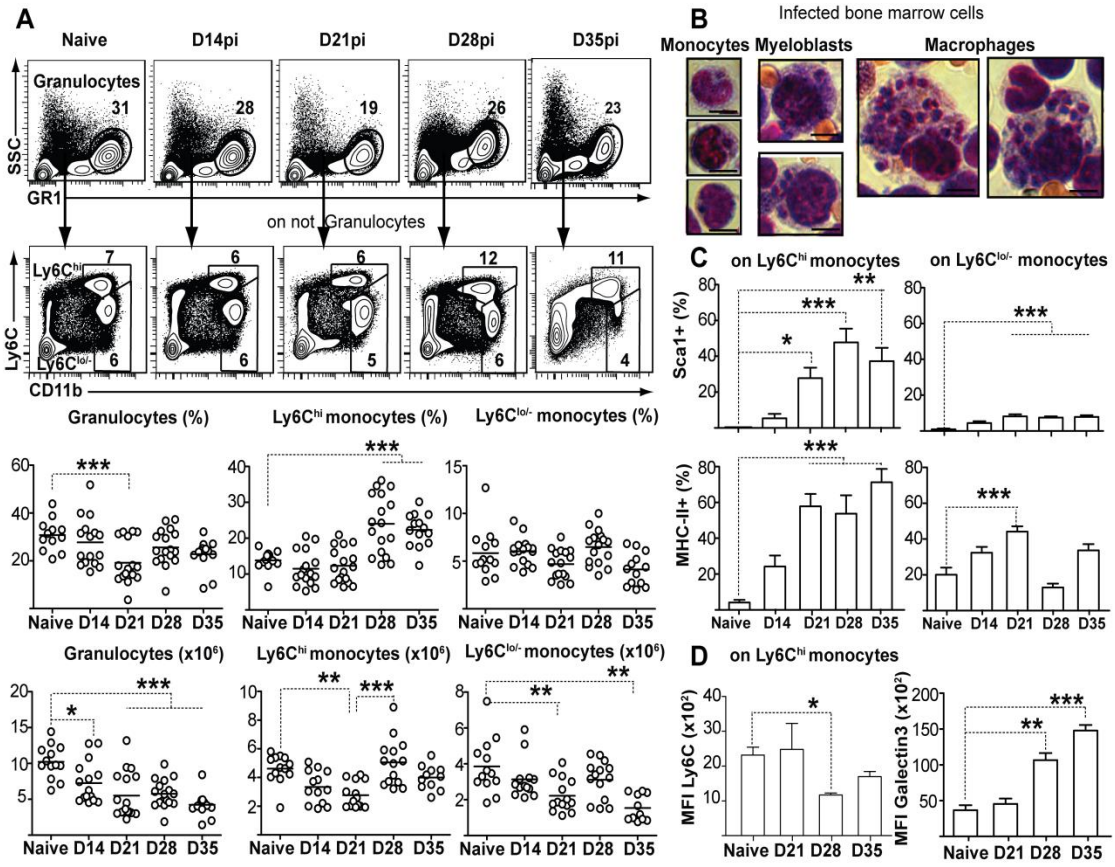

Figure 3. *Leishmania* parasite expansion promotes myeloid output in the bone marrow

(A) Representative flow cytometry data to demonstrate gating strategy for myeloid cell subsets in the bone marrow. Graphs show percentage and numbers of granulocytes ($GR1^{hi}$ SSC^{hi}), mature monocytes ($Ly6C^{hi}CD11b^{+}$) and remaining immature/resident myelo-monocytes ($Ly6C^{lo/-}$ $GR1^{lo/-}$ $CD11b^{+}$). Data are pooled from three independent experiments, with each individual dot representing one mouse. Horizontal lines represent the sample mean. See also Figure S3. (B) Giemsa-stained infected bone marrow monocytes, myeloblast-like cells and macrophages bearing intracellular *Leishmania* amastigotes (100 X under oil immersion lens). Scale bar=5µm. (C) Sca-1 and MHC-II expression on $Ly6C^{hi}$ and $Ly6C^{lo/-}$ monocyte subsets. (D) Galectin and Ly6C expression (MFI) on $Ly6C^{hi}$ monocytes. All bar graphs represent mean + SEM with 4 mice per group coming from one single infection. Similar results were obtained in a second, independent experiment. *P<0.05; **P<0.01; ***P<0.001.

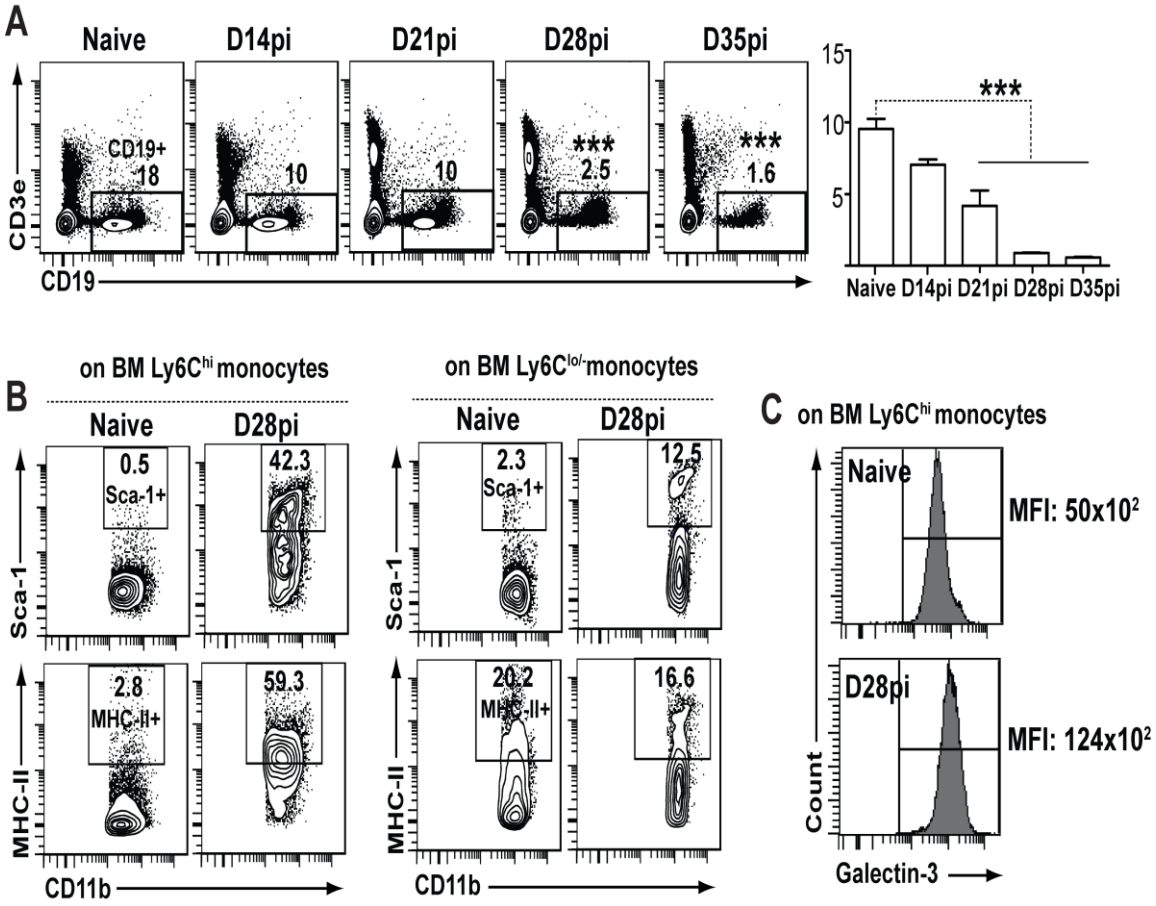

Figure S3. Analysis of bone marrow B cell and myeloid cells during infection (Related to Figure 3).

(A) Representative flow cytometry plots and gating strategy for $CD19^+$ B cells in the bone marrow of infected mice. Data represent mean + SEM from four mice at each time point. Similar results were obtained in a second, independent experiment. *P<0.05; **P<0.01; ***P<0.001. (B) Representative flow cytometry data for MHC-II and Sca-1 expression on bone marrow $Ly6C^{hi}$ and $Ly6C^{lo/-}$ monocytes. (C) Representative flow cytometry data for Galectin-3 expression on bone marrow $Ly6C^{hi}$ monocytes.

4.3. *Fzd6* promotes bone marrow response to *L. donovani*

We have previously shown that the absence of the Wnt signaling receptor Frizzled6 (*Fzd6*) dampens HSC expansion during LPS-induced emergency hematopoiesis (Abidin *et al.*, 2015b). Given that Wnt signaling was induced in *L. donovani* –activated HSCs (Fig. 1H), we sought to evaluate if *Fzd6* was also important for the bone marrow response to the parasite, and if we could use *Fzd6*-deficient mice as a tool to investigate the importance of the bone marrow response in promoting parasite expansion. There was no significant difference in bone marrow LSK or HSC numbers between *Fzd6*$^{-/-}$ and *Fzd6*$^{+/+}$ mice on day 14, before the expansion (Fig. 4A) or on day 21 (Fig. S4). However, expansion was blunted in *Fzd6*$^{-/-}$ mice on day 28 post-infection (Fig. 4A), which corresponds to the peak of expansion (Fig. 1G). The difference in HSPC accumulation was not due to decreased proliferation, as *Fzd6*$^{-/-}$ HSCs entered the cell cycle at least as efficiently as their *Fzd6*$^{+/+}$ counterparts (Fig. 4B). If anything, there was a decrease in the proportion of quiescent HSCs in *Fzd6*$^{-/-}$ bone marrow on day 28 (Fig. 4B), correlating with their previously reported self-renewal defect (Abidin *et al*., 2015), and indicating that the *Fzd6*$^{-/-}$ bone marrow responded to the infection. However, the response did not result in as great an accumulation of HSPCs. There was also no significant difference in β-catenin activation (Fig. 4C), similar to what we had previously reported at steady state (Abidin *et al*., 2015). These results suggested that *Fzd6*$^{-/-}$ mice could provide a model to investigate the impact of *L. donovani* –adapted hematopoiesis on parasite expansion.

Fzd6$^{-/-}$ mice display no specific defects in the bone marrow MPC compartment at steady state (Fig. S5) or on day 14 post-infection, prior to the parasite-induced changes in hematopoiesis (Fig. 4D, 4E). However, on day 28 we observed a two-fold decrease in GMPs (Fig. 4D) and more specifically in Sca1^{+} emergency GMPs (Fig. 4E). Similar to HSCs, there was no difference in cell cycle (Fig. 4F) or β-catenin activity (Fig. 4G) between *Fzd6*$^{-/-}$ and *Fzd6*$^{+/+}$ GMPs; nor was GM-CSF receptor expression altered (Fig. S6). We also evaluated the presence of common monocyte precursors (cMoPs; Lin^{-} CD115^{+}cKit^{hi}Flt3^{-}Ly6C^{+}CD11b^{-}, Fig. S5), and found that the number of cMoPs was slightly higher in *L. donovani* –infected bone marrow when compared to uninfected controls (Fig. 4H). cMoPs acquired Sca1 expression in the infected marrow, similar to GMPs and Ly6C^{hi} monocytes (Fig. 2D, 2E, 3C). However, *Fzd6*$^{-/-}$ cMoPs were not expanded in response to *L. donovani* and a lower proportion had acquired Sca1 (Fig. 4H). Myeloid colony formation in culture was also decreased, with HSPCs isolated from

infected *Fzd6*$^{-/-}$ bone marrow generating 3-times fewer CD11b^{+} myeloid cells than *Fzd6*$^{+/+}$ controls (Fig. 4I). Together these data show that the presence of *Fzd6* promotes parasite-induced HSPC expansion and skewing towards an emergency phenotype.

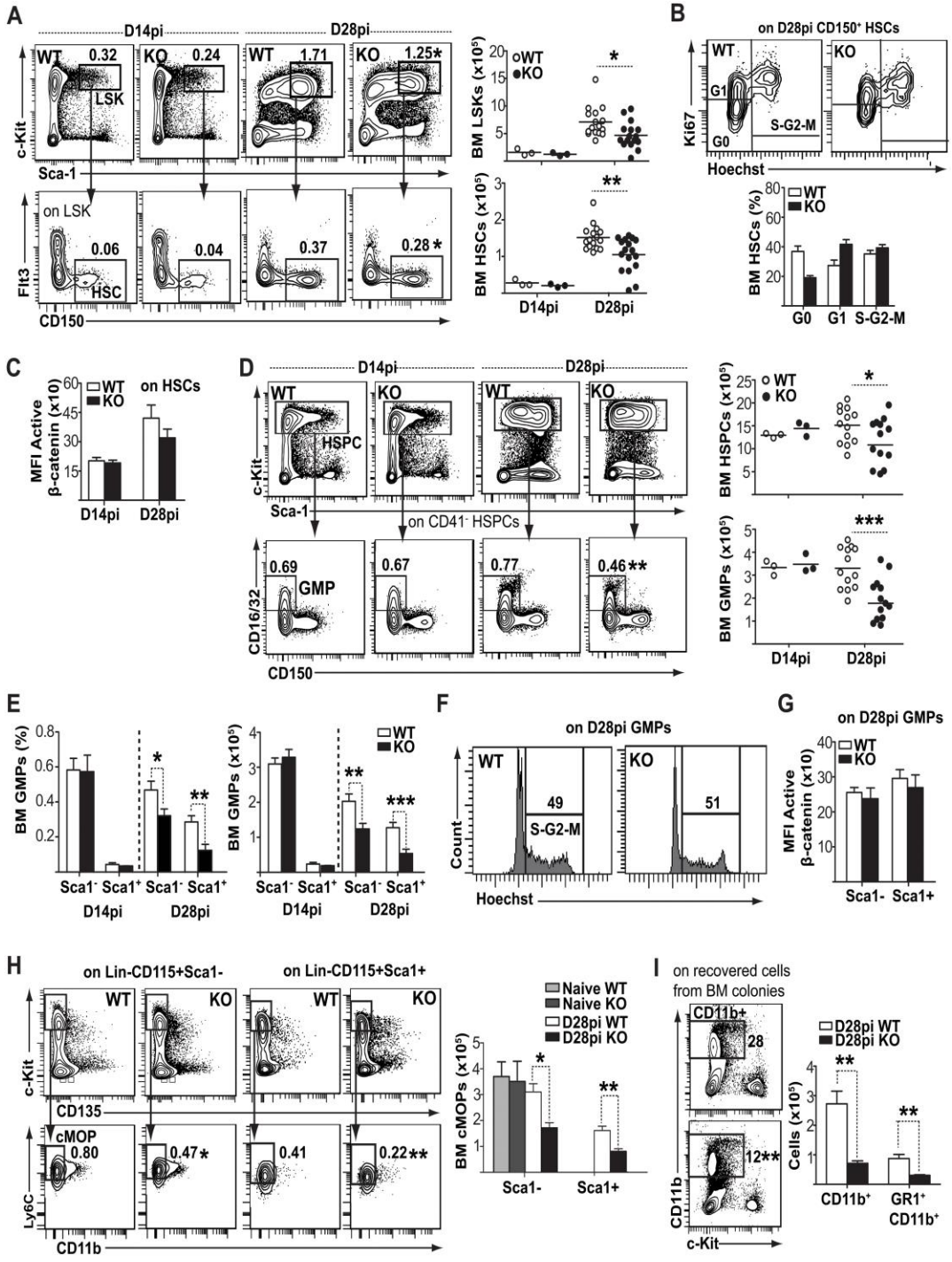

Figure 4. Frizzled-6 is required for parasite-induced expansion and myeloid differentiation of HSPCs

(A) Analysis of bone marrow LSK and HSC compartments in infected *Fzd6*$^{-/-}$ (KO) and *Fzd6*$^{+/+}$ (WT) mice. Mean percentage for LSKs and HSC-like cells within total bone marrow are indicated in the corner of each histogram. Graphs show the numbers of LSKs and HSCs on days 14 and 28. Day 28 data are pooled from three independent experiments, with each individual dot representing one mouse. Horizontal lines represent the sample mean. (B) Representative flow-cytometry plots for cell cycle analysis of WT

and KO HSCs on day 28. Graph shows percent CD150$^+$ HSC like cells in the G0, G1 and S-G2-M phases of cell cycle. (C) Intracellular active β-catenin levels (MFI) in WT and KO CD150$^+$ HSCs on days 14 and 28. (D) Flow cytometry analysis of BM GMPs. Numbers in the histograms represent mean percentage for WT and KO GMPs. Graphs show the numbers of HSPCs and GMPs at day 14 and 28. Day 28 data are pooled from three independent experiments, with each individual dot representing one mouse. See also Figures S4, S5, S6. (E) Percentage and numbers of Sca-1$^-$ and Sca-1$^+$ GMPs on day 14 and 28 pi. (F) Cell cycle analysis of WT and KO GMPs on day 28. Similar results were obtained from six individual mice for each group. (G) Intracellular active β-catenin levels (MFI) in different GMP subsets on day 28. (H) Gating strategy and representative flow cytometry plots for common monocyte progenitors (cMoPs). BM cells were first gated on Lin$^-$ (CD3e$^-$B220$^-$NK1.1$^-$Ly6G$^-$) CD115$^+$ and then subdivided according to Sca1. cMoPs were identified as cKit$^+$CD135$^-$Ly6C$^+$CD11b$^-$ within Lin$^-$ CD115$^+$ cell populations. Mean percentage for cMoPs within total bone marrow is depicted in flow cytometry plots, and the graph shows absolute numbers (mean + SEM from six mice per group). To note, there were no Sca1$^+$ cMoPs in naïve mice. (I) Flow cytometry analysis of cells recovered from myeloid colony forming assays. Numbers shown in different quadrants indicate the mean percentage in CD11b$^+$ cells. Histogram represents pooled data from one single infection for a total of five mice. Similar results were obtained in a second, independent experiment. All bar graphs represent mean + SEM with 10 mice per group for day 28 pooled from two independent experiments and 3 mice per group for day 14 unless otherwise noted. *P<0.05; **P<0.01; ***P<0.001.

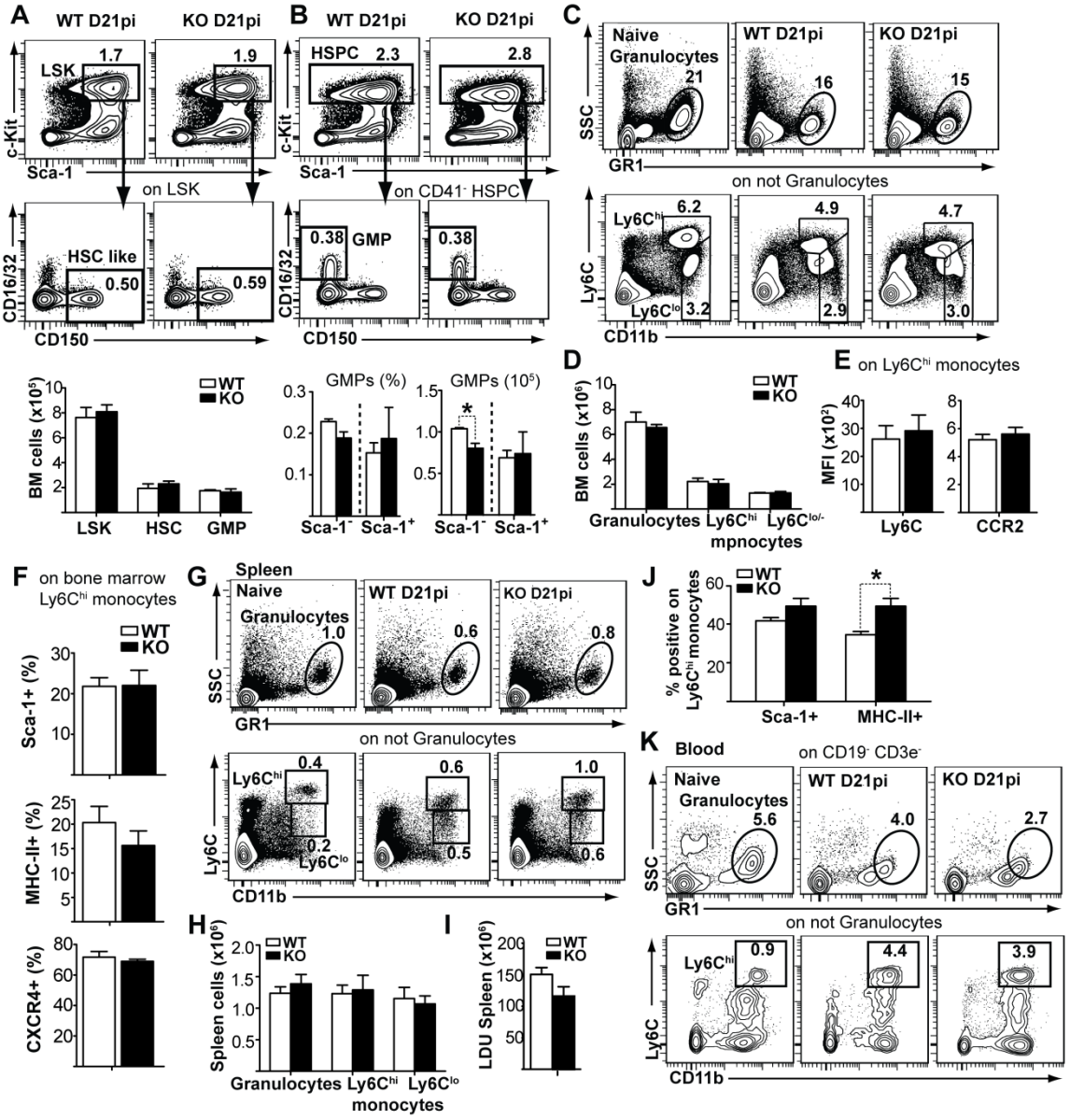

Figure S4. Analysis of emergency hematopoiesis in infected *Fzd6*$^{-/-}$ (KO) and *Fzd6*$^{+/+}$ (WT) mice on day 21 (Related to Figure 4 and Figure 5)

Analysis of (A-F) bone marrow and (Choi *et al.*) splenic HSPCs and myeloid cell subsets in infected *Fzd6*$^{-/-}$ (KO) and *Fzd6*$^{+/+}$ (WT) mice on day 21 post-infection. (A) Flow cytometry data for LSK and HSC compartments in the bone marrow. (B) Flow cytometry analysis of BM GMPs. Numbers in the histograms represent mean percentage. (C) Analysis of bone marrow myeloid subsets in the bone marrow of infected KO and WT mice. (D) Graph shows numbers of granulocytes and monocytes in the bone marrow. (E) Ly6C and CCR2 expression (MFI) on bone marrow Ly6C^{hi} monocytes. (F) Percentage of Sca-1^{+}, MHC-II^{+} and CXCR4^{+} cells within bone marrow Ly6C^{hi} monocytes. (G) Flow cytometry analysis of myeloid cells in the spleen. (H) Numbers of myeloid cell subsets in the spleen. (I) Parasite burden in the spleen. (J) Percentage of Sca-1^{+} and MHC-II^{+} cells within Ly6C^{hi} monocytes in the spleen. (K) Flow cytometry data for myeloid cell subsets in the blood. Numbers indicate mean percentage of

granulocytes and Ly6C^{hi} monocytes. All bar graphs represent mean + SEM with 3 mice per group for day 21pi coming from one single infection. *P<0.05; **P<0.01; ***P<0.001.

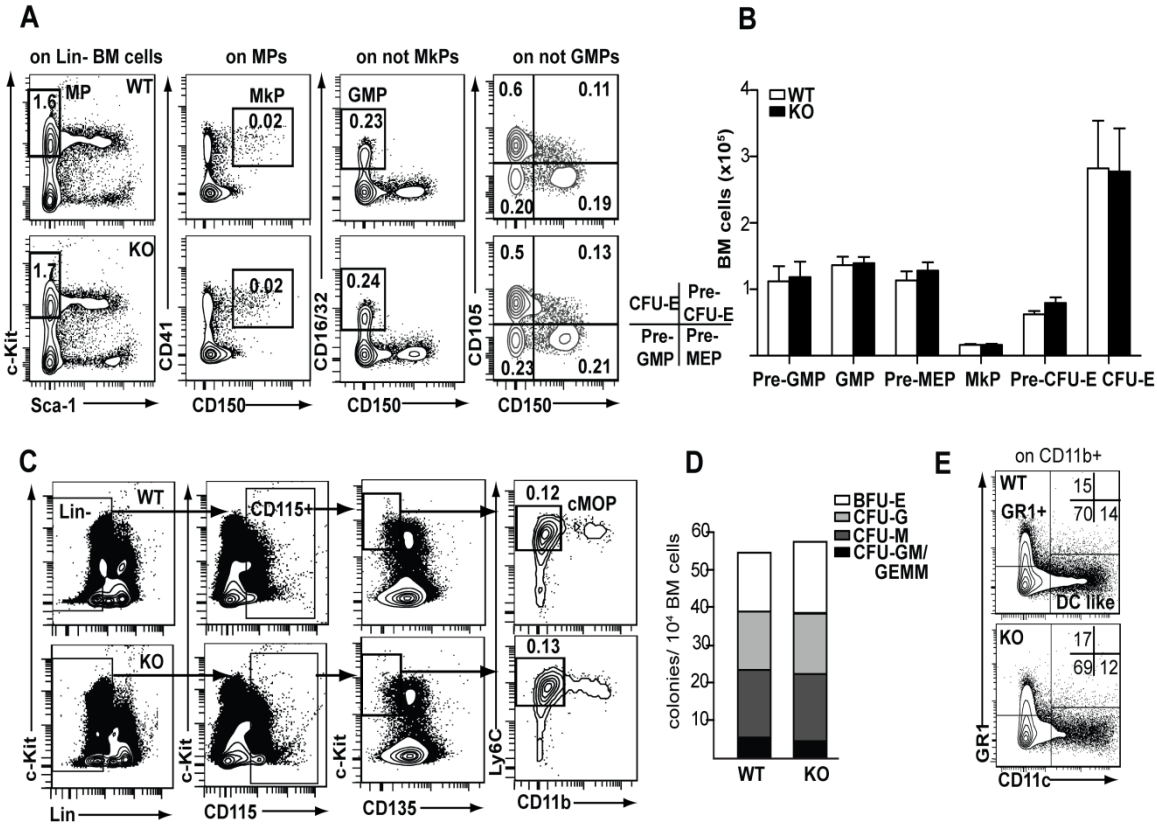

Figure S5. Steady-state myelopoiesis does not require *Fzd6* in the bone marrow (Related to Figure 4)

(A) BM cells were first gated on Lin$^-$ (B220$^-$CD3e$^-$CD11b$^-$GR1-Ter119$^-$) and subdivided according to the expression of CD41, CD150, CD16/32 and CD105 as depicted in the representative FACS plots: CD41$^+$CD150$^+$, Megakaryocyte progenitors (MkP); CD16/32^{hi}, CD150$^-$, granulocyte-monocyte progenitors(GMP); CD105$^+$CD150$^+$, pre-CFU-E; CD105$^+$ CD150$^-$, CFU-E; CD105$^-$CD150$^+$, Megakaryocyte-erythrocyte progenitors (MEP); CD105$^-$ CD150$^-$ CD41$^-$CD16/32$^-$, pre-GMP (or CMP). (B) Total numbers for different myeloid progenitor subsets per bone marrow. (C) Representative flow cytometry data and mean percentages of cMOPs in naïve *Fzd6*$^{+/+}$ and *Fzd6*$^{-/-}$ bone marrow. (D) Colony forming ability of *Fzd6*$^{+/+}$ and *Fzd6*$^{-/-}$ bone marrow cells. Colonies were classified as erythroid (BFU-E), granulocyte (CFUG), macrophage (CFU-M), granulocyte-macrophage (CFU-GM) and granulocyte/erythroid/megakaryocyte/ macrophage (CFU-GEMM) according to size and morphology on day 12. (E) Flow cytometry analysis of cells recovered from CFU assays. Numbers shown in different quadrants indicate the mean percentage in CD11b$^+$ cells. All histograms represent pooled data from at least three independent experiments for a total of at least five mice per group.

4.4. Enhanced myelopoiesis correlates with increased parasite burden

To further investigate the impact of HSPC expansion on downstream myeloid differentiation during infection, we evaluated the acquisition of regulatory markers by *Fzd6*$^{-/-}$ Ly6C^{hi} monocytes. There was no difference in the proportion of granulocytes (SSC^{hi} GR1^{hi}/Ly6G^{+}), mature Ly6C^{hi} monocytes, or Ly6C^{lo} monocytes/macrophages at steady state (Fig. S7) or on day 14 (Fig. 5A) or day 21 (Fig. S4). However, there was a specific decrease in the output of both GR1^{hi} and Ly6C^{hi} myeloid cells (Fig. 5A) and F4-80^{+} CD11b^{int} macrophages (Fig. 5B) in the *Fzd6*$^{-/-}$ bone marrow on day 28. In contrast to *Fzd6*$^{+/+}$ cells, *Fzd6*$^{-/-}$ Ly6C^{hi} monocytes did not downregulate Ly6C or Ccr2 (Fig. 5C, S8) and expressed higher levels of Cxcr4 (Fig. 5D, S8). Ccr2 and Ly6C downregulation are generally associated with monocyte differentiation into macrophages (Morias *et al.*, 2015, Phillips *et al.*, 2005, Zhou *et al.*, 1999), while Cxcr4 expression has been recently shown to correspond to a transitional stage between cMoPs and mature monocytes (Chong *et al.*, 2016). Together, these data suggest that *Fzd6*$^{-/-}$ Ly6C^{hi} monocytes were more immature and perhaps less likely to differentiate locally into macrophages.

Both *Fzd6*$^{-/-}$ and *Fzd6*$^{+/+}$ Ly6C^{hi} monocytes expressed elevated Sca1 and MHCII (Fig. 5D, S8), indicative of an inflammatory microenvironment. In further support of their potential regulatory function, Ly6C^{hi} monocytes from both groups upregulated Arginase1 expression, and expressed IL-10 (Fig. 5E, S8). Furthermore, there was no upregulation of iNOS by Ly6C^{hi} monocytes or F4-80^{+} bone marrow macrophages from either *Fzd6*$^{-/-}$ or *Fzd6*$^{+/+}$ mice (Fig. 5F, S8). Thus, *Fzd6*$^{-/-}$ Ly6C^{hi} monocytes are produced in lower numbers, presumably due to decreased expansion of upstream progenitors, but they do not significantly differ in the expression of activation markers, such as MHCII, Sca1, or iNOS. However, *Fzd6*$^{-/-}$ mice present with lower parasite burden in the bone marrow (Fig. 5G), suggesting that the decreased production of “safe targets” in the form of Ly6C^{hi} monocytes might be sufficient to dampen parasite expansion, independent of their functional capacity. In further support of this hypothesis, we established a statistical correlation between bone marrow parasite burden and the number of CD150^{+} HSCs (Fig. 5H) as well as bone marrow Ly6C^{hi} monocytes (Fig. 5I), independent of mouse genotype or the stage of infection (D14-D28).

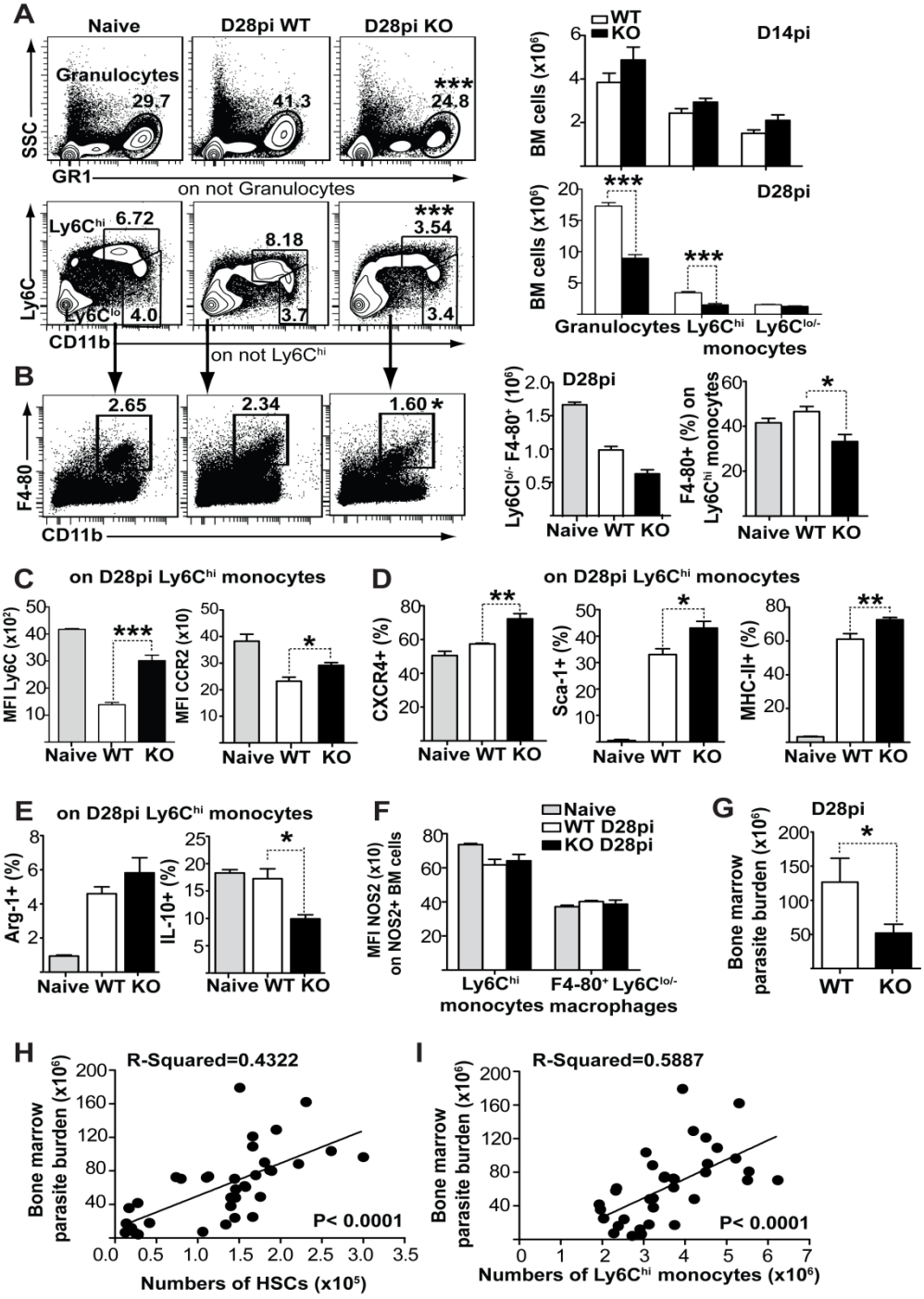

Figure 5. Diminished myeloid output in *Fzd6*$^{-/-}$ mice correlates with a reduced parasite burden during the chronic phase of infection.

(A) Analysis of bone marrow myeloid subsets infected *Fzd6*$^{-/-}$ (KO) and *Fzd6*$^{+/+}$ (WT) mice. Mean percentage for each cell subset is indicated within flow cytometry plots. Graphs show numbers of granulocytes and monocytes on day 14 and 28. See also Figures S4, S6, S7. (B) Numbers within flow cytometry plots indicate mean percentage of Ly6C$^{lo/-}$ F4-80^{+} bone marrow macrophages. Histograms show total numbers of macrophages and percent F4-80^{+} within Ly6C^{hi} monocytes (mean + SEM from seven mice per group). (C) Ly6C and CCR expression (MFI) on Ly6C^{hi} monocytes at day 28pi. (D)

Percentage of CXCR4^{+}, Sca-1^{+} and MHC-II^{+} cells within Ly6C^{hi} monocytes on day 28pi. See also Figure S8. (E) Percentage of Arginase-1 (Arg-1) and IL-10 expressing cells within Ly6C^{hi} monocytes. (F) NOS2 expression (MFI) on Ly6C^{hi} monocytes and Ly6C$^{lo/-}$ F4-80^{+} bone marrow macrophages at day 28pi. (G) Parasite burden determined by the limiting dilution assay in WT and KO bone marrow at day 28. Data shown were pooled from two independent infections with 10 mice per genotype. (H-I) Pearson's correlation coefficient was used to assess correlation between bone marrow parasite burden and bone marrow HSCs (H) and Ly6C^{hi} monocytes (I) during the course of the infection. Data for correlation were pooled from C57BL/6, *Fzd6*$^{+/+}$ and *Fzd6*$^{-/-}$ mice at day 14, 21 or 28. All bar graphs represent mean + SEM with 6 mice per group for day 28 and 3 mice per group for day 14 unless otherwise noted. Similar results were obtained from three independent experiments for day 28. **P*<0.05; ***P*<0.01; ****P*<0.001.

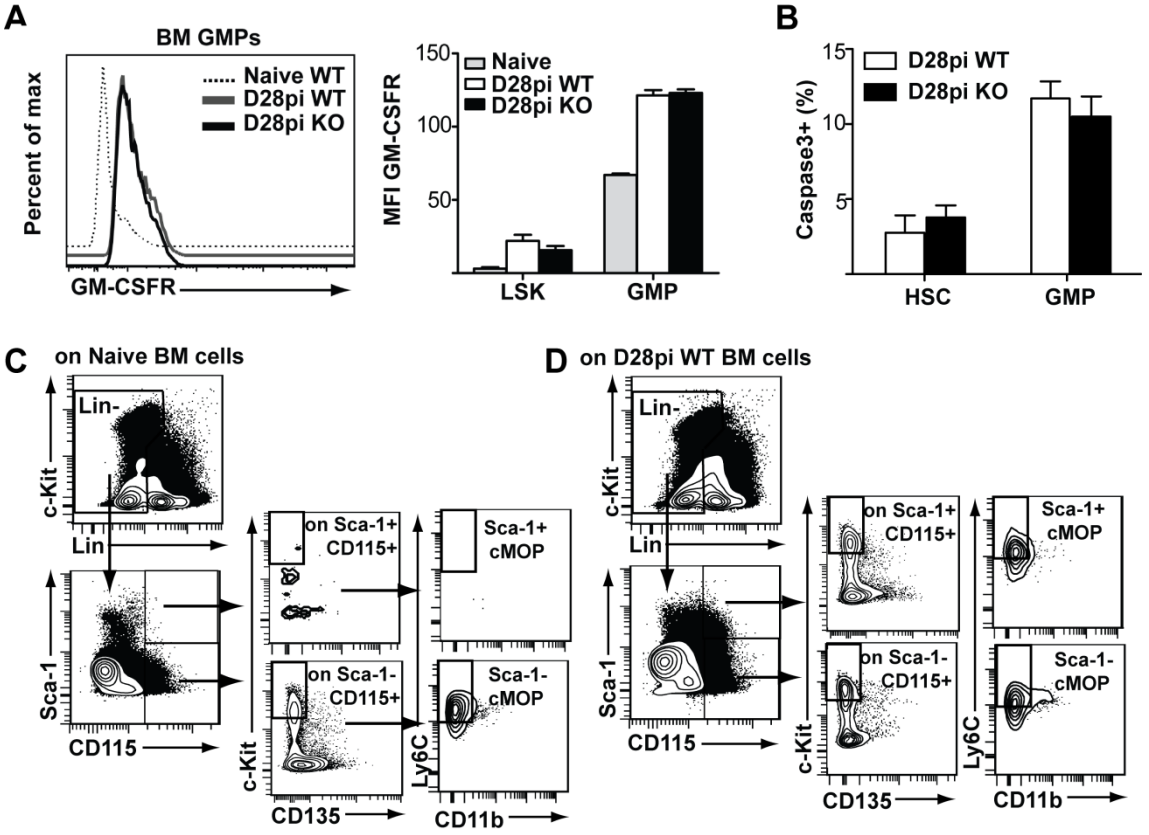

Figure S6. Analysis of myeloid progenitor cells in infected *Fzd6*$^{+/+}$ and *Fzd6*$^{-/-}$ bone marrow. (Related to Fig. 4 and Fig. 5)

(A) Representative flow cytometry data showing GM-CSFR expression on *Fzd6*$^{+/+}$ (WT) and *Fzd6*$^{-/-}$ mice (KO) bone marrow LSKs and GMPs at day 28pi. Mean fluorescence intensities are depicted in the graph on the right (Mean + SEM from five mice per group). (B) Apoptotic rate of WT and KO HSCs determined by caspase-3 activity at D28pi. (C-D) Representative flow cytometry plots for Sca-1^{-} and Sca-1^{+} common monocyte progenitors (cMOPs) in naïve and infected mice.

To determine whether the differences in bone marrow were also reflected in peripheral organs, we evaluated the recruitment of myeloid cells also to liver and spleen, two major target organs in visceral *Leishmania*sis. Similar to the bone marrow, spleen myeloid compartment was still unchanged on day 14, but the numbers of granulocytes and monocytes were all substantially increased by day 28 (Fig. 6A, 6B). Once more, there was no difference between *Fzd6*$^{-/-}$ and *Fzd6*$^{+/+}$ mice at steady state (Fig. S7), or earlier during the infection, before the onset of altered hematopoiesis (Fig. 6B, S4). We observed a specific decrease in *Fzd6*$^{-/-}$ GR1^{hi} and Ly6C^{hi} myeloid cells (Fig. 6A, 6B) with no difference in Ly6C^{lo} monocytes or F4-80^{+} CD11b^{int} red pulp macrophages (Fig. 6A-C). There was also a corresponding decrease in Ly6C^{hi} monocytes in the liver (Fig. S9). The decrease in myeloid cells correlated with decreased parasite burden on day 28 in *Fzd6*$^{-/-}$ spleen (Fig. 6D) and liver (Fig. S9). Wnt signaling has been reported to affect leukocyte infiltration (Chae *et al.*, 2016); however, there was no difference in the proportion of Ly6C^{hi} monocytes in peripheral blood (Fig. S4) or in the relative ratio of Ly6C^{hi} monocytes present in spleen as compared to bone marrow (Fig. 6E), suggesting that the decrease in *Fzd6*$^{-/-}$ peripheral monocytes was mainly due to differences in hematopoiesis. There was no difference in the expression of Sca1, MHCII, Ly6C, or Ccr2 between *Fzd6*$^{-/-}$ and *Fzd6*$^{+/+}$ Ly6C^{hi} monocytes in spleen (Fig. 6F, 6G) or liver (Fig. S9). *Fzd6*$^{+/+}$ Ly6C^{hi} monocytes expressed IL-10 (Fig. 6H, S8) but did not upregulate iNOS (Fig. 6H), thus supporting the theory that they may have regulatory functions. A smaller proportion of *Fzd6*$^{-/-}$ Ly6C^{hi} monocytes were IL-10^{+}. Wnt5a has been reported to directly induce IL-10 production in dendritic cells through a non-canonical pathway (Oderup *et al.*, 2013), while β-catenin activation promotes TLR-mediated IL-10 secretion by human monocytes (Martin *et al.*, 2005). Overall these results suggest that the decreased monocyte output by the *Fzd6*$^{-/-}$ bone marrow translates into decreased monocytes numbers in the periphery, decreased IL-10 production, and an apparently improved control of parasite expansion.

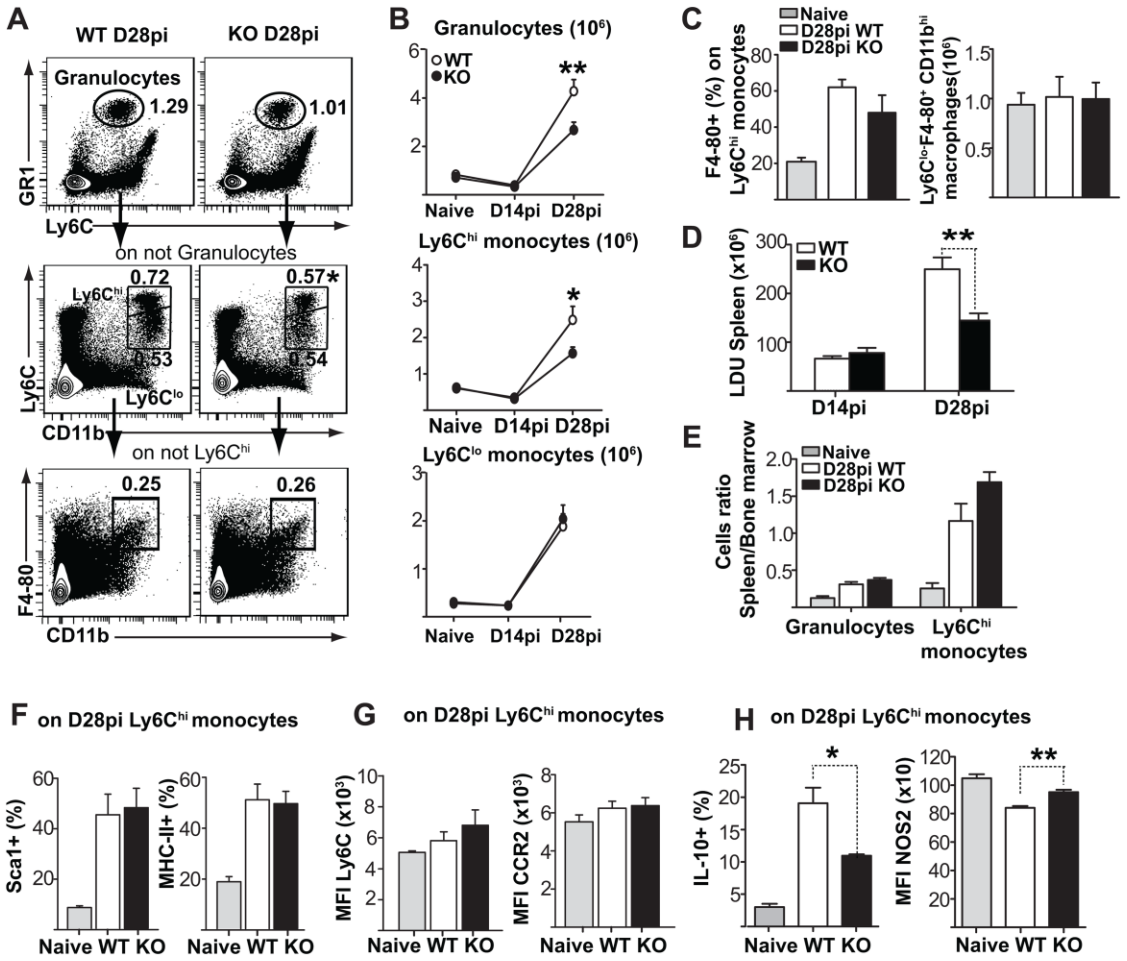

Figure 6. Decreased accumulation of myeloid cells is accompanied with reduced parasite burden in *Fzd6*$^{-/-}$ spleen

(A) Analysis of myeloid subsets in the spleen of infected *Fzd6*$^{-/-}$ (KO) and *Fzd6*$^{+/+}$ (WT) mice. Mean percentage for each cell subset is indicated within flow cytometry plots. See also Figures S7, S8 and S9. (B) Graphs show numbers of granulocytes and monocytes on day 14 and 28. (C) F4-80^{+} cells within Ly6C^{hi} monocytes and numbers of Ly6C$^{lo/-}$ F4-80^{+} macrophages in the spleen. (D) Parasite burden expressed as *Leishmania donovani* Units (LDU) in spleen on days 14 and 28pi. (E) Ratio of splenic to bone marrow granulocytes and Ly6C^{hi} monocytes in infected KO and WT mice on day 28 (mean + SEM from six mice per group). (F) Percentage of Sca-1^{+} and MHC-II^{+} cells within Ly6C^{hi} monocytes on day 28pi. (G) Ly6C and CCR2 expression (MFI) on Ly6C^{hi} monocytes at day 28pi. (H) Percentage of IL-10 expressing cells and NOS2 expression (MFI) on Ly6Chi monocytes at day 28pi. All bar graphs represent mean + SEM with 18 mice per group for day 28 pooled from three independent experiments and 3 mice per group for day 14 unless otherwise noted. **P*<0.05; ***P*<0.01; ****P*<0.001.

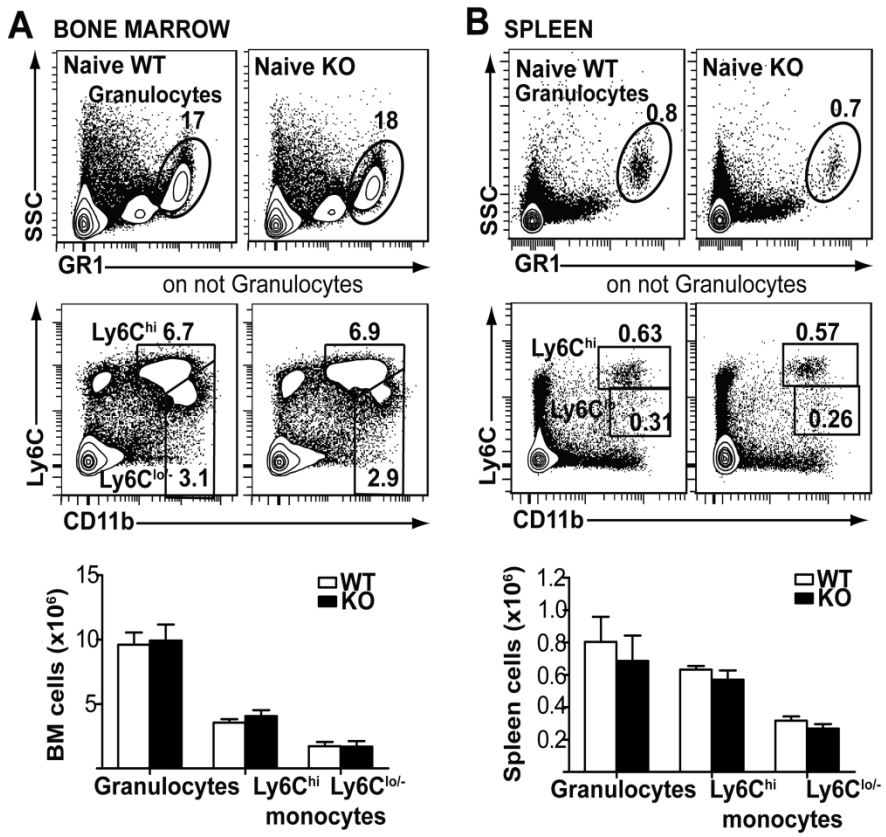

Figure S7.Steady-state myeloid maturation and migration to spleen do not require *Fzd6* (Related to Fig. 5 and Fig. 6)

Representative flow cytometry analysis of granulocytes (GR1^{hi}SSC^{hi}), mature monocytes (Ly6C^{hi}CD11b^{+}) and remaining immature/resident myelo-monocytes (Ly6Cl$^{o/-}$ CD11b^{+}) in the bone marrow (A) and spleens (B) of naive *Fzd6*$^{+/+}$ (WT) and *Fzd6*$^{-/-}$ mice (KO). Numbers represent the mean percentage of total bone marrow cells. Bar graphs show numbers of myeloid cell subsets in bone marrow (mean+SEM from at least three experiments for a total of at least five mice per group).

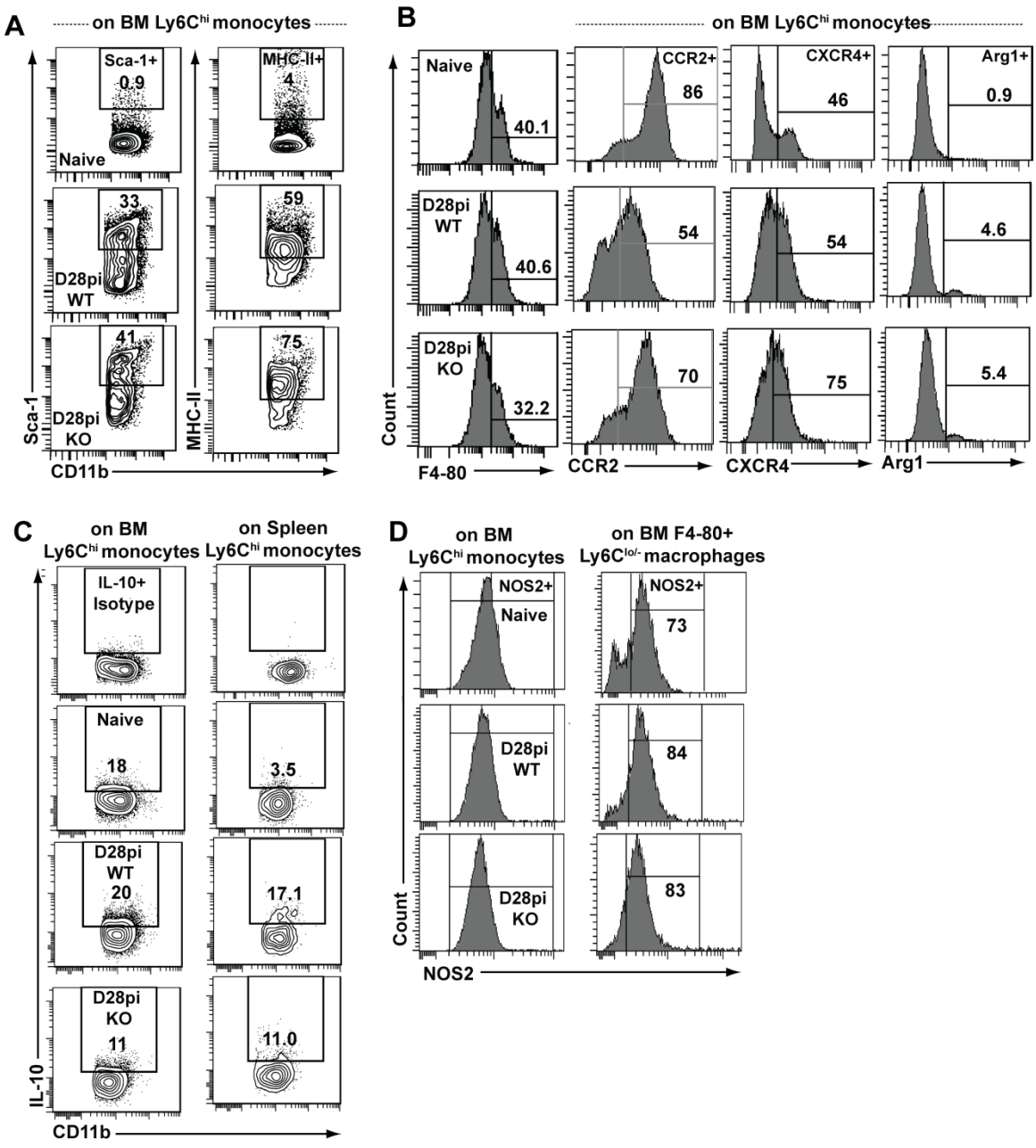

Figure S8. Flow cytometry analysis of bone marrow myeloid cells subsets in WT and KO mice at D28pi (Related to Fig. 5 and Fig. 6)

Representative flow cytometry data for (A) MHC-II and Sca-1; (B) F4-80, CCR2, CXCR4 and Arginase-1; (C) IL-10; and (D) NOS2 on Ly6C^{hi} monocytes at D28pi.

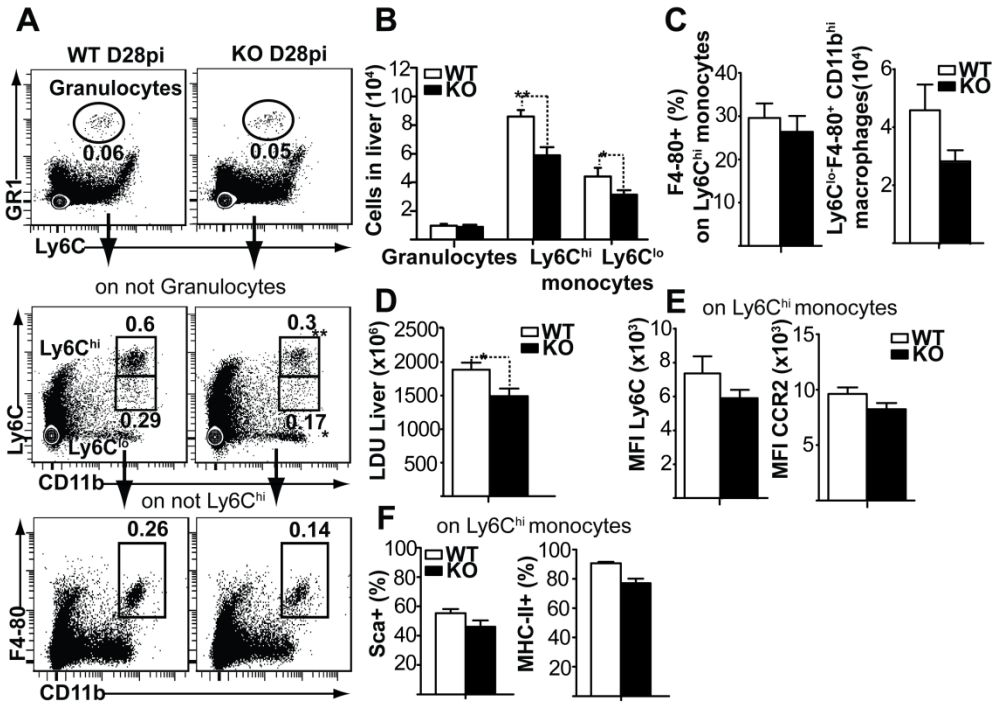

Figure S9. Liver monocytes are also decreased in number in *Leishmania*-infected *Fzd6*$^{-/-}$ mice (Related to Fig. 6)

Analysis of myeloid cell subsets in the livers of infected *Fzd6*$^{-/-}$ (KO) and *Fzd6*$^{+/+}$ (WT) mice on day 28pi. (A) Representative flow cytometry data shows granulocytes, monocytes and macrophages in the liver. Mean percentage for each cell subset is indicated within flow cytometry plots. (B) Graph show numbers of granulocytes and monocytes. (C) Percentage of F4-80^{+} cells within Ly6C^{hi} monocytes and numbers of Ly6C$^{lo/-}$ F4-80^{+} macrophages in the liver. (D) Parasite burden expressed as LDU in the liver on day 28pi. (E) Ly6C and CCR2 expression (MFI) on Ly6C^{hi} monocytes in the liver. (F) Percentage of Sca-1^{+} and MHC-II^{+} cells within Ly6C^{hi} monocytes in the liver. All bar graphs represent mean + SEM with 7 mice per group for day 28pi coming from one single infection. Similar results were obtained in a second, independent experiment. *P<0.05; **P<0.01; ***P<0.001.

4.5. Decreased parasite expansion in *Fzd6*$^{-/-}$ mice is not due to enhanced T lymphocyte activity

Improved T lymphocyte function (Bankoti *et al.*, 2012, Belkaid *et al.*, 2002, Glennie *et al.*, 2015, Romano *et al.*, 2015) or macrophage-mediated parasite killing (Arango Duque *et al.*, 2015) could also contribute to diminished parasite expansion, independent of changes in myeloid differentiation. We first investigated T lymphocyte recruitment to the bone marrow and their ability to produce cytokines. There is no major difference in T lymphocyte development in the absence of *Fzd6* (Abidin *et al*., 2015). There was also no major difference in the numbers of T lymphocytes in the bone marrow or spleen of *Fzd6*$^{-/-}$ mice when compared to controls (Fig. 7A, 7B, S10), as we observed a similar increase in CD4^{+} T lymphocytes in both groups. There was a slight but statistically significant increase in CD8^{+} T lymphocytes in the *Fzd6*$^{-/-}$ spleen. However, CD8+ T cells become progressively exhausted in chronic visceral *Leishmania*sis (Joshi *et al.*, 2009a). There was also no increase in cytokine production by either bone marrow or splenic T lymphocytes in response to parasite presented by bone marrow-derived dendritic cells (Fig. 7C, 7D), indicating that T lymphocytes were unlikely to explain the difference in parasite burden.

To investigate the contribution of macrophages, we first generated bone marrow-derived macrophages (BMDMs) in culture from *Fzd6*$^{-/-}$ and *Fzd6*$^{+/+}$ progenitors. We had not observed any striking differences the Ly6C^{lo} CD11b^{+} population that includes bone marrow macrophages at steady state (Fig. S7), although the F4-80^{+} CD11b^{int} macrophage population is decreased in mice infected with *L. donovani* (Fig. 5A, 5B). Similarly, there was no difference in the expression of CD11b, F4/80, MHCII or Sca1 between *Fzd6*$^{-/-}$ and *Fzd6*$^{+/+}$ macrophages at baseline (Fig. S10). There was also no difference in their upregulation of MHCII in response to IFNγ (Fig. S10), and we detected no significant changes in parasite uptake using fluorescence-labeled amastigotes (Fig. 7E, S10). However, there was an improvement in parasite control 72h post-infection in the presence of IFNγ (Fig. 7F, 7G), suggesting that *Fzd6*$^{-/-}$ bone marrow macrophages could partially contribute to the decreased parasite burden. Interestingly, exposure to the parasite resulted in massive upregulation of Sca1 on BMDMs (Fig. S10), while IFN-γ alone had no impact. Sca1 expression in the Ly6C^{lo} bone marrow population could therefore represent a biomarker for active infection rather than the IFN-dependent acquisition of a regulatory phenotype.

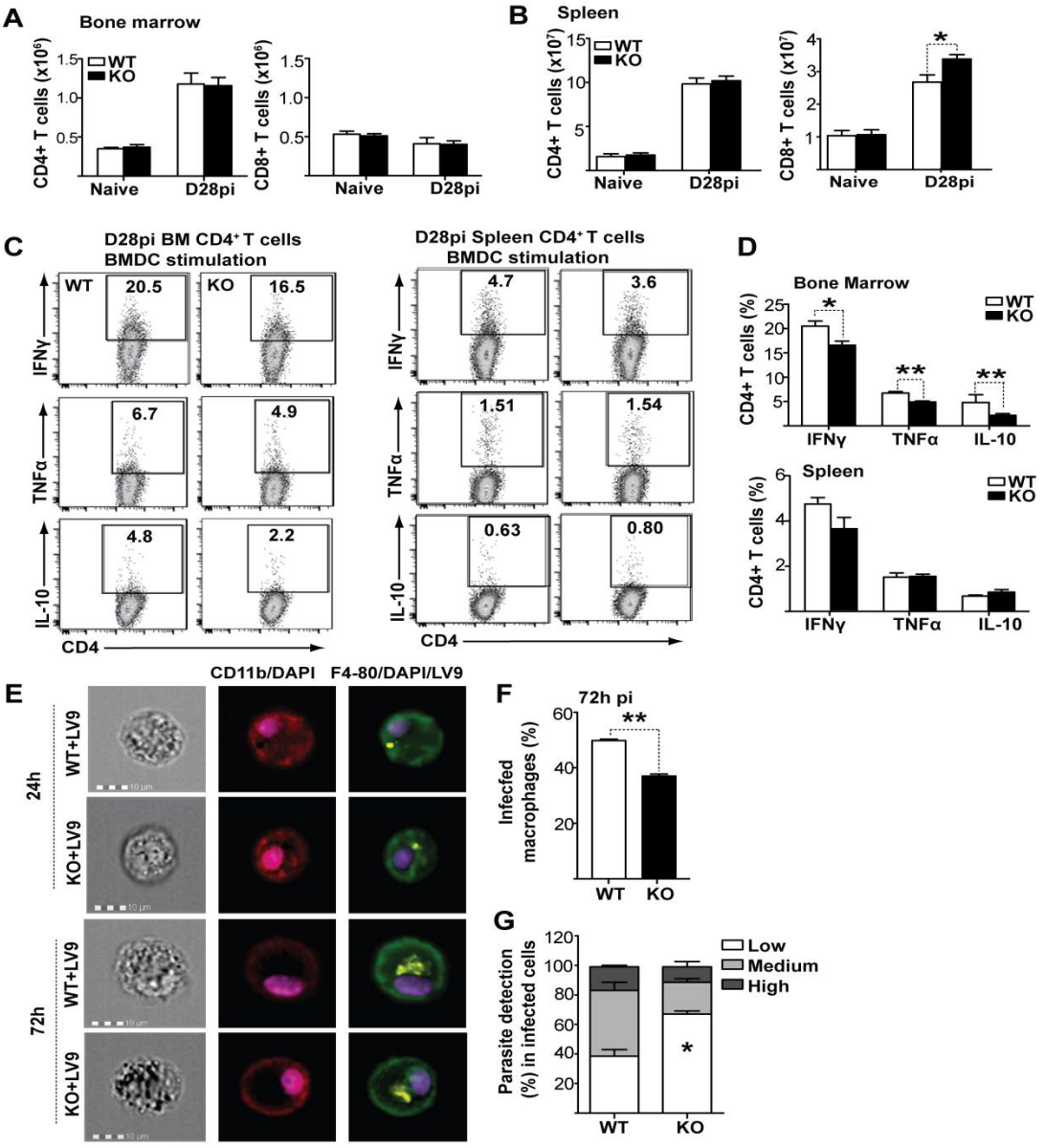

Figure 7. *Fzd6*$^{-/-}$ T lymphocytes are functionally indistinguishable from their *Fzd6*$^{+/+}$ counterparts

(A-B) Numbers of CD4^{+} and CD8^{+} T cells in BM (A) and spleen (B) of naïve and infected mice on day 28. (C-D) Cytokine production by bone marrow and spleen CD4^{+} T cells isolated from infected mice and stimulated *ex vivo* with parasite-pulsed bone marrow dendritic cells. Representative flow cytometry data are shown in (C). Graph in (D) shows compiled results from a representative experiment. See also Figure S10. All bar graphs represent mean + SEM with 7 mice per group for day 28. (E) Macrophages derived from naïve *Fzd6*$^{-/-}$ (KO) and *Fzd6*$^{+/+}$ (WT) bone marrow were either left untreated, stimulated with IFN-γ alone or first primed with IFN-γ and then infected with PKH26-labeled *L. donovani* amastigotes. See also Figure S10. Imaging flow cytometry analysis of macrophages infected with fluorescent *Leishmania* parasites, showing multiple parasites within both *Fzd6*$^{-/-}$ (KO) and *Fzd6*$^{+/+}$ (WT) macrophages. (F) Histograms represent percentage of infected macrophages and (G) parasite numbers in infected macrophages 72h post-infection. Low=1-3, medium=4-10, high= >10 parasites / cell. Bar graphs represent mean + SEM from two independent experiments.

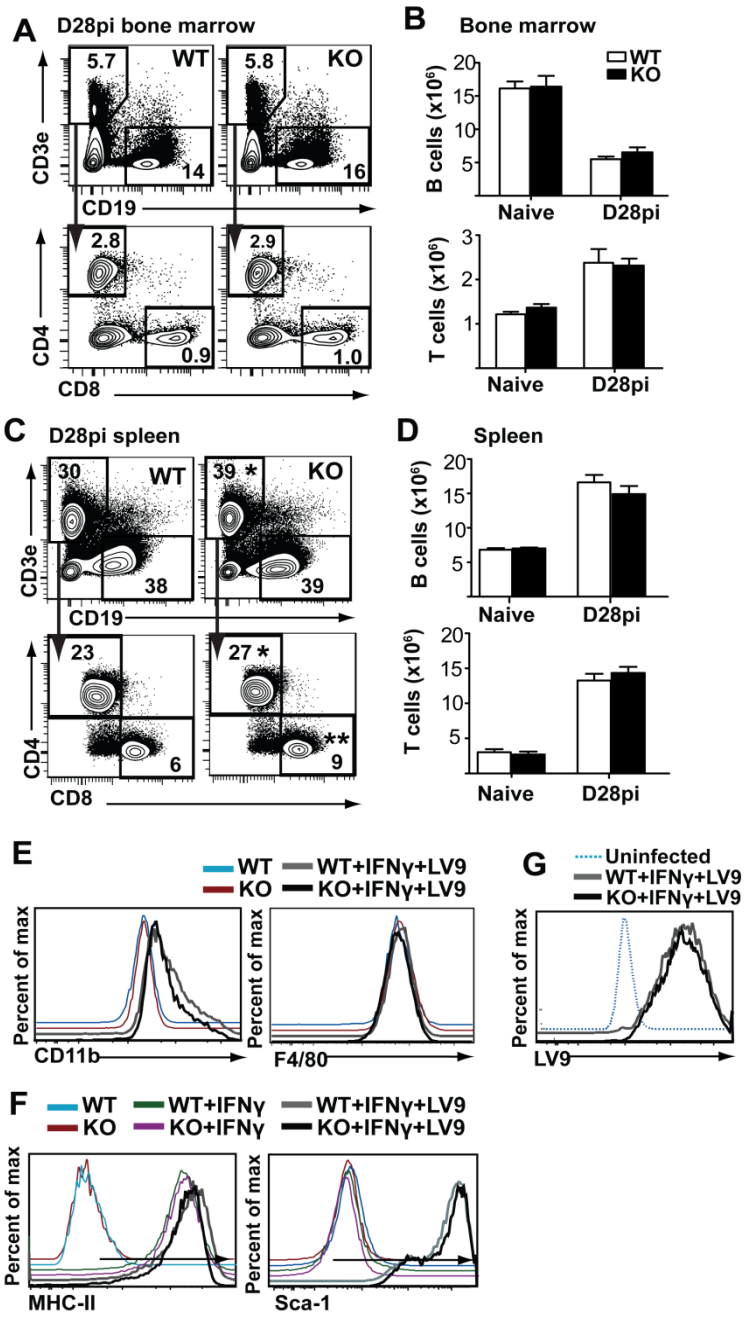

Figure S10. Analysis of T lymphocytes and bone marrow-derived macrophages from *Fzd6*$^{-/-}$ and *Fzd6*$^{+/+}$ mice. (Related to Fig. 7)

(A, C) Representative flow cytometry data for lymphoid cell subsets in (A) bone marrow and (C) spleen of naïve and infected *Fzd6*$^{-/-}$ (KO) and *Fzd6*$^{+/+}$ (WT) mice. Numbers in flow cytometry plots indicate mean percentage for CD19^{+} B cells, total CD3ε^{+} T cells and CD3ε^{+} CD4^{+} and CD3ε^{+}CD8^{+} T cells within BM. (B, D) Numbers of CD19^{+} B cells and CD3ε^{+} T cells in (B) BM and (D) spleen of naïve and infected mice on day 28. (E) Representative flow cytometry histograms showing uniform CD11b and F4/80 expression on untreated and infected macrophages. (F) MHC-II and Sca-1 expression on untreated, IFN-γ stimulated and infected macrophages. (G) Flow cytometry analysis of parasite uptake at 24h. Similar results were obtained from three independent experiments.

4.6. Bone marrow cytokine environment promotes the generation of permissive monocytes

L. donovani-infected macrophages produce TNF-α and GM-CSF, which have been suggested to promote myelopoiesis (Cotterell *et al*., 2000b). However, stromal macrophages represent a relatively small proportion of all bone marrow cells, with the vast majority of the marrow being occupied by the developing myeloid, lymphoid and erythroid cells. To obtain a more comprehensive picture of the bone marrow cytokine environment during infection, we used the supernatant from freshly harvested total bone marrow cells as a surrogate for the extracellular milieu and compared uninfected mice to *L. donovani*-infected mice at different time points. The levels of MIP1α/Ccl3, IL-1, and IFNγ-inducible factors, such as ICAM-1, IP10/Cxcl10 and Cxcl9 were all increased in the chronic phase of the infection (Fig. 8A). Both IFN-α and IFN-γ were also upregulated on day 21 (Fig. 8A, 8B), at the beginning of the chronic phase, and could thus contribute to HSPC expansion in the bone marrow (Baldridge et al., 2010, Buechler et al., 2013, de Bruin et al., 2013, Matatall et al., 2014). However, IFN-γ levels appeared to decrease back to or even below baseline levels by day 28. There was no significant difference in the levels of IFN-γ, IFN-α, or myeloid growth factors GM-CSF, M-CSF, IL-3, or IL-6 between *Fzd6*$^{-/-}$ and *Fzd6*$^{+/+}$ mice. However, *Fzd6*$^{-/-}$ bone marrow environment demonstrated an even more dramatic upregulation of MIP1α and IL-1, together with a stronger IFNγ response. These observations together with data from Figures 4 and 5 suggest that the reduced parasite burden in *Fzd6*$^{-/-}$ mice is at least partially the result of a different inflammatory microenvironment that could contribute to its own maintenance via altered myeloid differentiation.

To investigate this hypothesis in culture, we used the bone marrow supernatants as source of growth factors and evaluated the expansion and differentiation of both *Fzd6*$^{-/-}$ and *Fzd6*$^{+/+}$ bone marrow progenitor cells over four days. Supernatants from both *Fzd6*$^{-/-}$ and *Fzd6*$^{+/+}$ infected bone marrow stimulated the emergence of GR1^{+}/Ly6C^{hi}/CD11b^{+} cells (Fig. 9A, S11); however, their numbers were significantly lower in cultures that had been exposed to the *Fzd6*$^{-/-}$ environment. Similar results were obtained also with GMPs and LSKs, indicating that the cytokine environment present in the *Fzd6*$^{-/-}$ bone marrow significantly reduces HSPC expansion. The differentiation patterns were very similar when *Fzd6*$^{-/-}$ progenitor cells were used (Fig. S11), suggesting that the initial steps of the progenitor cell response were not affected, similar to what we observed *in vivo* on day

14 (Fig. 4A-4E), or over the first few days after transplant (Abidin *et al*., 2015). Cell-intrinsic self-renewal defects would only appear after a much longer period. Thus, although *L. donovani* induces the production of factors that promote myeloid differentiation in both *Fzd6*$^{-/-}$ and *Fzd6*$^{+/+}$ bone marrow, the *Fzd6*$^{-/-}$ cytokine environment reduces HSPC expansion, possibly via myelosuppressive factors, which in turn results in the generation of fewer GR1^{+}/Ly6C^{hi} monocytes both in culture (Fig. 9) and *in vivo* (Fig. 5).

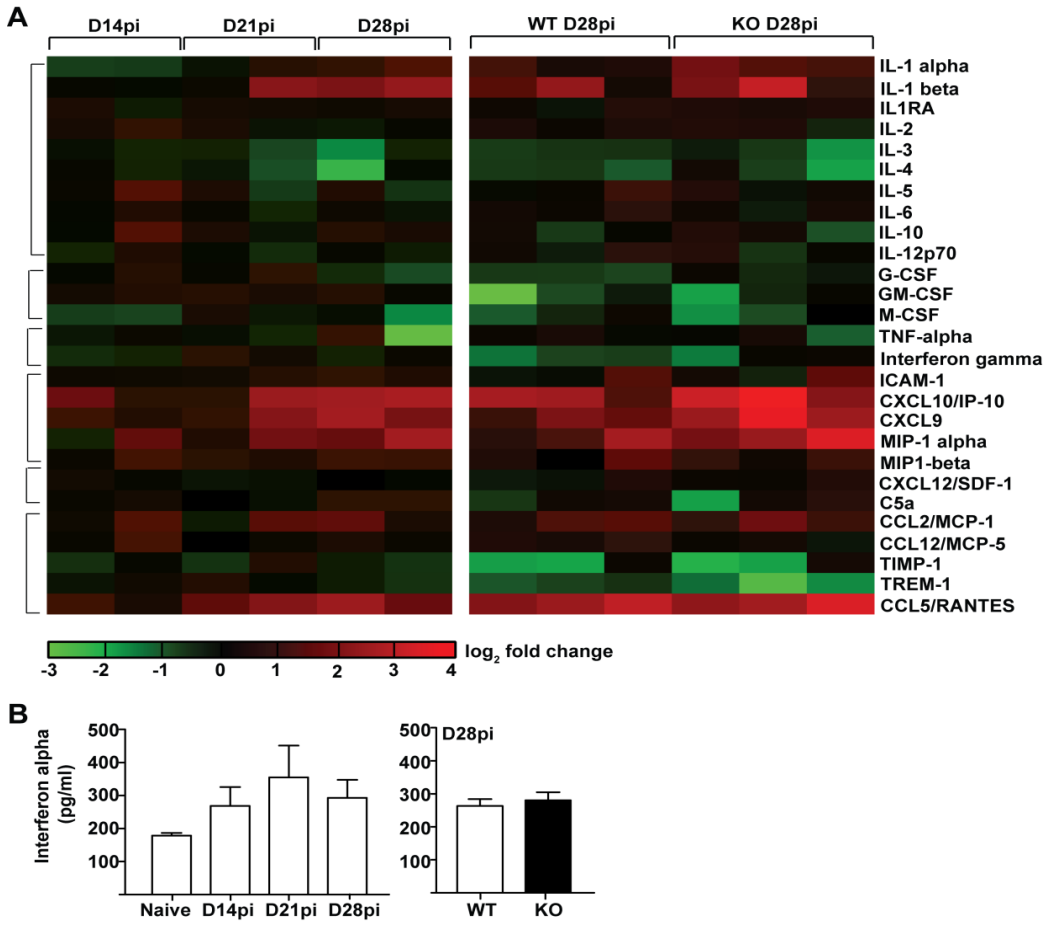

Figure 8. *Fzd6*$^{-/-}$ bone marrow microenvironment is enriched in pro-inflammatory cytokines and chemokines

(A) Fold change in cytokine/chemokine levels in *Fzd6*$^{-/-}$ (KO) and *Fzd6*$^{+/+}$ (WT) bone marrow extracellular milieu on day 28pi as compared to untreated mice. Heatmap represents log 2 fold change in mean pixel density per cytokine/chemokine in at various time points of infection as compared to untreated mice. Each square represents one individual experiment coming from a pooled sample of 4-6 mice per group. (B) Interferon alpha levels in bone marrow supernatant obtained from naïve and infected mice.

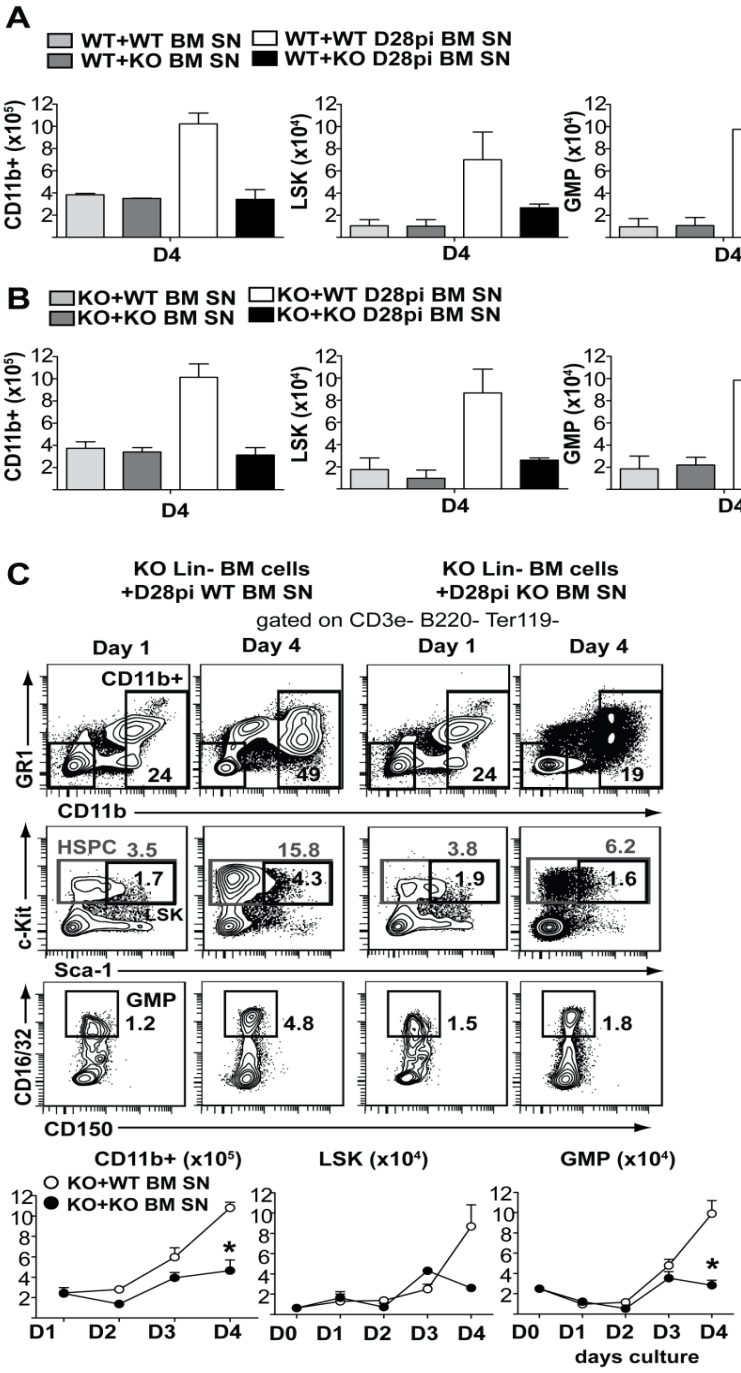

Figure S11. Extracellular fluid from naive bone marrow does not promote myeloid differentiation (Related to Fig. 9)

Impact of bone marrow supernatants from naïve mice as compared to *L. donovani* –infected mice after four days of culture on (A) Lin$^-$ *Fzd6*$^{+/+}$ (WT) BM cells and (B) Lin$^-$ *Fzd6*$^{-/-}$ (KO) BM cells. (C) Freshly isolated lineage-depleted *Fzd6*$^{-/-}$ (KO) BM cells were cultured in complete medium supplemented with 30% BM supernatant as indicated. Representative flow cytometry data show the gating strategy for CD11b$^+$, LSK and GMP populations. Graphs show numbers of cell recovered per 5x10^5 cells seeded for each subset.

To directly test whether the monocytes generated in the infected bone marrow environment were functionally different from normal *in vitro* –differentiated monocytes, we exposed the cultures to fluorescence-labeled *L. donovani* amastigotes. Less than 5% Ly6Chi monocytes generated in the naïve setting became infected and there was no increase in the proportion of infected cells from 24h to 72h (Fig. 9C-9E), although the proportion of monocytes containing efficiently replicating parasites increased over that period. In contrast, there was a 10-fold increased in the proportion of infected monocytes derived in the presence of supernatants coming from the infected bone marrow (Fig. 9C-9E), and this proportion increased over time. In both cultures, the proportion of $Ly6C^{hi}$ cells increased between 24h and 72h (Fig. 9B), which could indicate either continued differentiation or the loss of $Ly6C^{lo/-}$ cells due to cell death or increased adhesion. In summary, these results show that the *L. donovani* –infected bone marrow environment not only promotes myelopoiesis but also renders the newly generated cells more permissive to infection.

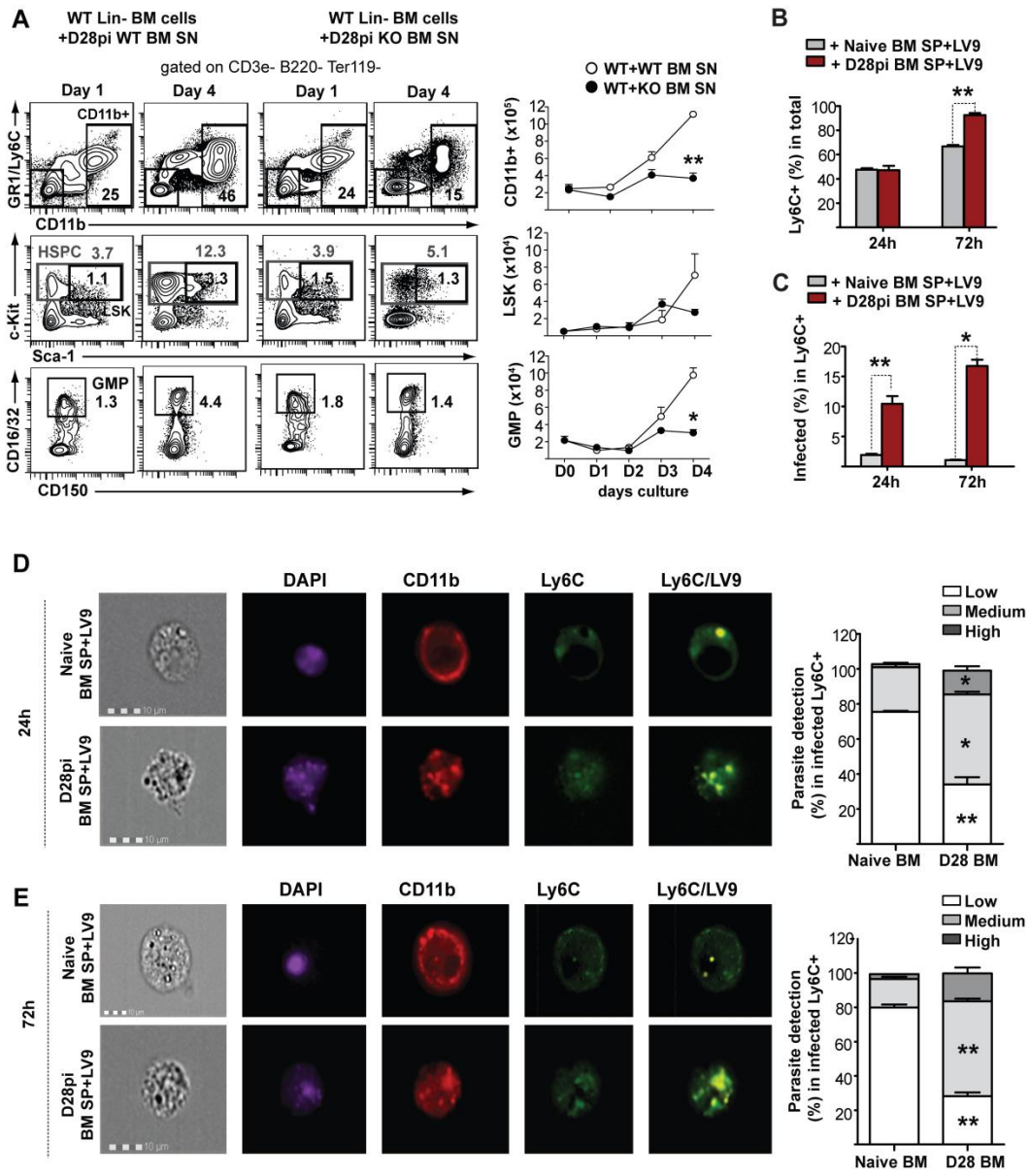

Figure 9. Infected bone marrow microenvironment directly promotes HSPC expansion and the generation of permissive monocytes..

Freshly isolated lineage-depleted *Fzd6*$^{+/+}$ (WT) BM cells were cultured in complete medium supplemented with 30% BM supernatant as indicated. **(A)** Representative flow cytometry data show the gating strategy for CD11b^{+}, LSK and GMP populations. Graphs show numbers of cell recovered per 5x10^{5} cells seeded for each subset. Lin^{-} *Fzd6*$^{+/+}$ (WT) BM cells cultured with BM supernatant obtained from infected *Fzd6*$^{-/-}$ (KO) and *Fzd6*$^{+/+}$ (WT) mice. See also Figure S11. Data were pooled from three independent experiments. **(B)** Percentage of Ly6C^{+} monocytes in differentiation cultures following infection on day 4, and **(C)** the proportion of infected Ly6C^{+} monocytes after 24h and 72h. **(D-E)** Imaging flow cytometry analysis of monocytes infected with fluorescent *Leishmania* parasites, showing multiple parasites at **(D)** 24h and **(E)** 72h post-infection. Histograms represents parasite uptake in infected macrophages. Low=1-3, medium=4-10, high= >10 parasites respectively. Bar graphs represent mean + SEM from three independent experiments. **P*<0.05; ***P*<0.01.

5. DISCUSSION

We have examined here the role of HSPC expansion and altered myeloid differentiation in promoting parasite proliferation during experimental visceral *Leishmania*sis. We demonstrate that *L. donovani* induces HSPC proliferation in the bone marrow, and promotes the generation of $Sca1^{+}$ emergency GMPs and their differentiation into $Ly6C^{hi}$ monocytes expressing regulatory markers, such as Sca1, MHCII, Galectin-3 and IL-10. In contrast, an inefficient emergency response and diminished accumulation of emergency GMPs and $Ly6C^{hi}$ monocytes in *Fzd6*$^{-/-}$ mice corresponds to lower parasite expansion in bone marrow and in the periphery. We further show that the bone marrow cytokine environment is sufficient to promote HSPC expansion and myeloid differentiation, and to render the newly produced monocytes more susceptible to infection. Collectively our data support the hypothesis that *L. donovani* modulates the host hematopoietic program to support its own expansion.

Leishmania parasites have developed multiple mechanisms to survive inside their classical targets, macrophages (Arango Duque *et al.*, 2015), and to promote the recruitment of more monocytes to the site of infection (Passos *et al.*, 2015). *Leishmania* infection has also been associated with increased extramedullary myelopoiesis in the spleen (Cotterell *et al.*, 2000b, Matnani *et al.*, 2016, Mirkovich *et al.*, 1986), which has been proposed to generate “safe targets”, or permissive monocytes for the parasite to replicate in (Mirkovich *et al.*, 1986). Thus the parasite could not only modify its target cells to its liking but also promote their generation. *L. donovani* establishes a chronic infection in the bone marrow (Cotterell *et al*., 2000b), the predominant site of adult hematopoiesis, and chronic *Leishmania*sis is associated with pancytopenia and may ultimately lead to bone marrow failure (de Freitas *et al.*, 2016, Matnani *et al.*, 2016). Despite all this, the role of bone marrow in the pathogenesis of visceral *Leishmania*sis remains obscure. We have now characterized in detail the changes to the bone marrow HSPC compartment during experimental visceral *Leishmania*sis, and demonstrate that HSC-like cells and emergency GMPs are substantially expanded during the early phases of chronic infection. Emergency GMPs are associated with superior proliferative potential when compared to their classical $Sca1^{-}$ counterparts, and would thus result in the generation of more progeny in the periphery (Buechler *et al*., 2013). In addition, our data provide functional evidence that *L. donovani* –induced adaptations to hematopoiesis are indeed important for parasite expansion: bone marrow cytokine

environment from infected mice was not only sufficient to induce HSPC expansion and myeloid differentiation in culture but also promoted the infection of newly generated monocytes. Conversely, parasite burden directly correlated with bone marrow HSPC and Ly6C^{hi} monocytes numbers. Thus put together, our data strongly support the hypothesis that infection-adapted emergency myelopoiesis directly promotes parasite expansion and the persistence of a chronic infection.

Monocytes have been proposed as safe targets for *L. major* (Mirkovich *et al.*, 1986), and alternative monocytes contribute to the pathogenesis of *L. brasiliensis* (Passos *et al.*, 2015); however, the role of monocytes in the pathogenesis of visceral *Leishmania*sis is not well established (Kaye *et al.*, 2011). Development of liver granulomas and the clearance of *L. donovani* in the liver are dependent on incoming monocytes and can be promoted with GM-CSF (Cervia *et al.*, 1993, Murray *et al.*, 1995), but the contribution of monocytes to the progression of infection in bone marrow and spleen is not known. Our results suggest that in contrast to liver, the accumulation of Ly6C$^{hi/int}$ monocytes in spleen and bone marrow is detrimental to the host, resulting in higher parasite burden. We observed an increased frequency of IL-10^{+} and Arginase1^{+} Ly6C^{hi} monocytes in *L. donovani*-infected mice with no upregulation of iNOS, suggesting that the myeloid cells adopt indeed an M2-like phenotype. We also show in another study investigating the importance of tissue hypoxia that splenic CD11b^{+} cells are able to suppress Th1 responses in an antigen-independent manner (AH, SS, manuscript submitted). This could be due to the different environmental factors that promote their conversion to a regulatory phenotype (Askenase *et al.*, 2015), permissive to infection but unable to kill the parasite.

Bone marrow cytokine environment was responsible for mediating HSPC expansion and myeloid differentiation in culture. Our finding correlates with previous reports, suggesting that infected macrophages produce myeloid growth factors, such as GM-CSF, in culture (Cotterell *et al.*, 2000b). However, our approach takes into account not only the infected macrophages but also other components of the bone marrow microenvironment, including the immature myeloid cells that compose the bulk of the marrow. When comparing the cytokine and chemokine environment from infected mice to uninfected controls, we saw that GM-CSF levels were actually downregulated in chronic infection, which could correlate with decreased leishmanicidal activity (Murray *et al.*, 1995). We also saw a corresponding increase in GM-CSFR expression on the

surface of both GMPs and more mature myeloid cells, suggesting decreased ligand availability. These data suggest that the impact of a chronic *in vivo* infection with *L. donovani* on cytokine production may be very different from what is seen in culture. They also emphasize the need for more in depth analysis of various microenvironmental niches, as the cytokines present in different organs or even between separate sites within the same organ may have opposing effects.

Chronic *Leishmania*sis is associated with anemia and pancytopenia, signs of diminished HSC function and bone marrow failure. We observed a decrease in SDF-1/Cxcl12 and an increase in MIP-1α/Ccl3 in the infected bone marrow environment. Cxcl12 is required for HSC maintenance in the bone marrow (Sugiyama *et al.*, 2006), and a decrease in Cxcl12 levels is associated with HSC activation and release to peripheral sites, for example, during acute bacterial infection (Takizawa et al., 2011, Ueda et al., 2005). Conversely, Ccl3 has been shown to modify bone marrow HSC niches, thus promoting the maintenance of leukemic stem cells, for example, at the expense of normal HSCs (Baba *et al.*, 2013, Schepers *et al.*, 2013). Ccl3 can also promote the settlement of macrophages to the bone marrow (Li *et al.*, 2015). Together, the decrease in Cxcl12 and the concomitant increase in Ccl3 observed in the bone marrow of *L. donovani* –infected mice are coherent with HSC activation and a gradual loss of function, and represent potential targets for intervention to prevent bone marrow failure.

A better understanding of the signaling pathways underlying bone marrow alterations during visceral *Leishmania*sis may also result in the development of immunotherapies. For example, it should be possible to develop complementary therapies to be used in combination with parasitostatic treatment that would selectively promote the generation of monocytes capable to controlling and ultimately eliminating the parasite. Alternatively, it might be possible to interfere with monopoiesis in a short-term, preventive treatment, targeted for travellers visiting an endemic area. Lastly, long-term trained immunity (Cheng *et al.*, 2014, Netea *et al.*, 2011, Saeed *et al.*, 2014) almost certainly involves epigenetic alterations at the HSPC level. Identifying the factors that stimulate HSPC expansion and the skewing of myeloid differentiation towards a permissive phenotype could thus pave the way for the design of vaccines dependent on innate immune cell function.

Taken together, we report here a substantial activation and accumulation of HSC-like cells and Sca1^{+} emergency GMPs during the early stages of chronic *L. donovani* infection, concomitant with the sudden increase in bone marrow parasite burden. We further demonstrate that reduced HSPC expansion is associated with lower parasitemia and thus provide mechanistic evidence that *L. donovani* indeed subverts bone marrow hematopoiesis *in vivo* to support its own expansion. Our results support the hypothesis that emergency hematopoiesis directly contributes to the pathogenesis of visceral *Leishmania*sis, and suggest that HSPCs could represent an interesting therapeutic target.

6. EXPERIMENTAL PROCEDURES

Experimental Animals and Parasites

C57BL/6 mice were purchased from The Jackson laboratory (Bar Harbor, ME). Mice deficient in Frizzled 6 (*Fzd6*$^{-/-}$) were first backcrossed to C57BL/6 for ten generations and then maintained under specific pathogen-free conditions in sterile ventilated racks at the animal facility of INRS-Institut Armand-Frappier (CNBE), as described (Abidin *et al*., 2015). Female *Fzd6*$^{-/-}$ mice were compared to sex-matched *Fzd6*$^{+/+}$ littermates unless otherwise noted. *Leishmaniadonovani* (strain LV9) were maintained by serial passage in *B6.129S7-Rag1tm1Mom* mice, and amastigotes were isolated from the spleens of infected animals. Experimental mice were infected by injecting 2×10^{7} amastigotes via the lateral tail vein. Bone marrow and splenic parasite burdens were determined either by limiting dilutions or by examining methanol-fixed, Giemsa stained tissue impression smears (Hammami et al., 2015). Data are presented as number of parasites per bone marrow (one femur and one tibia) or as Leishmani Donovan Units (LDU).

Ethics statement

All procedures were in accordance with the Canadian Council on Animal Care guidelines and approved by the Comité institutionnel de protection des animaux of the INRS (CIPA #1411-02, 1210-06, 1510-02).

Flow Cytometry

Bone marrow was harvested by flushing tibiae and femora in phosphate-buffered saline (PBS), and the cells were then passed through a 25-gauge needle to obtain a single cell suspension. PBS was supplemented with 0.1% bovine serum albumin (BSA) and 0.5mM ethylene-diamine-tetra-acetic acid (EDTA) for flow cytometry staining. See Table S1 for a complete list of antibodies. For intracellular staining, surface stained cells were fixed and permeabilized using the Foxp3 staining kit (eBioscience, San Diego, CA) and then incubated with appropriate antibodies. For cell cycle analysis, cells were first incubated for 30 min at 37°C with Hoechst #33342 (Sigma-Aldrich, Oakville, ON, Canada) in DMEM supplemented with 10% Premium FBS (Wisent Bioproducts, St-Bruno, QC, Canada) and 1 mM HEPES (Life Technologies, Burlington, ON, Canada), followed by staining with surface antibodies and intracellular anti-Ki67 as described above. Samples were acquired with a four-laser LSR Fortessa flow cytometer (BD Biosciences, Mountain View, CA) and analyzed using BD FACS Diva software (BD Biosciences) or FlowJo (for histogram overlays; Tree Star).

Wright-Giemsa Staining

Freshly isolated bone marrow cells from infected mice were collected on slides using a cytospin centrifuge (Hettich, Tuttlingen, Germany) at 800rpm for 5 min. The smears were air dried and stained with modified Wright-Giemsa stain (Protocol Hema3; Thermo Fisher Scientific) as per manufacturer's instructions. Slides were mounted using Fluoromount-G (SouthernBiotech) and coverslips sealed with nail polish. Analyses of infected cells were performed using a Nikon Eclipse E800 microscope (Nikon) with a 100x oil immersion lens and images were captured with a digital camera (COOLPIX 990; Nikon).

Colony Assays

Freshly harvested cells were seeded in duplicate into 35mm non-adherent petri dishes at a density of 10^4 cells/dish in methylcellulose medium containing stem cell factor, IL-3, IL-6, and Erythropoietin (Methocult GF M3434, Stem Cell Technologies, Vancouver, BC, Canada). The cultures were incubated at 37°C in 5% CO2 for 10 days and hematopoietic colonies were identified based on morphology under an inverted microscope.

Table S1. Antibodies used in flow cytometry

Antigen	Fluorochrome	Clone	Supplier
Active caspase-3	Alexa Fluor 488	-	Cell Signaling Technologies
Active β-catenin	Unconjugated	D13A1	Cell Signaling Technologies
Arginase-1	PE	-	R&D Systems, Minneapolis, MN
CCR2	Alexa Fluor 700	-	R&D Systems
CD105	Pacific Blue	MJ7-I8	eBioscience, San Diego, CA
CD115	PE-Cy7, FITC	AFS98	eBioscience
CD117 (cKit)	PE	2B8	BD Biosciences, Mississauga, ON, Canada
CD11b	Biotin	M1/70	BD Biosciences
CD11b	Alexa Fluor 647	M1/70	eBioscience
CD135 (Flt3)	PerCP–eFluor 710	A2F10	BD Biosciences
CD150	Alexa Fluor 647	TC15-12F12.2	BD Biosciences; BioLegend, San Diego, CA
CD16/32	PerCP-Cy5.5	93	eBioscience
CD19	PE	1D3	eBioscience
CD3e	Biotin	145-2C11	BD Biosciences
CD3e	PE-Cy7	145-2C11	eBioscience
CD4	APC-eFluor 450	RM4-5	eBioscience
CD41	PE, FITC	MWReg30	BD Biosciences
CD45/B220	Biotin	RA3-6B2	BD Biosciences
CD48	PerCP-Cy5.5	HM48-1	BioLegend
CD8	eFluor 450	53-6.7	eBioscience
CXCR4	PE	2B11	BD Biosciences
F4/80	PE-Cy7	BM8	BioLegend
Galectin3	PE	eBioM3/38	eBioscience
GM-CSFR Rα	Alexa Fluor 700	-	R&D Systems
GR1	Biotin	RB6-8C5	BD Biosciences
GR1	APC-eFluor 780	RB6-8C5	eBioscience
Hoechst		33342	Sigma-Aldrich, Oakville, ON, Canada
IFN-γ	APC	-	BD Biosciences
IL-10	PE	-	BD Biosciences
iNOS	APC	CXNFT	eBioscience
Ki67	PE–eFluor 610	SoIA15	eBioscience
Ly6C	PerCP-Cy5.5, FITC	AL-21	eBioscience; BD Biosciences
Ly6G	Biotin	A8	BioLegend
MHC-II	PE	MS/114.15.2	eBioscience
NK-1.1	Biotin	PK136	BD Biosciences
Sca-1 (Ly6A/E)	PE-Cy7, BV711	D7	BD Biosciences
Streptavidin	BD Horizon V500, FITC	-	BD Biosciences
Ter119	Biotin	-	BD Biosciences
TNF-α	PE-Cy7	MP6-XT22	BD Biosciences

T cell stimulation

To analyze endogenous CD4 T cell responses, bone marrow-derived dendritic cells (BMDCs) were pulsed with fixed parasites for 24 h at 37℃. Splenocytes or bone marrow cells from infected animals were then added to BMDCs and incubated for 2 h at 37℃. Brefeldin A (BD Pharmingen) was added for an additional 4 h, after which cells were stained with appropriate antibodies as described above.

Macrophage infection and parasite survival

Macrophages were derived from total bone marrow cells in IMDM supplemented with 10% very low endotoxin FBS and 15% L929-cell conditioned medium (Heinonen *et al.*, 2006). Purity of macrophage cultures was determined by flow cytometry 7 days after culture. Macrophages were then plated 2-3x10^6 cells per 35mm non-adherent petri dish and either left untreated or stimulated with 10ng/ml IFN-γ for 2h. IFN-γ-primed macrophages were infected with PKH26-labeled *L. donovani* amastigotes at MOI of 5:1 for 24h or 72h in the continued presence of IFN-γ. Macrophages were harvested from cultures and analyzed by flow cytometry. For imaging flow cytometry, cells were first surface-stained with anti-CD11b and anti-F4/80 and then fixed and counterstained with DAPI (Life Technologies). Samples were acquired with Amnis Imagestream Mark II imaging flow cytometer (EMD Millipore) and analyzed with IDEAS v6.1 software using spot count function.

Bone marrow cytokine/chemokine analysis

Bone marrow cell supernatants were collected from naïve or *L. donovani* infected mice by harvesting cells from both hind legs into 2 ml PBS, followed by centrifugation. Supernatants were pooled from at least four mice per sample, and analyzed using a membrane-based Proteome profiler mouse cytokine/chemokine array kit (R&D Systems). Array images were further analyzed using the NIH ImageJ image analysis software. Samples were normalized by subtracting pixel intensities from negative controls, and the fold changes for infected mice were determined as a ratio over naive mice of the same genotype. Levels of mIFN-α were determined by using mouse IFN-alpha Platinum ELISA (eBioscience).

In vitro differentiation assay

Bone marrow cells were first enriched for progenitor cells using a Mouse hematopoietic progenitor cell enrichment kit (StemCell Technologies, Vancouver, BC), and their purity was determined by flow cytometry. $5x10^5$ Lin- BM cells per condition were divided in ten wells of a non-adherent 96-well plate (BD Falcon), and cultured in IMDM supplemented with 10% very low endotoxin FBS (Wisent) and 30% bone marrow supernatant in the presence of 10ng/ml IL-3, IL-6 and SCF for up to 4 days. Cells were harvested as indicated and their number and differentiation stage were analyzed by flow cytometry. Culture-derived monocytes were infected with PKH26-labeled *L. donovani* amastigotes at MOI of 5:1 for 24h or 72h. Cells were surface-stained with anti-CD11b and anti-Ly6C and then fixed and counterstained with DAPI (Life Technologies) for analysis by imaging flow cytometry as detailed above.

Statistical analysis

Statistical significance was determined using ANOVA (for multiple comparisons) or two-tailed student's t test (for comparing $Fzd6^{+/+}$ and $Fzd6^{-/-}$). $P < 0.05$ was considered significant.

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CHAPTER 4

GENERAL DISCUSSION

1. *Fzd6* is indispensable for the self-renewal and repopulation of hematopoietic stem cells

Hematopoietic stem/progenitor cells (HSPCs) located in the BM, are responsible for the lifetime production of blood cells. Blood homeostasis depends on the appropriate balance between self-renewal and differentiation of HSPCs, allowing tightly controlled generation of mature blood cells as well as the maintenance of stem cell function at steady state and during regeneration (Yamamoto *et al*., 2013, Wilson *et al*., 2008). The polarity establishment plays a critical role in cell fate determination and patterning in tissues with high turnover rates, such as the hematopoietic system (Carolina Florian *et al.*, 2010, Pham *et al.*, 2014). The core PCP receptor, *Fzd6* plays a crucial role in the regulation of cell planar polarity at precursor cell level to establish an orientation axis for molecular asymmetry in different tissue cells through interactions with neighboring cell surfaces (Cantilena et al., 2011, Chang et al., 2016, Oozeer et al., 2017). Recent studies have identified *Fzd6* as a predictive factor of a *poor* prognosis and invasion in diverse human cancers (Corda *et al.*, 2017a). Although *Fzd6* is known to be highly expressed on HSPCs, its precise role in hematopoiesis had not been previously defined. In the Chapter-2 of this thesis, we show that cell-autonomous *Fzd6* signaling is indispensable for the long-term maintenance and self-renewal of HSPCs. Our results further demonstrate that Wnt/*Fzd6* signaling acts as guardian of integrity of hematopoietic stem cell pool under transplantation stress.

Mouse HSPCs, including LT-HSCs, ST-HSCs and LMPPs can be identified within the LSK (Lin-Sca1+c-Kit) subset of BM cells (Adolfsson *et al*., 2001, Morrison, 2002, Muller-Sieburg *et al*., 2012). Evaluating the steady-state frequencies of each of LSK cell populations under steady-state conditions, we found that whereas lack of *Fzd6* did not appreciably affect the frequency and numbers of HSCs in weanling (3 wk) or young adult (6 wk) mice, *Fzd6* deficiency resulted in a modest, approximately two-fold increase in the number of LSKs with high cell–surface Flt3 (LMPPs) in young adult *Fzd6*$^{-/-}$ BM (Figure 1-publication no.1). We also observed a higher frequency of Flt3 expressing progenitor cells within Lin^{-}cKit^{+}Sca1^{lo} (SL) fraction of *Fzd6*$^{-/-}$ BM (data not shown). These results suggest an overall increase in Flt3 staining intensity in BM in the absence of *Fzd6*. It has been previously shown that upregulation of Flt3 expression in BM stem cell compartment is accompanied by loss of self-renewal capacity of HSCs (Adolfsson *et al.*, 2001). Considering also the fact that proliferating fetal HSCs shifts to a

quiescent adult phenotype *3–4 weeks* after birth (Bowie *et al.*, 2007) and no significant differences were observed at *3 week-old* mice in our analysis, it is possible to speculate that *Fzd6* might be involved in the regulation of HSC turnover. It is well known that a similar developmental switch occurs in HSCs during aging, which is characterized by increased proliferation of non-functional stem cells (Rossi *et al.*, 2007). Our observation that age-dependent HSC expansion was significantly diminished in early-aging (12-14 wk) *Fzd6*$^{-/-}$ mice (Figure 1), further suggests that *Fzd6* plays a critical role in the regulation of homeostatic maintenance and self-renewal divisions of HSCs throughout the adult life.

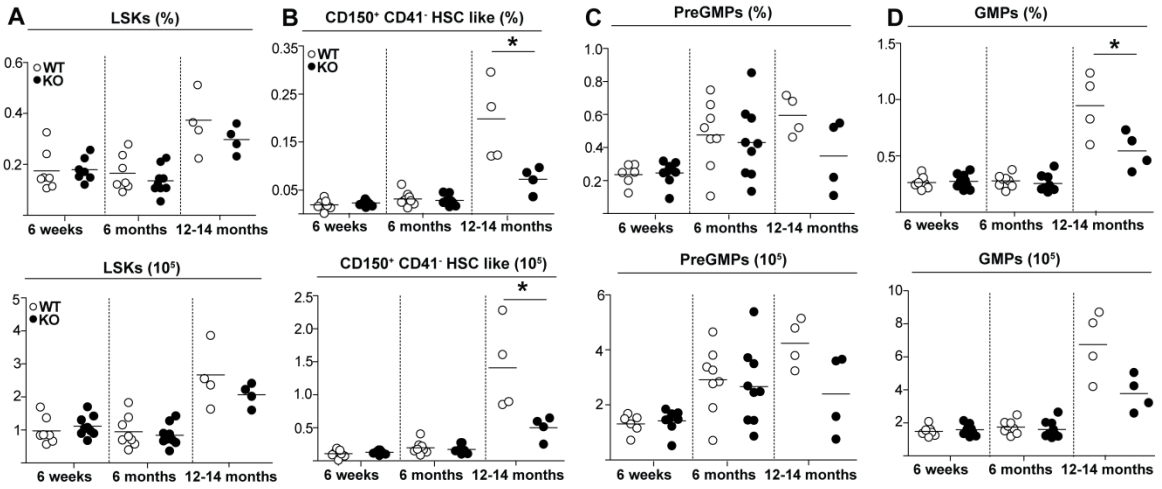

Figure 1. The percentage and numbers of HSPC populations in wild-type and *Fzd6* deficient young and early aging adult mouse bone marrow.

Quiescence is crucial for the proper maintenance of HSCs and its loss is often linked to HSC exhaustion (Pietras *et al.*, 2011). The establishment of cell polarity plays a critical role in the maintenance of a quiescent state in HSCs. Consistently, the loss of core PCP components in HSPCs including *Celsr1*/*Fmi* and *Fzd*8 alters stem cell pool size, self-renewal and engraftment (Sugimura et al., 2012). Enriched *Fzd6* expression in the slow-dividing fraction of primitive human HSCs suggests a similar role for *Fzd6* in the regulation of quiescence and self-renewal of HSPCs (Wagner et al., 2004). Unexpectedly, *loss of Fzd6* did not affect quiescence in our study, but rather resulted in a slight decrease in the proportion of proliferating HSCs (Figure 1-publication no.1).

These results suggest that *Fzd6* might regulate cell cycle progression and proliferation in HSCs, a finding consistent with previous reports in other cell types (Cantilena et al., 2011, Corda et al., 2017b, Wu et al., 2009a).

Although we observed that *loss of Fzd6* slightly increased the LMPP/HSC ratio within BM stem cell compartment, it did not significantly disrupt hematopoiesis in homeostatic state. It is now established that the majority of HSCs remain dormant and divide only five times during the lifetime of a mouse under steady-state conditions (Foudi et al., 2009, Wilson et al., 2008). Recent evidence shows that even the depletion of LT-HSCs from the hematopoietic system does not significantly compromise steady-state (Schoedel et al., 2016). While homeostatic hematopoiesis is mainly sustained by proliferating hematopoietic progenitors, hematopoietic stress can induce proliferation of LT-HSC pool (Busch et al., 2015, Sun et al., 2014). Indeed, it has been shown that quiescent and proliferating HSCs have a distinct gene expression signature as shown in transplantation settings (Venezia et al., 2004). Because, only a few HSCs are required to sustain homeostatic hematopoiesis (Busch et al., 2015, Schoedel et al., 2016, Sun et al., 2014), and the existence normal numbers of steady-state HSCs does not certainly reflect their functional capacity (Chambers et al., 2007, Desponts et al., 2006, Juntilla et al., 2010, McMahon et al., 2007, Sun et al., 2010, Ueno et al., 2009), the detection of the functional HSCs require more fastidious approaches. This prompted us to test the role of *Fzd6* in the regulation of HSC function under transplantation-induced stress. With this end, we examined the relative BM reconstitution efficiency of *Fzd6*$^{-/-}$ BM in a competitive setting (Figure 3-publication no.1). Analysis of PB chimerism in recipients showed a progressive loss of *Fzd6*$^{-/-}$ donor-derived BM cells in recipient mice between 4 to 16 weeks post-transplant. This was accompanied by a gradual loss of *Fzd6*$^{-/-}$GR1^{hi} granulocytes that was directly linked to the low numbers of *Fzd6*$^{-/-}$ HSCs in the BM from the primary recipient mice. Consequently, *Fzd6*$^{-/-}$ HSC exhibited a strong defect in their ability to reconstitute short term- and long term-hematopoiesis in primary and secondary transplants. Following transplantation, engraftment of HSCs occurs as a multistep process involving homing of donor cells to the BM cavity and stem cell adhesion in the niche (Srour *et al.*, 2001). Loss of cell polarity is often associated with impaired homing ability and consequent defective repopulation. The Rho family of GTPases (Cdc42, Rac and Rho) was shown to be activated through stimulation of CXCR4/CXCL12 signaling. Consistently, the deletion of Rac-1 leads to reduced migration in response to CXCL12 and diminishes the homing of HSCs to the endosteum (Williams *et al*., 2008). Likewise,

Cdc42 deficient HSCs show defective homing due to altered reorganization response to CXLCL2 as well as defective adhesion and trans-endothelial migration (Yang *et al*., 2007). The core PCP receptor Ptk7 is also involved in directing homing of HSPCs to the BM following transplantation. Given the fact that *Fzd6*$^{-/-}$ BM cells exhibited poor reconstitution capacity in the vast majority of primary recipient mice already at 4 weeks post-transplant, we speculated that this could be an early event after transplantation. A previous study has shown that recruitment of HSCs to the BM is completed during the first 12 to 24 hours following transplantation (Lewandowski *et al.*, 2010). Our results show that comparable frequencies of *Fzd6*$^{-/-}$ and *Fzd6*$^{+/+}$ LSKs and HSCs are found in recipient BM 16 hours post-transplant. Moreover, our colony-forming assays revealed no difference in *in vitro* myeloid differentiation capacity of *Fzd6*$^{-/-}$ progenitor cells at this time point (Figure 5-publication no.1). These data indicate that loss of *Fzd6* does not affect initial homing and differentiation potential of ST-HSCs/MPPs *in vivo*. It was previously reported that HSC proliferation occurs on the second or third day following transplantation, self-renewal divisions appear to take place on between 5 to 7 days post-transplant for the massive expansion of HSPCs (Lewandowski *et al.*, 2010). Consistent with these results, *Fzd6*$^{+/+}$ HSPCs progressively multiplied their numbers between day 4 and day 8 following injections that was correlated with increased cell cycle progression. In contrast, the numbers of *Fzd6*$^{-/-}$ HSPCs remain unchanged in the first week of transplantation (Figure 6-publication no.1). Therefore, *Fzd6* might regulate self-renewal divisions and proliferation of ST-HSC/MPPs and long-term maintenance of HSC pool.

Recent studies indicate that stress hematopoiesis, which occurs following BM transplantation is subject to distinct molecular and cellular regulatory networks (Rossi *et al*., 2012). When HSCs exit quiescence and enter the cell cycle, self-renewal occurs through SCD and ACD. Numb protein, a negative regulator of Notch signaling, is known to modulate SCD/ACD. Increased inheritance of Numb in one of two daughter cells points to ACD, while Numb protein segregation in both daughter cells indicates SDD and sustained low levels of Numb in both daughter cells marks SCD (Schroeder, 2007a). During homeostasis, the quiescent stem cell pool intrinsically restricted to ACD while stress conditions can promote both ACD and SCD. Regulation of appropriate balance between these two fate decisions is the essence of the hematopoiesis as increased differentiation can result in exhaustion of HSC pool. Polarity is an attractive candidate mechanism for the regulation of the outcome (self-renewal versus differentiation) of HSPC divisions under stress conditions. Evidence indicates that loss polarity can result

in the stem cell defects by altering the ratio of self-renewal divisions. For instance, dynein binding protein *Lis1*, which regulates inheritance of fate determinants in daughter cells during the cell divisions, plays a crucial role in cell polarity determination. The loss of Lis1 leads to increased inheritance of Numb and results in increased frequency of asymmetric HSC divisions, consequently a bloodless phenotype (Zimdahl *et al*., 2014). Reduction in essential components of the cell polarity signaling has been indicated during stress hematopoiesis. For instance, reduced expression levels of non-canonical Wnt components Fmi and *Fzd*-8 in LT-HSCs in response to 5FU treatment (Sugimura et al., 2012), may account for the loss of self-renewal in LT-HSCs due to excessive proliferation. These studies suggest the repression of gene sets involved in HSC polarity results in reduced symmetric self-renewal and increased symmetric differentiation divisions, subsequently leading to progressive depletion of functional HSCs (Will *et al*., 2013). It will be of interest to investigate whether the loss of *Fzd6* favors SDDs in HSCs leading to exhaustion of stem cell pool in response to transplantation stress.

Because active cross talk between the BM niche and HSPCs is essential for stable hematopoietic repopulation, it is also crucial to understand how donor *Fzd6*$^{-/-}$ HSPCs interacts with different components of recipient BM microenvironment. *Fzd6* is highly expressed in OBs (Spencer *et al*., 2006), MSCs (Okoye *et al*., 2008, Etheridge, 2004) and endothelial cells (Goodwin *et al*., 2006), which are important components of the BM microenvironment. Adhesion of donor HSCs within the endosteal niche, particularly their attachment to OBs plays a crucial role in the expansion of HSPCs following transplantation (Jung *et al*., 2007, Sahin *et al*., 2012), thus decreased adhesion could lead to a defective engraftment and subsequent loss of the stem cell pool. Indeed, our data demonstrate that inadequate expansion of *Fzd6*$^{-/-}$ HSCs was a consequence of strong caspase-3 activation that was followed by almost total depletion of HSCs as early as 8 days after transplantation. Fmi and *Fzd*8 have been shown to mediate a homophilic interaction between N-cadherin^{+} OBs and HSCs at the interface of these cells (Sugimura et al., 2012). Therefore, one can speculate that *Fzd6* might be also involved in such complex interactions. Consistent with this notion, we observed that *Fzd6* potentially restrict the size of Rho-related small GTPase Cdc42 clustering in HSPCs (Figure 2-publication no.1). Cdc42 is known to play a central role in cell polarization, adhesion and migration by dictating cell fate decisions via regulation of actin cytoskeleton or the microtubule network. Considering the fact that constitutively increased Cdc42 activity in HSPCs is associated with the activation of JNK-mediated apoptotic machinery and

defective engraftment (Wang *et al*., 2006a) and loss of *Fzd6* in HSPCs also results in higher levels of phosphorylated active JNK, it is likely that *Fzd6* serve as negative regulator of Cdc42/JNK signaling in HSPCs and support survival and expansion of HSC pool. Future studies are needed to investigate the role of *Fzd6* in polarity in the establishment and mode/outcome of HSPCs.

Previous studies indicated that *Fzd6* act as a negative regulator of β-catenin-dependent canonical Wnt pathway in other cell types (Cantilena *et al*., 2011, Golan *et al*., 2004b, Mirkovic *et al*., 2011b). In our study, although we did not detect any differences in active β-catenin expression at the baseline, elevated levels of activated β-catenin *Fzd6*$^{-/-}$ HSPCs following transplantation indicate that *Fzd6* might be a negative regulator of β-catenin-dependent Wnt signaling during regeneration (Figure 6-publication no.1). It was previously shown that while proper amount canonical Wnt activity maintains a functional stem cell pool, sustained activation of β-catenin in HSCs results in the down-regulation HSC self-renewal genes and subsequent loss of functional HSCs (Luis et al., 2011, Scheller et al., 2006a). Thus, the constitutive β-catenin activation in the absence of *Fzd6* might be the cause of impaired self-renewal and multi-lineage defects observed in recipient mice.

2. *Fzd6* regulates endotoxin-induced expansion and myeloid differentiation of hematopoietic stem/progenitor cells

Mouse models of steady-state and bone-marrow (BM) transplantation-induced stress hematopoiesis have provided valuable information to identify key molecules and signaling pathways that regulate hematopoietic stem/progenitor cell (HSPC) function *in vivo*. Acute inflammation is characterized by rapid generation and mobilization of myeloid effector cells from the BM, leading to inflammatory leukocytosis. Myeloid effector cells, particularly granulocytes and monocytes act as the first line host defense against pathogenic agents (Lacy *et al*., 2015). Even though HSCs predominantly remain quiescent under steady-state conditions, recent studies suggest that they directly respond to infections by expanding the progenitors and producing myeloid cells capable of destroying the microbial pathogens (Scumpia *et al*., 2010, Ueda *et al*., 2005). Pro-inflammatory and inflammatory cytokines directly affect expansion and myeloid

differentiation of HSPCs during infections (Baldridge *et al.*, 2011). Thus, balanced production of these factors plays a critical role in preserving an intact stem cell pool, a critical feature of host defense. Sepsis is one of the best examples of excessive production of pro-inflammatory cytokines, which promote HSPC exhaustion and myelosuppression that can eventually lead to death (Zhang *et al.*, 2016). Lipopolysaccharide (LPS) or endotoxin from gram-positive bacteria accounts for almost 60% of sepsis cases (Cohen, 2002). However, by which mechanisms HSPCs respond to inflammation is incompletely understood. In this thesis, we investigated the role of Wnt/*Fzd6* signaling in response to LPS-induced acute inflammation. Our findings demonstrate that *Fzd6* regulates the expansion and myeloid outcome of HSPCs through limiting hyper-immune response during endotoxin-induced inflammation (see Appendix).

During steady-state hematopoiesis, *Fzd6*$^{-/-}$ deficient BM was present with increased numbers of LMPPs but the potential lymphoid bias was largely absorbed (Figure S1-publication no.1). Conversely, HSPCs derived from *Fzd6*$^{-/-}$ BM showed a substantial decrease in *in-vitro* granulocyte-macrophage colony formation in response to myeloid cytokines GM-CSF and IL-3 (Figure A1), suggesting the defective growth and differentiation of myeloid progenitors in the absence of *Fzd6*. Considering the importance of myeloid cells in host defense, we investigated the hematopoietic changes in *Fzd6*$^{-/-}$ mice in response endotoxin-induced inflammation. With this end, we challenged *Fzd6*$^{-/-}$ and *Fzd6*$^{+/+}$ mice with two sub-lethal doses of LPS by two days intervals. As previously reported (Scumpia *et al.*, 2010), a single dose of LPS induced a rapid LSK expansion beginning 24h post-infection and lasting 48h before the second dose (Figure 7-publication no.1 and Figure A2). This increase was more pronounced in the LT-HSC (CD41^{-}CD150^{+}CD48^{-} LSKs) subset whose numbers gradually increased over the course of 72h LPS challenge. Indeed, LPS treatment promoted the recruitment of LT-HSCs from the dormant into an activated state and less than 50% of this cell subset was found to be quiescent 24h after the first dose of LPS (Figure A2). Increased expansion of LT-HSCs was accompanied by myeloid differentiation, as the expression of CD48 increased in CD150^{+} HSC-like cells and a gradual increase is also observed in the numbers of myeloid-biased MPPs (MMP2:CD41^{-}CD48^{-}CD150^{-}LSKs) (Wilson et al., 2008, Zhang et al., 2016). Although the initial LPS response was not impaired in *Fzd6*$^{-/-}$ HSPCs at 24h, *Fzd6*$^{-/-}$ LT-HSCs failed to expand and generate efficient numbers of myeloid biased MPPs beginning 48h after the first dose and 24h after the second dose of LPS injection (Figure A2). Since the cell-cycle progression of *Fzd6*$^{-/-}$ LT-HSCs were

similar to *Fzd6*$^{+/+}$ HSCs, it was likely due to the reduced proliferation capacity rather than lack of activation of *Fzd6*$^{-/-}$ HSCs. It was previously shown that toll-like receptor (TLR) signaling plays an active role in HSC activation in response to pathogen-induced stimulation during emergency hematopoiesis (Clapes *et al*., 2016). TLR4 is specific for LPS recognition and highly expressed on murine HSCs and GMPs (Takizawa *et al*., 2012). However, no differences are observed in TLR4 expression levels on the surface of HSCs from LPS-treated *Fzd6*$^{+/+}$ and *Fzd6*$^{-/-}$ mice (Figure A3). This data indicates that LPS component was sufficiently recognized on the surface of *Fzd6*$^{-/-}$ HSPCs.

We have previously shown that *Fzd6* act as a negative regulator of β-catenin activation under BM transplantation stress (Figure 6-Publication no1). The involvement of Wnt/β-catenin signaling in the LPS-induced inflammatory responses of other cell types has been shown in culture (Jang *et al.*, 2017). Moreover, the induction of β-catenin-dependent Wnt signaling in HSCs was found to accompany HSC expansion in response 5-FU treatment (Sugimura et al., 2012). However, we did not detect any increase in intracellular active β-catenin levels in HSCs from LPS-treated mice (data not shown), suggesting that low dose of LPS is not sufficient to induce activation of canonical Wnt signaling in HSCs.

Sca-1, also known as Ly6a (lymphocyte antigen 6 complex, locus A) is one of the cell surface proteins whose expression is highly enriched in HSPCs and myeloid progenitors are found in the Lin$^-$Sca-1$^-$c-Kit$^+$ cell subset within the BM during steady-state hematopoiesis (Morrison, 2002). Bacterial infections and LPS have been shown to induce Sca-1 expression in not only in LSKs but also in myeloid progenitors (Buechler *et al*., 2013, Shi *et al.,* 2013). Up-regulation of Sca-1 expression correlates with HSC activation and increased myeloid differentiation in inflammatory settings (Essers *et al.*, 2009). Thus, disruption of Sca-1 impairs myeloid-colony formation of HSPCs at steady state (Ito *et al.*, 2003) and during inflammation (Melvan *et al.*, 2011). In agreement with these studies, we observed increased upregulation of Sca-1 in Lin$^-$c-Kit$^+$ HSPCs (Figure A4). As previously reported (Buechler *et al*., 2013), there was a decline in Sca-1$^-$ GMPs over the course of treatment while a gradual increase was observed in the numbers of Sca-1$^+$ emergency GMPs (eGMPs) in *Fzd6*$^{+/+}$ BM (Figure A4). In contrast, *Fzd6*$^{-/-}$ BM were present with significantly fewer numbers of Sca-1$^+$ HSPCs and Sca-1$^+$ eGMPs at 24h after the second dose of LPS injection. This could be explained by the increased myeloid differentiation of *Fzd6*$^{-/-}$ eGMPs. As expected, *Fzd6*$^{+/+}$ mice exhibited a rapid decline in BM cellularity and a sustained reduction in in the absolute numbers of

lymphoid cells, particularly CD19+ B cells and LMPPs in the BM at 24h after the first dose of LPS (data not shown). The numbers of granulocytes was also declined at 24h post-injection. This was accompanied by a relative increase of myeloid effector cells, particularly granulocytes and Ly6C^{hi} inflammatory monocytes in the blood circulation, "injection" site, peritoneal cavity and the extramedullary hematopoietic site, spleen (Figure A5 and Figure A6). Conversely, we observed an increase in BM myeloid cell numbers at 48h after the first injection that became more pronounced at 24h following the second injection. This increase was presumably associated with appearance and rapid expansion of Sca-1+ emergency GMPs as the increased numbers of eGMPs reached to a plateau at 48h post-infection (Figure A4). However, LPS-treated *Fzd6*$^{-/-}$ mice had overall reduced accumulation of myeloid cells in the BM, spleen, peritoneal cavity (Figure A5 and FigureA6). It was previously reported that lethal dose of LPS administration increases HSC death and lead to a decline in HSPC cell numbers (Zhang *et al.*, 2016). Yet, no differences were observed in the caspase-3 activity in *Fzd6*$^{-/-}$ LSKs. HSCs, GMPs and myeloid cell subsets following LPS treatment when compared to *Fzd6*$^{+/+}$ BM (data not shown). Therefore, it can be suggested that *Fzd6* deficiency not only alters the expansion of HSPCs but also decreases the myeloid output without affecting the survival of progenitors in response to LPS.

In addition to cell-intrinsic regulation, BM microenvironment also actively contributes to the activation and response of HSPCs during infection (Boettcher *et al.*, 2014, van Lieshout *et al.*, 2012). Modulation of stromal-derived signals by certain pathogens has been shown to induce expansion and myeloid differentiation of HSPCs (Zhou *et al.*, 2012). LPS is known to induce the production of G-CSF by endothelial cells, macrophages, epithelial cells and fibroblasts in the BM microenvironment (Bendall *et al.*, 2014). Increased G-CSF levels induce the recruitment of myeloid cells to the inflammation sites that maximizes pathogen clearance (Bendall *et al.*, 2014). Elevated G-CSF levels were shown to alter BM microenvironment by suppressing CXCL12 production in stromal cells (Day *et al.*, 2015, Eash *et al.*, 2010, Semerad *et al.*, 2005). Down-regulation of CXCL12 allows HSPC mobilization from the BM into the circulation (Johns *et al.*, 2012). The spleen serves as an extramedullary hematopoietic organ during inflammation and infection. Although the numbers of HSPCs remain very low in homeostasis, infection induce the accumulation these cells in the spleen (Burberry *et al.,* 2014).Our results show that *Fzd6*$^{-/-}$ BM is able to upregulate G-CSF production, which was accompanied by down-regulation of CXCL12 at similar levels compared to *Fzd6*$^{+/+}$

in response to LPS (Figure A7). Moreover, similar numbers of HSPCs observed in the spleen following LPS stimulation point that inadequate expansion HSCs and myeloid progenitor was not due the increased extramedullary accumulation. LPS stimulation also increased the expression levels of the monocyte chemoattractant protein-1 (MCP-1/CCL2). Evidence show that CAR cells and Nestin^{+} MSCs are the main producers of MCP-1 in response to LPS (Shi *et al.*, 2011a). MCP-1 plays a critical role in monocyte mobilization and trafficking in response to low dose LPS (Shi *et al.*, 2011a). Therefore, sufficient upregulation observed in MCP-1 levels in the BM and its ligand CCR2 on Ly6C^{hi} monocytes in *Fzd6*$^{-/-}$ mice further indicate that the overall decreased numbers of monocytes cannot be explained by defective accumulation or trafficking of this cell subset (Figure A7). Interestingly, other pro-inflammatory factors, particularly MIP-1α, MIP-2 and TNF-α were only increased in the BM of *Fzd6*$^{-/-}$ mice following LPS stimulation. While the production of MIP-1α is restricted to hematopoietic cells, particularly activated macrophages (Berkman *et al.*, 1995, Christman *et al.*, 1992), MIP-2 can be produced by different cellular sources including macrophages (Wolpe *et al.*, 1989), endothelial cells (Li *et al.*, 2000) and mast cells (Biedermann *et al.*, 2000) in response to bacterial infections. The release of MIP-1α and MIP-2 from hematopoietic and non-hematopoietic cells in the BM is not only crucial for myeloid cell recruitment but also enhanced phagocytic function necessary for the clearance of pathogens (Fahey *et al.*, 1992, Tumpey *et al.*, 2002, Walley *et al.*, 1997). However, elevated levels of these two chemokines have been reported to supress not only the proliferation but also myeloid differentiation of HSPCs (Broxmeyer *et al.*, 1990, Cook, 1996, Eaves *et al.*, 1993). Likewise, excessive production of TNF-α, the major regulator of endotoxin induced immune response, not only inhibit HSPC proliferation (Pronk *et al*., 2011) but also leads to a block in myeloid differentiation (Sade-Feldman *et al*., 2013) during infections. Increased levels of MIP-1 α, MIP-2 and TNF- α, are often observed during severe sepsis in patients and experimental models (Brueckmann *et al.*, 2004, Lv *et al.*, 2014, Walley *et al.*, 1997). Indeed, significantly higher amount of body weight loss (≥ 20%) is observed in *Fzd6*$^{-/-}$ mice 24h after a lethal dose of LPS injection, thus indicating the severity of sepsis in these mice (data not shown). Our reciprocal transplantation assays revealed that *Fzd6* signaling regulates the differentiation and activation of myeloid cells in a cell autonomous fashion (Figure A8). Considering monocyte/macrophages are generated from donor HSPCs following transplantation and the initial response of *Fzd6*$^{-/-}$ HSPCs to LPS appear to be similar to *Fzd6*$^{+/+}$ mice, it is

likely that increased production of hematopoietic suppressive factors such as MIP-1α, MIP-2 and TNF-α from *Fzd6*$^{-/-}$ macrophages had a secondary role in decreased expansion and myeloid differentiation of HSPCs in our model. However, the functional relevance and activity of *Fzd6*$^{-/-}$ macrophages had yet to be elucidated.

3. *Fzd6* signaling accelerates the progression of *Leishmania donovani* parasite infection in mice

Visceral *Leishmania*sis (VL) is a potentially life-threating chronic disease, which is characterized by long-term impairment of the immune system. VL is caused by the infestation of macrophages with *Leishmania donovani* parasites (Badaro *et al.*, 1986, Faleiro *et al.*, 2014). Because the liver and spleen are the main targets of in VL, most studies have investigated the mechanisms underlying organ-specific immune-pathological changes associated with cell mediated immunity in these tissues (Faleiro *et al.*, 2014, Goto *et al.*, 2004). However, the involvement of the BM during the chronic phase of VL both in patients (Chandra *et al.*, 2013, Varma *et al.*, 2010) and experimental models (Cotterell *et al*., 2000a, Cotterell *et al*., 2000b)appears to be the major cause of progression from infection to chronic inflammatory disease. Recent reports suggest that HSPCs might play a critical role in coordinated host response to limit parasite infections by modulating hematopoietic activity (Belyaev *et al.*, 2010, Cotterell *et al.*, 2000a, Shi *et al.*, 2011c). However, the impact of the hematopoietic and stromal compartments of BM microenvironment on *Leishmania* parasite expansion is poorly understood. In chapter 3 of this thesis, we show that chronic *L. donovani* infection promotes expansion and myeloid differentiation of HSPC in the BM. Our results show that enhanced myeloid output in the BM plays a critical role in the chronic progression of VL. Our results further suggest that non-canonical Wnt/*Fzd6* signaling contributes to the establishment of persistent *L. donovani* infection through modulating HSC differentiation and cytokine production in the BM.

Leishmania parasites are intracellular protozoan parasites whose flagellated promastigotes exist within sandfly vector, while intracellular amastigotes are found in infected mammals. In infected mammals, *Leishmania* parasites preferentially infect mononuclear phagocytes, particularly macrophages, which can be found in the spleen, liver and BM (Stanley *et al.*, 2007). Experimental VL is initiated by intravenous injection of *L. donovani* amastigotes at high numbers. Following injection, *L. donovani*

amastigotes enter the liver, spleen and BM and rapidly multiply their numbers by invading host macrophages. Although parasite growth is controlled in the liver during the chronic phase (days 28-35) of infection (Paun *et al.*, 2011), a dramatic enhancement of parasite expansion in the spleen (Hammami *et al*., 2015) and BM (Cotterell *et al.*, 2000a) leads to the establishment of a persistent infection in these tissues.

It has been previously shown that *Leishmania* parasites does not directly infect HSPC, but their accumulation in the BM increases *in vitro* myeloid colony-forming ability of HSPCs during the chronic phase of experimental VL (Cotterell *et al*., 2000a). Other reports have pointed that *L. donovani* amastigotes induce *in vitro* production of myeloid growth factors and in turn enhance the numbers of mononuclear phagocytes, which are the main targets for parasite replication (Singal *et al*., 2005, Cotterell *et al*., 2000b). In this study, we showed that increased parasite growth in the BM during the chronic phase (days 21-35) of the infection enhance the numbers of myeloid cells, particularly Ly6C^{hi} monocytes in the BM and spleen (Figure 1-publication no.2). The importance of monocytes in defense against other Leishmania species (Passos *et al.*, 2015, Romano *et al.*, 2017) was previously demonstrated, however the role of monocytes in the pathogenesis of visceral Leishmaniasis was not well documented (Kaye *et al*., 2011). Our results suggest that myeloid cell precursors and monocytes are ideal secondary *in vivo* target cells of *L. donovani* amastigotes (Figure 3-publication no.2). Our *in vitro* infection assays further revealed that a remarkably higher proportion of infected monocyte were Ly6C^{+}, thus suggesting these immature inflammatory monocyte subset as the preferred mononuclear host cell similar to those observed in *L. major* infection (Romano *et al.*, 2017). Upregulation of IL-10 and Arginase1 on Ly6C^{hi} monocytes from infected mice at D28 pi suggested a regulatory function for this subset of monocytes during Leishmania infection (Figure 5-publication no.2). In contrast to naïve monocytes, Ly6C^{hi} monocytes at this stage expressed high levels of MHC-II and Sca-1 that resembles IFN-γ response (Askenase *et al.*, 2015). Indeed, increased IFN-γ production has been reported during the chronic phase of VL both in patients (Khoshdel *et al.*, 2009) and mice (Lehmann *et al.*, 2000). Consistently, we observed an increase in IFN-γ expression in the BM microenvironment at day 21pi which was accompanied by increased production of IFNγ-inducible factors, such as ICAM-1, IP10/Cxcl10 and Cxcl9 at days 21-28pi (Figure 8-publication no.2). IFN-γ is known to induce activation and myeloid differentiation of HSPCs in infection settings (Baldridge *et al.*, 2010a, Matatall *et al.*, 2014). In agreement with these studies, enhanced parasite growth in the BM

activated LT-HSCs and promoted their expansion and differentiation with a contaminant increase in the numbers of ST-HSCs. Increased intracellular levels of active β-catenin in HSCs suggest the contribution of canonical Wnt signaling to HSC activation in response to Leishmania infection (Figure 1-publication no.2). Enhanced expansion of HSCs directly correlated with the parasite growth in the BM (Figure 5-publication no.2), thus indicating a role for HSC expansion in host defense. Appearance of Sca-1^{+} GMPs and cMOPs during the chronic stage (Figure 2 and Figure 4-publication no.2) supports the idea that sensing of danger signals by these progenitors directly affects the generation $Ly6C^{hi}$ monocytes with a regulatory phenotype.

In addition to enhanced IFN-γ-inducible factors, we also observed dramatically increased production of MIP-1α, IL-1 (IL-1α and IL-1β) and MCP-1 in the BM microenvironment. MIP-1α and MCP-1 do not only act as a chemoattractant to promote the recruitment of monocytes (DiPietro *et al.*, 1998, Shi *et al.*, 2011b) but contributes to parasite uptake and phagocytic activity of monocytes/macrophages (Dey *et al.*, 2005, Sato *et al.*, 1999) during L. donovani infection. Likewise, increased production of IL-1 accompanies enhanced parasitization and killing ability of monocyte/macrophages during *in vitro L. donovani* (Reiner *et al.*, 1990) and *L.major* infection (Cillari *et al.*, 1989). Yet, IL-1 is also known to induce myeloid differentiation of HSPCs (Pietras *et al.*, 2013). In line with these results, exposure to BM supernatant obtained from infected mice at day 28pi, induced myeloid differentiation of Lin depleted HSPCs and generation of $Ly6C^{hi}$ monocytes in culture (Figure 9-publication no.2). Moreover, our results demonstrated that infected BM supernatant increased the parasite uptake of monocytes, indicating susceptibility to intracellular *L. donovani* infection. In contrast, infected *Fzd6*$^{-/-}$ BM supernatant showed significantly elevated levels ($P<0.05$) of MIP-1α, CXCL9, IL-1 (IL1a and IL1b) when compared infected *Fzd6*$^{+/+}$ BM. In addition MIP-1α, chronic exposure to elevated levels of CXCL9 (Lu *et al.*, 2012) or IL-1 (Pietras et al., 2016) can also lead to the depletion of HSC pool and myelosuppression. Consequently, *Fzd6*$^{-/-}$ mice presented with significantly fewer numbers of BM HSCs and myeloid progenitors and a dramatic reduction in the accumulation of myeloid cells, particularly $Ly6C^{hi}$ monocytes in the BM, spleen and liver at day 28pi compared to those in *Fzd6*$^{+/+}$ mice. Decreased myeloid output in *Fzd6*$^{-/-}$ mice directly correlated with a reduction in the parasite growth due to the corresponding decrease in safe targets in these mice (Figure 5-publication no2). Furthermore, *Fzd6* deficiency also enhanced parasite killing ability of macrophages in culture (Figure 7-publication no.2). A previous study has shown that

non-canonical Wnt5a signaling intermediates Rac1 and Rho kinase and inhibits *L. donovani* growth in infected macrophages *in vitro (Chakraborty et al., 2017)*. It will be interesting to determine the interaction of Fzd6 signaling with Wnt5a and down-stream signaling during parasite infection.

4. Conclusion: a new role for *Fzd6* signaling in homeostatic and stress-induced hematopoiesis

Homeostasis and regeneration of the hematopoietic system upon injury depend on the balanced fate decisions of HSCs regarding to quiescence, self-renewal and survival. The reduction or depletion of functional stem cell population can be potentially devastating and contribute to the development of malignancies such as leukemia and lymphoma. This raised the questions of which signaling mechanisms control cell-fate decisions and function of these clinically valuable cells, what goes awry in hematological malignancies and how to enhance marrow engraftment after BM transplantation. In recent years, non-canonical Wnt signaling components have emerged as critical regulators of HSC function in homeostasis and in regeneration. The deregulation of this pathway has been also shown to promote leukemia development. The current study gives new insights into the role of non-canonical Wnt/Fzd6 signaling in homeostatic and stress-induced hematopoiesis. We provide evidence for the requirement of cell autonomous Fzd6 signaling in the expansion, self-renewal and survival of HSPCs following BM transplantation. Considering the clinical utility of HSCs in the hematologic disease and to restore hematopoiesis in cancer patients, we anticipate that Fzd6 signaling is a promising therapeutic target to modulate HSC engraftment and survival for new treatment concepts to overcome the limitations of BM transplantation.

Although BM transplantation remains to be a gold standard to assess proliferation and multi-lineage differentiation of HSCs *in vivo*, new efforts focus on understanding how HSC function is regulated in response to natural signals, particularly those stemming from the interplay between infectious agents and the host immune system. Recent evidence shows that the ability of HSPCs to respond to infections by expanding the progenitors and producing myeloid effector cells capable of destroying the microbial pathogens, while preserving an intact stem cell pool, is a critical feature of host

defense. However, there are still many outstanding questions about which networks (cell types and molecular mediators) contribute to the regulation of hematopoietic lineage specification and myeloid cell production and how the crosstalk between BM microenvironment and HSPCs affects hematopoietic output at steady state and during chronic inflammation. Elements of non-canonical Wnt signaling, which are known to be crucial in lineage specification of HSCs during development, have recently been identified as the important modulators of the inflammatory immune response. Yet, the precise role of this signaling pathway in HSC response and emergency myelopoiesis during acute and chronic inflammatory stress had not been studied. The current study extends the role of Wnt/Fzd6 signaling in emergency myelopoiesis and regulating innate effector functions by using mouse models of endotoxin-induced inflammation and chronic visceral leishmaniasis. Findings of this study demonstrate that during chronic parasitic infection, the cytokine milieu from BM microenvironment directly regulate HSC expansion and promotes the generation of distinct myeloid progenitors with a regulatory phenotype, which generates regulatory immune effector cells to enhance host-defense. Our study further identifies a potential feedback mechanism in which Fzd6 signaling may fine tune the activation and effector function of antigen presenting cells, thereby protecting the hematopoietic system from hyper-activation during pathogen-induced inflammation. Further studies needs to be undertaken to elucidate the molecular mechanisms *Fzd6* action in the function and lineage specification of HSCs and immune response. Filling the gaps in understanding the regulatory networks that balance stem cell expansion and lineage differentiation will enhance the treatment options for pathological conditions such as chronic infections and inflammatory diseases.

CHAPTER 5 REFERENCES

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ANNEX-I

The role of *Fzd6* in endotoxin-Induced inflammation

Hematopoietic Frizzled-6 signaling regulates myeloid differentiation through modulating immune response during endotoxin-induced inflammation

1. SUMMARY

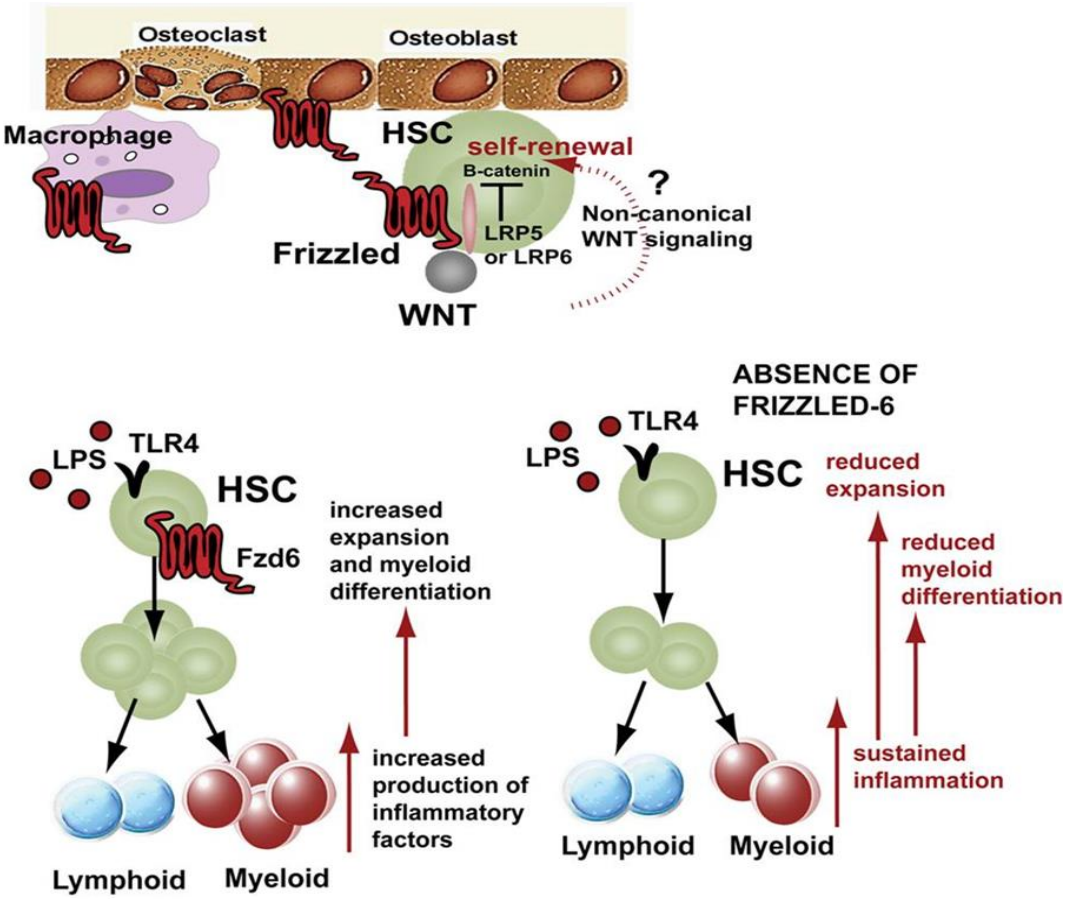
The ability of hematopoietic stem/progenitor cells (HSPCs) to respond to infections by expanding the numbers of myeloid cells plays a crucial role in host defense. Excessive immune response during bacterial sepsis results in severe depletion of HSPCs and myelosuppression, which can eventually lead to death. Lipopolysaccharide (LPS) or endotoxin from gram-positive bacteria accounts for almost 60% of sepsis cases, but the molecular mechanism of modulating inappropriate immune response during LPS-induced inflammation remains incompletely understood. Elevated levels of Wnt ligands have been implicated in both patients with septic shock and experimental models of LPS-induced endotoxemia. Here we show that non-canonical Wnt/Fzd6 signaling regulates emergency myelopoiesis and the establishment of balanced immune responses during endotoxin-induced inflammation. Our findings demonstrate that Fzd6 signaling fine tune the activation and effector function of antigen presenting cells, thereby protecting the hematopoietic system from hyper-activation during pathogen-induced inflammation.

Keywords: hematopoietic stem cells, LPS, inflammation, Wnt signaling, emergency myelopoiesis

Key Points

- *Fzd6* deficient mice are more susceptible to endotoxin shock
- Increased production of pro-inflammatory factors by *Fzd6*$^{-/-}$ hematopoietic cells impairs the expansion and myeloid differentiation of HSPCs in response to LPS

2. GRAPHICAL SUMMARY



3. INTRODUCTION

Inflammatory response of the immune system is a multistep process, which involves the recognition of pathogens by myeloid effector cells via chemokine receptors and PRRs on their surface, the recruitment of these cells to the infection sites and the clearance of pathogenic agents (Lacy *et al*., 2015). Due to their short life span, myeloid cells have to be continuously supplied during infections by the expansion of myeloid progenitors in the bone marrow (BM). (Furze *et al.*, 2008, Suratt *et al.*, 2001). This demand-driven hematopoietic state is a component of “emergency myelopoiesis”, which

is characterized by rapid generation and mobilization myeloid effector from the BM leading to inflammatory leukocytosis (Takizawa *et al.*, 2012). The fine balance between inflammatory and anti-inflammatory immune responses plays a crucial role in controlling infections while preventing excessive damage to host tissues. Dysregulation of pro-inflammatory responses and myelosuppression are the major cause death from bacterial sepsis (Cohen, 2002). Endotoxins, or termed bacterial lipopolysaccharide (LPS), is recognized as the most potent mediator of sustained inflammation and increased hyper-responsiveness in the pathogenesis of sepsis and septic shock (Cohen, 2002). LPS has been previously shown to induce hematopoietic stem/progenitor cell (HSPC) depletion and reduce myeloid cell generation in the BM (Zhang *et al.*, 2016), but molecular mechanisms underlying LPS-induced HSC exhaustion and myelosuppression are incompletely understood.

Elements of Wnt/Frizzled signaling pathways, which are known to play a crucial role in hematopoiesis (Lento *et al.*, 2013), have recently been identified as the modulators of the inflammatory immune response. Accumulating evidence indicates that Wnt/Frizzled (Fzd) signaling not only modulates to the pro- and anti-inflammatory immune responses (Blumenthal *et al.*, 2006, Christman *et al.*, 2008, Sen *et al.*, 2000), but also contributes to the tissue injury/regeneration (Irvine *et al.*, 2015, Tian *et al.*, 2003) in bacterial infections and chronic inflammatory disorders. Elevated and reduced levels of different Wnt ligands have also been reported in the blood of patients with septic shock (Gatica-Andrades *et al.*, 2017, Pereira *et al.*, 2008, Schulte *et al.*, 2015) and experimental mouse models during endotoxin-induced inflammation (Gatica-Andrades *et al.*, 2017). The activation of canonical Wnt/β-catenin signaling in response to LPS has been shown to enhance the production of pro-inflammatory cytokines, which promotes severe systemic immune response (Gatica-Andrades *et al.*, 2017). Hence, the suppression of canonical Wnt/ signaling dampens *in vitro* (Jang *et al.*, 2017) and *in-vivo* (Gatica-Andrades *et al.*, 2017) pro-inflammatory cytokine production in response to LPS. Likewise, the blocking of β-catenin-independent Wnt ligand, Wnt5a results in the suppression of *in vitro* LPS-induced inflammatory response (Jang *et al.*, 2017). However, the role of Wnt signaling in the activation and myeloid output of HSCs in response to endotoxin-induced inflammation has not been studied. We recently reported a crucial role for Wnt/Frizzled-6(Fzd6) signaling in the expansion mouse BM HSCs under replicative transplantation stress and in response to sub-lethal LPS challenge (Abidin *et*

al., 2015). In this study, we examined the role of *Fzd6* in the activation and myeloid lineage fate determination of HSCs in response sub-lethal and lethal LPS administration. Our results show that non-canonical Wnt/Fzd6 signaling is cell-autonomously required for the expansion and enhanced myeloid output of HSPCs during LPS-induced emergency myelopoiesis. Here, we also provide evidence that Wnt/Fzd6 signaling fine tune the activation of pro-inflammatory responses and protect the hematopoietic system from hyper-activation in response to endotoxin. Our findings suggest that *Fzd6* signaling may be a promising therapeutic target for the treatment of sepsis and other inflammatory disorders.

4. RESULTS AND DISCUSSION

4.1. *Fzd6* deficiency impairs the cytokine response of myeloid progenitors

Myeloid commitment and differentiation of HSPCs are a multistep process. Under steady-state conditions, myeloid subsets of immune cells are produced by committed progenitors, which include common myeloid progenitors (CMPs) and granulocyte/macrophage progenitors (GMPs). GMPs give rise to unipotent precursor cells that generate terminally differentiated granulocytes and monocytes/macrophages (Adolfsson *et al*., 2001, Morrison, 2002, Muller-Sieburg *et al*., 2012). We have previously shown that *Fzd6* deficiency does not affect the frequency and the numbers of myelo-erythroid progenitors, which include common myeloid progenitors (CMP), granulocyte/macrophage progenitors (GMP) and megakaryocyte/erythrocyte progenitors (MkP, Pre-CFU-E and CFU-E) in the BM (Figure S5-publication no2). Here we assessed *in vitro* colony forming activity of *Fzd6*$^{-/-}$ BM on semi-solid medium supplemented with GM-CSF and IL-3, which promote myeloid differentiation of multipotent, bipotent and unipotent progenitor cells. The BM cells derived from *Fzd6*$^{-/-}$ mice showed a substantial decrease in the granulocyte-macrophage colony formation in response to GM-CSF or IL-3 (Figure A1). The percentage of GR1^{hi} myeloid cells emerging from the BM myeloid progenitors in the presence of GM-CSF or IL-3 was also decreased in the absence of *Fzd6*. These results suggest an intrinsic role for *Fzd6* in differentiation of from bi-potent GMP progenitors to unipotent CFU-G and CFU-M precursor cells.

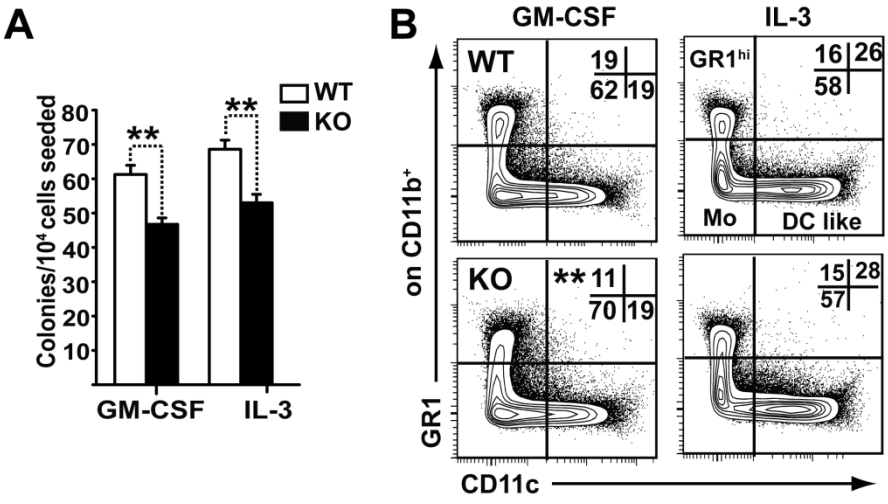


Figure A1. *Fzd6* is required for the growth and differentiation of myeloid progenitor cells

Freshly isolated BM cells at a density of 10^4 cells are seeded on semi-solid methylcellulose medium (Methocult GF M3231) supplemented with either 10 ng/ml GM-CSF or IL-3. The cultures were incubated at 37°C in 5% CO2 for 7–10 days and hematopoietic colonies (CFU-C, colony forming units) were counted under an inverted microscope. Harvested colony forming cells were further stained with antibodies against CD11b, CD11c and GR1 and analysed by flow-cytometry. A) The numbers of granulocyte-monocyte colonies generated by *Fzd6*$^{-/-}$ (KO) and *Fzd6*$^{+/+}$ (WT) BM cells in response to GM-CSF and IL-3. (B) Flow cytometry analysis of cells recovered from CFU assays. Numbers shown in different quadrants indicate the mean percentage in CD11b$^+$ cells. All histograms represent pooled data from at least three independent experiments for a total of at least five mice per group. **P<0.01.

4.2. *Fzd6* regulates LPS-induced expansion and differentiation of hematopoietic stem/progenitor cells

Because demand-adapted myeloid cell production is the hallmark of host defense (Boettcher *et al.*, 2016, Takizawa *et al.*, 2012), we examined the role of *Fzd6* in emergency myelopoiesis by using a LPS-induced systemic inflammation model. Our previous work showed that *Fzd6*$^{-/-}$ mice fail to achieve the expected expansion of HSPCs in response sub-lethal LPS-induced inflammation (Abidin *et al*., 2015), but the underlying mechanisms remain unclear. In this study, we challenged *Fzd6*$^{-/-}$ and *Fzd6*$^{+/+}$ mice with two sub-lethal doses of LPS by two days intervals and analyzed the hematopoietic compartment during 72 hours LPS challenge (Figure A2a). Similar to our previous findings, *Fzd6*$^{+/+}$ mice responded to LPS with a significant increase in the frequency and absolute number of BM Lin$^-$Sca-1$^+$c-Kit$^+$ (LSK) subset. Although *Fzd6*$^{-/-}$ mice behaved in a manner similar to *Fzd6*$^{+/+}$ mice at 24h and 48h following the first LPS injection, *Fzd6*$^{-/-}$

BM presented with fewer numbers of LSK cells at 24h following the second dose of LPS (Figure A2b). The LSK subset contains distinct cell HSPC populations, which can be further divided into long-term HSCs (LT-HSC; $CD41^{-}CD48^{-}CD150^{+}$), short-term HSCs (ST-HSCs; $CD41^{-}CD48^{+}CD150^{+}$) and myeloid-biased multi-potent progenitor cells (MPP2; $CD41^{-}CD48^{+}CD150^{-}$) as previously described (Wilson et al., 2008). Previous studies have shown that emergency hematopoiesis induced by LPS awakens the dormant LT-HSCs and promotes their entry into the cell cycle (Boettcher et al., 2012, Scumpia et al., 2010). In agreement with these studies, *Fzd6*$^{-/-}$ and *Fzd6*$^{+/+}$ mice responded to LPS with a significant increase in the numbers of LT-HSCs, following the first dose of LPS (Figure A2c and d). Notably, a parallel expansion was also seen in both frequency and absolute numbers of ST-HSC and MPP2 cell subsets when compared to PBS injected mice. Moreover, more than half of those LT-HSCs were found in G1 or S-G2-M phases of the cell cycle at 24h following first LPS challenge (Figure A2e). These results suggest that even a low dose of LPS is sufficient to activate and promote the expansion and differentiation of LT-HSCs. Although, we observed a gradual increase in the numbers of *Fzd6*$^{+/+}$ LT-HSCs and MPP2s over the course of 72h LPS challenge, *Fzd6*$^{-/-}$ mice exhibited no such expansion of LT-HSCs or MPP2s. This could be explained by either lack of self-renewal or an ineffective response to LPS. Because detection of LPS component by TLR4 on HSPCs is a crucial step in the initiation of immune response (Rodriguez *et al.*, 2009, Takizawa *et al.*, 2012), we analyzed TLR4 expression on HSCs and GMPs from LPS-injected mice following the second LPS challenge (Figure 3a). TLR4 expression levels on LPS-treated *Fzd6*$^{-/-}$ and *Fzd6*$^{+/+}$ HSPCs appeared to be similar in our assay. In agreement with previous studies (Burberry *et al.,* 2014, Esplin *et al.,* 2011), LPS treatment induced the accumulation of HSCs in the spleen, which serves as an extramedullary site (Figure 3b), In contrast to BM, LSKs and $CD150^{+}$ HSCs in the *Fzd6*$^{-/-}$ spleen were present at numbers comparable to *Fzd6*$^{+/+}$ mice following the second LPS injection. Of note, we did not also any difference in the apoptotic rate of Fzd6$^{-/-}$ and Fzd6$^{+/+}$ HSPCs in response to LPS (data not shown). These results suggest that lower numbers of HSCs in the Fzd6$^{-/-}$BM at 72h post-injection cannot be explained simply by impaired recognition of LPS component, increased apoptosis and/or extramedullary accumulation.

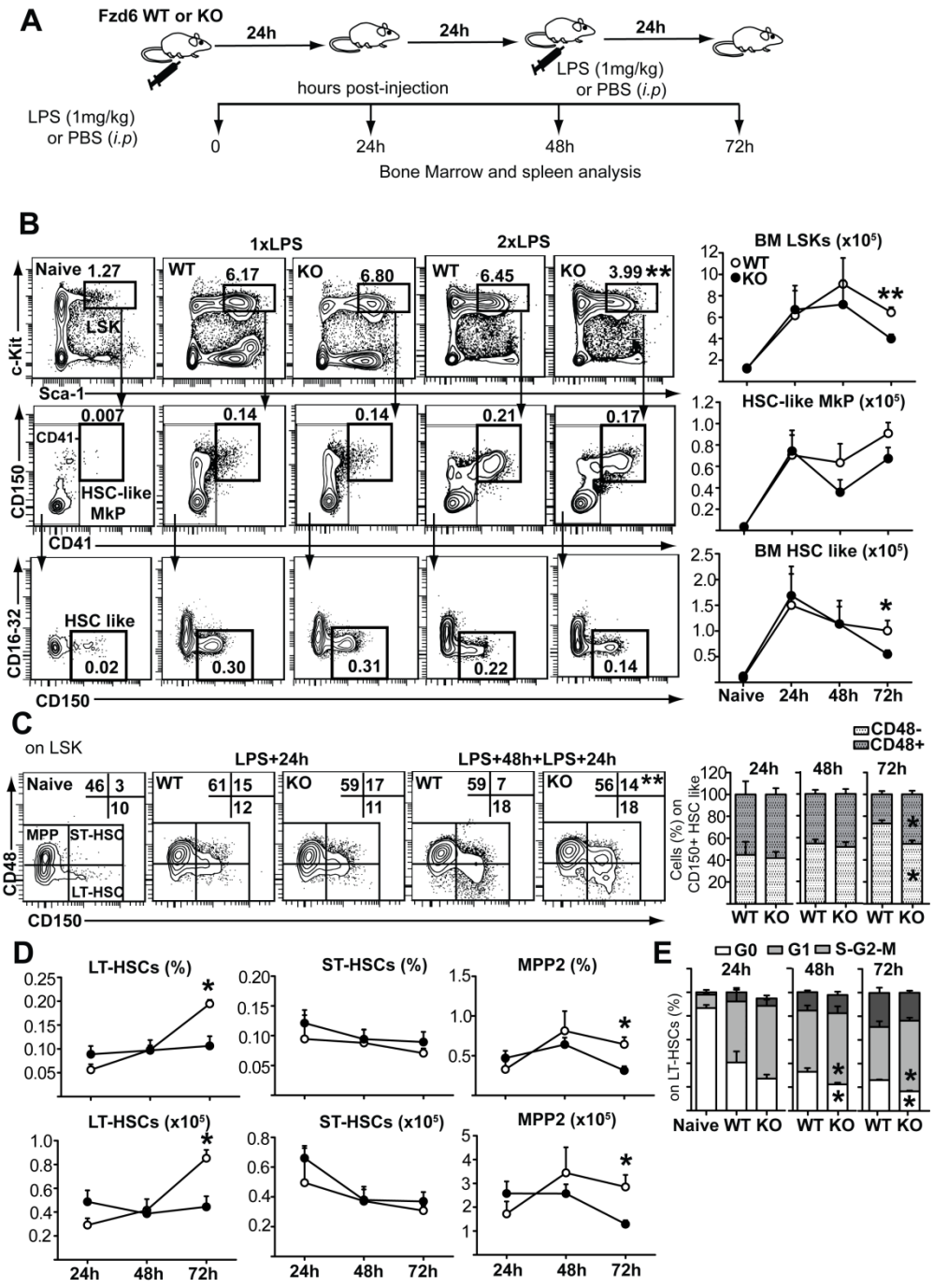


Figure A2. *Fzd6* deficiency impairs LPS-induced HSC expansion

A) To evaluate HSPC expansion LPS-induced inflammation, *Fzd6*$^{-/-}$(KO) and *Fzd6*$^{+/+}$ (WT) mice were given two i.p. injections of g-irradiated LPS (E. coli) at a dose of 1 mg/kg body weight with 48h interval. Their BM was harvested for flow cytometry and functional analysis every 24 h during 72h LPS challenge. (B) Representative flow cytometry data and gating strategy of LPS-treated BM HSPCs of *Fzd6*$^{-/-}$ and *Fzd6*$^{+/+}$ mice at various time points. BM cells were first gated on Lin- (B220^{-}CD3e^{-}CD11b^{-}GR1^{-}Ter119^{-}) and identified according to Sca1 and cKit (CD117) expression. Megakaryocyte progenitors (MkP) were identified as CD41^{+}CD150^{+} within Lin^{-}cKit^{hi}Sca1^{+} (LSK) population. Hematopoietic stem cells (HSCs) were

gated positive for CD150 (SLAM) and negative for CD16/32 and CD41 within LSKs. Graphs show absolute numbers of LSKs, HSC-like MkPs and CD150^{+} HSC-like cells in BM. (C) CD48^{-}CD150^{+} LT-HSCs, CD48^{+}CD150^{+} ST-HSCs and CD48^{+}CD150^{-} myeloid bias multipotent progenitor (MPP2) subsets were analysed within CD41^{-}LSK population. Histograms show the contribution of CD48^{-} and CD48^{+} subsets in total CD150^{+} HSCs. (D) Absolute numbers of different HSPCs progenitors at various time points. (E) E) Ki-67/Hoechst co-staining was used to distinguish the G0, G1, and S/G2/M cell cycle phases of CD48^{-} CD150^{+} LT-HSCs. *P<0.05; **P<0.01; ***P<0.001.

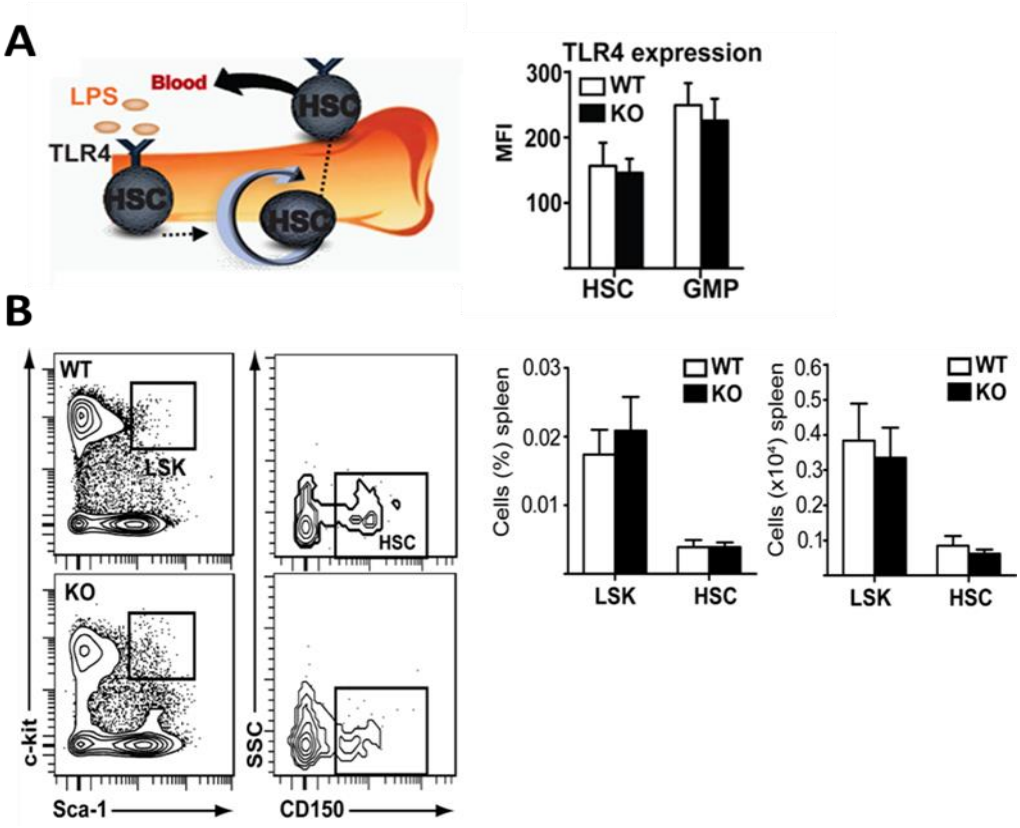


Figure A3. Impaired expansion of *Fzd6*$^{-/-}$ HSCs in the bone marrow is not due to impaired Toll-like receptor-4 (TLR4) expression or increased accumulation in the spleen.

(A) Bacterial lipopolysaccharide (LPS) is recognized by TLR4 on hematopoietic stem cells (HSCs) and granulocyte-monocyte progenitors (GMPs). Graphs show TLR4 expression levels of *Fzd6*$^{+/+}$ (WT) and *Fzd6*$^{-/-}$ (KO) CD150^{+} HSCs and Lin^{-}c-Kit^{+}CD16/32^{+}CD41^{-}CD150^{-} granulocyte-monocyte progenitors (GMPs) analysed by flow-cytometry. (B) Representative flow cytometry data and gating strategy of LSK and HSC subsets in the spleen of Fzd6 WT and KO mice at 24h following the second LPS injection. Graphs represent percentage and absolute numbers of LSK and CD150^{+} HSCs in the spleen.

4.3. *Fzd6* regulates expansion and differentiation of myeloid progenitor cells in response to LPS

Under steady-state conditions, HSCs give a balanced lymphoid-myeloid lineage output, while HSC differentiation shifts towards emergency myelopoiesis during infection, leading to increased myeloid cell expansion (Ueda et al., 2005, Zhang et al., 2016a). Although myeloid progenitor does not express stem cell antigen-1 (Sca-1) during homeostasis, upregulation of stem cell antigen-1 (Sca-1) expression have been reported in response to IFN type I exposure (Buechler *et al*., 2013). In our study, systemic LPS administration resulted in total depletion of classical $Sca1^{-}$ myeloid progenitors (Figure A4a-c), which includes CMPs and GMPs (Figure A4d) but dramatically increased the numbers of $Sca1^{+}$ emergency GMPs (eGMPs) (Figure A4d). We next analyzed different myeloid cells including granulocytes, $Ly6C^{hi}$ immature and $Ly6C^{lo/-}$ mature monocytes in the bone marrow, spleen and injection site, peritoneal cavity. Even though BM granulocytes were dramatically declined in WT BM at 24h post-infection (Figure A5a), this was probably due to their recruitment to spleen (Figure A5b) and peritoneal cavity (Figure A6a and b). In the following days, however, we observed significant increase granulocytes at 48h post-infection. Similar increase was observed in $Ly6C^{hi}$ inflammatory monocytes following second dose of LPS (Figure A5a). Increased numbers of myeloid cells well correlated with the appearance of eGMPs as their numbers reached to a plateau at 48h post-injection, suggesting the generation of new myeloid cells from these progenitors. Conversely, $Fzd6^{-/-}$ HSPCs generated significantly fewer numbers of eGMPs compared to those in $Fzd6^{+/+}$ BM in response to LPS (Figure A4d). Although no differences were observed in the numbers of granulocytes in $Fzd6^{-/-}$ BM compared to $Fzd6^{+/+}$, $Fzd6^{-/-}$ peritoneal cavity and spleen presented with significantly fewer numbers of granulocytes following the second LPS injection. Moreover, the numbers of $Ly6C^{hi}$ inflammatory monocytes remained unchanged in $Fzd6^{-/-}$ BM over the course treatment. No difference was detected in the frequency of myeloid cells in $Fzd6^{-/-}$ and $Fzd6^{+/+}$ blood after the second LPS administration (Figure A6c), indicating that the observed reduction in these cells was not due to their accumulation in the circulation. Thus, our results suggest that *Fzd6* deficiency results in impaired expansion and differentiation of myeloid progenitor cells in response LPS that is reflected by an overall reduced accumulation of terminally differentiated granulocytes and monocytes.

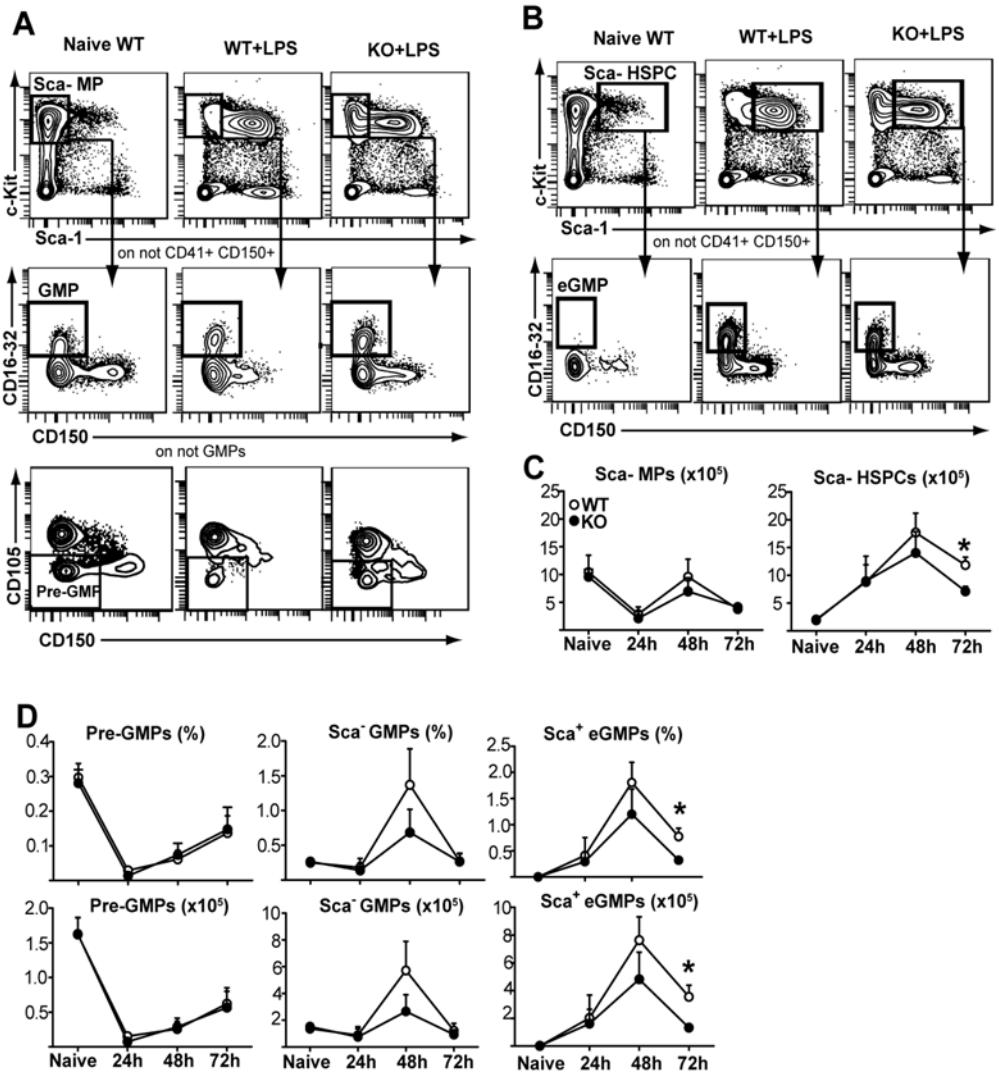


Figure A4. ***Fzd6*** **deficiency reduce the generation of emergency granulocyte-monocyte progenitors (eGMPs) in response to LPS**

(A-B) Representative flow cytometry data and gating strategy of myeloid progenitors in the BM. Steady state BM myeloid progenitor (MP) cells were gated on $Lin^-Sca1^-c\text{-}kit^{hi}$ and then subdivided according to the expression of CD41, CD150 and CD16/32. GMPs were identified positive for CD16/32 and negative for CD41 and CD150. Due to the inflammation induced shift in Sca-1 expression on HSPCs, GMPs were analyzed within Lin^-ckit^{hi} HSPC population during LPS-induced inflammation. (C) Graphs show absolute numbers of Sca-1^- myeloid progenitors (MP) and Sca-1^+ hematopoietic stem/progenitor cells in ***Fzd6 WT and KO*** **BM at different time points. (D) Graphs show absolute numbers of $Lin^-Sca1^-c\text{-}Kit^+CD16/32^-CD105^-CD150^-$ pre-GMPs (CMP), and $Sca1^-$ homeostatic GMPs and $Sca1^+$ eGMPs in** ***Fzd6*** **WT and KO mice.**

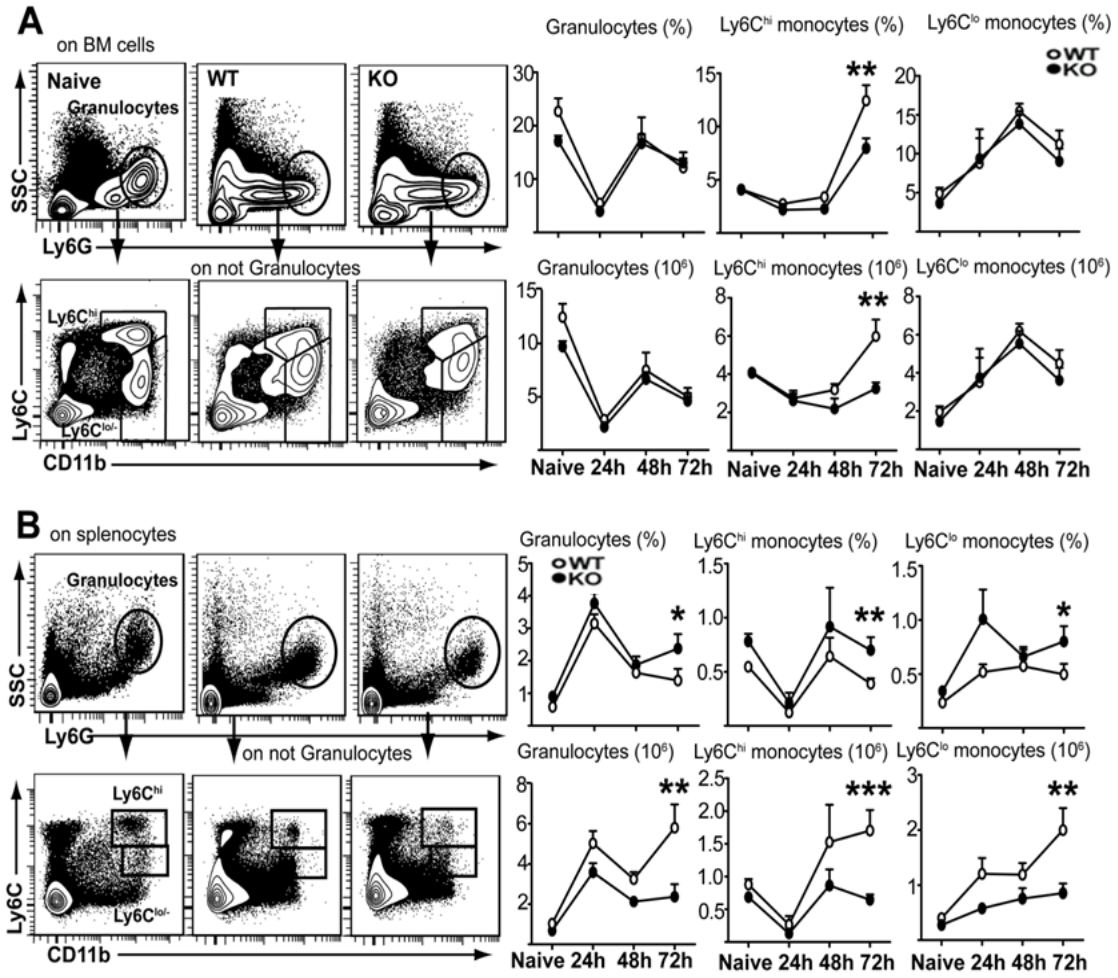


Figure A5. LPS-treated *Fzd6*$^{-/-}$ mice are present with decreased myeloid output

Representative flow cytometry data to demonstrate gating strategy for myeloid cell subsets in BM (A) and spleen (B) at 24h following the second dose of LPS injection. Graphs shows percentage and numbers of granulocytes (Ly6G^{+}SSC^{hi}) and mature monocytes (Ly6C^{hi}CD11b^{+}) and remaining immature/resident myelo-monocytes (Ly6C$^{lo/-}$ CD11b^{+}) gated on not granulocytes at various time points of 72 h LPS challenge.

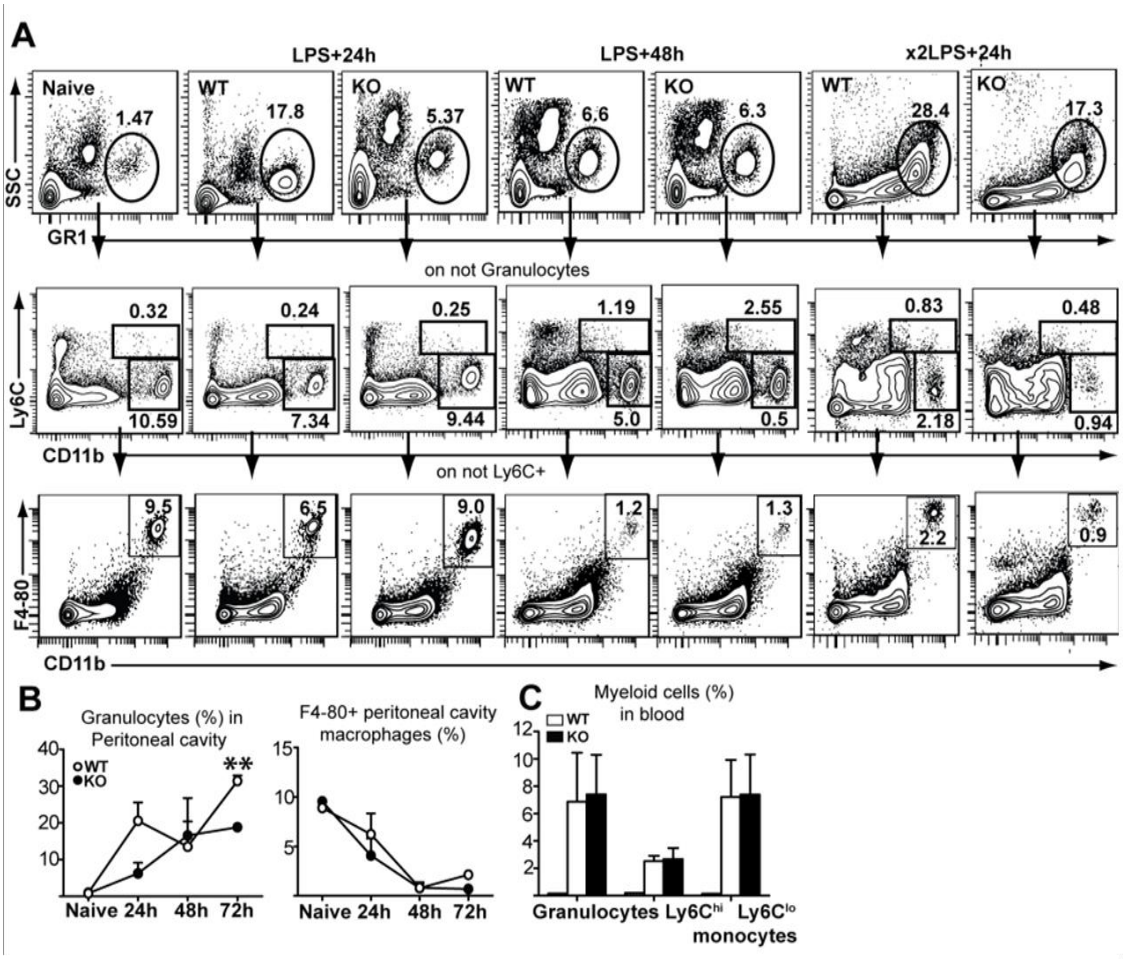


Figure A6. *Fzd6* deficiency reduce the accumulation of granulocytes in the infection site (peritoneal cavity) but not in the blood

(A) Representative flow cytometry data for myeloid cell subsets in the peritoneal cavity of WT and *Fzd6* KO mice at different time points following LPS injections. (B) Graphs shows percentage of granulocytes ($GR1^{hi}$ SSC^{hi}) and macrophages ($Ly6C^{-}CD11b^{+}F4\text{-}80^{+}$) gated on not granulocytes. (C) The frequency of granulocytes, mature and immature monocytes in the blood at 24h following the second LPS injection.

4.4. *Fzd6* modulates hyper-immune response during endotoxin-induced inflammation

In addition to cell-intrinsic mechanisms in HSCs, the hematopoietic microenvironment regulates inflammation-induced expansion and lineage specification of HSCs through the secretion of inflammatory cytokines (Boettcher *et al*., 2014, van Lieshout *et al*., 2012). Therefore, now the question that remains to be answered is does *Fzd6* regulate emergency myelopoiesis in a HSC cell-autonomous fashion or by an indirect mechanism of action through the alterations of the BM microenvironment?

G-CSF is known to influence HSC activation and immune response during endotoxin-induced inflammation (Bendall *et al*., 2014). Indeed, LPS treatment induced an increase in BM stromal G-CSF expression, which was at higher levels in *Fzd6*$^{-/-}$ when compared to *Fzd6*$^{+/+}$ BM (Figure A7). Thus, in *Fzd6*$^{-/-}$ mice LPS-induced LSK expansion was abrogated despite high levels of G-CSF. Elevated G-CSF levels were shown to alter BM microenvironment by suppressing CXCL12 production in stromal cells (Day et al., 2015, Eash et al., 2010, Semerad et al., 2005). Down-regulation of CXCL12 allows HSPC mobilization from the BM into the circulation (Johns et al., 2012). Consistent with these findings, there was a decrease in BM production of CXCL12 but no differences were observed between *Fzd6*$^{-/-}$ and *Fzd6*$^{+/+}$ mice. Although we cannot exclude the possible effect of other cytokines such as GM-CSF, M-CSF, IL-3 and IL-6, which are known with their positive impact on HSC expansion and myeloid differentiation, we did not detect any increase in their BM expression levels in response to LPS. Indeed, it is well established that LPS can stimulate myelopoiesis without stimulating the production of hematopoietic growth factors but rather acting on responsive myeloid progenitors (Moore *et al.*, 1980). However, the expression levels of the monocyte chemoattractant protein-1 (MCP-1/CCL2) were increased in LPS-treated *Fzd6*$^{-/-}$ and *Fzd6*$^{+/+}$ BM microenvironment. This increase was more pronounced in *Fzd6*$^{-/-}$ BM. MCP-1 and its ligand CCR2 regulates monocyte recruitment and trafficking during inflammation and infection (Shi *et al.*, 2011a). Nevertheless, there was no decrease in CCR2 expression of *Fzd6*$^{-/-}$ Ly6C^{hi} inflammatory monocytes (Figure AS1), suggesting that inadequate accumulation of Ly6C^{hi} monocytes in *Fzd6*$^{-/-}$ BM and spleen was rather a consequence of defective differentiation. Unexpectedly, the levels of MIP-1α/Ccl3, macrophage Inflammatory Protein-2 (MIP-2) and TNF-α were only increased in *Fzd6*$^{-/-}$ BM (Figure A9). The chemokines MIP-1α and MIP-2 are critical chemoattractants, which contribute to monocyte/neutrophil recruitment to the inflammation site and the clearance of

pathogens during infection (Fahey *et al.*, 1992, Tumpey *et al.*, 2002, Walley *et al.*, 1997). However, supressing effects of these two chemokines on HSPC proliferation and myelopoiesis have been reported (Broxmeyer *et al.*, 1990, Cook, 1996, Eaves *et al.*, 1993). Likewise, excessive production of TNF-α not only restricts HSPC expansion (Pronk *et al*., 2011) but also leads to a block in myeloid differentiation (Sade-Feldman *et al*., 2013) during inflammation. Since Increased levels of MIP-1 α, MIP-2 and TNF- α, are often observed during severe sepsis in patients and experimental models (Brueckmann *et al.*, 2004, Lv *et al.*, 2014, Walley *et al.*, 1997), we evaluated the endotoxemia response of *Fzd6*$^{-/-}$ and *Fzd6*$^{+/+}$ with a lethal dose of LPS. In agreement with hypersensitive immune response in *Fzd6*$^{-/-}$ mice, significantly higher amount of body weight loss (≥ 20%) were observed 24h post-injection, indicating the severity of sepsis in these mice (not shown). These results suggest that *Fzd6* acts as a suppressor to modulate pro-inflammatory immune response during endotoxin-induced inflammation and plays a critical role in the susceptibility to endotoxins.

4.5. *Fzd6* act as a cell-intrinsic factor in the regulation of generation and activity of myeloid effector cells

Although there is evidence that MIP-1α, MIP-2 and TNF-α, are predominantly expressed by monocyte/macrophage lineage cells in response to LPS (Björkbacka *et al.*, 2004, Ha *et al.*, 2011, Xaus *et al.*, 2000), their source in *Fzd6*$^{-/-}$ BM has yet to be identified. To exclude the possibility that non-hematopoietic cells also contribute to the LPS-induced hypersensitivity in *Fzd6*$^{-/-}$ mice, we performed reciprocal transplantation assays (Figure A7a). Comparable reconstitution levels were achieved in *Fzd6*$^{-/-}$ and *Fzd6*$^{+/+}$ mice at 12 weeks post-transplant (Figure A7b). When we further challenged these mice with two sub-lethal doses of LPS as previously described, the numbers of HSPC subsets and myeloid cells in BM and spleen were also comparable in *Fzd6*$^{-/-}$ and *Fzd6*$^{+/+}$ recipient mice (Figure A10b). In agreement with these findings, the changes in pro-inflammatory cytokines/chemokines in *Fzd6*$^{-/-}$ recipient BM appear to be similar to those seen in LPS-treated *Fzd6*$^{+/+}$ mice with the exception of G-CSF. Since the main source of G-CSF production is known to be endothelial cells in BM microenvironment during emergency myelopoiesis (Boettcher *et al*., 2014). This could be explained by damaging effect of irradiation on this cell type (Lee, 2012).

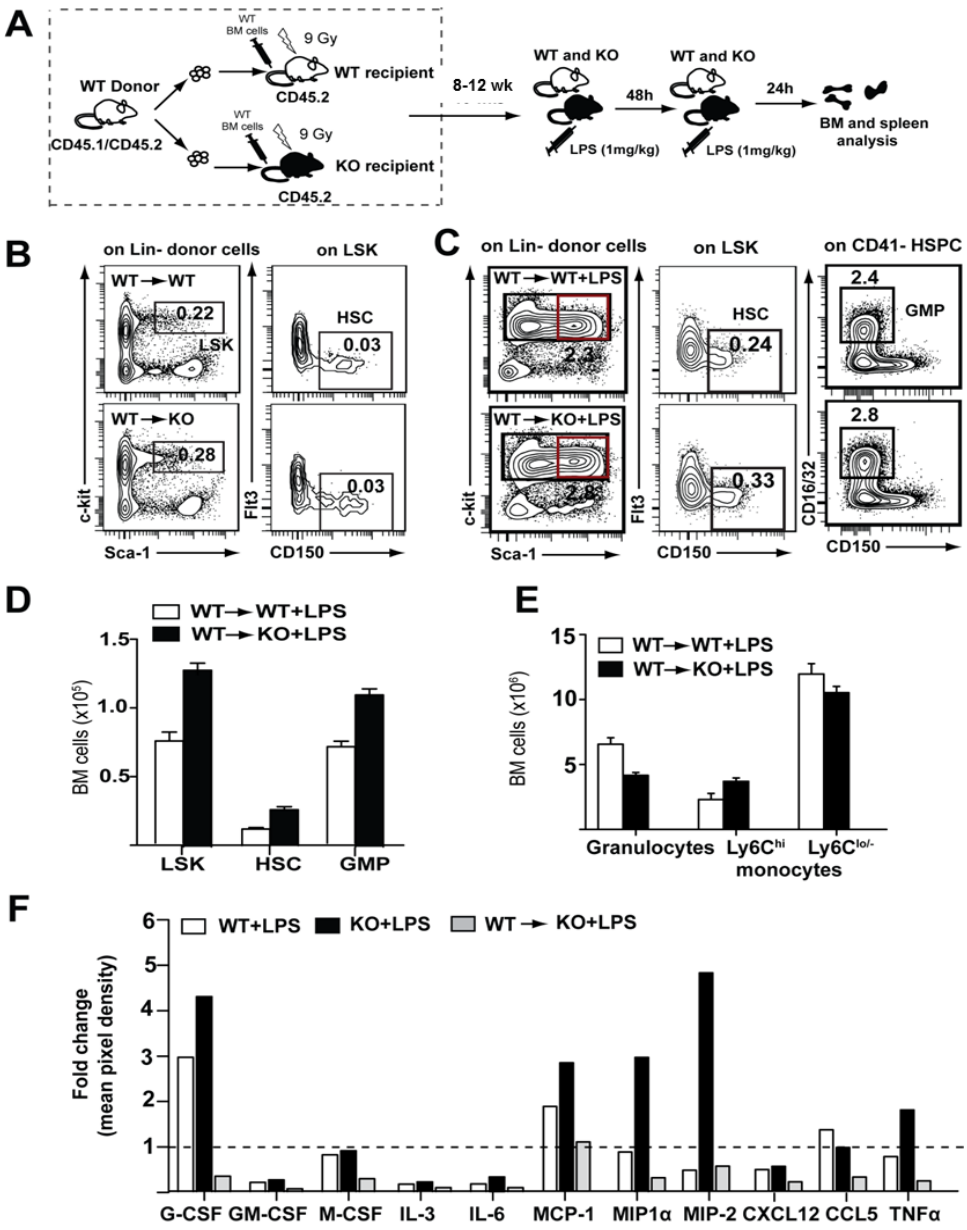


Figure A7. ***Fzd6*** **act as a cell-intrinsic factor in the regulation of HSC response and cytokine production during LPS-induced inflammation**

(A) For reciprocal transplantation experiments, 5x10^6 WT bone marrow cells (CD45.1+ or CD45.1+/CD45.2+) injected into the lateral tail vein of lethally irradiated Fzd6$^{-/-}$ or Fzd6$^{+/+}$ recipient mice (CD45.2+). For LPS-induced inflammation, recipient mice were further challenged with two i.p. injections of LPS at 48h interval at 8-12 wk post-transplant. Mice were sacrificed 24 h following the second injection and BM and spleens were harvested for flow-cytometry analysis. Representative flow cytometry data from the BM 12 wk after transplant (B) and following LPS challenge (C). Numbers shown in different quadrants indicate the mean percentage of LSKs and HSCs from four Fzd6$^{-/-}$ or Fzd6$^{+/+}$ recipient mice. Absolute numbers of bone marrow HSPC subsets (D) and myeloid cells (E) are shown in the graphs. (F) Fold change in cytokine/chemokine levels in Fzd6$^{-/-}$ and Fzd6$^{+/+}$ bone marrow extracellular milieu at 24 h following the second LPS injection compared to PBS injected mice. Each histogram represents data coming from a pooled sample of 4-6 mice per group.

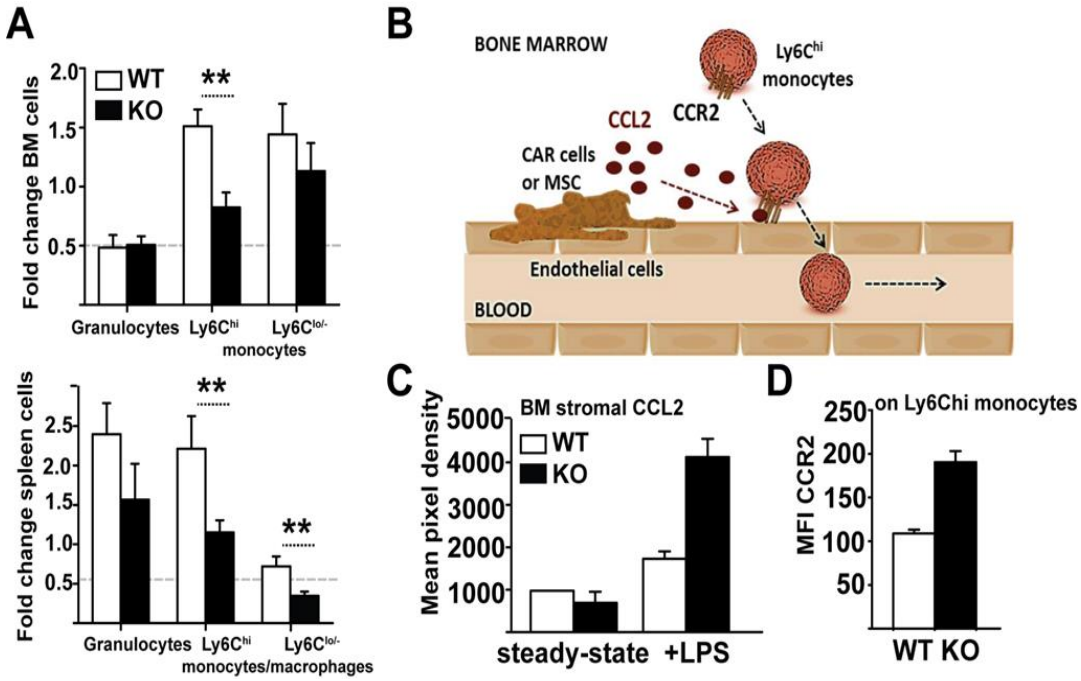


Figure AS1. ***Decreased expansion of Ly6Chi monocytes in Fzd6$^{-/-}$ bone marrow in response to LPS is not due to altered CCL2/CCR2 signaling or the accumulation of these cells in the spleen***

(A) **Fold change in the numbers of myeloid cell subsets in the bone marrow and spleens of Fzd6$^{-/-}$ and Fzd6$^{+/+}$ mice at 24 h following the second LPS injection compared to uninjected control mice. (B) Pro-inflammatory cytokine the monocyte chemoattractant protein-1 (MCP-1/CCL2) its ligand CCR2 on Ly6C^{hi} monocytes regulates monocyte recruitment and trafficking during inflammation and infection. Graphs show the stromal MCP-1/CCL2 expression at steady-state and LPS-challenged bone marrow from Fzd6 WT and KO mice (C) and CCR2 expression on Ly6C^{hi} monocytes from LPS-injected mice.**

Our results suggest that the observed reduction in the myeloid differentiation and increased immune response in *Fzd6*$^{-/-}$ is cell-autonomous. Considering monocyte/macrophages are generated from donor HSPCs following transplantation and initial response of *Fzd6*$^{-/-}$ HSPCs to LPS appear to be similar to *Fzd6*$^{+/+}$ mice, it is likely that increased production of hematopoietic suppressive factors such as MIP-1α, MIP-2 and TNF-α from *Fzd6*$^{-/-}$ macrophages had a secondary role in the decreased expansion and myeloid differentiation of HSPCs in our model. The inhibitory role of non-canonical Wnt signaling LPS stimulated macrophages and in patients with sepsis caused by LPS-containing, gram-negative bacteria were previously reported (Bergenfelz *et al.*, 2012). *Fzd6* also appear to inhibit JNK activation, which is reported to mediate LPS-induced TNF-α (Comalada *et al.*, 2003) and MIP-2 (Ha *et al.*, 2011) production and induce

apoptosis in monocyte/macrophages. Thus, it can be suggested that *Fzd6* might be involved in a feedback inhibition in our model, resulting in the decreased myeloid cell accumulation. Although *in vivo* functional relevance of *Fzd6*$^{-/-}$ monocyte/macrophages is yet be elucidated, our results demonstrate that *Fzd6* is a potential modulator of inflammatory response and susceptibility to endotoxins. We anticipate that Wnt/*Fzd6* signaling may be one of the promising therapeutic targets in the management of endotoxemia-induced inflammation and sepsis.

5. EXPERIMENTAL PROCEDURES

Experimental Animals

Fzd6$^{+/-}$ mice were backcrossed to C57Bl/6 for 10 generations to minimize genetic variability and then maintained as *Fzd6*$^{+/-}$ to *Fzd6*$^{+/-}$ intercrosses. *Fzd6*$^{-/-}$ mice were compared to sex-matched *Fzd6*$^{+/+}$ littermates unless otherwise noted. All mice were bred and housed under specific pathogen-free conditions in sterile ventilated racks at the animal facility of INRS-Institute Armand Frappier (CNBE). All procedures were in accordance with the Canadian Council on Animal Care guidelines.

Flow-cytometry Analysis and Cell-Sorting

Bone marrow (BM) was harvested by flushing tibias and femurs from the hind limbs in phosphate-buffered saline (PBS). The cells were passed through 25-gauge needles to obtain single cell suspensions. Single cell suspensions were prepared in PBS containing 0.1% bovine serum albumin (BSA) and 0.5mM ethylene-diamine-tetra-acetic acid (EDTA). Flow cytometry analyses were performed as previously described in Chapter 2 and Chapter 3.

Colony Assays

Single bone marrow suspensions were prepared in IMDM containing 10% very low endotoxin FBS (Wisent). Bone marrow cells were seeded into 35mm non-adherent petri dishes at a density of 10^4 cells/dish in methylcellulose medium containing stem cell factor, IL-3, IL-6, and Erythropoietin (Methocult GF M3434, Stem Cell Technologies,

Vancouver, BC, Canada). For single cytokine experiments, 10 ng/ml GM-CSF and or IL-3 (Peprotech) were added in methylcellulose medium (Methocult GF M3231). The cultures were incubated at 37°C in 5% CO2 for 7–10 days and hematopoietic colonies (CFU-C, colony forming units in culture) were counted and identified based on morphology under an inverted microscope. The average of duplicate cultures was calculated. Harvested colony forming cells were further stained with antibodies against CD11b, CD11c, CD117/c-Kit and GR1. Stained cells were analyzed as described in Chapter 3.

LPS-induced inflammation

To evaluate LPS-induced HSPC expansion and emergency myelopoiesis, *Fzd6*$^{-/-}$ and *Fzd6*$^{+/+}$ mice were given two i.p. injections of PBS or g-irradiated LPS (E. coli 0111:B4; Sigma-Aldrich) at a dose of 1 mg/kg body weight, 48 h apart. Their BM and spleen was harvested every 24 h during the course of 72 h LPS challenge. For endotoxemia experiments, *Fzd6*$^{-/-}$ and *Fzd6*$^{+/+}$ mice were injected with a lethal dose of LPS (3mg/kg) and were sacrificed at 24 h following the injection.

Reciprocal Transplantation Assays

For reciprocal transplantation experiments, 5x10^6 WT bone marrow cells (CD45.1$^+$ or CD45.1$^+$/CD45.2$^+$) injected into the lateral tail vein of lethally irradiated (9 Gy as a split dose of two times 4.5 Gy, 16 hr apart) *Fzd6*$^{-/-}$ or *Fzd6*$^{+/+}$ recipient mice (CD45.2$^+$). Sixteen weeks after transplantation, mice were euthanized and analyzed for reconstitution in BM and spleen. For LPS-induced inflammation, recipient mice were further challenged with two i.p. injections of g-irradiated LPS as described above at 8-12 wk post-transplant. Mice were sacrificed 24 h following the second injection and BM and spleens were harvested for flow-cytometry analysis.

Bone marrow cytokine/chemokine analysis

Bone marrow cell supernatants were collected from PBS or *LPS* injected mice at 24 h after the second LPS injection by harvesting cells from both hind legs into 2 ml PBS, followed by centrifugation. Supernatants were pooled from at least four mice per sample, and analyzed using a membrane-based Proteome profiler mouse

cytokine/chemokine array kit (R&D Systems). Array images were further analyzed using the NIH ImageJ image analysis software. Samples were normalized by subtracting pixel intensities from negative controls, and the fold changes for injected mice were determined as a ratio over PBS injected mice of the same genotype. Levels of mIFN-α were determined by using mouse IFN-alpha Platinum ELISA (eBioscience).

Statistical analysis

Each value represents at least three independent experiments. Two-tailed student's t test was used to determine statistical significance. $P < 0.05$ was considered significant.

ANNEX-I

List of communications

Meetings and Conferences

Oral Presentations

1. **Belma M. Abidin**. “Frizzled-6 deficiency limits parasite survival in Leishmania Donovani infection”. The 16th Annual Quebec Molecular Parasitology Symposium of Centre for Host-Parasite Interactions-CHPI, McGill University, Montreal, June, 2016.
2. **Belma M. Abidin**. “Diminution de la différenciation des cellules myéloïdes et réduction de la charge parasitaire dans les souris Frizzled-6 déficientes”. Congres Armand Frappier, Orford, Quebec, November 2015.
3. **Belma M. Abidin**. “Frizzled 6 is required for maintenance and lineage priming of murine hematopoietic stem/progenitor cells”. 1st Montréal Immunology Meeting, Montreal, November, 2014
4. **Belma M. Abidin** “Frizzled 6 promeut l’engagement vers le lignage granulo-macrophagique et inhibe la différenciation lymphocytaire”. Congres Armand Frappier, Orford, Quebec, November 2013.

Poster Presentations

1. **Belma M. Abidin**, Akil Hammami, Simona Stager and Krista Heinonen*. Bone marrow emergency response favors the progression of visceral leishmaniasis. 6th World Leish Congress on Leishmaniasis, Spain, May, 2017. (*Presenting author)

2. Akil Hammami, **Belma M. Abidin**, Tania Charpentier, Aymeric Fabié, Krista Heinonen and Simona Stager. HIF-1α is critical for inducing MDSC-like functions and M2 macrophage polarization during chronic visceral leishmaniasis. 6th World Leish Congress on Leishmaniasis, Spain, May, 2017.

3. Akil Hammami, **Belma M. Abidin**, Tania Carpenter, Aymeric Fabie, Krista Heinonen and Simona Stager. HIF-1α impairs monocyte’s effector function and induces M2 macrophage polarization during chronic Leishmania donovani infection. International Congress of Immunology, *Melbourne, Australia, August, 2016*.

4. Roxann Hétu-Arbour, **Belma M. Abidin**, Edward Kwarteng and Krista Heinonen Progenitor cell-intrinsic Wnt4 contributes to lymphoid priming.. 30th Annual CSI Conference, Banff, 2017.

5. **Belma M. Abidin** and Krista Heinonen. Frizzled-6 regulates inflammation-induced hematopoietic stem/progenitor cell response. 29th Annual CSI Conference, Ottawa, April, 2016.

6. **Belma M. Abidin**, Akil Hammami, Simona Stager and Krista Heinonen. Frizzled-6 deficiency protects mice against Leishmania donovani due to reduced myeloid differentiation. 29th Annual CSI Conference, Ottawa, April, 2016.

7. **Belma M. Abidin**, Roxann Hétu-Arbour, Krista Heinonen*. Progenitor cell-intrinsic Wnt4 contributes to T lymphoid priming. 29th Annual CSI Conference, Ottawa, April, 2016. (*presenting author)

8. **Belma M. Abidin**, Akil Hammami, Simona Stager and Krista Heinonen.Frizzled-6 regulates inflammation- induced myeloid differentiation. 2nd Montréal Immunology Meeting, Montreal, November, 2015.

9. Akil Hammami, **Belma M. Abidin**, Tania Charpentier, Aymeric Fabié, Krista Heinonen, Simona Stäger. HIF-1α impair INFϒ+CD4+ T cell expansion and induces M2 macrophages during chronic Leishmania donovani infection. 2nd Montréal Immunology Meeting, Montreal, November, 2015.

10. **Belma M. Abidin**, Edward Owusu-Kwarteng and Krista M. Heinonen. Frizzled 6 is required for maintenance and lineage priming of murine hematopoietic stem /progenitor cells. 1st Montréal Immunology Meeting, Montreal, November, 2014.

11. **Belma M. Abidin**, Edward Owusu-Kwarteng and Krista M. Heinonen. Frizzled6 regulates murine hematopoietic stem/progenitor cell function and granulocytic lineage choice. 27emes Entredients Jacques Cartier, Stem Cells and Cancer, Montreal, October, 2014.

12. **Belma M. Abidin**, Edward Owusu-Kwarteng and Krista M. Heinonen. Frizzled-6 maintains hematopoietic stem cells and regulates lympho-myeloid lineage choice. 27th Annual Spring Meeting of the Canadian Society for Immunology, Quebec, March 2014.

13. **Belma M. Abidin**, Josée-Anne Boucher and Krista M. Heinonen. Frizzled-6 signaling regulates lympho- myeloid lineage choice. Immunology Montreal-2013 Annual Fall Symposium, Montreal, December 2013.

14. **Belma M. Abidin**, Edward Owusu-Kwarteng and Krista M. Heinonen*. Planar cell polarity-like Wnt signaling in early hematopoiesis and immune function. CIHR New Investigator Forum 2013, Lac Delage, QC, May 2013. (*Presenting author)

15. **Belma M. Abidin** and Krista M. Heinonen*. Non-canonical Wnt4/Fzd6 signaling regulates granulocyte- monocyte lineage choice. 26th Annual Spring Meeting of the Canadian Society for Immunology, British Columbia, April 2013. (*Presenting author)