Understanding pattern recognition receptor signaling and its effect on hematopoietic stem cells

by

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DEDICATION

To my parents, Mitchell and Barbara.

Thank you for all your love and encouragement.

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LIST OF ABBREVIATIONS

AMP	Antimicrobial peptide
APC	Antigen presenting cell
CD	Crohn's disease
CFU	Colony forming unit
DC	Dendritic cell
FL	Fetal liver
G-CSF	Granulocyte-colony stimulating factor
GVHD	Graft-versus-host disease
HEK293	Human embryonic kidney 293
Hif-1α	Hypoxia inducible factor-1α
HSC	Hematopoietic stem cell
HSPC	Hematopoietic stem and progenitor
IκB	Inhibitor of NF-κB
KO	Knockout
LPS	Lipopolysaccharide
LSK	Lineage$^{-/lo}$ Sca1^{+} cKit^{+}
LTMR	Long term multilineage repopulating
MAPK	Mitogen activated protein kinase
MDP	Muramyl dipeptide

MPP	Multipotent progenitor
Myd88	Myeloid differentiation primary response 88
NCF1	Neutrophil cytosolic factor 1
NEMO	NF-κB essential modifier
NF-κB	Nuclear factor-κB
NLR	NOD-like receptor
NOD2	Nucleotide-binding oligomerization domain containing 2
NT	Non-targeting
PAMP	Pathogen associated molecular pattern
PRR	Pattern recognition receptor
RIPK2	Receptor-interacting serine-threonine kinase 2
RNF31	Ring finger protein 31
ROS	Reactive oxygen species
RU	Repopulating units
SCF	Stem cell factor
siRNA	Small interfering ribonucleic acid
TLR	Toll-like receptor
TRIF	TIR domain containing adaptor inducing interferon beta
UC	Ulcerative colitis
WBM	Whole bone marrow
WT	Wild type

ABSTRACT

Pattern recognition receptors (PRRs) sense unique and conserved structures common to many types of microorganisms and activate pro-inflammatory signaling cascades that are important for mounting effective immune responses, but aberrant regulation of PRR signaling can lead to autoimmunity. Polymorphisms in *NOD2*, an intracellular PRR, are associated with increased risk of developing the inflammatory bowel disorder Crohn's disease. To gain insight into how NOD2 signaling is regulated, we performed a genome-wide siRNA screen in which NOD2-induced NF-κB activation was assessed using a luciferase reporter system. Using this strategy, we identified and validated hundreds of novel regulators of the NOD2 and NF-κB signaling pathway, some of which were previously implicated in Crohn's disease susceptibility. By comparing our results with publicly available protein-protein interaction databases, we were able to visualize networks of genes, including the linear ubiquitin chain assembly complex, that are important for mediating NOD2-dependent responses. Classically, PRRs are thought to function in differentiated cells to orchestrate inflammation, but recent evidence suggests that hematopoietic stem cells (HSCs), which maintain production of all blood cells throughout life, express PRRs and could be important for supporting immune responses during infection though the mechanisms underlying this function are unclear. We found that systemic infection of mice with the gram negative bacterium *Escherichia coli* resulted in moderate reduction of HSC activity in bone marrow and vastly expanded

HSC activity in spleen, indicative of extramedullary hematopoiesis. Expansion of splenic HSCs was reduced in mice deficient for the PRR TLR4 or the adaptor protein RIPK2, which is required for NOD1 and NOD2 signaling, implicating PRRs in the regulation of HSCs during infection. PRR signaling in radio-resistant cells was important for promoting progenitor expansion in spleen, suggesting an indirect mechanism. We found that expansion of splenic HSCs was dependent on dual activation of NOD1 and TLR4 and production of granulocyte-colony stimulating factor. Altogether, these results provide a global view of the mechanisms that regulate PRR signaling and provide insight into the pathways that facilitate extramedullary hematopoiesis in the context of infection.

CHAPTER I

Introduction

Overview of the hematopoietic system

The hematopoietic system can be broadly divided into two groups of cells, including red blood cells (RBCs; erythrocytes) whose main role is to transport oxygen throughout the body and white blood cells (WBCs; leukocytes), which defend the host against invading organisms and contribute to processes of wound healing. WBCs are further subdivided into either innate or adaptive immune cells. Leukocytes recognize infection or cellular damage by sensing and responding to conserved components of foreign organisms, termed pathogen associated molecular patterns (PAMPs), and alarmins, which comprise endogenous cellular structures that are typically shielded from immune recognition, but are released during injury and non-programmed cell death (Bianchi, 2007). Recognition of harmful agents and subsequent interplay between different cell types via cytokines, chemokines and lipid mediators is essential for directing recruitment, activation, and resolution of the inflammatory response (Medzhitov, 2007).

Many types of innate immune cells contribute to host defense, including antigen presenting cells (APCs) such as macrophages and dendritic cells (DC), megakaryocyte-derived platelets, natural killer (NK) cells, and granulocytes including neutrophils, mast

cells, eosinophils and basophils. Although their roles are varied, each cell type can be classified by their general function. At sites of infection or damage, PAMPs or alarmins are recognized by resident macrophages and DCs that initiate the immune response by secreting inflammatory cytokines and chemokines, engulfing invading microorganisms or damaged cells and processing ingested components for presentation to cells of the adaptive immune system (Aderem and Underhill, 1999; Soehnlein and Lindbom, 2010; Vyas et al., 2008). Platelets initiate the coagulation cascade to form clots at sites of injury and coat bacteria to facilitate uptake by APCs (Projahn and Koenen, 2012; Verschoor et al., 2011). NK cells kill host cells that attempt to evade the immune system such as tumorigenic cells and those infected with viruses, bacteria or parasites (Di Santo, 2006). Granulocytes limit the spread of microorganisms by exposing them to reactive oxygen species, proteolytic enzymes and anti-bacterial peptides present in their granules and by releasing cytokines to manipulate the inflammatory microenvironment and to prime adaptive immune responses (Geering et al., 2013; Pham, 2006; Stone et al., 2010). Together these innate immune cells form the first line of defense against potentially harmful organisms and agents.

Cells of the innate immune system are considered innate because their genes encoding proteins utilized for host defense do not require rearrangement to function. In contrast, cells of the adaptive immune system, including B-cells and T-cells, require rearrangements in genes encoding antibodies and T-cell receptors, respectively, to form functional proteins and to fully mature (Germain, 2002; Hardy and Hayakawa, 2001; Schatz and Ji, 2011). Combinatorial rearrangement allows for the generation of a highly diverse repertoire of B-cells and T-cells that can be selectively expanded once a

particular antigen is encountered. Thus, the successful clearance of invading pathogens frequently involves cooperation between both branches of the immune system, with innate immune cells limiting the initial invasion, signaling infection and promoting cell recruitment, while adaptive immune cells generate pathogen specific and sustained responses.

Both host defense and oxygen transport require extraordinary amounts of cellular proliferation and turnover. Billions of damaged or defective RBCs are removed from circulation daily and must be replaced to sustain the body's unyielding demand for oxygen (Higgins and Mahadevan, 2010). Removal of potentially harmful stimuli such as invading bacteria, viruses, fungi, and damaged or tumorigenic cells requires expansion of immune populations, yet unrestricted cellular proliferation and inflammation can lead to cancer or autoimmunity (Barnes, 2008; de Visser et al., 2006; Geering et al., 2013; Michlewska et al., 2007; Rahman, 2011). To support this massive requirement for new cells and to provide multiple levels of regulation, the hematopoietic system has evolved as a tiered hierarchy (**Figure 1.1**).

At the base of the hematopoietic hierarchy are the innate and adaptive effector cells described above, which have limited replicative potential and are only capable of differentiating into specialized subsets. Effector cells are immediately derived from lineage restricted progenitors, which differentiate into one or a few different effector cell types. Lineage restricted progenitors arise from oligopotent progenitors with more relaxed differentiation capacity, such as common myeloid progenitors (CMPs) that give rise to erythrocytes, platelets, granulocytes and macrophages (Akashi et al., 2000) and common lymphoid progenitors (CLPs) that produce NK cells, B-cells and T-cells (Kondo

et al., 1997). Interestingly, DCs derive from CMPs and CLPs with equal potential, but because CMPs outnumber CLPs under steady state conditions, the majority of DCs arise from myeloid specific progenitors (Manz et al., 2001a; Manz et al., 2001b; Traver et al., 2000). A population of lymphoid-primed multipotent progenitors (LMPPs) has been described that gives rise to NKs, B-cells, T-cells, DCs, macrophages and granulocytes, but lacks the ability to produce erythrocytes and megakaryocytes (Adolfsson et al., 2005). The precursors of oligopotent progenitors are multipotent progenitors (MPPs) that by definition have the capability to produce all cells of the hematopoietic system, yet have a limited replicative lifespan ranging from a few weeks to a few months (Morrison and Weissman, 1994; Oguro et al., 2013).

At the zenith of the hematopoietic hierarchy lie hematopoietic stem cells. Like MPPs, HSCs have the capacity to give rise to all cells of the blood system, but in contrast to MPPs they possess the ability to self-renew, where cell division results in at least one daughter cell with equal differentiation potential as the parent. Self-renewal divisions allow HSCs to maintain production of all hematopoietic cells over the lifespan of an organism (Orford and Scadden, 2008). Impressively, HSCs from young mice are capable of surviving four or more rounds of serial transplantation before they die or differentiate (Allsopp et al., 2003). This indicates that HSCs can support hematopoiesis nearly indefinitely and are able to survive multiple rounds of stress induced by transplantation, thereby outliving their original organism. Consequently, therapies that utilize HSCs have proved beneficial in the clinic, where hematopoietic transplantation is used to treat a variety of disorders including severe combined immunodeficiency, leukemia, solid tumors, sickle cell anemia, and autoimmune disorders (Li and Sykes, 2012).

In summary, the hematopoietic system contains multiple subsets of effector cells that have both unique and redundant functions in limiting the spread of infection, killing tumorigenic cells and supporting essential biological functions such as oxygen transport and wound healing. Proper generation and regulation of effector cells is essential for health, as aberrant inflammation and cell proliferation can lead to autoimmunity and tumorigenesis. Continuous and regulated production of effector cells is accomplished through a tiered system of highly proliferative progenitors that are maintained by a small pool of self-renewing HSCs. Understanding how HSCs function under normal and pathologic conditions offers great promise for improving their use in treating human disease.

Recognition of foreign organisms by PRRs

In the perpetual conflict between macroscopic and microscopic organisms, a class of proteins termed pattern recognition receptors have evolved to recognize molecular motifs conserved in many types of microbes and trigger pro-inflammatory signaling cascades. These PRRs include Toll-like receptors (TLRs), nucleotide-binding oligomerization domain-containing (NOD)-like receptors (NLRs), retinoic acid-inducible gene (RIG-I)-like receptors (RLRs) and C-type lectin receptors (CLRs). RLRs dwell in the cytoplasm and sense viral-derived double-stranded RNA (Yoneyama and Fujita, 2007). CLRs are either secreted or reside in the plasma membrane where they recognize carbohydrates common to many types of pathogens (Geijtenbeek and Gringhuis, 2009). TLRs are membrane spanning proteins found in the plasma membrane or endosomal

membranes that recognize components of bacterial and fungal cell wall, bacterial and viral DNA and RNA, and endogenous alarmins (Akira et al., 2006). Once activated, TLRs recruit the adaptor proteins Myd88 (utilized by TLR1, 2, and 4-9) and TRIF (utilized by TLR3 and 4), which lead to signaling via nuclear factor-κB (NF-κB), mitogen activated protein kinases (MAPK) and interferon regulatory factor pathways that result in the transcription of genes important for inducing inflammation and killing invading organisms (Akira and Takeda, 2004).

The founding members of the NLR family, NOD1 and NOD2, sense cleavage fragments of bacterial cell wall-derived peptidoglycan, whose minimal activating structures are *meso*-diaminopimelic acid (meso-DAP) and muramyl dipeptide (MDP), respectively (Chamaillard et al., 2003; Inohara et al., 1999; Inohara et al., 2003; Ogura et al., 2001b). Signaling downstream of NOD1 and NOD2 is dependent on the adaptor protein receptor-interacting serine-threonine kinase 2 (RIPK2) and converges on NF-κB and MAPK pathways to promote inflammation (Girardin et al., 2001; Inohara et al., 1999; Ogura et al., 2001b). Dual stimulation with NLR and TLR agonists can result in the synergistic production of inflammatory cytokines and can promote maturation of antigen presenting cells, which is due in part to the upregulation of NOD1 and NOD2 after TLR stimulation (Fritz et al., 2005; Tada et al., 2005; Takada et al., 2002; Takahashi et al., 2006). Together, NLRs and TLRs can cooperate to initiate a robust immune response.

Whereas clearance of invading organisms frequently relies on mounting an inflammatory response, excessive cytokine production and inflammation can damage host tissues and lead to septic shock. Tolerance, or the lack of immune response

following antigen encounter, is especially important at mucosal surfaces such as the digestive tract, where host cells are in close proximity to beneficial commensal bacteria (Steele et al., 2012). Macrophages become tolerogenic to TLR signaling following initial stimulation with LPS, but maintain responsive to NLR agonists (Kim et al., 2008). This provides a mechanism in which immune cells can effectively ignore signals emanating from commensals while maintaining vigilance against pathogens that attempt to breach the epithelial barrier. Alteration of this delicate balance in the gut can predispose humans to autoimmune disorders such as Crohn's disease (CD).

Crohn's disease and NOD2

Inflammatory bowel disease (IBD), encompassing both CD and ulcerative colitis (UC), affects nearly 2 persons out of 1,000 in populations of European descent (Cho, 2008). Patients suffering from IBD characteristically exhibit excessive destruction of the intestinal epithelium, which presents clinically as severe abdominal pain, bloody stool and excessive weight loss. Inflammation in CD involves all layers of the bowel and is discontinuous, affecting isolated regions throughout the digestive tract while preferentially focusing in the terminal region of the small intestine. In contrast, inflammatory lesions observed in UC involve only the luminal surface of the bowel and extend continuously from the rectum to the colon. Treatment of IBD has classically involved the use of corticosteroids, which kills cells of the immune system, and more recently involves the use of antibodies that inhibit the inflammatory cytokine TNFα (Cheifetz, 2013). Since UC is restricted to the distal bowel, in some cases it can be cured

by surgical resection of the inflamed colon (Rokke et al., 2011). As CD-associated inflammation can recur in multiple regions of the gastrointestinal tract, surgery is not curative for that condition (McLeod et al., 1997; Rutgeerts et al., 1990).

Genome-wide association studies comparing IBD patients and healthy controls have linked inactivating mutations of *NOD2* to CD, but not UC (Hampe et al., 2002; Hugot et al., 2001; Ogura et al., 2001a). Mutations in both copies of the *NOD2* gene confer a 20-40 fold enhanced susceptibility to CD (Hugot et al., 2001). Still, biallelic inactivation of *NOD2* does not invariably lead to CD and approximately 60% of CD patients do not have mutations in *NOD2* (Cho and Abraham, 2007; Linde et al., 2003). This indicates that NOD2 plays an important role in preventing CD, but many other factors also contribute to CD susceptibility. Indeed, polymorphisms in genes such as *IL23R*, *IL12B*, *STAT3*, *JAK2* and the autophagy related gene *ATG16L1* as well as smoking and a western diet are all risk factors for CD (Cho, 2008). Moreover, mouse models of IBD require the presence of commensal bacteria, indicating that signals emanating from the gut microbiota are essential for promotion of CD (Elson et al., 2005). Altogether, CD is a multifactorial disorder that can affect all regions of the digestive tract and has a complex etiology involving both environmental and genetic components.

Meta-analyses have revealed the existence of over 1000 independent CD susceptibility loci and the majority of these loci contain multiple genes of which the etiological factor is unknown (Barrett et al., 2008; Franke et al., 2010; Rivas et al., 2011). Given the importance of NOD2 signaling in CD, it is possible that proteins which regulate signal transduction downstream of NOD2 may also influence CD vulnerability and a global view of the NOD2 signaling pathway could assist in determining which

genes within CD susceptibility loci are likely to be the causative factors. Functional characterization of NOD2 regulators may also provide insight into the pathogenesis of CD and could elucidate novel targets for therapeutic intervention. Interestingly, hematopoietic transplantation has emerged as a potential therapy for CD patients that are refractory to conventional treatments (Burt et al., 2010; Cassinotti et al., 2008; Hommes et al., 2011), so a better understanding of how infection and autoimmunity influence hematopoietic stem cell function may lead to improvements in current clinical practice.

Prospective isolation of hematopoietic stem cells

The concept that blood cells are derived from a small population of stem cells was first introduced by Till and McCulloch in 1961 based on their observation that transplantation of whole bone marrow (WBM) from untreated mice into irradiated mice resulted in the formation of nodal colonies in the spleens of recipients within ten days, which they termed colony forming unit-spleen (CFU-S) (Till and McCulloch, 1961). Supporting the idea that stem cells were responsible for the CFU-S activity, the number of transplanted bone marrow cells correlated linearly with the number of colonies formed and the colonies contained both undifferentiated and differentiated cells of clonal origin (Till and McCulloch, 1961).

Later it was realized that most cells with CFU-S activity are not actually HSCs, but their more proliferative MPP progeny. Yet it wasn't until 1988 when the Weissman group used fluorescence activated cell sorting to fractionate whole bone marrow from mice to enrich for a population of hematopoietic cells with the capacity to protect lethally

irradiated recipients and contribute to myeloid and lymphoid lineages beyond six months that definitive HSCs could be isolated and studied with precision (Spangrude et al., 1988). This HSC-enriched population was originally identified by the surface marker profile Thy1.1^{lo} Sca1^{+} Lineage^{-}, where Lineage (or Lin) represents a combination of surface markers present on differentiated blood cells that typically include CD3, B220, CD11b and TER119, which mark T-cells, B-cells, myeloid cells and erythrocytes, respectively. To date, the gold standard for assessing HSC function in mice involves transplantation of donor cells (containing a putative HSC population) along with a fixed number of competitive whole bone marrow cells (containing a defined number of functional HSCs) into a conditioned (usually irradiated) recipient and assessing production of blood cells from both myeloid and lymphoid lineages over the course of four months, a time consistent with exhaustion of all non-HSCs in the donor graft (Purton and Scadden, 2007). If HSCs in the test fraction are impaired in relation to competitor HSCs, they will either fail to engraft the recipient or exhibit diminished output of differentiated blood cells, indicative of a functional defect.

Consistent with the finding that cKit mutant mice die perinatally due to hematopoietic defects, all HSCs have been shown to express the growth factor receptor cKit on their surface and require cKit signaling for survival (Bernstein et al., 1990; Ikuta and Weissman, 1992). By subdividing the Lineage^{-} Sca1^{+} cKit^{+} (LSK) population, which contains both HSCs and MPPs, based on expression of CD34, it was shown that 21% of single CD34$^{-/lo}$ LSK from mouse bone marrow could provide long term multilineage reconstitution (LTMR) when co-injected with radioprotective cells, allowing extensive *in vitro* and *in vivo* analysis of HSC self-renewal at the single cell level (Ema et al., 2005;

Ema et al., 2000; Osawa et al., 1996). Whereas the combination of CD34 and LSK are sufficient to robustly isolate functional HSCs by flow cytometry, these surface markers are not amenable to *in situ* staining and therefore are insufficient to visualize HSCs in their endogenous setting.

In 2005, the Morrison group fractionated bone marrow based on positive or negative expression of the signaling lymphocyte activation molecule (SLAM) family members CD150 (Slamf1) and CD48 (Slamf2) and showed that all LTMR activity was contained within the $CD150^{+}$ $CD48^{-}$ population (Kiel et al., 2005). Using the simple combination of $CD150^{+}$ $CD48^{-}$ and $CD41^{-}$ (to exclude contaminating megakaryocytes), the authors showed that 45% of single-sorted bone marrow cells and 33% of single-sorted spleen cells had LTMR activity (Kiel et al., 2005). Moreover, using this combination of antibodies to stain sections of bone marrow, the authors were able to visualize putative HSCs throughout the bone marrow and frequently found these cells near vascular endothelium (Kiel et al., 2005). Work from other labs has verified the utility of SLAM markers for staining HSCs under steady-state conditions and have revealed that the level of CD150 expression positively correlates with the ability of HSCs to self-renew (Morita et al., 2010; Weksberg et al., 2008). Whether the SLAM markers accurately identify HSCs during periods of stress remains to be determined.

Maintenance of hematopoietic stem cells in their niche

In mice, the first definitive multilineage blood forming cells arise in the extra-embryonic yolk sac and placenta around E7.5 and the intra-embryonic aorto-gonadal

mesonepherous around E9.5 (Boisset and Robin, 2012; Mikkola and Orkin, 2006). Following their generation, embryonic HSCs migrate through the blood stream to the fetal liver, thymus and spleen and reside there just before birth, whereupon they transit to the bone marrow (Mazo et al., 2011). In adults, the vast majority of HSCs are found in the bone marrow where they are surrounded by an interconnected system of supporting cells termed the "stem cell niche". Niche cells are responsible for supplying soluble and/or membrane bound factors that are important for maintaining HSC survival, quiescence and retention (Morrison and Spradling, 2008). Quiescence, or exit from the cell cycle, is essential for the long term maintenance of HSCs, as deletion of genes that promote HSC quiescence leads to exhaustion of the stem cell pool and can result in lethality (Cheng et al., 2000; Takubo et al., 2010), while deletion of genes that inhibit HSC quiescence can improve HSC longevity and reconstitution potential (Yu et al., 2006). In the bone marrow, many cell types have been implicated as putative members of the stem cell niche including osteoblasts, osteoclasts, perivascular cells, mesenchymal progenitors, megakaryocytes, macrophages, neural cells, and endothelial cells but the necessity for each of these cell types in supporting HSC function is a subject of continuing debate (Kiel and Morrison, 2008; Park et al., 2012).

For a cell or cell-derived factor to be considered part of the HSC niche, it must be demonstrated that some aspect of HSC biology is altered when the entity is removed. **Table 1.1** summarizes growth factor and receptor pairs important for maintenance of adult HSCs within the bone marrow niche. A prime example of an essential pair of niche components are the secreted and/or membrane bound protein Stem Cell Factor (SCF; Kit-ligand; Steel factor) and it's receptor cKit on the surface of HSCs (Ikuta and Weissman,

1992). Mice deficient for SCF or cKit die near birth due to anemia, underscoring the importance of these genes in maintaining hematopoiesis (Bernstein et al., 1990; Broudy, 1997). To study the role of SCF in adult hematopoiesis, the cre/loxP system, in which a gene can be deleted by placing loxP sites around essential exons and expressing cre-recombinase from a specific promoter to excise the "floxed" gene, was used to delete SCF after birth. When SCF was deleted in all cells using a ubiquitously expressed tamoxifen-inducible cre in adult mice, the number of HSCs (defined as $CD150^+$ $CD48^-$ LSK) was reduced more than 90% in the bone marrow and a reduction in HSC activity was confirmed by competitive transplantation (Ding et al., 2012). Using cell-type-specific deletion of SCF, it was shown that SCF from $Tie2^+$ endothelial cells and $Lepr^+$ blood vessel-lining perivascular cells was required for HSC maintenance, whereas SCF from $Vav1^+$ hematopoietic cells or $Col2.3^+$ osteoblasts was not required (Ding et al., 2012). Thus, SCF is a bona-fide HSC niche factor and endothelial and perivascular cells are bona-fide members of the HSC niche.

In addition to local growth factors, microenvironmental features such as hypoxia, or low oxygen tension, influence HSCs in their niche. HSCs are enriched in regions of bone marrow that stain highly with pimonidazole, a compound that marks hypoxic cells (Parmar et al., 2007; Takubo et al., 2010; Varia et al., 1998). Hypoxia inducible factor 1 alpha (Hif1α) is a transcription factor that is upregulated when cells are exposed to hypoxia or inflammation and controls gene networks important for adaptation to low oxygen conditions such as upregulation of glycolytic enzymes and synthesis of angiogenic factors (Nizet and Johnson, 2009). Using poly (I:C)-inducible Mx-1 cre to conditionally delete Hif1α from all hematopoietic cells ($Hif1\alpha^{f/f} \rightarrow Hif1\alpha^{\Delta/\Delta}$), it was shown

that following transplantation, Hif1α$^{\Delta/\Delta}$ HSCs give rise to more progeny than wild type (WT) controls after 4 months, but Hif1α$^{\Delta/\Delta}$ HSCs give rise to vastly reduced progeny when the cells were re-isolated and transplanted again, suggesting a loss of self-renewal (Takubo et al., 2010). Consistent with this, in mice that received equal numbers of WT and Hif1α$^{f/f}$ cells that were subsequently exposed to poly (I:C), Hif1α$^{\Delta/\Delta}$ HSCs outcompeted WT HSCs after 4 months, but were outcompeted by WT HSCs after 11 months (Takubo et al., 2010). These results suggested that responsiveness to hypoxia by Hif1α is important for HSC maintenance long term.

The authors went on to show that Hif1α$^{\Delta/\Delta}$ HSCs are less quiescent, having a lower proportion of cells in G_0 (Takubo et al., 2010). Deletion of the E3 ubiquitin ligase von Hippel-Lindau (vHL$^{\Delta/\Delta}$), which promotes the degradation of Hif1α, in hematopoietic cells resulted in the stabilization of Hif1α, expansion of the HSC pool and reduced HSC output (Takubo et al., 2010). This was due to decreased homing of vHL$^{\Delta/\Delta}$ HSCs to the bone marrow after transplantation and increased quiescence, which would presumably limit the ability of HSCs to differentiate (Takubo et al., 2010). The enhanced numbers and reduced output of vHL$^{\Delta/\Delta}$ HSCs was completely rescued in Hif1α$^{\Delta/\Delta}$: vHL$^{\Delta/\Delta}$ double-deficient HSCs, indicating that the effects of vHL deletion on HSC quiescence and function were Hif1α dependent (Takubo et al., 2010). Altogether, these results indicate that precise control of Hif1α levels in HSCs is required to avoid inescapable quiescence or exhaustion.

These and other studies have made it clear that maintenance of HSCs within their bone marrow niche involves a complex interplay between extracellular factors, hematopoietic and non-hematopoietic cell types and their surrounding microenvironment.

In general, any phenomenon that alters the bone marrow is likely to either directly or indirectly affect the HSCs within it. Therefore, it is important to view alterations in HSC activity not only as consequence of changes within HSCs themselves, but also as a function of their local surroundings. As I will discuss in the following sections, retention of HSCs within the bone marrow is also an important feature and manipulation of the bone marrow niche can result in egress of HSCs to the periphery.

Localization and migration of hematopoietic stem cells

Trafficking to the bone marrow in later stages of embryogenesis and residency within the bone marrow during adulthood is crucial for proper maintenance of HSCs. **Table 1.2** summarizes factors that contribute to the localization and retention of adult HSCs in the murine bone marrow niche. The first indication that a chemokine/receptor pair was important for HSC function came from mice constitutionally deficient in CXCL12 (also known as stromal derived factor-1; Sdf-1) or its g-protein coupled receptor CXCR4. Mice lacking either CXCL12 or CXCR4 have nearly identical deficiencies in B-cell development during embryogenesis and B-cell and myeloid development during adulthood that correlates with massively reduced numbers of colony forming units in the bone marrow of these mice (Ma et al., 1998; Nagasawa et al., 1996; Zou et al., 1998). To assess a role for CXCR4 in adult HSCs and to circumvent limitations associated with reduced bone marrow homing, CXCR4 was conditionally deleted from adult hematopoietic cells using poly (I:C)-inducible Mx-1 cre, which resulted in decreased frequency of HSCs in the bone marrow (Sugiyama et al., 2006).

HSCs from these mice also exhibited reduced function, a feature that was shown to be cell autonomous by transplanting $CXCR4^{fl/fl}$ cells along with WT cells and then deleting CXCR4, which resulted in the WT HSCs outcompeting those that lacked the receptor (Sugiyama et al., 2006). Complimenting these findings, two separate groups conditionally deleted CXCL12 from $Tie2^{+}$ endothelial cells or $Prx\text{-}1^{+}$ mesenchymal progenitors and observed a reduced frequency and function of HSCs in the bone marrow (Ding and Morrison, 2013; Greenbaum et al., 2013). Together, these results indicate that CXCL12 production from endothelial cells and mesenchymal progenitors within the bone marrow niche promote the retention of CXCR4-expressing HSCs during adulthood and that retention of adult HSCs within the bone marrow is essential for their optimal function.

It has been known for some time that hematopoietic stem and progenitors (HSPCs) are present in the blood of adult mice (Dorie et al., 1979; Goodman and Hodgson, 1962). To study physiologic trafficking of adult HSCs, some groups have utilized parabiosis studies, in which the circulatory systems of two genetically distinguishable mice are surgically linked. Unlike competitive transplantation assays, where HSCs within the recipient mouse are killed by lethal irradiation in order to open niches for the donor HSCs to freely engraft, parabiosis assays test the ability of HSCs to migrate from one mouse, through the blood stream, to the conjoined mouse and compete with endogenous HSCs for residency in their niches. When congenic mice were linked for 10 days to allow for normalization of their circulatory systems followed by surgical separation and sacrifice after five months, it was shown that about 1 to 5% of HSCs had migrated to the connected host, successfully engrafted and had given rise to progeny

beyond five months (Wright et al., 2001). This demonstrated that a small fraction of adult HSCs regularly traffic through the blood stream and can compete for available niches. More recently, functional HSCs have been isolated from lymphatic ducts, indicating that in addition to trafficking through the blood, HSCs transit through peripheral tissues and have the potential to contribute to local cell production at sites of inflammation (Massberg et al., 2007).

What processes control the physiologic release of HSCs from the bone marrow? Circadian rhythms integrate signals from light/dark cycles to control gene expression in nearly all tissues and are important for regulating energy utilization and controlling bodily processes such as preparing for food intake (Levi and Schibler, 2007). Both HSPCs and HSCs exhibit rhythmic release from the bone marrow that parallels regular light-dark cycles and requires the essential clock gene Bmal-1 (Mendez-Ferrer et al., 2008). Surgical denervation or lack of the beta3 adrenergic receptor also disrupted the rhythmic egress of HSPCs to the blood stream and this was largely dependent on cyclic downregulation of CXCL12 within the bone marrow (Mendez-Ferrer et al., 2008). While multiple facets of the niche influence retention of adult HSCs within bone marrow, the CXCL12/CXCR4 signaling axis appears to be the most dominant.

A splenic reservoir of hematopoietic stem cells exists in adult mice

The spleen is a multi-functional organ that simultaneously serves as a site for phagocytosis of defective erythrocytes, participates in erythropoiesis and acts as a massive lymph node to promote interactions between resident APCs and cells of the

adaptive immune system (de Porto et al., 2010). Splenectomized mice and humans are viable, but suffer an enhanced susceptibility to encapsulated bacteria such as *Streptococcus pneumonia* due to a lack of innate B-cells (B-1a), which produce natural antibodies targeting capsular polysaccharides and require the spleen for persistence and antibody generation (Baumgarth, 2011; Kruetzmann et al., 2003; Wardemann et al., 2002). The spleen is also a reservoir for myeloid progenitors that can be recruited to sites of injury and aid in wound healing (Leuschner et al., 2012; Swirski et al., 2009). During periods of stress, the spleen can act as a site of extramedullary hematopoiesis (O'Malley et al., 2005). As noted in previous sections, HSCs can be isolated from murine spleen at later stages of embryogenesis and into adulthood, but their characteristics are less well defined as compared to bone marrow HSCs.

Work from the Nakauchi group has provided the most detailed characterization of adult splenic HSCs under steady-state conditions. When equal numbers of WBM or splenocytes were mixed with competitor bone marrow and transplanted, it was shown that splenic HSCs are approximately 15-fold less frequent than bone marrow HSCs on a percent basis (Morita et al., 2011). By competitively transplanting single HSCs, defined as $CD34^{-/lo}$ LSK, from bone marrow or spleen, the authors found no difference in the ability of these cells to repopulate recipient mice long term, indicating no inherent functional defects in splenic HSCs (Morita et al., 2011). Importantly, the authors observed that $CD34^{-/lo}$ LSK from the spleen were nearly twice as proliferative as their bone marrow counterparts and this enhanced rate of proliferation was determined by the tissue itself, as bone marrow-derived $CD34^{-/lo}$ LSK that were transplanted into irradiated hosts and subsequently homed to the spleen exhibited higher rates of proliferation than

those which homed back to bone marrow (Morita et al., 2011). This suggested that the splenic microenvironment drives a greater percentage of $CD34^{-/lo}$ LSK to proliferate regardless of their previous origin.

Perhaps the most interesting finding came from parabiosis studies, where the circulatory systems of two mice are linked. After 7 weeks of parabiosis, the percentage of partner-derived circulating blood cells was approximately 50%, which is expected given unimpeded trafficking between each mouse (Morita et al., 2011). By comparison, less than 15% of $CD34^{-/lo}$ LSK in the bone marrow and spleen were derived from the adjoined partner (Morita et al., 2011). This indicated that while cells in the blood stream had free access between each mouse, the majority of $CD34^{-/lo}$ LSK in bone marrow and spleen remained in their original niche throughout the 7 week experiment. Thus, under steady-state conditions there exists a small population of resident splenic HSCs that are more proliferative yet functionally comparable to bone marrow resident HSCs. Whether resident splenic HSCs contribute to immune responses during infection is unknown.

Transplantation and mobilization of hematopoietic stem and progenitors

While studies in the mouse have proved useful to dissect mechanisms underlying the regulation of HSCs in their niche, ultimately it will be necessary to translate this knowledge into improved therapies for humans. Often, the goal of clinical hematopoietic transplantation therapies is to ablate the patient's hematopoietic system, which might contain mutations that have led to cancer, autoimmunity, or lack of a particular hematopoietic lineage, and to replace it with HSCs from a compatible donor that do not

contain the deleterious mutation and will faithfully recapitulate all cells of the hematopoietic system throughout the patient's lifetime. Many factors can influence the outcome of hematopoietic transplantation. In particular, the source of donor cells, the disease status of the recipient and the conditioning regimen used to ablate the offending hematopoietic cells can dictate the speed and success of engraftment as well as the risk of subsequent disease (summarized in **Figure 1.2**) (Arai and Klingemann, 2003; Myers and Davies, 2009). HSCs isolated from different sources offer characteristic benefits and limitations.

There are three main sources of donor HSCs, including those isolated from umbilical cord blood, bone marrow, or peripheral blood following mobilization, which describes the induced migration of a cell population from the bone marrow to the blood stream. Umbilical cord blood is saved at birth and is used in the same individual or matched recipient later in life; this is associated with lower occurrences of graft-versus-host disease (GVHD), a condition in which the transplanted hematopoietic cells attack recipient cells due to antigen incompatibilities. However, umbilical cord blood transplantations are frequently associated with graft failure because of lower numbers of HSCs in the umbilical cord (Gluckman et al., 2001; Wagner et al., 2002). Bone marrow is a rich source of HSCs, but isolation requires a painful procedure and hematopoietic reconstitution can be slower, resulting in an enhanced risk of bacterial infections and a requirement for more platelet transfusions (Arai and Klingemann, 2003). By comparison, mobilization and isolation of HSCs from peripheral blood is relatively well tolerated by donors and results in faster hematopoietic reconstitution, fewer instances of bacteremia and reduced initial hospital stay (Arai and Klingemann, 2003). Long term follow-up

studies have shown that peripheral blood transplants are more likely than bone marrow transplants to result in chronic GVHD and systemic fungal or viral infections (Anderson et al., 2003; Schmitz et al., 2006). While mobilized HSCs have become the preferred source for hematopoietic transplantation (To et al., 2011), it is debatable whether the short term benefits outweigh the long term risks. A better understanding of the mechanisms that promote mobilization should lead to improvements in our ability to harvest HSCs and to mitigate harmful side effects.

Though many cytokines, niche-disrupting agents and stress-inducing phenomenon can induce the mobilization of HSPCs (summarized in **Table 1.3**), granulocyte-colony stimulating factor (G-CSF) is the mobilizing agent of choice for autologous and allogeneic transplantation, since it is well tolerated and efficacious in the majority of hematopoietic donors (To et al., 2011). Even so, approximately 5-30% of donors fail to mobilize sufficient numbers of HSCs for transplantation following G-CSF treatment and mobilization failure is correlated with increased age, prior exposure to cytotoxic drugs and genetic polymorphisms in critical niche components (Bogunia-Kubik et al., 2009; Hill et al., 2011; Hosing et al., 2009; Kumar et al., 2009; Martin-Antonio et al., 2011). Recently, the CXCR4 antagonist AMD3100 has been successfully used in many patients that fail to mobilize with G-CSF alone (Calandra et al., 2008; Liles et al., 2003). While G-CSF treatment is associated with downregulation of CXCL12 in the bone marrow, mobilization of HSPCs with G-CSF and AMD3100 is synergistic, indicating that G-CSF promotes mobilization through both CXCL12/CXCR4 dependent and independent mechanisms (Broxmeyer et al., 2005; Flomenberg et al., 2005; Petit et al., 2002). These include signals emanating from the sympathetic nervous system, signals from endosteal

macrophages and osteoblasts and also may involve the chemotactic lipid mediator sphingosine-1-phosphate (Christopher et al., 2011; Golan et al., 2012; Juarez et al., 2012; Katayama et al., 2006; Winkler et al., 2012; Winkler et al., 2010). Mobilization is an effective tool for harvesting clinically useful preparations of HSCs, but gaps in our knowledge still exist about how and why mobilizing agents work.

Hematopoietic transplantation is an attractive treatment for many diseases in which no other cure exists. Mobilization with G-CSF is a safe and effective alternative to harvesting HSCs directly from bone marrow, yet the enhanced risk of delayed infection and chronic GVHD in transplant recipients indicates that complimentary strategies to limit adverse outcomes are needed. The generation of AMD3100 and its growing clinical usage illustrates that identifying components of the HSC niche can be translated into better mobilization therapies. While procedures to mobilize HSCs have improved over the past decades, a subset of donors still fail to mobilize with dual administration of G-CSF and AMD3100 (To et al., 2011). Thus, the clinical community would benefit from a more comprehensive understanding of the mechanisms that facilitate HSC mobilization.

There are many outstanding questions concerning how PRR signaling is controlled and how PRR activation influences the hematopoietic system. The goal of **Chapter II** will be to identify novel regulators of the NOD2 and NF-κB signaling pathways. This will be accomplished by performing an unbiased genome-wide siRNA screen in cells that express human NOD2 and an NF-κB reporter gene. The goal of **Chapter III** will be to understand how bacterial infection affects the localization and function of HSCs and to determine which signaling pathways are responsible for these effects. This will be accomplished by generating a model of systemic infection followed

by analysis of the number and function of HSCs in wild type and knockout mice using flow cytometry and competitive transplantation assays. Finally, in **Chapter IV,** I will discuss the broader implications of our findings and suggest future avenues of research.

Figures and Tables

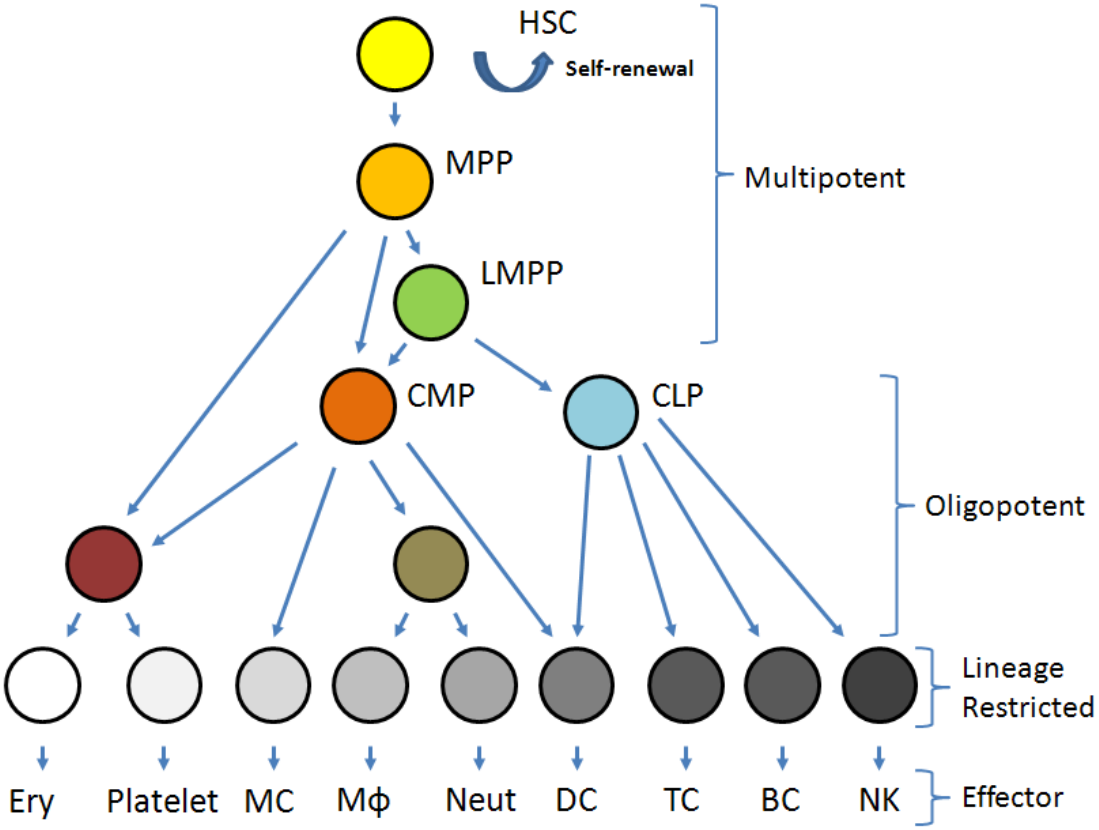

Figure 1.1 Hierarchy in the hematopoietic system
A tiered system of progressively restricted progenitors facilitates massive generation of blood cells and multiple levels for regulation while allowing a small number of rarely-dividing hematopoietic stem cells (HSC) to persist long term. Lines represent potential avenues of differentiation and may include intermediate populations. (MPP) multipotent progenitor. (LMPP) lymphoid primed MPP. (CMP) common myeloid progenitor. (CLP) common lymphoid progenitor. (Ery) Erythrocyte. (MC) mast cell. (Mϕ) macrophage. (Neut) neutrophil. (DC) dendritic cell. (TC) T-cell. (BC) B-cell. (NK) Natural killer cell.

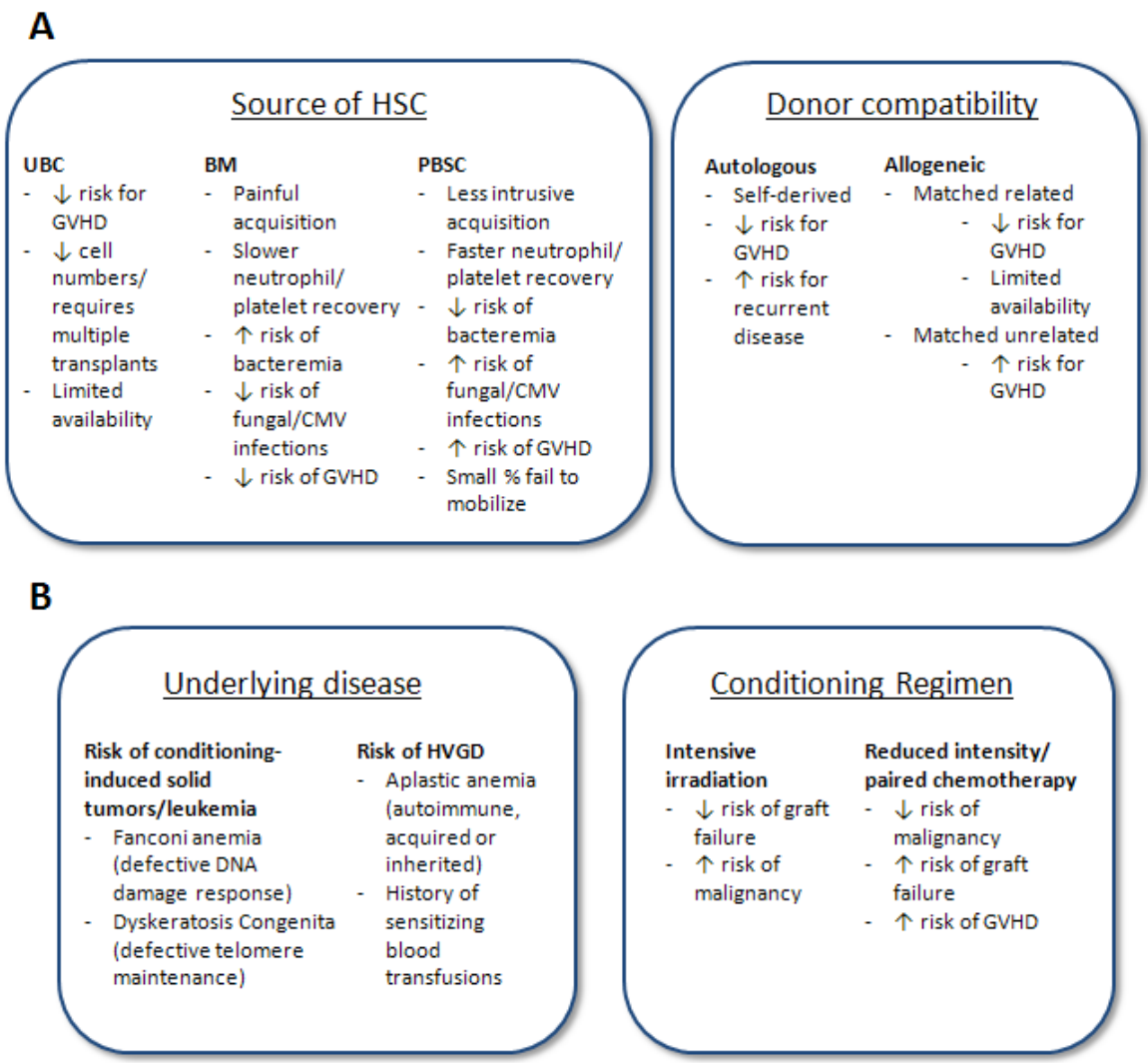

Figure 1.2 | Considerations for clinical hematopoietic stem cell transplantation
(A) Considerations for donor. (UBC) Umbilical cord blood. (BM) Bone marrow. (PBSC) Peripheral blood stem cells (mobilized). (GVHD) Graft versus host disease. (CMV) Cytomegalovirus. **(B)** Considerations for recipient. (HVGD) Host versus graft disease. Data summarized from (Arai and Klingemann, 2003; Myers and Davies, 2009) and references therein.

Table 1.1 Growth factor and receptor pairs important for maintenance of adult HSCs in mice

Gene	Effect on fetal HSCs	Effect on adult HSCs	References
Stem Cell Factor (SCF)	• Required for HSC maintenance in fetal liver (FL) • KO is embryonic lethal (hematopoietic defects)	• Important for HSC maintenance in BM • Conditional KO has massive reduction in BM HSCs	(Ding et al., 2012; McCarthy et al., 1977)
cKit	• Required for HSC maintenance in FL • KO is embryonic lethal (hematopoietic defects)	• Important for HSC maintenance in BM • Blocking antibody allows HSC engraftment in absence of irradiation	(Czechowicz et al., 2007; Ogawa et al., 1991; Ogawa et al., 1993)
Thrombopoietin (THPO)	• KO have enhanced LSK in FL • Functional requirement unknown	• Important for promoting HSC quiescence and maintenance in BM • KO exhibit enhanced cycling, reduced HSCs in bone marrow	(Qian et al., 2007)
cMPL	• KO have enhanced LSK in FL • Functional requirement unknown	• Important for promoting HSC quiescence and maintenance in BM • KO have reduced LSK in BM • Blocking antibody reduces quiescent HSC and allows HSC engraftment in absence of irradiation	(Qian et al., 2007; Yoshihara et al., 2007)
Angiopoietin 1 (Ang1)	• KO are embryonic lethal (vasculogenesis defects) • Functional requirement unknown	• Addition *in vitro* promotes HSC quiescence and maintenance	(Arai et al., 2004; Suri et al., 1996)
Tie2	• KO are embryonic lethal (vasculogenesis defects) • KO have normal fetal hematopoiesis	• Important for maintenance • KO FL HSCs are outcompeted in adult chimeras	(Dumont et al., 1994; Puri and Bernstein, 2003)

Table 1.2 Regulators of HSC localization in murine bone marrow

Gene	Effect on fetal HSCs	Effect on adult HSCs	References
CXCL12 (Sdf-1)	• Important for transition to BM • KO have impaired engraftment in fetal BM (extrinsic)	• Important for BM retention and maintenance • Conditional KO have reduced HSCs in BM	(Ara et al., 2003; Ding and Morrison, 2013; Greenbaum et al., 2013)
CXCR4	• Important for transition to BM • KO have impaired engraftment in fetal BM (intrinsic)	• Important for BM retention and maintenance • Conditional KO have reduced HSCs in BM and increased cycling	(Sugiyama et al., 2006; Zou et al., 1998)
Calcium sensing receptor (CaR)	• Important for transition to BM • KO have impaired engraftment in fetal BM (intrinsic)	• KO is perinatally lethal (hypercalcemia) • KO FL HSCs are at a competitive disadvantage in adult recipients and do not localize to bone interface	(Adams et al., 2006)
Circadian Rhythms (clock gene Bma-1)	• unknown	• Rhythmic entry and exit from bone marrow to blood is disrupted in KO (extrinsic, dependent on CXCL12)	(Mendez-Ferrer et al., 2008)

Table 1.3 Stimuli capable of inducing HSPC mobilization

Category	Stimuli	References
Cytokines/Chemokines	• G-CSF, GM-CSF, Flt3-L, CXCL2 (Gro-β), IL-1, IL-8, VEGF, CXCL12, SCF	(Duhrsen et al., 1988; Fibbe et al., 1992; Fukuda et al., 2007; Hattori et al., 2001; Kollet et al., 2006; Liu et al., 1997; Molineux et al., 1991; Socinski et al., 1988)
Niche-disrupting agents	• AMD3100 (CXCR4 antagonist), proteases, α-integrin Ab, α-selectin Ab, α-VLA4 Ab	(Craddock et al., 1997; Frenette and Weiss, 2000; Liles et al., 2003; Papayannopoulou et al., 2001; Vos et al., 1972)
Stress-inducing phenomena	• Bleeding, exercise, chemotherapy, zymosan, LPS	(Barrett et al., 1978; Cheshier et al., 2007; Richman et al., 1976; Vos et al., 1972; Vos and Wilschut, 1979)

CHAPTER II

A Genome-Wide siRNA Screen Reveals Positive and Negative Regulators of the NOD2 and NF-κB Signaling Pathways

SUMMARY

The cytoplasmic receptor NOD2 senses peptidoglycan fragments and triggers host defense pathways that lead to inflammatory immune responses. Dysregulation of NOD2 signaling is associated with inflammatory diseases, such as Crohn's disease. We used a genome-wide, small interfering RNA (siRNA) screen to identify regulators of the NOD2 signaling pathway. Several genes associated with Crohn's disease risk were identified in the screen, supporting a role for NOD2 and NF-κB pathways in the pathogenesis of Crohn's disease. A comparison of hits from this screen with other "omics" data sets revealed interconnected networks of genes implicated in NF-κB signaling. Secondary assays, including the measurement of interleukin-8 secretion, served to validate many of the regulators. Knockdown of putative regulators in HEK293 cells followed by stimulation with tumor necrosis factor α revealed that most of the genes identified were general regulators of NF-κB signaling. Overall, the genes identified here provide a resource to facilitate the elucidation of the molecular mechanisms that regulate NOD2- and NF-κB–mediated inflammation.

INTRODUCTION

To combat microbial infections, mammals mount a sophisticated immune response. Initial recognition of microorganisms by the innate immune system is mediated by various host-encoded pattern recognition receptors (PRRs). In many cases, PRRs stimulate both antimicrobial and pro-inflammatory responses. The nucleotide-binding oligomerization domain (NOD)-like receptor family of intracellular PRRs consists of multidomain scaffolding proteins, many of which are dysregulated in inflammatory diseases (Chen et al., 2009). NOD2 is found in various cell types, including monocytes and intestinal epithelial cells, and it is capable of sensing bacterial peptidoglycan fragments containing muramyl dipeptide (MDP) (Girardin et al., 2003; Inohara et al., 2003). Upon sensing MDP, NOD2 undergoes a conformational change that leads to the recruitment of receptor-interacting protein serine-threonine kinase 2 (RIPK2), a step that is essential for the activation of downstream signaling (Kobayashi et al., 2002). Subsequently, Lys^{63}-linked poly-ubiquitination of RIPK2 and nuclear factor κB (NF-κB) essential modulator (NEMO), a regulatory subunit of the inhibitor of κB (IκB) kinase (IKK) complex, leads to the recruitment of transforming growth factor–β (TGF-β)-activating kinase 1 (TAK1), a kinase that is required for the activation of mitogen-activated protein kinases (MAPKs) and NF-κB signaling (Abbott et al., 2007; Hasegawa et al., 2008; Yang et al., 2007). Together, these pathways trigger a potent antimicrobial and inflammatory host response.

The role of NOD2 in maintaining inflammatory homeostasis is underscored by the association of loss-of-function *NOD2* mutations with increased susceptibility to CD,

an inflammatory disease of the gastrointestinal tract (Hugot et al., 2001; Ogura et al., 2001a). Conversely, gain-of-function mutations in *NOD2* are associated with Blau syndrome and early onset sarcoidosis, two relatively rare disorders characterized by inflammation of the eyes, skin, and joints (Kanazawa et al., 2005; Miceli-Richard et al., 2001). Genetic variation in the gene encoding NOD2 is, of all of the loci identified, the strongest known genetic risk factor in the development of CD. Because more than 70 loci have been identified that predispose to CD (Franke et al., 2010), it is possible that some of the genes associated with CD are involved in the regulation of the NOD2 signaling pathway. Thus, increased understanding of the NOD2 signaling pathway may provide insight into the pathogenesis of CD.

Genome-wide, small interfering RNA (siRNA) screens are effective tools for identifying previously uncharacterized modulators of biological processes. Using a human cell line expressing human *NOD2* and an NF-κB–responsive luciferase reporter, we performed a genome-wide siRNA screen aimed at identifying regulators of the NOD2 signaling pathway. Our screen revealed many regulators of the NOD2 and NF-κB signaling pathways, including previously uncharacterized networks of positive and negative regulators. Additionally, we linked several genes associated with CD risk to the NOD2 signaling pathway. Together, our results provide a framework for understanding how gene networks contribute to the regulation of NOD2-mediated inflammatory signaling and provide insight into the genetic mechanisms of CD pathogenesis.

MATERIALS AND METHODS

Primary siRNA screen

Human embryonic kidney (HEK) 293 cells stably expressing human *NOD2* (HEK293-NOD2) and an NF-κB luciferase reporter were cultured on 384-well plates (Greiner Bio-One) in Dulbecco's modified eagle medium with 10% fetal bovine serum, 2 mM glutamine, 1 mM sodium pyruvate, and 1X Pen Strep (Gibco). Pools of four distinct siGENOME siRNAs (Thermo Scientific) were reverse transfected at a concentration of 20 nM with Lipofectamine 2000 (Invitrogen) diluted in Opti-MEM (Invitrogen) for 48 hours followed by stimulation with MDP (20 ng/ml, Bachem) for 18 hours. A PHERAstar plate reader (BMG Labs) was used to quantify both the cell viability assays (Cell Titre Fluor, Promega) and the NF-κB luciferase assays (Steady Glo, Promega), according to the manufacturer's directions.

Secondary validation assays

Reporter cells were reverse transfected with 40 nM ON-TARGETplus siRNA pools (Thermo Scientific) for 48 hours, followed by stimulation with either MDP (20 ng/ml) or human TNF-α (10 ng/ml, R&D Biosystems) and cells were analyzed as described earlier.

Bacterial Inoculation

E. faecalis (ATCC 47077) or *S. aureus* (strain 8325-4, a gift from Timothy Foster, Trinity College, Dublin, Ireland) were inoculated into Brain Heart Infusion media and incubated overnight at 37°C (for *E. faecalis*) or 30°C (for *S. aureus*) with shaking. Overnight cultures were sub-cultured into fresh media and incubated at 37°C with shaking for approximately 2 hours until reaching an OD_{600} of 0.6. The bacteria were washed and diluted in sterile phosphate-buffered saline (PBS). 40,000 bacteria were added to each well giving a bacterium to HEK293-NOD2 cell ratio of approximately 1:1. After a 1 hour infection, gentamicin (50 ng/µl, Gibco) was added to the media. Supernatants were collected after 17 hours. Luciferase and viability assays were carried as described for the primary screen.

Epistasis

HEK 293 cells were reverse transfected with 20 nM siRNA (siGENOME) as described earlier. After 48 hours, cells were transfected with pcDNA3, pcDNA3-NOD2ΔLRR, pcDNA3-RIPK2ΔCT-FPK3, or pcDNA3-RIPK1ΔCT-FPK3 together with NF-κB luciferase and *Renilla* luciferase reporter plasmids. After 6 hours, some wells received 100 nM AP1510 (ARIAD) to induce dimerization. Eighteen hours later, cells were lysed in passive lysis buffer (Promega) and analyzed with the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions.

Cell viability assay

Cell viability was analyzed with the CellTitre Fluor reagent (Promega) and read with a PHERAstar plate reader (BMG Labs) at the University of Michigan Center for Chemical Genomics. All cell viability values were calculated relative to multiple control wells of cells that received either non-targeting (NT) siRNA or Passive Lysis Buffer (PLB, Promega); such controls were present on each assay plate. The average value of the wells receiving NT siRNA was considered as 100%, and the average value of wells receiving PLB was considered as 0%. Therefore, wells with increased cell number had values greater than 100%, whereas those with decreased cell numbers relative to those of controls treated with NT siRNA had values less than 100%.

NF-κB luciferase assay

Activation of the NF-κB luciferase reporter was quantified with the Steady Glo reagent (Promega) and a PHERAstar plate reader in the University of Michigan Center for Chemical Genomics. The average relative luminescence values for each well were calculated relative to those of multiple control wells present on each plate. RIPK2-specific siRNA was used as a positive control for plates in which cells were stimulated with MDP, whereas NT siRNA was used as a negative control. For each plate, the average value of wells receiving RIPK2-specific siRNA was considered as 100% inhibition, and the average value of wells receiving NT siRNA was considered as 0%. Therefore, test siRNA that resulted in decreased luciferase (relative to that of wells receiving NT siRNA) resulted in values greater than 0, whereas test siRNA that resulted

in increased luciferase (relative to that of wells receiving NT siRNA) resulted in values less than 0. For assay plates of cells stimulated with TNF-α, RelA-specific siRNA was used as the positive control.

Measurement of IL-8 by ELISA

Secretion of IL-8 into culture media was analyzed by Joel Whitfield in the University of Michigan immunology core facility. For assay plates in which cells were stimulated with MDP, normalized amounts of IL-8 were calculated relative to those of cells that received RIPK2-specific siRNA as well as those that received NT siRNA. For each plate, the average amount of IL-8 (in pg/ml) of wells receiving RIPK2-specific siRNA was considered as 100% inhibition, whereas the average amount of IL-8 in wells that received NT siRNA was considered as 0% inhibition. Therefore, test siRNA that resulted in decreased IL-8 secretion (relative to that of cells that received NT siRNA) resulted in values greater than 0, whereas test siRNA that resulted in increased IL-8 secretion (relative to that of cells treated with NT siRNA) resulted in values less than 0. For assay plates stimulated with TNF-α, the average value of IL-8 secreted by cells that received RelA-specific siRNA was considered as 100% inhibition.

Preparation of BMDMs

B6 (Cg)-*Ncf1*^{m1J}/J mice (*ncf1* mutant mice) were obtained from the Jackson Laboratory. Mice were housed in a pathogen-free facility, and all animal studies were

conducted under protocols approved by the University of Michigan Committee on Use and Care of Animals. BMDMs were isolated as previously described (Celada et al., 1984). Briefly, femurs and tibia were removed from euthanized mice after sterilization with 70% ethanol. Bone marrow cells were resuspended in L-cell-conditioned complete Iscove's Modified Dulbecco's media (Gibco) and cultured for 5 to 6 days before overnight stimulation with MDP (10 μg/m) and subsequent analysis of IκB and MAPK proteins by Western blotting (Cell Signaling Technology Inc.).

Bioinformatics and statistical analysis

A custom-built relational database (M-screen) was developed and used for data storage, display, analysis, and queries (available at: http://mscreen.lsi.umich.edu/index.php). Assay quality was determined by calculating mean, standard deviation, coefficient of variation, and Z' factor (Zhang et al., 1999) values for each plate. After initial normalization to positive and negative controls on each plate, an additional normalization step was performed to normalize the percentage knockdown luciferase values to those for viability. Because the normalized knockdown luciferase values ranged from negative values to >100%, to convert to the luciferase scale, we first subtracted the values from their maximum rather than from 100. After normalization with the percentage viability, we then converted back to the percentage knockdown scale.

The specific formula used was: $\max(L) - (100(\max(L) - L) / V)$, where L = the normalized percentage knockdown of luciferase activity, and V = the percentage viability.

Values were then normalized to the median of each plate, because substantial shifts in plate-to-plate medians were observed, and then global median normalization was used as a final step to make the samples comparable. To calculate *P* values, we tested for normalized knockdown luciferase values that were statistically significantly higher or lower than the overall median. We used an empirical Bayes method (a moderated t-test) that takes into account the relationship between the percentage knockdown of luciferase activity and variance to calculate *P* values (Sartor et al., 2006), enabling more accurate sample variation estimates for each gene. *P* values were adjusted for multiple testing with the Bonferroni correction. Secondary validation analyses were performed in triplicate on 313 hits from the primary screen spread over two separate assay plates. For each of these plates, we analyzed both luciferase and IL-8 data by following the same steps as described earlier, except that no plate-to-plate normalization was performed.

RESULTS

Genome-wide siRNA screen identifies regulators of NOD2 signaling

To identify regulators of the NOD2 signaling pathway, we performed a genome-wide siRNA screen. We engineered a luciferase-based reporter cell line derived from highly transfectable HEK 293 cells, and used this line to quantitatively measure NOD2-dependent signaling (**Figure 2.1A**). HEK 293 cells are a well-established model for interrogating NOD2 signaling because they do not naturally have detectable amounts of NOD2 and they are normally unresponsive to stimulation with MDP (Inohara et al.,

2003). However, stimulation of HEK 293 cells stably expressing *NOD2* with MDP resulted in NF-κB activation, phosphorylation of p38 MAPK, and secretion of the pro-inflammatory chemokine interleukin-8 (IL-8) (**Figure 2.2**). We developed a bioluminescence assay to monitor NF-κB activation with a stably incorporated luciferase reporter gene downstream of tandem copies of a consensus NF-κB–binding site. The screen was performed in triplicate with a commercially available siRNA library targeting 18,110 genes (**Figure 2.1B**). The extent of luciferase activity after stimulation of cells with MDP was measured and displayed relative to that of cells transfected with control siRNAs present on each plate. On average, across all of the assay plates used in the screen, cells transfected with non-targeting (NT) siRNA and stimulated with MDP resulted in ~50-fold induction in luciferase activity. In comparison, the extent of luciferase induction upon silencing RIPK2, a serine and threonine protein kinase required for NOD2-dependent NF-κB activation (Kobayashi et al., 2002) was on average ~7 fold, representing an 86% reduction in the extent of NOD2 signaling.

To enable comparisons to be made between plates, we normalized both luciferase and viability data sets with multiple positive and negative control wells present on each assay plate (**Figure 2.3**). For both the luciferase and viability assays, the data gave a normal distribution (**Figure 2.4**). Most of the *Z*' factor scores, a measure of assay quality (Zhang et al., 1999), for each assay plate were above 0.5 for both the luciferase and viability data sets, indicating a signal-to-noise ratio sufficient for performing robust high-throughput screens (**Figure 2.5**).

We classified genes as NOD2 regulators if their silencing reproducibly decreased the induction of the NF-κB luciferase reporter (positive regulators) or increased luciferase

activity (negative regulators) relative to that of controls treated with NT siRNA (**Figure 2.1C and Figure 2.4**). To avoid nonspecific effects of gene silencing on cell number, we used a fluorescence-based viability assay (Niles et al., 2007). Control wells present on each assay plate were used to calculate the percentage of viable cells (**Figure 2.1D**). The luciferase signal weakly correlated with cell viability, especially for genes whose knockdown resulted in reduced viability, which most likely reflected a lack of sufficient cell numbers to produce luciferase (**Figure 2.6**). Therefore, we considered those genes whose silencing resulted in either (i) enhanced luciferase signal and increased viability (>120%) or (ii) decreased luciferase signal and decreased cell viability (<70%) to be inconclusive with respect to their effect on NOD2 signaling because of their nonspecific effects on cell viability. For example, knockdown of genes involved in core house-keeping functions, such as transcription and translation (**Figure 2.7A**) and cell survival (**Figure 2.7B**), resulted in decreases in both cell viability and luciferase activity. Together, these results demonstrate the importance of using a cell viability control to avoid false positives and to provide increased confidence in the ability of our screen to properly identify genes involved in the regulation of the NOD2–NF-κB signaling pathway.

Numerous NOD2 regulators interact with core components of the NF-κB pathway

To confirm the validity of our experimental approach, we examined how known components of the NOD2 signaling pathway behaved in the screen. Indeed, knockdown of RIPK2 and other core components of the NF-κB signaling pathway, such as NEMO,

NF-κB1, and avian reticuloendotheliosis viral oncogene homolog A (RelA) substantially diminished NOD2-dependent luciferase activity (**Figure 2.1E**), thereby validating our approach. In addition, two serine and threonine kinases involved in NF-κB activation, protein kinase C δ (PRKCD) (Lallena et al., 1999) and casein kinase 2 alpha' (CSNK2A2) (Bird et al., 1997), were both classified as positive regulators in the screen (**Figure 2.1E**). Notably, Klotho (KL), an anti-inflammatory protein that suppresses NF-κB signaling (Zhao et al., 2011), and PDZ and LIM domain protein 2 (PDLIM2), a RelA-binding protein that inhibits NF-κB signaling through its E3 ubiquitin ligase activity against RelA (Tanaka et al., 2007), were each classified as negative regulators in the screen (**Figure 2.1E**).

Consistently, many proteins known to interact with core components of the NOD2 and NF-κB signaling pathways were categorized as positive and negative regulators in the screen (**Figure 2.8**). For example, the NOD2-interacting protein carbamoyl phosphate synthetase/aspartate transcarbamylase/dihydroorotase (CAD) was revealed as a negative regulator in the screen, consistent with previous reports (Gewurz et al., 2012; Richmond et al., 2012). Furthermore, PPP2R5E (protein phosphatase 2, regulatory subunit B', epsilon isoform), another NOD2-interacting protein (Nimmo et al., 2011), was categorized as a positive regulator of NOD2 signaling. Similarly, numerous RIPK2-, NEMO-, and RelA-interacting proteins were identified as either positive or negative regulators (**Figure 2.8A and B**). Furthermore, enrichment analysis of the top 700 positive regulators from the screen revealed components of the proteasome (**Figure 2.8C**) and nuclear pore complex (**Figure 2.8D**) were present more often than what would be expected by chance consistent with the respective roles of these complexes in mediating

inhibitor of κB (IκB) degradation and translocation of NF-κB to the nucleus. Overall, the ability of our screen to identify many proteins already implicated in the regulation of the NOD2 and NF-κB signaling pathways validated our screening approach and provided increased confidence in our discovery of the many putative regulators not previously connected with NOD2 signaling.

Analysis of intersecting data sets reveals networks of putative NOD2 regulators

Next, we used additional lines of biochemical and functional data from the literature to identify experimental evidence supporting connections among hits from the screen. In one approach, we used the Search Tool for the Retrieval of Interacting Genes (STRING) database (Szklarczyk et al., 2011) to reveal numerous protein-protein interactions (PPIs) among hits from the screen (**Figure 2.9**). For example, PPIs between two members of the endosomal sorting complex required for transport (ESCRT)-1 complex, tumor susceptibility gene 101 (TSG101) and vacuolar protein sorting 28 (VPS28), and three proteins involved in autophagy, ATG4A, ATG4B, and γ-aminobutyric acid receptor-associated protein (GABARAP), were identified among putative NOD2 negative regulators. In addition, multiple components of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex, including neutrophil cytosolic factor (NCF1), cytochrome b-245 beta (CYBB), and NADPH oxidase activator 1 (NOXA1), were each classified as positive regulators in the screen. Together, these types of analysis with orthogonal data sets provide additional experimental support for a

number of putative regulators and offer potential mechanistic insight into the level at which these regulators may act to regulate NOD2 signaling.

Given the link between NOD2 and CD, we compared genes categorized as hits in our screen with those associated with CD risk by genome-wide association studies (GWAS). Fifteen genes associated with CD risk were identified as hits in our screen (**Figure 2.10**). In addition, multiple genes whose products interact with these CD risk factors were also categorized as hits in our screen. These results not only support a role for these gene products in NOD2 signaling, but they also suggest that these regulators of the NOD2 and NF-κB signaling pathways may play a role in the pathogenesis of CD.

Multiple NOD2 regulators were confirmed in secondary validation assays

To verify that the hits identified in our primary screen were bona fide regulators of NOD2 signaling, we carried out a series of secondary validation experiments with alternative siRNA pools that targeted 313 genes from our primary screen (**Figure 2.11**). In addition to measuring the induction of NF-κB–dependent luciferase activity, we used enzyme-linked immunosorbent assay (ELISA) analysis to measure IL-8 secretion, whose production is partially dependent on NF-κB activation (Yasumoto et al., 1992). We found that 25% of the putative positive regulators and 33% of the putative negative regulators tested were concordant between the primary and secondary screens, a confirmation rate similar to those reported for other genome-wide siRNA screens (**Figure 2.11B**) (Chiang et al., 2012; Gewurz et al., 2012).

For example, siRNA-mediated knockdown of NCF1, a critical component of the NADPH complex that regulates the generation of reactive oxygen species, diminished NOD2-dependent NF-κB activity in both the primary and secondary screens (**Figure 2.12A and B**). Given that many molecules that interact with NCF1 were identified as positive regulators in the primary screen (**Figure 2.9A**), we continued to validate these results with primary bone marrow–derived macrophages (BMDMs) from *Ncf1*-deficient (*Ncf1*$^{-/-}$) mice stimulated with MDP and analyzed for activation of the NOD2 pathway by Western blotting. BMDMs from *Ncf1*$^{-/-}$ mice exhibited impaired degradation of IκB compared to those from wild-type control mice (**Figure 2.12C**); however, p38 MAPK activation was not affected (**Figure 2.12D**). Moreover, the accumulation of phosphorylated IκB (pIκB) in *Ncf1*-deficient cells (**Figure 2.12C**) further supports an inability to properly degrade IκB, which is consistent with a role for NCF1 in mediating MDP-induced NF-κB activation.

Stimulation of cells with TNF-α identifies general regulators of NF-κB signaling

Because NOD2 signals through NF-κB, we were interested in separating candidate regulators that specifically regulate NOD2 signaling from those that are more general regulators of NF-κB signaling. To distinguish between these two possibilities, we measured NF-κB activation in response to stimulation with either MDP or TNF-α. As predicted, knockdown of RIPK2 inhibited MDP-induced, but not TNF-α–induced, NF-κB–dependent luciferase activity, whereas knockdown of RIPK1 inhibited TNF-α–induced, but not MDP-induced, NF-κB–dependent luciferase activity (**Figure 2.11C**).

Comparison of the responses to MDP and TNFα for each gene revealed that most of the tested genes affected both MDP- and TNF-α–induced NF-κB signaling, suggesting that most of the validated hits are likely downstream of RIPK2 and act as general regulators of the NF-κB pathway.

Apart from RIPK1 and RIPK2, we did not observe genes exhibiting robust specificity for either MDP or TNF-α. However, knockdown of ring finger protein 31 (RNF31) and zinc finger, DHHC-type containing 2 (ZDHHC2) exhibited more substantial inhibition of MDP-induced luciferase activity than of TNF-α–induced luciferase activity (**Figure 2.11C**). To explore the level at which ZDHHC2 and RNF31 regulated NOD2 signaling, we performed epistasis analysis with various constructs that stimulate NF-κB signaling from different points in the pathway (**Fig 2.11D and Figure 2.13**). We used a constitutively active (CA) version of NOD2 that lacks the inhibitory C-terminal leucine-rich repeats, as well as the inducibly active (IA) fusion proteins RIPK2-ΔCARD-Fpk3 (RIPK2 IA) and RIPK1-ΔCARD-Fpk3 (RIPK1 IA) whose CARD domains are replaced by tandem FK506 binding protein (FKBP)-related dimerization domains. Previous studies showed that enforced oligomerization of RIPK2 or RIPK1 induces NF-κB activation that is dependent on the FKBP-specific dimerization agent AP1510 (Inohara et al., 2000). Using this approach, we showed that siRNA-mediated knockdown of RIPK2 inhibited activation of NF-κB signaling when stimulated with CA NOD2 or IA RIPK2, but not with IA RIPK1 alone, consistent with a specific requirement for RIPK2 in NOD2 signaling (Fig. 5D). However, knockdown of RNF31 (**Figure 2.11D**) and ZDHHC2 (**Figure 2.13**) inhibited NF-κB signaling induced by all three

constructs, indicating that both RNF31 and ZDHHC2 are capable of affecting NF-κB signaling downstream of RIPK1 and RIPK2.

Members of LUBAC positively mediate NOD2 signaling

The ubiquitin conjugation machinery plays a critical role in the regulation of NF-κB signaling (as reviewed by Liu and Chen, 2011). Linear ubiquitination mediated by the linear ubiquitin chain assembly complex (LUBAC) plays a role in the regulation of canonical NF-κB signaling in response to various stimuli, such as TNF-α, interleukin-1β (IL-1β), and lipopolysaccharide (LPS) (reviewed by Emmerich et al., 2011). LUBAC is composed of three components: RanBP-type and C3HC4-type zinc finger containing 1 (RBCK1), SHANK-associated RH domain–interacting protein (SHARPIN), and RNF31, the latter two of which were identified as positive regulators of NF-κB in our screen. Analysis of protein-protein interaction databases identified a network of LUBAC-interacting proteins among hits from our primary screen linking LUBAC to core components of the NF-κB signaling pathway (**Figure 2.14A and B**). For example, two of the proteins in this network, RNF31 and ubiquitin-conjugating enzyme E2 D3 (UBE2D3), are known to be involved in the degradation of IκB (Gonen et al., 1999) and the ubiquitination of NEMO (Tang et al., 2003), and both were validated in our secondary assays (**Figure 2.14C**). Knockdown of RNF31 resulted in diminished MDP-induced NF-κB luciferase activity and IL-8 secretion but had only a weak effect on TNF-α–induced NF-κB luciferase activity and IL-8 secretion, whereas UBE2D3 was a positive

regulator of MDP-induced NF-κB luciferase activity, but had no effect on MDP-induced IL-8 secretion or TNF-α–induced signaling.

To further examine roles for RNF31 and UBE2D3 in stimulating NF-κB activation, we treated our reporter cells with two different Gram positive bacteria that activate NOD2 signaling. We chose the common intestinal commensal bacterium *Enterococcus faecalis*, given the required role of NOD2 in mediating a cytokine response (Kim et al., 2011), and *Staphylococcus aureus*, another commensal organism that triggers a NOD2-mediated cytokine response both in vitro (Hruz et al., 2009) and in vivo (Deshmukh et al., 2009). We found that RNF31 was a positive regulator of bacterially-induced NF-κB luciferase activity and IL-8 secretion in response to *S. aureus*, whereas UBE2D3 positively regulated *S. aureus*-induced NF-κB luciferase activity and IL-8 secretion, as well as *E. faecalis*-induced NF-κB luciferase activity, but not IL-8 secretion (**Figure 2.14D**). Together, these results support a critical role for LUBAC as a general regulator of NF-κB signaling and specifically in the modulation of NF-κB signaling downstream of NOD2.

DISCUSSION

Coordination of the inflammatory response is mediated by a wide range of effectors, and proper balance of responses is required for the maintenance of health (Medzhitov, 2008). NOD2 signaling is capable of activating the NF-κB pathway, leading to an inflammatory response to bacteria that is dysregulated in human diseases such as Crohn's disease (Wullaert et al., 2011). In the quiescent state, NF-κB family members

reside within the cytoplasm where they are kept inactive because of physical associations with IκB family members. Degradation of IκB by the proteasome exposes a nuclear localization signal in NF-κB, which mediates its relocalization to the nucleus where it can bind to DNA and regulate the transcription of various target genes. Multiple core components of NOD2 and NF-κB signaling pathways including RIPK2, NEMO, NF-κB1, RelA, and subunits of the proteasome and the nuclear pore complex were classified as hits in our screen, providing evidence that our screen accurately detected bona fide NOD2 regulators. In addition, our genome-wide siRNA screen expanded the number of genes implicated in the regulation of the NOD2 and NF-κB signaling pathways.

Many steps in the NF-κB signaling pathway are regulated by protein ubiquitination (Liu and Chen, 2011). For example, Lys^{63}-linked poly-ubiquitination of NEMO, an IKK scaffolding and regulatory protein, mediates the recruitment of the IKK-activating kinase TAK1. Consistent with this, our screen identified protein phosphatase magnesium-dependent 1 like (PPM1L), a phosphatase that binds to and dephosphorylates TAK1 (Li et al., 2003), as an inhibitor of NOD2 signaling analogous to its previously reported role as an inhibitor of NF-κB activation downstream of TNF-α (Li et al., 2006). Furthermore, we observed decreased NOD2 signaling upon knockdown of two components of LUBAC (RNF31 and SHARPIN), which is consistent with reports showing a role for LUBAC in the regulation of NOD2 signaling (Damgaard et al., 2012). Our results extend these observations by showing a role for RNF31 in the activation of the NOD2 pathway in response to bacterial stimulation. In vivo, s*harpin* deficient mice exhibit excessive inflammation (Seymour et al., 2007), impaired secretion of pro-inflammatory cytokines, and a reduction in NF-κB activation (Tokunaga et al., 2011;

Tokunaga et al., 2009; Wang et al., 2012), features that are somewhat analogous to the symptoms of CD. Biochemically, SHARPIN interacts with RNF31, and together they play a role in NF-κB activation by acting as an E3 ubiquitin ligase to catalyze the linear ubiquitination of NEMO (Gerlach et al., 2011; Ikeda et al., 2011; Niu et al., 2011; Rahighi et al., 2009), which agrees with our secondary validation data showing a positive role for RNF31 in TNF-α–induced NF-κB activation.

Although RBCK1, the third known component of the LUBAC complex, was not individually identified as a hit in our screen, Parkinson protein 2 (PARK2), a protein with the same domain structure as RBCK1 and that was previously implicated in NF-κB signaling (Henn et al., 2007), was classified as a positive regulator of NOD2 signaling. Notably, PARK2, like NOD2 and RIPK2, is linked to susceptibility to leprosy (Mira et al., 2004; Zhang et al., 2009). Furthermore, UBE2D3, an E2 ubiquitin conjugation enzyme that interacts with RBCK1 (Zenke-Kawasaki et al., 2007) was validated as a stimulator of NOD2 signaling in our screen. In addition, two ubiquitin-specific proteases (USP), USP2 and USP36, previously associated with TNF-α–induced NF-κB signaling (Metzig et al., 2011), were classified as positive regulators of NOD2 signaling. Together, these data show a role for protein ubiquitination and, in particular, LUBAC, in NOD2 signaling.

Since protein-protein interactions mediate the assembly and regulation of signal transduction pathways, we were pleased to identify numerous proteins known to interact with NOD2 and other core components of the NF-κB signaling pathway as hits in our screen. In addition, our screen implicated several additional protein complexes in the regulation of NOD2 signaling. For example, our identification of ESCRT-1 as having a

role in the inhibition of NOD2 signaling is supported by the enhanced IL-8 secretion in response to NOD1 signaling that was observed upon knockdown of ESCRT-I complex subunit tumor susceptibility gene 101 (TSG101) (Yeretssian et al., 2011). In addition, several genes associated with CD appear to regulate oxidative stress (Khor et al., 2011), consistent with our identification of several components of the NADPH oxidase complex as regulators of NOD2 signaling. Finally, multiple interacting components of autophagy, an evolutionarily conserved process required for the degradation and recycling of cellular contents that can limit infection by certain intracellular pathogens, were identified as inhibitors of NOD2 signaling in our screen, the relevance of which is increased given evidence of their biochemical links to NOD2 (Homer et al., 2010; Travassos et al., 2010) coupled with the observation that multiple components of the autophagy network are associated with an increased risk of CD (reviewed by Fritz et al., 2011). Together, these examples illustrate the use of overlaying siRNA screening data with protein-protein interaction networks to provide insight into the regulatory mechanisms by which these proteins may influence NOD2 signaling, and to generate models worth pursuing by more traditional hypothesis-based research.

The NOD2 signaling pathway is just one of many upstream inputs that converge on the IKK complex and regulate the evolutionarily conserved NF-κB stress response pathway. These branches are unlikely to function in isolation in vivo. Therefore, we suggest that the relatively large number of genes identified to influence NOD2 signaling by our screen likely includes direct regulators as well as gene products in parallel pathways that indirectly affect NF-κB signaling. Indeed, our TNF-α counter screen revealed that apart from RIPK2, most of the hits identified are general regulators of NF-

κB signaling. This lack of NOD2-specific components supports the idea that different danger-sensing receptors of the innate immune system likely use a small number of specific components before converging on a more general core NF-κB response.

Biologically, activation of the NOD2 and NF-κB signaling pathways is critical for host defense against pathogens (reviewed by Franchi et al., 2009). Tight control of both the strength and duration of this response is necessary to achieve a balance between uncontrolled infection and tissue damage associated with chronic inflammation (Ruland, 2011). Our screen identified many inhibitors of the NF-κB pathway. For example, MDP-induced activation of NF-κB was enhanced upon silencing PDLIM2, a protein that interacts with RelA and mediates its degradation (Tanaka et al., 2007). Furthermore, we validated the RNA-binding protein FUS and its interacting partner FUSIP1 as inhibitors of NOD2 signaling in our siRNA screen. The systematic identification of genes whose products inhibit NF-κB signaling will improve our understanding of how this critical pathway is restricted and may provide new avenues for therapeutic intervention.

In conclusion, we have identified numerous genes that regulate the NOD2 and NF-κB signaling pathways, providing a resource for future hypothesis-based studies aimed at understanding the detailed molecular mechanisms that regulate these pathways. It is becoming increasingly clear that complex networks of interacting proteins, and not simple linear pathways, are involved in the regulation of innate immunity. Our study offers a systems-level glimpse of the NOD2 signaling pathway, which will increase our understanding of how this pathway regulates inflammatory homeostasis and provides insight into the genetic basis for CD.

ACKNOWLEDGEMENTS

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FIGURES

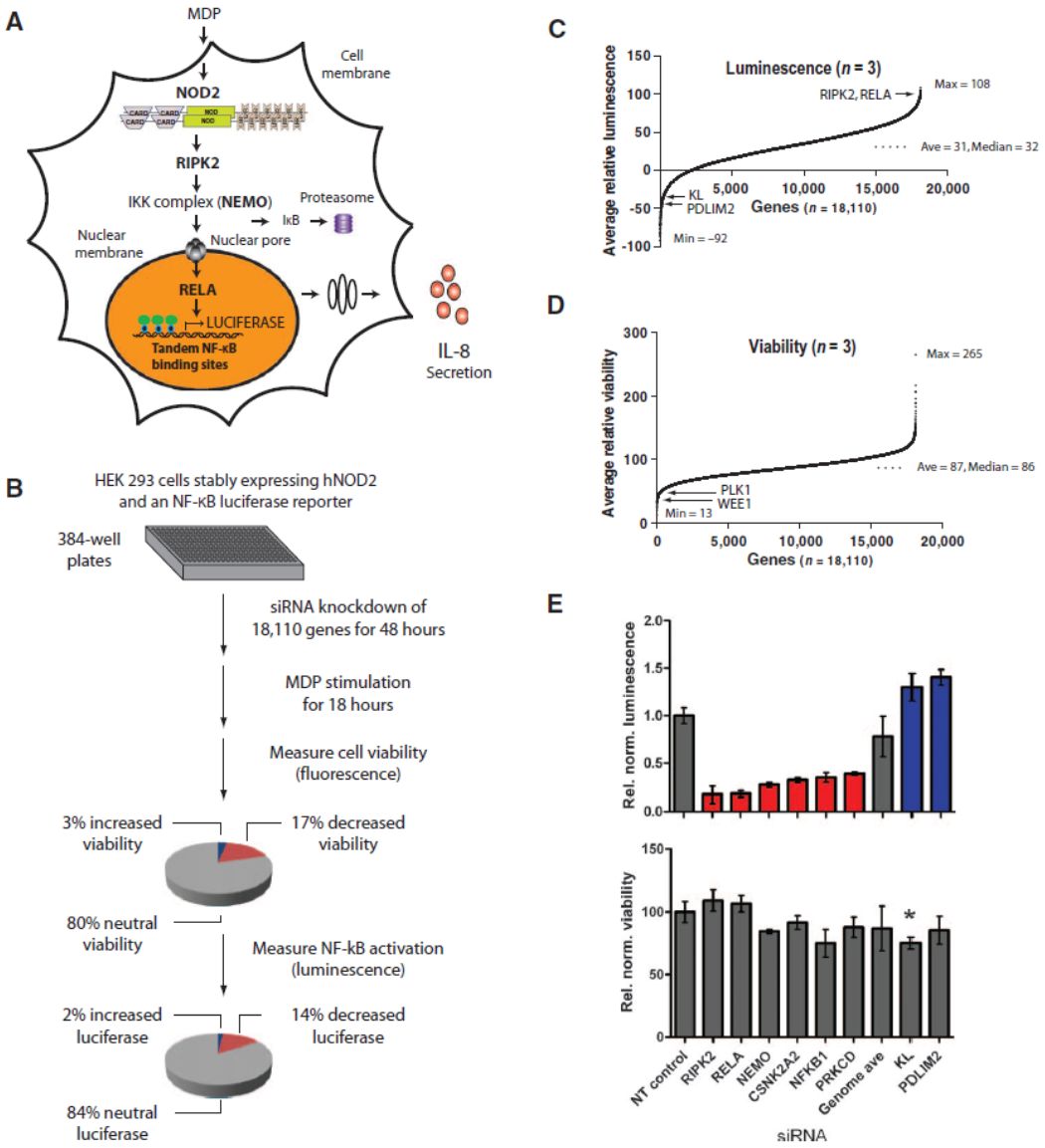

Figure 2.1 | **A genome-wide siRNA screen identifies regulators of NOD2 signaling**
(**A**) Schematic representation of the reporter cell line engineered to quantitatively assess MDP-induced NOD2 signaling together with known components of the NOD2 signaling pathway. (**B**) The screen was carried out in triplicate with pools of four distinct siRNAs specific for each gene. Pie charts summarize the percentages of genes whose silencing affected cell viability and the percentages of genes not affecting viability that either decreased (red) or increased (blue) luminescence. (**C**) Rank-order plot of the average normalized NF-B luciferase reporter activity displayed relative to positive (RIPK2) and negative (nontargeting, NT) siRNA controls present on each assay plate. (**D**) Rank-order plot of the average percentage cell viability displayed relative to NT siRNA and lysed cell controls present on each assay plate. (**E**) siRNA-mediated silencing of known components of the NOD2 signaling pathway led to decreased (red) or increased (blue) NF-B luciferase activity relative to that of NT control siRNA cells (upper panel) without major effects on cell viability (lower panel). All luminescence values were statistically significant with $P < 0.001$. The asterisk indicates viability measurements with $P < 0.001$

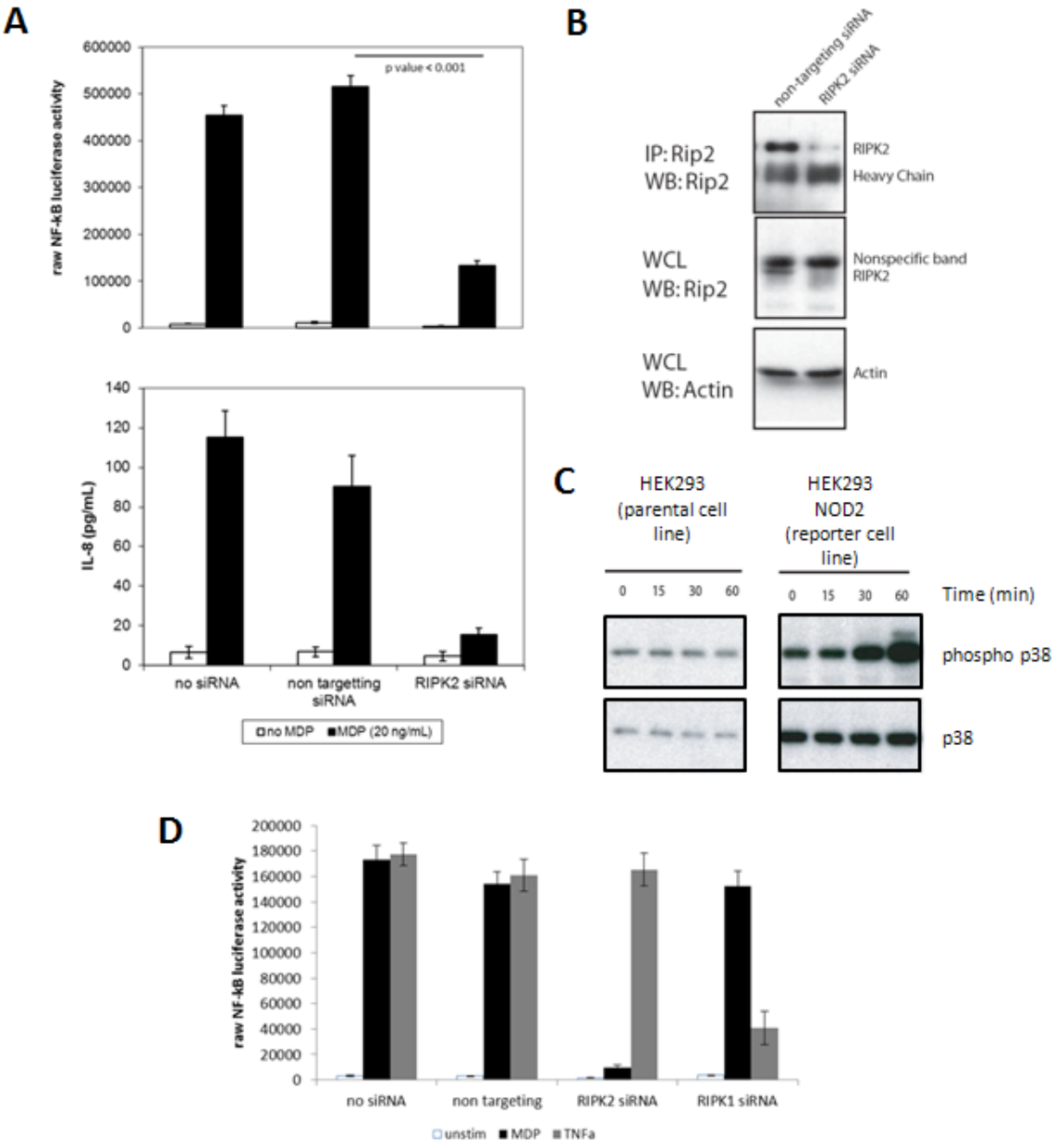

Figure 2.2 | Characterization of the NOD2 reporter cell line used in the genome-wide siRNA screen.
A reporter cell line derived from HEK 293 cells was engineered to stably express human NOD2 and a luciferase reporter gene downstream of tandem copies of a consensus NF-κB–binding site. (**A**) MDP-induced NOD2 signaling was dependent on RIPK2 as assessed by NF-κB luciferase assays (top) and measurement of IL-8 by ELISA (bottom). (**B**) siRNA-mediated depletion of RIPK2 was performed with a pool of four distinct siGENOME siRNA reagents targeting RIPK2 at a concentration of 20 nM. Western blotting (WB) analysis performed on both RIPK2 immunoprecipitated (IP) samples (top) and whole cell lysates (WCL, middle) was performed with anti-RIPK2 antibody. Actin was used as a loading control (bottom). (**C**) The reporter cell line also exhibited MDP- and NOD2-dependent activation of p38 MAPK . Western blot experiments were performed in duplicate with one representative example shown. (**D**) Stimulation of NF-κB activity in the reporter cell line by TNF-α was independent of RIPK2, but dependent on RIPK1, whereas MDP-dependent stimulation was independent of RIPK1.

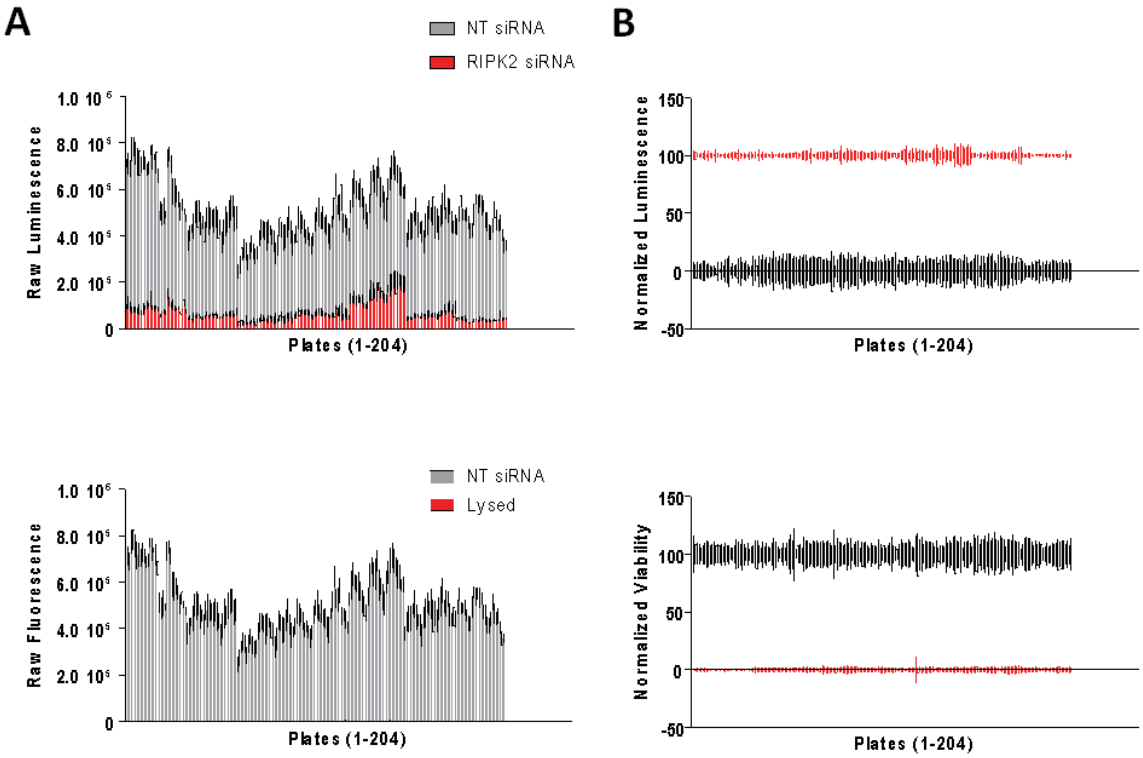

Figure 2.3 | Plate normalization

(**A and B**) Raw bioluminescence data (NF-κB luciferase assay, Steady Glo, Promega, upper panel) and fluorescence data (cell viability assay, Cell Titer Fluor, Promega, lower panel) were collected for each barcoded assay plate with an automated plate reader over a two month period. The siRNA library was spread over 68 plates, with each plate assayed in triplicate to give a total of 204 plates. (**A**) The average raw values for the positive (red) and negative (black) controls on each plate are shown. (**B**) The positive controls were set to 100% and the negative controls were set to 0% to normalize values to enable sample comparison across assay plates.

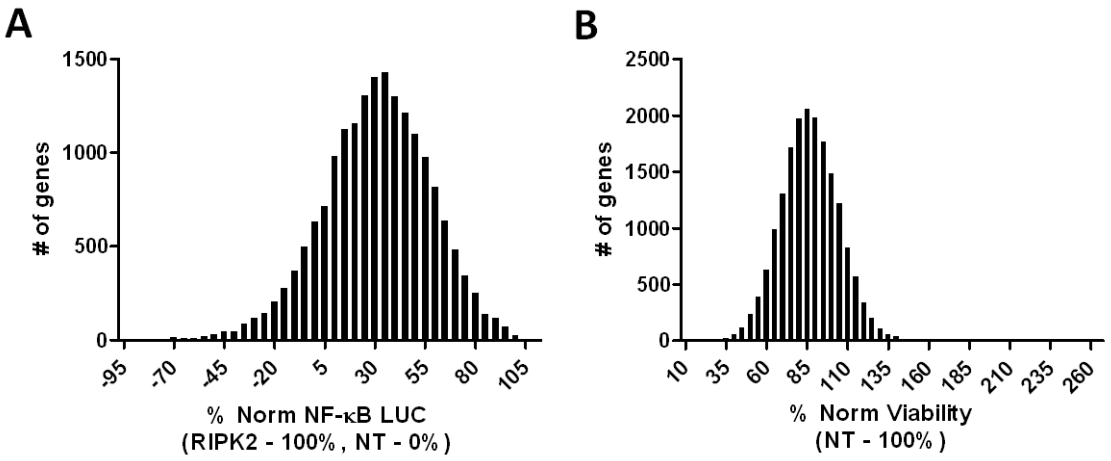

Figure 2.4 | Data distribution

(**A and B**) Histogram analysis of the normalized average (n=3) (**A**) luminescence and (**B**) viability readings for each of the 18,110 genes assayed were binned, revealing a normal distribution of data from the genome-wide screen. Most of the genes had only mild effects on NF-κB luciferase and viability relative to those of the NT control siRNA, whereas a relatively small number of genes were at the tail ends of the distribution.

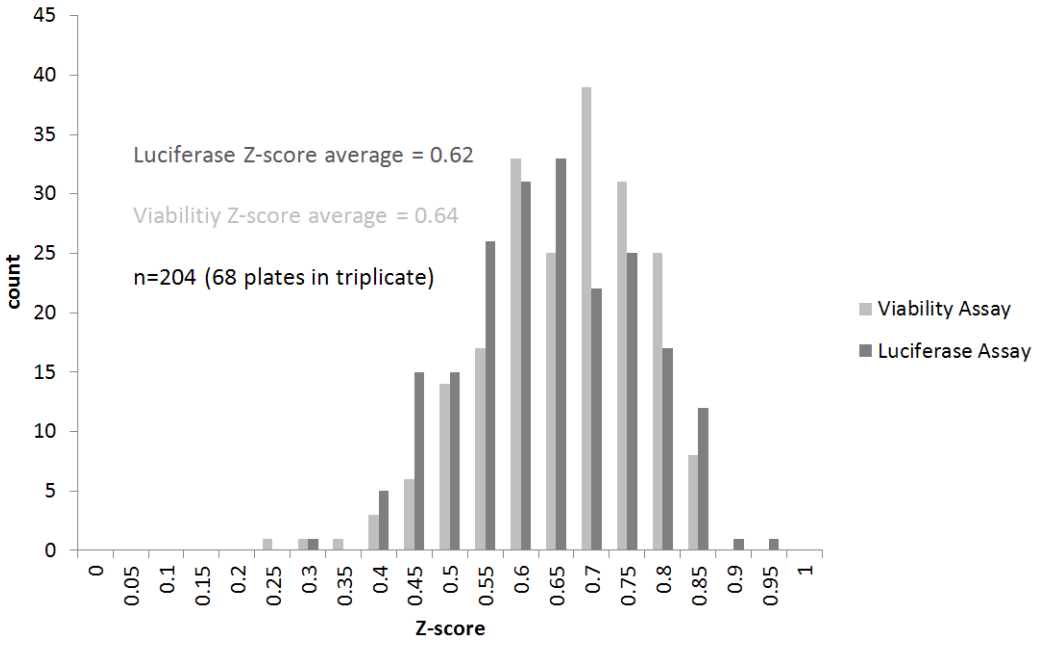

Figure 2.5 | Screening assay robustness
Z-score analysis, with positive and negative controls on each assay plate, was performed for all 204 assay plates for both the NF-κB luciferase (dark) and cell viability assays (light). The Z-scores were nearly all > 0.5, which is generally considered acceptable for high-throughput screening.

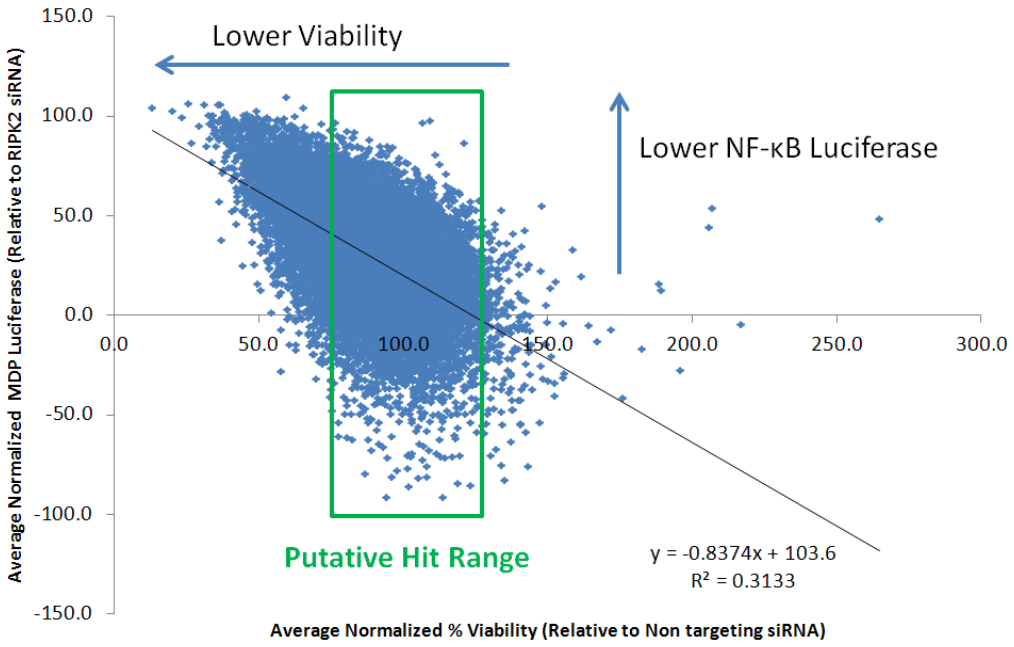

Figure 2.6 | MDP-induced NF-κB luminescence weakly correlates with cell viability
A weak correlation between average relative normalized viability and average relative normalized luminescence (where NT siRNA represents 100% viability and RIPK2 siRNA represents 100% luminescence reduction) was revealed when values for all 18,110 genes were plotted. The line of best fit and linear correlation (R^2) coefficient is shown. Genes with a particularly low cell viability tended to have reduced NF-κB luminescence, which was likely a result of a lack of cells to produce sufficient luciferase signal. This indicates a need to correct for genes whose silencing resulted in extremely low viability. We focused our analysis on genes with average viability between 70% and 120% of controls (green box).

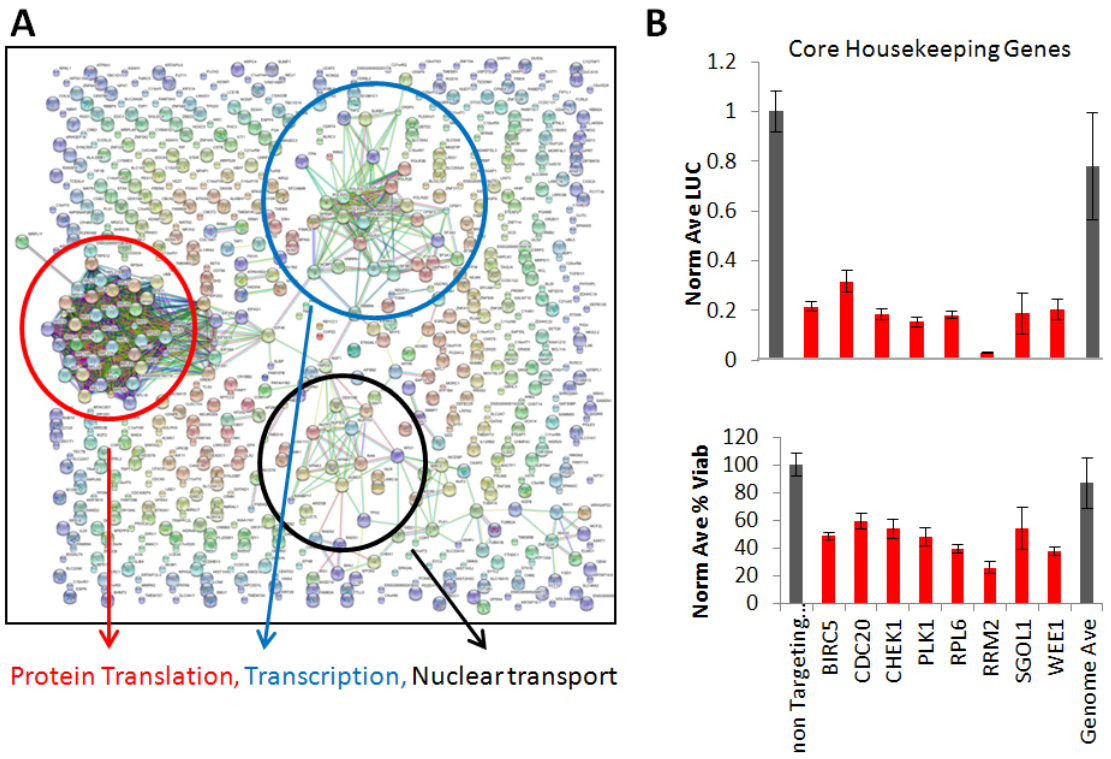

Figure 2.7 | Cell viability analysis helps reduce false positives

(**A**) STRING-based analysis reveals multiple connections between genes that had an average relative normalized viability of less than 60%. Only high-confidence interactions (confidence threshold >0.7) are depicted. Three highly connected sub-networks involving components of core housekeeping complexes, such as the ribosome (translation), RNA polymerase II (transcription), and cell cycle progression (the kinetochore complex and the anaphase-promoting complex) are highlighted. (**B**) The knockdown of the serine and threonine kinase PLK1 and its substrate WEE1 as well as of several other genes that are required for cell survival significantly reduced NF-κB luciferase (upper panel) and cell viability (lower panel) (P <0.001). These genes would have been identified as stimulators of NF-κB signaling without accounting for reduced cell viability.

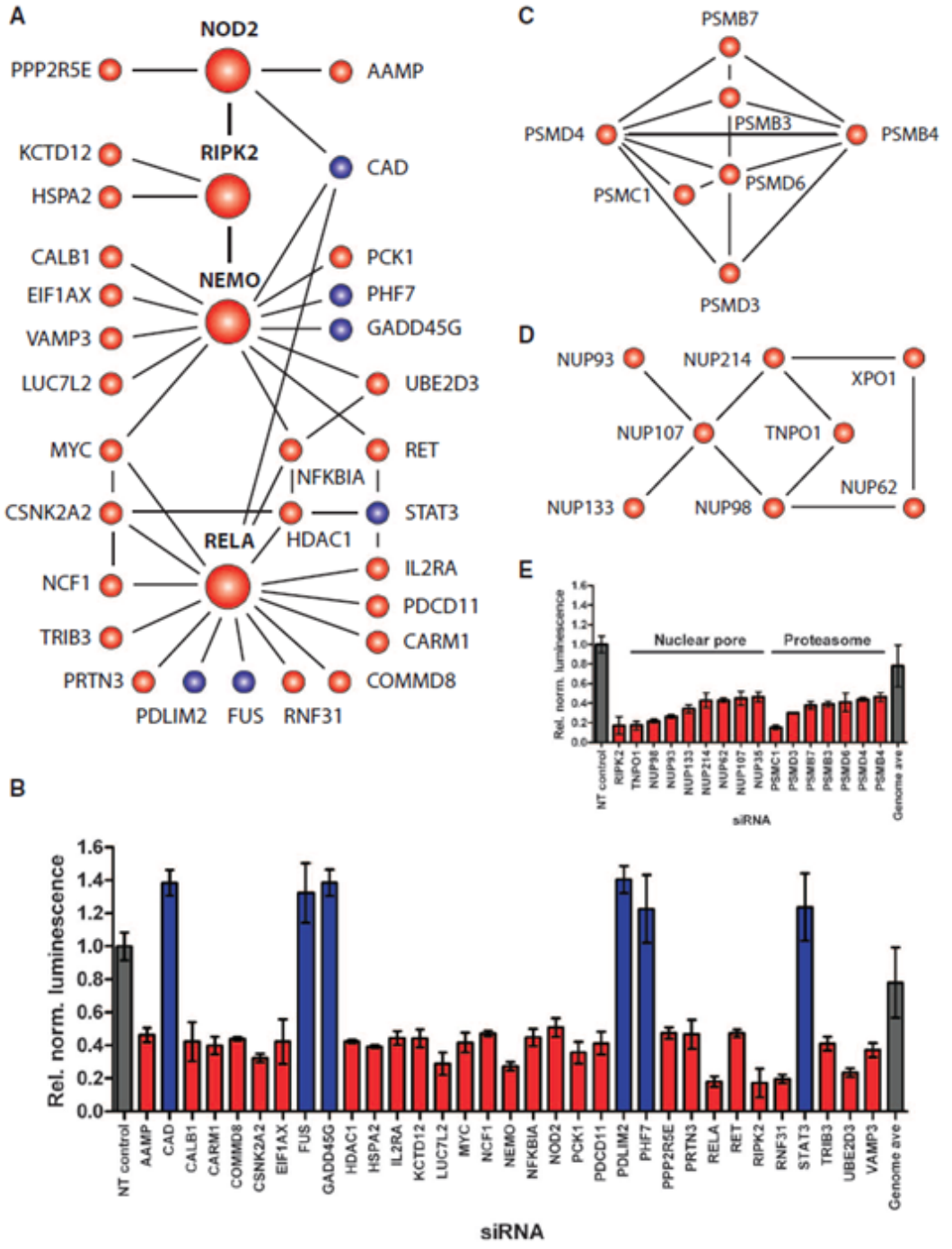

Figure 2.8 | Genome-wide screen identifies known regulators of NOD2 and NF-B signaling pathways
(**A**) Numerous positive (red) and negative (blue) regulators identified in the siRNA screen interact with core components of the NOD2 and NF-B signaling pathways (larger circles labeled in bold). Protein-protein interactions depicted by black lines were taken from publically available databases, such as STRING, with a confidence score of 0.4. (**B**) siRNA-mediated gene silencing led to decreased (red) or increased (blue) NF-B luciferase activity upon stimulation with MDP relative to that of cells treated with NT control siRNA, without major effects on cell viability. (**C to E**) In addition, several components of protein complexes known to affect NF-B signaling by mediating (**C**) the proteasomal degradation of IκB or (**D**) the nuclear translocation of RelA were significantly enriched among (**E**) positive regulators in the screen. All luminescence values were statistically significant with $P < 0.001$.

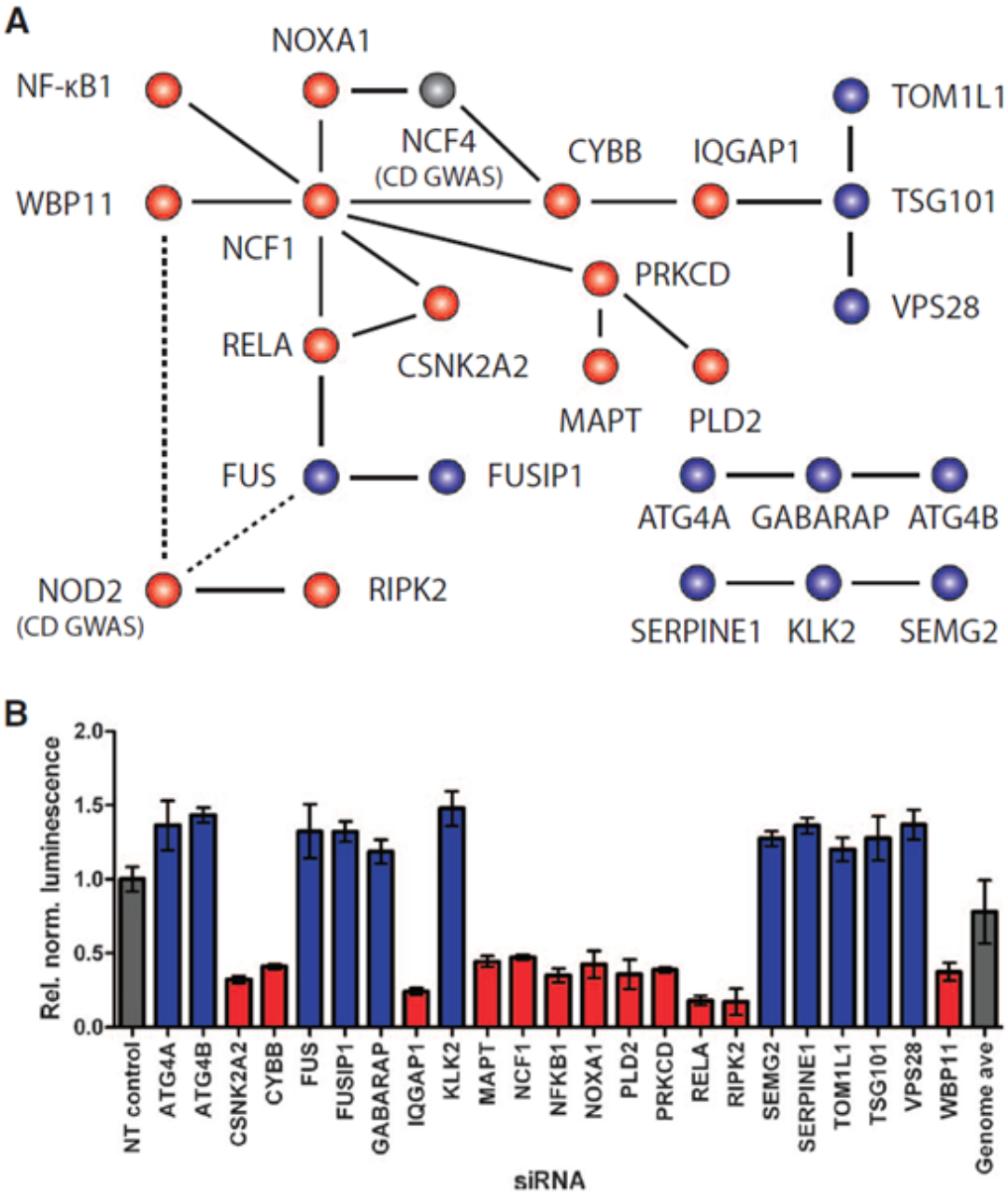

Figure 2.9 | Genome-wide screen identifies networks of positive and negative regulators of the NOD2 and NF-B signaling pathways

(**A**) Schematic depiction of selected protein-protein interactions among hits from the siRNA screen identified with a confidence score of 0.4 from the STRING database. Published interactions are depicted by black lines, whereas dashed lines indicate interactions with NOD2 from a NOD2 pull-down. (**B**) The relative effect on MDP-induced NF-B signaling is shown for each gene in the network with positive and negative regulators shown in red or blue, respectively. All luminescence values were statistically significant; $P < 0.001$.

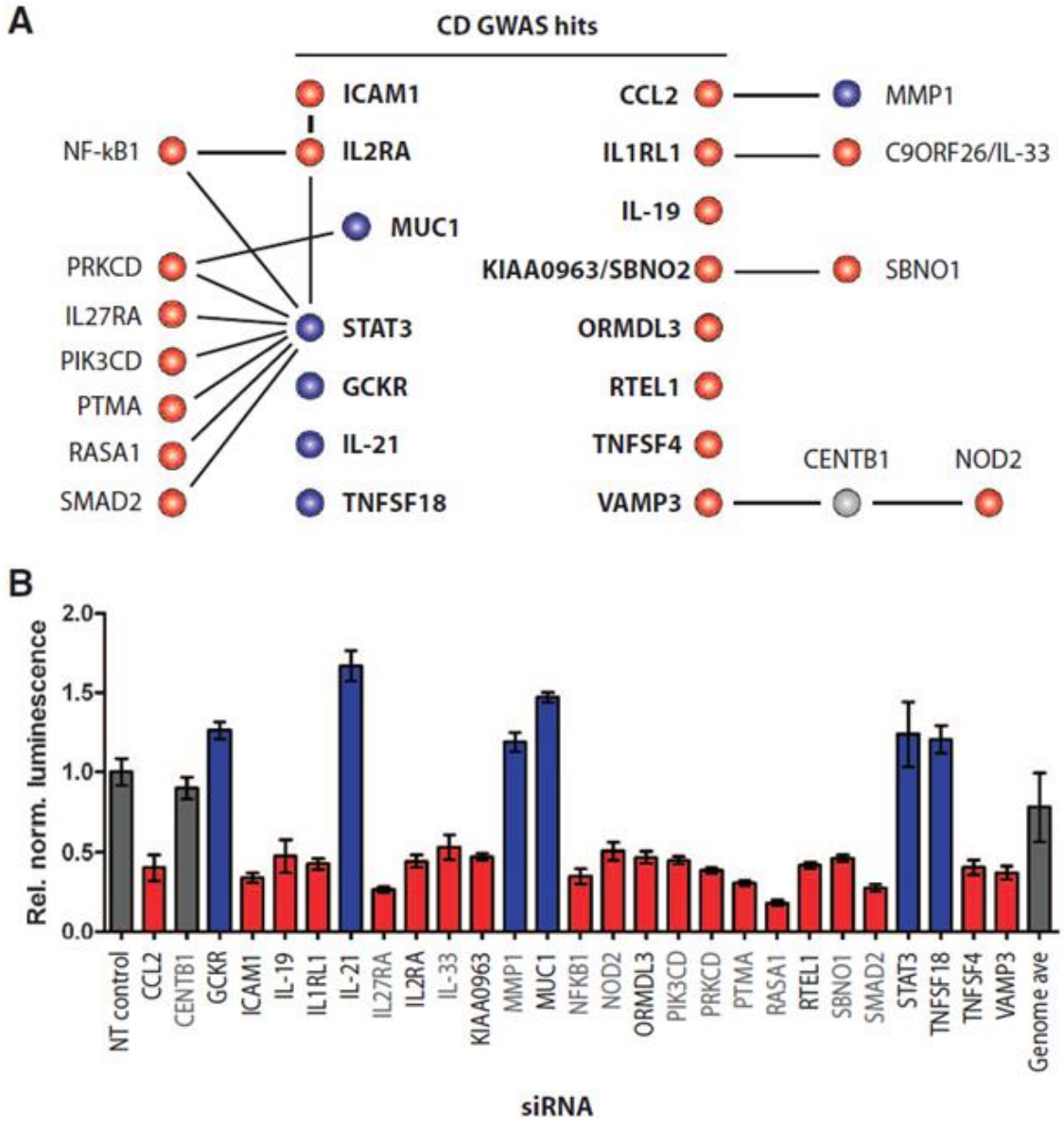

Figure 2.10 | Many genes associated with Crohn's disease risk affect NOD2 signaling
(**A**) Fifteen genes (labeled in bold) whose silencing either diminished (red) or enhanced (blue) NOD2 signaling in our siRNA screen have been identified as risk factors in the development of Crohn's disease by GWAS. Black lines indicate published protein-protein interactions, taken from the STRING database, with other putative NOD2 regulators identified in the primary siRNA screen. A putative link between the Crohn's disease risk factors VAMP3 and NOD2 is provided through their mutual interacting protein CENTB1, which was not identified as a hit in our screen (gray). (**B**) siRNA-mediated silencing led to decreased (red) or increased (blue) NOD2-dependent NF-B luciferase activity relative to that of cells treated with NT control siRNA, without major effects on cell viability. All luminescence values were statistically significant; $P < 0.001$.

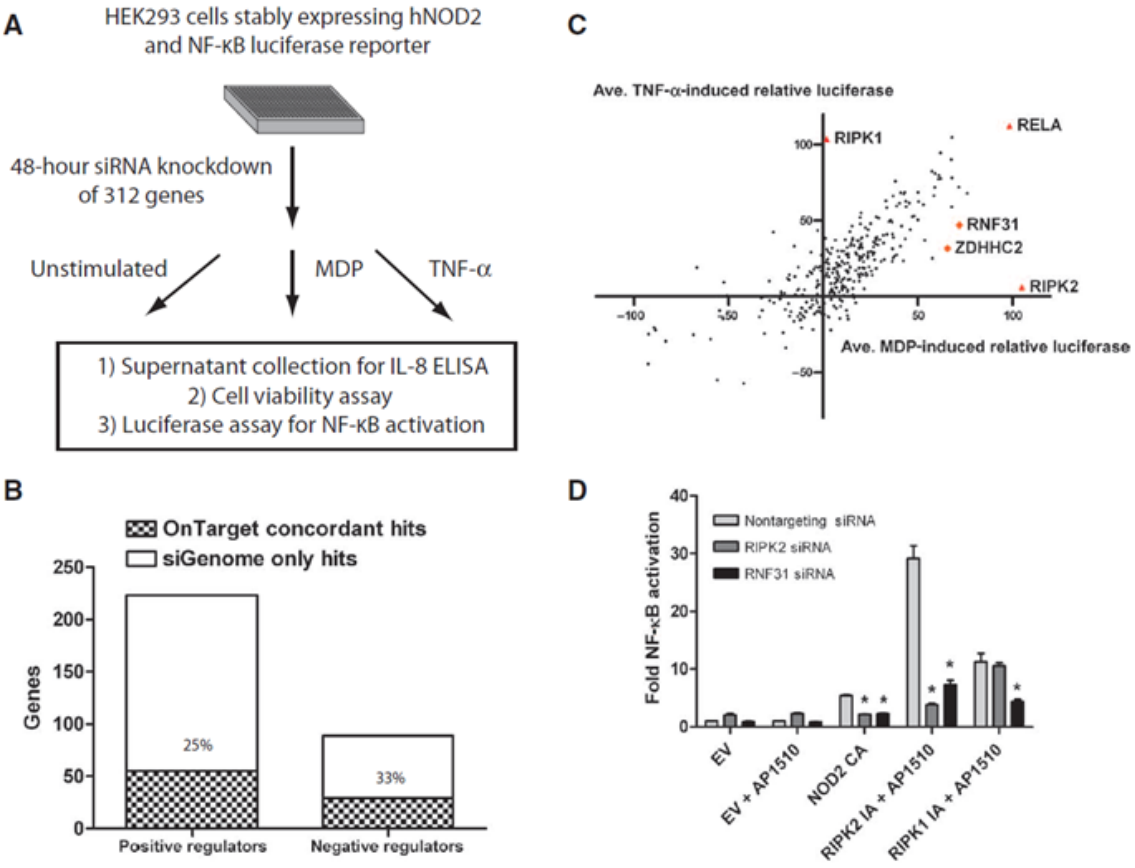

Figure 2.11 | Secondary validation of hits with independent siRNA pools
(**A**) Workflow summarizing the secondary validation screen with alternative sets of ON-TARGETplus siRNA pools consisting of four distinct siRNA sequences for each gene. (**B**) Summary of the secondary validation rate of positive and negative regulators tested for MDP-induced NF-B luciferase activity. (**C**) Comparison of relative NF-B luciferase activity after stimulation with MDP or TNF-α. Positive controls (RIPK2, RIPK1, and RelA) are highlighted along with two selected validated hits. (**D**) Epistasis analysis reveals that RNF31 is a general regulator of NF-B signaling. HEK 293 cells were transfected with the indicated siRNA pools. After 48 hours, a second transfection with an NF-B luciferase reporter and a *Renilla* reporter (for normalization) was used to monitor NF-B pathway activation with (i) a constitutively active NOD2 construct (NOD2 CA), (ii) an inducibly active (IA) RIPK2 construct (RIPK2-IA), and (iii) an inducibly active RIPK1 construct (RIPK1-IA) relative to a pcDNA3 empty vector (EV) control. The dimerization agent AP1510 (ARIAD) was added as indicated to induce signaling. Experiments were performed in triplicate, of which one representative example is shown. $*P < 0.05$ by one-way analysis of variance (ANOVA) and Tukey's multiple comparison test performed with GraphPad Prism software.

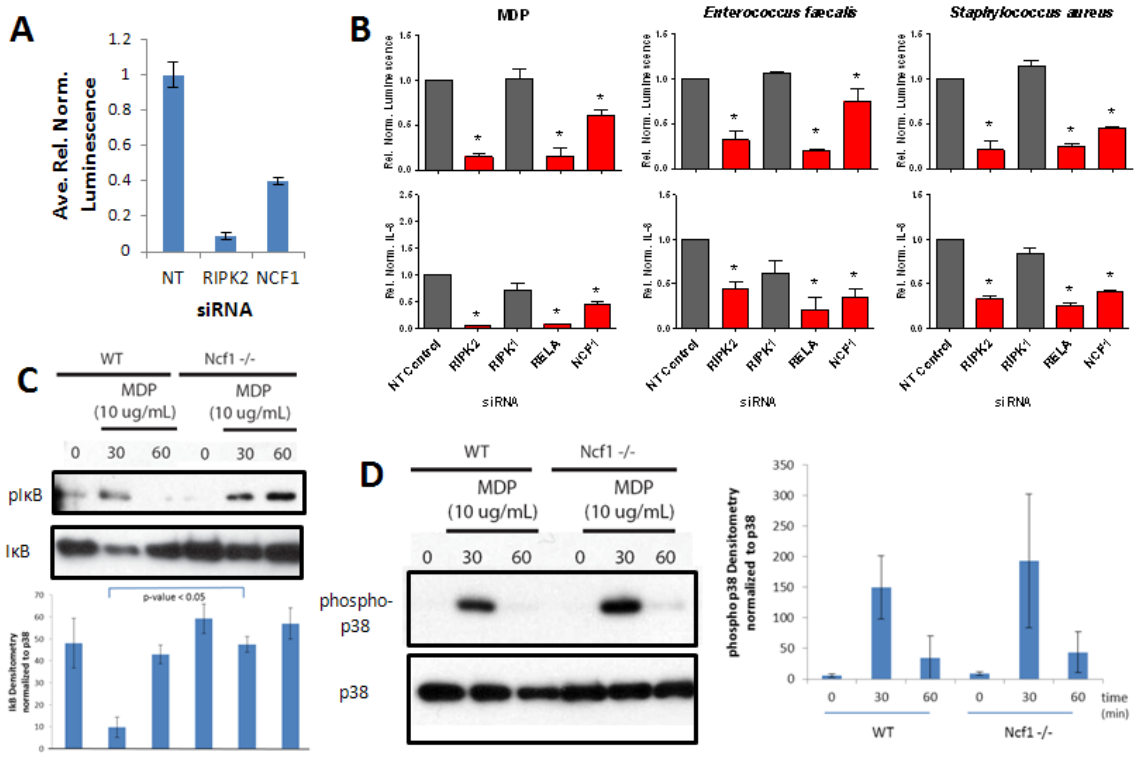

Figure 2.12 | NCF1 is a positive regulator of NF-κB signaling

(**A**) An siGENOME siRNA pool targeting NCF1 from our primary screen reduced NOD2-dependent, MDP-induced NF-κB luciferase activity compared to a non-targeting siRNA control. (**B**) Secondary validation experiments with ON-TARGETplus siRNA pools were used to silence the indicated genes, and NF-κB activation (top) or IL-8 secretion (bottom) were tested after stimulation with MDP, *E. faecalis,* or *S. aureus*. (**P* <0.05). Primary bone marrow–derived macrophages cultured from WT or *Ncf1* deficient mice were stimulated with MDP for 30 or 60 min. (**C and D**) Western blotting analysis was used to monitor NOD2 pathway activation with specific antibodies against (**C**) IκB and pIκB or (**D**) p38 and phosphorylated p38. Representative western blots are shown and pooled densitometry data was used to quantify the relative protein abundances from two independent experiments normalized to the abundance of p38 protein, which was used as a loading control. The amount of IκB in WT cells was significantly lower than that in *Ncf1*$^{-/-}$ cells 30 min after stimulation with MDP, as determined by the student's t-test. No statistically significant difference was observed in the extent of p38 phosphorylation between WT and *Ncf1*$^{-/-}$ cells.

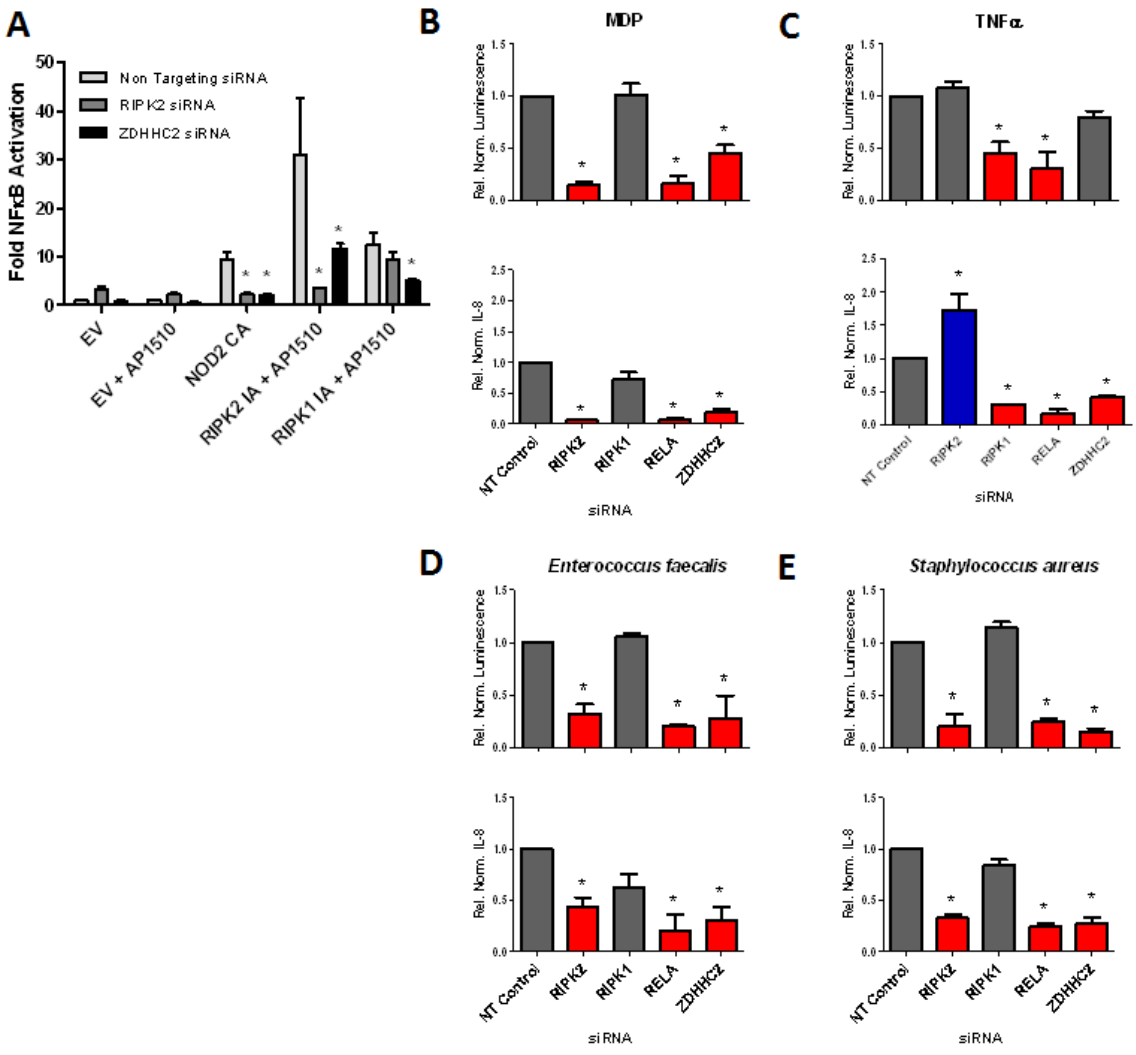

Figure 2.13 | ZDHHC2 is a general regulator of NF-κB signaling
(**A**) Epistasis analysis was performed with siGENOME siRNA targeting RIPK2 and ZDHHC2 coupled with NF-κB–activating constructs as described in the Materials and Methods. Knockdown of ZDHHC2 reduced NF-κB–induced luciferase activity stimulated by all of the constructs tested. In secondary validation experiments, ON-TARGETplus siRNA pools were used to silence the indicated genes, and NF-κB activation (top) and IL-8 secretion (bottom) were tested after stimulation with (**B**) MDP, (**C**) TNF-α, (**D**) *E. faecalis*, or (**E**) *S. aureus*. (*$P < 0.05$).

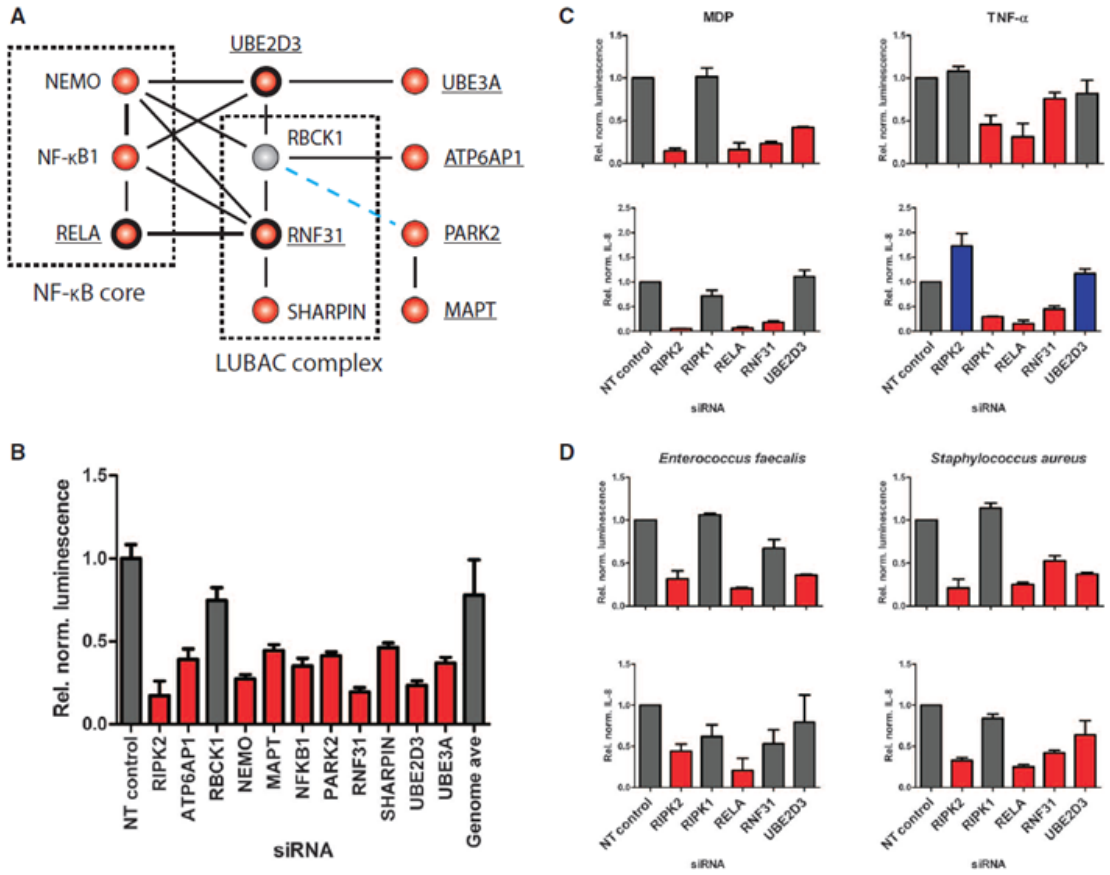

Figure 2.14 | Components of the LUBAC complex positively regulate NOD2 signaling
Two components of the LUBAC complex (RNF31 and SHARPIN) and many of their associated proteins were revealed as positive components of MDP-induced NOD2 signaling in the primary siRNA screen. (**A**) Protein-protein interactions are depicted by black lines, whereas a dashed blue line shows that PARK2 is a protein homolog of RBCK1 and that they share a similar domain structure. Factors required for MDP-induced activation of the NF-B pathway are shaded in red, whereas those genes having no statistically significant effect are gray. Underlined names indicate genes tested by secondary screening, and bold circles indicate genes that were validated. (**B**) Induction of MDP-induced NF-B luciferase activity relative to that of cells treated with NT siRNA from the primary screen is shown for each component of the protein-protein interaction network. All luminescence values were statistically significant; $P < 0.001$. (**C and D**) Induction of NF-B luciferase reporter activity (upper panel) or IL-8 secretion (lower panel) after knockdown with ON-TARGETplus siRNA pools in response to (C) stimulation with MDP (left) or TNF-α (right) or (D) infection with two Gram-positive bacteria, *E. faecalis* (left) or *S. aureus* (right). Bars shaded in red and blue indicate $P < 0.05$.

CHAPTER III

Infection Expands Extramedullary Hematopoietic Stem Cells Through NOD-like-Receptor and Toll-like Receptor Signaling via G-CSF

SUMMARY

Adult hematopoietic stem cells (HSCs) are maintained primarily in specialized niches within the bone marrow and may engage in extramedullary hematopoiesis during periods of stress, yet the underlying mechanisms are largely unknown. We show that infection with the gram negative bacterium *Escherichia coli* resulted in marginal reduction of bone marrow HSCs, but induced robust expansion of functional HSCs in spleen. Most HSC activity was retained within the $CD150^{+}$, $CD48^{-}$, $Lineage^{-/low}$, $Sca1^{+}$ and $cKit^{+}$ fractions while subsets of HSCs had increased expression of CD48 and Lineage markers during infection. Expansion of splenic HSCs was diminished in TLR4 deficient and RIPK2 deficient mice, implicating TLRs and NOD1/NOD2 signaling in the activation of HSCs after infection. Dual stimulation of NOD1 and TLR4 expanded HSCs and progenitors in spleen, which was dependent on G-CSF and correlated with reduced CXCL12 in bone marrow. Expression of TLR4 and RIPK2 in radio-resistant cells was required, while TLR4 expression on HSCs was dispensable. Our studies highlight a role for NLR and TLR signaling in radio-resistant cells to induce the production of endogenous G-CSF and subsequent extramedullary HSC expansion. Furthermore, our

results indicate that bacterial infection has minimal effect on bone marrow HSCs, but enhances extramedullary HSC activity.

INTRODUCTION

Hematopoietic stem cells are defined by their ability to give rise to all cells of the blood system and to self-renew, where cell division results in at least one daughter that retains the full developmental potential of its parent. The majority of HSCs reside in the bone marrow where they are surrounded by a network of supporting cells, collectively termed the stem cell niche. A smaller subset of HSCs reside in the spleen, which serves as a site for hematopoiesis during embryogenesis and periods of duress (Morrison and Spradling, 2008). While residency in a niche is essential for HSC maintenance, HSCs regularly traffic through the blood stream in a process that may facilitate competition for niches to ensure a robust pool of stem cells (Wright et al., 2001). Moreover, HSCs have been isolated from lymphatic ducts, indicating that HSCs travel through peripheral tissues and have the potential to provide a local source of cell production (Massberg et al., 2007). While much is known about HSC activity under homeostatic conditions, how HSCs function during stress is less well understood.

Bacterial infection is a common form of stress that can induce profound effects on the fate of hematopoietic stem and progenitor cells (HSPCs). Host-derived pattern recognition receptors (PRRs) sense components of bacteria and respond by activating pro-inflammatory signaling pathways that aid in the defense against infection. *Escherichia coli* is a gram negative bacterium whose cell wall contains LPS, which is

sensed by Toll-like receptor 4 (TLR4), and peptidoglycan, whose cleavage products are sensed by the nucleotide-binding oligomerization domain containing (NOD)-like-receptors (NLRs) NOD1 and NOD2. TLR4 signals via the adaptor proteins myeloid differentiation primary response 88 (Myd88) and TIR-domain-containing adapter inducing interferons (TRIF), while NOD1 and NOD2 signaling requires the adaptor protein receptor-interacting serine-threonine kinase 2 (RIPK2), which leads to the activation of NF-κB and MAPK pathways (Franchi et al., 2009; Sartor, 2008).

TLR4 and TLR2 are expressed on the surface of Lineage$^{-/low}$ Sca1^{+} cKit^{+} (LSK) cells, which mark both HSCs and non-self-renewing progenitors, suggesting that HSPCs may actively participate in innate immune responses (Nagai et al., 2006). Activation of TLRs has been proposed to alter the fate and function of HSCs either directly, or indirectly via production of inflammatory cytokines or alterations in the bone marrow niche (Baldridge et al., 2010; Chen et al., 2010; Esplin et al., 2011; Essers et al., 2009; Johns et al., 2009; Rodriguez et al., 2009; Scumpia et al., 2010; Takizawa et al., 2011). Only one study tested the function of HSCs following live bacterial infection (Baldridge et al., 2010), but they did not assess HSC activity in unfractionated bone marrow or in sites of extramedullary hematopoiesis such as the spleen. Moreover, no study has characterized the surface marker profile of functional HSCs after infection. Thus, it is unclear whether bacterial infection alters the phenotype and function of HSCs in the bone marrow and spleen. Furthermore, it remains to be determined whether HSCs directly sense and respond to components of bacteria or whether infection merely alters the bone marrow microenvironment and modulates their fate indirectly.

Activation of TLRs can also affect the localization of HSPCs. Systemic administration of LPS results in the accumulation of HSPCs in the spleen (Esplin et al., 2011; Vos et al., 1972), but the signals and cell types responsible for driving this phenomenon are unknown. Repeated administration of granulocyte-colony stimulating factor (G-CSF), which can be produced in response to infection or LPS (Hareng and Hartung, 2002), induces the mobilization of HSPCs from bone marrow to peripheral blood and spleen and is the preferred mobilizing agent used in the clinic (Duhrsen et al., 1988; Molineux et al., 1990; Morrison et al., 1997; To et al., 2011). While the mechanisms of G-CSF induced mobilization are well characterized (Levesque and Winkler, 2008; Mazo et al., 2011), it is currently unclear whether the production of endogenous G-CSF contributes to alterations in HSC activity during infection. Here we investigated mechanisms underlying how bacteria influence the localization and function of HSCs.

MATERIALS AND METHODS

Mice

Wild type CD45.2 (C57BL/6) and WT CD45.1 (B6.SJL-Ptprc^{a} Pepc^{b}/BoyJ) mice were purchased from Jackson Laboratories. *Nod1*$^{-/-}$ mice were kindly provided by Grace Chen (University of Michigan, USA). *Csf3r*$^{-/-}$ (G-CSFR$^{-/-}$) mice were kindly provided by Daniel Link (Washington University, USA). *Myd88*$^{-/-}$, *Trif*$^{-/-}$ and *Tlr4*$^{-/-}$ were kindly provided by Shizuo Akira (Osaka University, Japan). All mice were maintained under

SPF conditions at the University of Michigan. All experiments were approved by the University of Michigan Committee on the Care and Use of Animals.

Reagents

KF1B and *E. coli* K12 were graciously provided by Naohiro Inohara (University of Michigan, USA). Ultrapure LPS (*E. coli* K12) was purchased from Invivogen. For experiments using bacterial compounds, 50 ug of KF1B and/or 100 ug of LPS was diluted in PBS and injected *i.p.* Anti-mouse G-CSF blocking antibody (clone 67604, R&D Systems, Minneapolis, MN, USA) and rat IgG1 isotype control were provided by Ian Wicks (WEHI, Australia) as a gift from CSL Limited, Australia, and used as described previously (Lawlor et al., 2004). For these experiments, 250 ug of antibody was diluted in PBS and injected *i.p.* one hour prior to injection of bacterial compounds.

Cell isolation and flow cytometry

Bone marrow cells were isolated by flushing one femur and tibia with 3 mL Ca2+ and Mg2+ free HBSS supplemented with 2% HI FBS and filtering through a 50 μm mesh and counted using a hemocytometer. Splenocytes were obtained by mashing the organ between two glass slides and filtering through a 100 μm mesh. Populations were visualized using combinations of antibodies listed in **Table 3.1.**

Long-term reconstitution assays

Ten week or older recipient mice were lethally irradiated using X-ray (Phillips RT250, Kimtron Medical) with two doses of 540 rad (total 1080) delivered between 3 and 24 hours apart. In some experiments, $3x10^5$ WBM or $1x10^6$ splenocytes were mixed with $3x10^5$ competitor whole bone marrow and transplanted via tail vein injection. To determine the surface marker profile of HSCs, total bone marrow or spleen was harvested from mice six days after infection, purity sorted to enrich for the positive or negative fraction, purity sorted again and mixed with $3x10^5$ WBM prior to retro-orbital injection. Recipient mice were maintained on antibiotic water (neomycin sulphate 1.11g/L and polymyxin B sulfate 0.121 g/L, 50 g/L sucrose) for at least 6 weeks. In experiments where live *E. coli* exposed tissues were transplanted, recipient mice received 5 mg/kg enrofloxacin (Bayer) *i.v.* at day 0 and *s.c.* at day 5 post transplant. Recipient mice were bled every four weeks to determine reconstitution. To generate reverse chimeras, $5x10^6$ WBM from WT, *Tlr4*$^{-/-}$ or *Ripk2*$^{-/-}$ mice was transplanted via tail vein into lethally irradiated WT, *Tlr4*$^{-/-}$ or *Ripk2*$^{-/-}$ mice and recipients were allowed to recover for 16 weeks prior to challenge.

Bacterial culture and infection

E. coli K12 was picked from frozen glycerol stock into LB broth, shaken at 37 C overnight, and sub-cultured in LB for another 2 hrs. Bacteria were washed multiple times in PBS and their number was estimated by optical density at 600 nm. Mice were

injected *i.p.* with 200 uL of bacteria diluted in PBS and residual bacteria was serially diluted in PBS and plated on LB agar to quantitate CFU.

Quantitative PCR

WBM was homogenized using a column (Omega) and RNA was isolated using a Total RNA kit (Omega). Eluted RNA was reverse transcribed into cDNA using High-Capacity RNA-to-cDNA kit (Applied Biosystems). Quantitative real-time PCR was performed using SYBR Green (Applied Biosystems). Gene expression was normalized to the house keeping gene GAPDH. Primers for CXCL12 were F-TGCATCAGTGACGGTAAACCA and R- GTTGTTCTTCAGCCGTGCAA. Primers for GAPDH were F- TGCGACTTCAACAGCAACTC and R-GCCTCTCTTGCTCAGTGTCC.

Methylcellulose culture

Splenocytes were counted using a hemocytometer, diluted in Methocult M3434 medium (Stem Cell Technologies) and incubated at 37 C for 8-12 days after which myeloerythroid colonies were quantified.

Statistical Analysis

All statistics were performed using Graphpad Prism software. Statistical tests used are described in Figure Legends.

RESULTS

Systemic *E. coli* infection reduces functional HSCs in bone marrow

To understand the mechanisms underlying the regulation of HSCs during infection, we developed a model of systemic infection using the gram negative bacterium *Escherichia coli* K12. This bacterium was chosen because systemic administration was sub-lethal in wild type (WT) mice for all conditions tested. To determine whether HSC activity is altered in the bone marrow after *E. coli* infection, we performed competitive transplantation experiments in which WT (CD45.1) mice were treated with PBS or *E. coli* and after six days, $3x10^5$ whole bone marrow (WBM) were mixed with $3x10^5$ untreated (CD45.2) WBM and transplanted into lethally irradiated (CD45.2) recipients and peripheral blood reconstitution was followed for 16 weeks (**Figure 3.1A and B)**. To quantitatively compare levels of reconstitution, we converted percent chimerism after 16 weeks into repopulating units (RUs) (Harrison et al., 1993). In two independent experiments, WBM from *E. coli* infected mice had on average 2.1-fold fewer RUs compared with PBS treated WBM (**Figure 3.1A**). The contribution of *E. coli* infected WBM to the B-lineage was significantly reduced, while contribution to myeloid and T-lineages trended toward a reduction (**Figure 3.1B**). These results indicate that total HSC activity in the bone marrow of mice infected with *E. coli* for six days was diminished compared to that of uninfected mice.

Systemic *E. coli* infection expands functional HSCs in spleen

The spleen can serve as a site for extramedullary hematopoiesis during periods of stress and previous studies have found that treatment of mice with LPS, which mimics some aspects of infection, expands HSPCs in the spleen (Esplin et al., 2011; Vos et al., 1972). To determine whether HSC activity is altered in the spleen after live bacterial infection, we treated WT (CD45.1) mice with PBS or *E. coli* and after six days mixed $1x10^6$ splenocytes with $3x10^5$ untreated (CD45.2) WBM and transplanted these cells into lethally irradiated (CD45.2) recipients and followed peripheral blood reconstitution for 16 weeks (**Figure 3.1C and D**). In two independent experiments, splenocytes from *E. coli* infected mice had on average 38.9-fold more RUs than splenocytes from PBS treated mice (**Figure 3.1C**). We defined long term multilineage reconstitution (LTMR) activity, characteristic of functional HSCs, as the presence of greater than 0.3% of donor-derived myeloid, T-lineage and B-lineage cells at 16 weeks post transplant. Whereas splenocytes from PBS treated donors contributed minimally to myeloid and lymphoid lineages over 16 weeks (2/9 LTMR), consistent with a very small resident population of splenic HSC under steady-state conditions, splenocytes from *E. coli* infected donors contributed robustly to myeloid and lymphoid lineages over 16 weeks (9/9 LTMR) (**Figure 3.1D**). These results indicate that functional HSC activity is enhanced in the spleens of mice infected with *E. coli* for six days compared with spleens of uninfected mice.

The majority of HSC activity is within the $CD150^+$, $CD48^-$, $Lineage^{-/low}$, $cKit^+$ and $Sca1^+$ fractions of bone marrow and spleen during infection

All functional HSC activity is present within the $CD150^+$ $CD48^-$ $Lineage^{-/low}$ $Sca1^+$ $cKit^+$ fraction of bone marrow under steady-state conditions (Kiel et al., 2005), yet administration of live bacteria, LPS or inflammatory cytokines can alter the expression of these surface markers on various cell types (Howie et al., 2002; Munitz et al., 2006; Zhang et al., 2008). Therefore we set out to determine whether functional HSCs retain their surface marker profile during infection. We performed competitive reconstitution experiments where bone marrow or spleen from *E. coli* infected mice was fractionated based on positive or negative expression of CD150 or CD48 (**Figure 3.2**). We transplanted donor cells based on the percentage of each population within infected bone marrow or spleen. The total number of cells transplanted for each condition is listed in **Table 3.2**. In two independent experiments, all mice that received $CD150^+$ bone marrow cells (10/10 LTMR) and spleen (7/7 LTMR) were long term multilineage reconstituted whereas all mice that received $CD150^-$ bone marrow cells (0/9 LTMR) and spleen (0/9 LTMR) exhibited transient myeloid reconstitution with sustained yet limited lymphoid reconstitution (**Figure 3.2A and B; Table 3.3**). This is consistent with previously published data indicating that $CD150^-$ bone marrow cells contain transiently reconstituting multipotent progenitors (Kiel et al., 2008).

Under steady-state conditions, all LTMR activity in adult bone marrow is contained within the CD48 negative fraction of cells (Kiel et al., 2005). In *E. coli* infected mice, while 7/10 and 9/10 recipients of $CD48^-$ bone marrow and spleen, respectively, were LTMR, we found that 2/10 and 2/10 recipients of $CD48^+$ bone marrow and spleen, respectively, were LTMR (**Figure 3.2C and D; Table 3.3**). These results

indicate that all HSC activity is present in the $CD150^+$ fraction and a majority is present in the $CD48^-$ fraction of bone marrow and spleen, while a minority of HSC activity is present in the $CD48^+$ fraction of bone marrow and spleen six days after *E. coli* infection.

We performed similar experiments by sorting bone marrow and spleen from infected mice based on positive or negative expression of Sca1, cKit or Lineage markers (**Figure 3.3**). We found that all HSC activity was present in the $Sca1^+$ and $cKit^+$ fractions and a majority of HSC activity was present in the $Lineage^{-/low}$ fraction of bone marrow and spleen during infection. A minority of HSC activity was present in the $Lineage^+$ fraction of the bone marrow but not the spleen (**Figure 3.3; Table 3.4**). Altogether, we find that the majority of functional HSC are contained within the $CD150^+$ $CD48^-$ LSK fraction of bone marrow and spleen six days after infection with *E. coli*.

Systemic *E. coli* infection dynamically affects HSPCs in bone marrow and spleen

Having confirmed the surface marker profile of HSCs following infection, we measured the frequency of HSPCs (LSK) and HSCs ($CD150^+$ $CD48^-$ $CD41^-$ LSK) in bone marrow and spleen over twelve days of infection (**Figure 3.4**). Although CD41 expression on HSCs increases with age, it is very low on HSCs from young adult mice and its inclusion helps to exclude contaminating megakaryocytes (Kiel et al., 2005). We observed moderate alterations in the surface marker profile of hematopoietic progenitors over twelve days of infection (**Figure 3.4A and B**). *E. coli* infection resulted in transient bone marrow hypocellularity at days 3 and 6 that normalized by day 9 along with mild splenomegaly that did not reach significance (**Figure 3.4C and D**). Total LSK cells

trended towards a reduction in the bone marrow at day 6 and recovered thereafter, while LSK cells in the spleen trended toward an increase at days 3 and 6 that diminished by day 12 (**Figure 3.4E and F**). Total $CD150^+$ $CD48^-$ $CD41^-$ LSK cells were reduced in the bone marrow at day 3 and recovered to near normal levels by day 9, while $CD150^+$ $CD48^-$ $CD41^-$ LSK cells in the spleen were significantly increased at day 6, peaked by day 9 and began to diminish by day 12 (**Figure 3.4G and H**). These results suggest that systemic infection induces a reduction and subsequent recovery of HSCs in bone marrow along with a delayed expansion of HSCs in the spleen.

Expansion of splenic HSCs during infection is dependent on PRRs

Systemic administration of LPS, which is sensed by TLR4, can promote extramedullary hematopoiesis in the spleen (Esplin et al., 2011; Vos et al., 1972), yet most preparations of LPS are contaminated with peptidoglycan that activate NLRs (Inohara et al., 2001). To determine whether TLRs and NLRs play a role in driving the changes observed in HSC populations during infection, we quantified $CD150^+$ $CD48^-$ $CD41^-$ LSK cells in bone marrow and spleen of WT, *Tlr4*$^{-/-}$ and *Ripk2*$^{-/-}$ mice before and after infection with *E. coli* (**Figure 3.5A and B**). We did not observe any differences in the total number of $CD150^+$ $CD48^-$ $CD41^-$ LSK cells in the bone marrow or spleens of uninfected *Tlr4*$^{-/-}$ or *Ripk2*$^{-/-}$ mice compared with uninfected WT mice (**Figure 3.5A and B**). Similar to WT mice, we observed a reduction of total $CD150^+$ $CD48^-$ $CD41^-$ LSK cells in bone marrow of *Ripk2*$^{-/-}$ mice three days after infection, but this was not observed in *Tlr4*$^{-/-}$ mice (**Figure 3.5A**). $CD150^+$ $CD48^-$ $CD41^-$ LSK cells were increased in the

spleen of *Ripk2*$^{-/-}$ mice at day 6 after infection, yet by day 9 after infection there were significantly fewer CD150^{+} CD48^{-} CD41^{-} LSK cells in spleens *Ripk2*$^{-/-}$ mice compared with WT mice (**Figure 3.5B**). Strikingly, we observed almost no accumulation of CD150^{+} CD48^{-} CD41^{-} LSK cells in spleens of *Tlr4*$^{-/-}$ at any point during infection (**Figure 3.5B**). These results implicate critical roles for TLR and NLR signaling in the expansion of extramedullary HSCs during infection.

NOD1 and TLR4 signaling act synergistically to expand splenic HSCs

To investigate whether activation of TLR and NLR signaling pathways are sufficient to expand CD150^{+} CD48^{-} CD41^{-} LSK cells in spleen, we treated WT mice with either ultrapure LPS, which is devoid of contaminating peptidoglycan, or KF1B, a bacterial dipeptide that specifically activates NOD1 (Masumoto et al., 2006), or both (**Figure 3.5C and D**). A single *i.p.* injection of ultrapure LPS or KF1B alone induced minimal alterations in the number of CD150^{+} CD48^{-} CD41^{-} LSK cells in bone marrow or spleen over six days, whereas dual administration of LPS and KF1B resulted in a reduction of CD150^{+} CD48^{-} CD41^{-} LSK cells in bone marrow after 2 days and an expansion of CD150^{+} CD48^{-} CD41^{-} LSK cells in spleen after six days (**Figure 3.5C and D**), reminiscent of changes observed after *E. coli* infection. To confirm that TLR4 and NOD1 stimulation expanded functional HSCs within the spleen, we treated WT (CD45.l) mice with PBS, LPS, KF1B or both and after six days mixed 1x10^{6} splenocytes with 3x10^{5} untreated (CD45.2) WBM and transplanted these cells into lethally irradiated (CD45.2) recipients and measured chimerism over 16 weeks (**Figure 3.5E and F**). In

three independent experiments, splenocytes from LPS or KF1B treated spleens had on average 2.2-fold (4/9 LTMR) and 2.5-fold (4/10 LTMR) more RUs, respectively, than PBS treated spleen (2/12 LTMR), while splenocytes from LPS and KF1B had on average 6.7-fold (7/12 LTMR) more RUs than PBS treated spleens, though these differences did not reach statistical significance due to high variability (**Figure 3.5E and F**). These results support the conclusion that dual activation of TLR4 and NOD1 cooperate to expand the pool of functional HSCs in spleen.

NOD1 signals via the adaptor protein RIPK2, whereas TLR4 signals via the adaptor proteins Myd88 and TRIF. To determine which signaling pathways are important for expansion of splenic HSCs following NOD1 and TLR4 activation, we challenged *Ripk2*$^{-/-}$, *Trif*$^{-/-}$ and *Myd88*$^{-/-}$ mice with PBS or LPS and KF1B and quantified CD150^{+} CD48^{-} CD41^{-} LSK cells after six days (**Figure 3.5G and H**). Accumulation of CD150^{+} CD48^{-} CD41^{-} LSK cells in the spleen after LPS and KF1B administration was dependent on RIPK2 and TRIF, but was inconclusive with respect to Myd88, since *Myd88*$^{-/-}$ mice had increased numbers of splenic CD150^{+} CD48^{-} CD41^{-} LSK cells at steady-state (**Figure 3.5G and H**). These results confirm that KF1B-induced activation of NOD1 signals through the adaptor RIPK2 in this setting and suggests that TLR4 signaling through TRIF is important for inducing the expansion of HSCs in the spleen.

TLR4 expression on HSCs is not required for their expansion in spleen

TLR4 is expressed on a subset of LSK cells (Nagai et al., 2006), yet it is unclear whether TLR4 expression on HSCs influences their response to LPS. Moreover, it has

been proposed that *Tlr4*$^{-/-}$ HSCs outcompete WT HSCs in competitive reconstitution assays (Ichii et al., 2010). To test whether expression of TLR4 on HSCs influences their reconstitution ability, we transplanted lethally irradiated WT (CD45.1) mice with a mixture of 2.5x10^6 WT (CD45.1) WBM and 2.5x10^6 *Tlr4*$^{-/-}$ (CD45.2) WBM and measured peripheral blood chimerism (**Figure 3.6A and B**). After 16 weeks, myeloid reconstitution was equivalent between WT and *Tlr4*$^{-/-}$ cells, while B-lineage reconstitution was unbalanced in favor of *Tlr4*$^{-/-}$ cells and T-lineage reconstitution was unbalanced in favor of WT cells (**Figure 3.6B**). The differences in T-lineage reconstitution at early points after transplant could be due to a functional deficit of *Tlr4*$^{-/-}$ T-lineage progenitors or could reflect the presence long lived recipient-derived T-cells. These results indicate that *Tlr4*$^{-/-}$ HSCs are functionally comparable to WT HSCs, but may harbor bias with respect to lymphoid reconstitution.

To determine whether TLR4 expression on HSCs themselves was required for expansion in spleen, we challenged the reconstituted mice with PBS or LPS and KF1B and enumerated CD150$^+$ CD48$^-$ CD41$^-$ LSK cells in bone marrow and spleen after six days (**Figure 3.6C and D**). In PBS treated mice, the levels of WT and *Tlr4*$^{-/-}$ CD150$^+$ CD48$^-$ CD41$^-$ LSK cells was roughly equivalent in bone marrow, consistent with our peripheral blood reconstitution data, yet we detected only minimal *Tlr4*$^{-/-}$ CD150$^+$ CD48$^-$ CD41$^-$ LSK cells in the spleen (**Figure 3.6C and D**). Conversely, in mice treated with LPS and KF1B, we found that *Tlr4*$^{-/-}$ CD150$^+$ CD48$^-$ CD41$^-$ LSK cells were expanded in the spleen (**Figure 3.6D**). To confirm these data functionally, we non-competitively transplanted 3x10^7 splenocytes from PBS or LPS and KF1B treated mice into lethally irradiated CD45.1 recipients and measured peripheral blood chimerism at 16 weeks

(**Figure 3.6E**). As expected, in recipients of PBS treated splenocytes, the majority of peripheral blood cells were WT derived, whereas in recipients of LPS and KF1B treated spleens, *Tlr4*$^{-/-}$ and WT peripheral blood cells were roughly equivalent (**Figure 3.6E**). These data indicate that expression of TLR4 on HSCs is not required for their accumulation in spleen after TLR4 and NOD1 stimulation.

NLR and TLR signaling in the radio-resistant compartment is important for expansion of splenic HSPCs

TLRs and NLRs are expressed on a variety of hematopoietic and non-hematopoietic cell types. To determine which cellular compartment is required for mediating TLR and NLR induced expansion of HSPCs, we generated reverse chimeras (**Figure 3.6F and G**). WBM from WT, *Ripk2*$^{-/-}$ or *Tlr4*$^{-/-}$ mice was transplanted into lethally irradiated WT, *Ripk2*$^{-/-}$ or *Tlr4*$^{-/-}$ recipients and were allowed to recover for at least 16 weeks. Chimeric mice were administered LPS and KF1B and after six days splenocytes were plated in Methocult 3434 medium to quantify myeloid and erythroid progenitors. The expansion of myeloid and erythroid progenitors in WT recipients transplanted with WT WBM was comparable to similarly treated non-irradiated WT mice (**Figure 3.6F and G**). *Ripk2*$^{-/-}$ recipients of *Ripk2*$^{-/-}$ WBM and *Tlr4*$^{-/-}$ recipients of *Tlr4*$^{-/-}$ WBM exhibited blunted expansion of myeloid and erythroid progenitors in spleen, consistent with a requirement for both TLR and NLR signaling in promoting extramedullary HSPC expansion (**Figure 3.6F and G**). Notably, expansion of myeloid and erythroid progenitors in the spleens of WT mice that received either *Ripk2*$^{-/-}$ or *Tlr4*$^{-/-}$

WBM was comparable to WT controls, whereas *Ripk2*$^{-/-}$ or *Tlr4*$^{-/-}$ mice that received WT WBM exhibited reduced expansion of myeloid and erythroid progenitors compared to WT controls (**Figure 3.6A and B**). These results indicate that TLR and NLR signaling in radio-resistant cells, but not radio-sensitive cells are required for expansion of HSPCs in the spleen and suggest that indirect factors are involved.

G-CSF signaling is required for NLR and TLR induced expansion of splenic HSCs

Repeated administration of G-CSF induces the mobilization of HSCs from bone marrow to peripheral blood and spleen in both mouse and man (Duhrsen et al., 1988; Molineux et al., 1990; Morrison et al., 1997). Systemic administration of LPS can increase G-CSF in the serum (Hareng and Hartung, 2002), but a role for endogenous G-CSF in LPS-induced extramedullary hematopoiesis has not been addressed. We found that *i.p.* injection of WT mice with ultrapure LPS resulted in massively increased levels of G-CSF in serum by 4 hours (186.2 ± 118.2 ng/mL) compared with KF1B (1.2 ± 0.3 ng/mL) or PBS (0.3 ± 0.1 ng/mL), and the level of G-CSF was synergistically enhanced with dual administration of LPS and KF1B (488.0 ± 50.0 ng/mL) (**Figure 3.7A**). Treatment of *Nod1*$^{-/-}$ mice with LPS and KF1B resulted in G-CSF levels (145.5 ± 164.9 ng/mL) similar to WT mice treated with LPS alone, confirming that KF1B acts exclusively through NOD1 (**Figure 3.7A**).

To test whether G-CSF was required for HSC accumulation in spleen, we injected WT mice with G-CSF blocking antibody or isotype control one hour prior to injection with LPS and KF1B and quantified $CD150^+$ $CD48^-$ $CD41^-$ LSK cells in bone marrow and

spleen after six days (**Figure 3.7B and C**). Antibody pre-treatment did not influence the levels of $CD150^+$ $CD48^-$ $CD41^-$ LSK cells in the bone marrow, yet accumulation of $CD150^+$ $CD48^-$ $CD41^-$ LSK cells in spleen was reduced in mice pre-treated with G-CSF blocking antibody but not isotype control antibody (**Figure 3.7B and C**). To test whether signaling through the G-CSFR was required, we treated *Csf3r*$^{-/-}$ (G-CSFR$^{-/-}$) mice with PBS or LPS and KF1B and found that the accumulation of $CD150^+$ $CD48^-$ $CD41^-$ LSK cells in spleens of these mice was almost completely blocked (**Figure 3.7C**). Finally, downregulation of CXCL12 in bone marrow is a common feature of G-CSF-mediated mobilization (Levesque and Winkler, 2008; Petit et al., 2002) so we tested whether expression of CXCL12 was altered in the bone marrow after challenge with bacterial products. Dual administration of LPS and KF1B resulted in reduced CXCL12 message in WBM after two days, while single administration of LPS or KF1B did not (**Figure 3.7D**). Thus, NOD1 and TLR4 act synergistically to induce HSC accumulation in the spleen via endogenous production of G-CSF.

DISCUSSION

Here we have demonstrated that systemic infection with the gram negative bacterium *Escherichia coli* drives the expansion of long term multilineage repopulating HSCs in the spleen along with a reduction of HSCs in the bone marrow. Fractionation and competitive transplantation of bone marrow and spleen cells from infected mice revealed that the majority of functional HSCs are $CD150^+$ $CD48^-$ $Lineage^{-/low}$ $Sca1^+$ and $cKit^+$, while a smaller fraction are CD48 and Lineage marker positive. Upregulation of

CD48 and Mac1, which was present in our Lineage cocktail, have been associated with activation of HSCs after treatment with the myeloablative drug 5-fuorouracil (Randall and Weissman, 1997; Venezia et al., 2004), suggesting that HSCs may be similarly affected during myeloablation and infection. Alternatively, inflammation may promote the de-differentiation of progenitors into self-renewing HSCs, with expression of CD48 and Lineage markers serving as a relic of their previously differentiated identity (Jopling et al., 2011). Expansion of splenic HSCs following infection was largely dependent on TLR4 signaling and partially dependent on NLR signaling via the adaptor protein RIPK2. Importantly, administration of ultrapure LPS alone was not sufficient to expand HSCs in the spleen, whereas dual administration of LPS and the synthetic NOD1 agonist KF1B was sufficient to expand splenic HSCs. Thus, our studies highlight a previously unappreciated role for NLR signaling in promoting extramedullary hematopoiesis in the context of infection.

The discovery that LSK cells express TLR4 and TLR2 raised the possibility that HSPCs can directly sense bacterial infection leading to alterations in lineage output (Nagai et al., 2006). A recent model proposed that during infection, inflammatory cytokines and bacterial components push HSCs to differentiate, while reductions in effector cell populations pull HSCs to differentiate (King and Goodell, 2011). Using reverse chimeras, we showed that signaling through PRRs in radio-sensitive cells was not important for driving HSPC expansion in spleen. Instead, TLR4 and NOD1 signaling in radio-resistant cells was sufficient to expand HSPCs in spleen. Consistent with this, another group found that TLR4 expression in radio-resistant cells was critical for production of G-CSF and induction of emergency myelopoiesis after systemic LPS

treatment (Boettcher et al., 2012). To unequivocally test whether TLR4 expression was required for HSC expansion in spleen, we generated chimeric mice with similar numbers of WT and *Tlr4*$^{-/-}$ derived hematopoietic cells. In these mice, *Tlr4*$^{-/-}$ HSCs expanded in the spleen after TLR4 and NOD1 stimulation and gave rise to progeny in irradiated recipients over 16 weeks. Altogether, our results strongly support a role for radio-resistant cells in driving the expansion of HSCs in spleen after PRR stimulation and indicate that TLR4 expression on HSCs is dispensable in this setting.

It is well established that repeated administration of recombinant G-CSF mobilizes functional HSCs from the bone marrow to blood and spleen (Duhrsen et al., 1988; Molineux et al., 1990; Morrison et al., 1997). Synergistic production of G-CSF following TLR4 and NOD1 activation correlated with downregulation of CXCL12 in the bone marrow and accumulation of HSCs in the spleen that was inhibited with either G-CSF blocking antibody or lack of the G-CSF receptor. These results suggest that mobilization of HSCs from bone marrow to the spleen may be responsible for the observed expansion of splenic HSCs during infection. Alternatively, since a small population of functional HSCs exists in the spleen under steady-state conditions (Morita et al., 2011), we cannot rule out the possibility that local proliferation also contributes to expansion of splenic HSCs during infection.

The spleen can serve as a site for extramedullary hematopoiesis during periods of stress, yet the spleen is not required for survival. The biological significance for an expanded pool of HSCs in the spleen during infection is not clear. Some studies have proposed that bacterial infection or administration of LPS damages HSCs in the bone marrow (Baldridge et al., 2010; Chen et al., 2010; Esplin et al., 2011; Rodriguez et al.,

2009). We observed a reduction of functional HSCs in bone marrow following *E. coli* infection along with expansion of functional HSCs in spleen. It is possible that excessive inflammatory signaling within the bone marrow microenvironment might transiently compromise the bone marrow niche and egress of HSCs could serve as a protective mechanism until the infection is cleared and the bone marrow niche is repaired.

Consistent with the notion that the bone marrow niche is altered during infection, we and others find that CXCL12 is downregulated in bone marrow after exposure to bacteria or their components (Delano et al., 2011; Johns and Borjesson, 2012; Ueda et al., 2005). Interestingly, CXCL12 also contributes to the retention of neutrophils in bone marrow and mobilization of neutrophils during polymicrobial sepsis is required for bacterial clearance and host survival (Delano et al., 2011). Physiologic cycling of HSPCs in and out of the blood steam is influenced by the level of circulating neutrophils and removal of aged neutrophils by bone marrow macrophages promotes HSPC mobilization (Casanova-Acebes et al., 2013). HSC egress during infection could merely represent a bystander effect secondary to an immediate requirement for peripheral granulocytes. Alternatively, given the immense need for cell replenishment during systemic infection, the splenic pool of HSCs may provide a lasting reservoir for effector cell production. This hypothesis is supported by work showing that HSCs traffic through peripheral tissue under steady-state conditions and that LPS can induce proliferation of HSPCs at local sites of inflammation (Massberg et al., 2007). Compared with the bone marrow niche, which preserves HSCs in a highly quiescent state, the splenic niche may provide a micro-environment more suitable for extensive cell division (Morita et al., 2011). Thus,

expansion of the splenic pool of HSCs may be important for limiting subsequent infections or providing a protective niche for HSCs during periods of stress.

The expansion of HSCs in the spleen during bacterial infection highlights the dynamic nature of stem cells and their niche(s) in the setting of stress. Our results support the hypothesis that direct sensing of infection by HSCs is not important for promoting extramedullary hematopoiesis. Rather, the initiation of extramedullary hematopoiesis during infection is more likely a consequence of alterations in the HSC microenvironment. A better understanding of the signals that influence HSCs and their niche(s) during infection may lead to improved methodologies for isolation and expansion of HSCs in the clinical setting.

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FIGURES AND TABLES

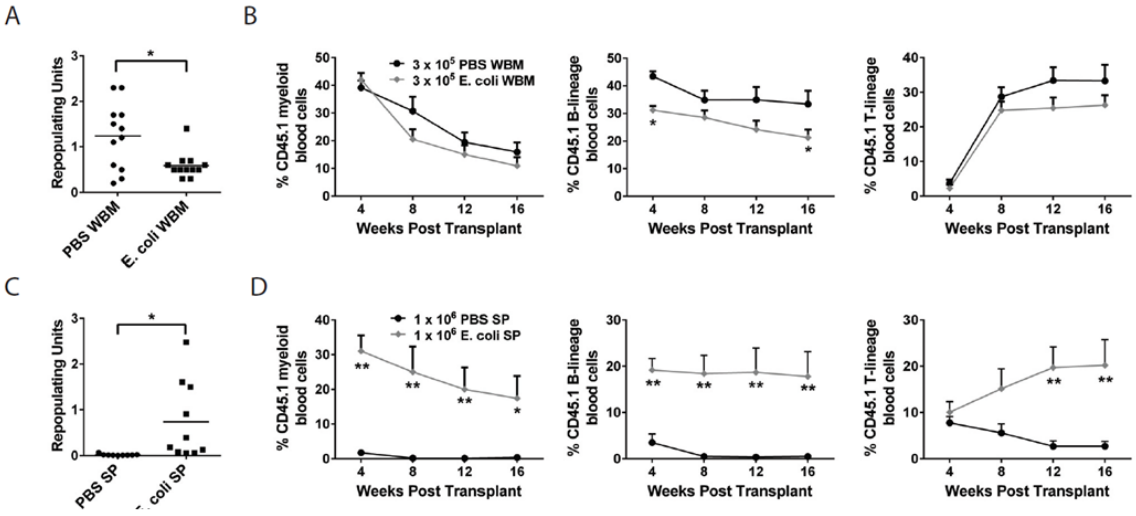

Figure 3.1 | Infection reduces HSC activity in bone marrow and increases HSC activity in spleen
(A-D) Wild type (CD45.1) mice were injected with ~$1x10^8$ E. coli K12 i.p and after six days **(A, B)** $3x10^5$ whole bone marrow (WBM) or **(C, D)** $1x10^6$ splenocytes were mixed with $3x10^5$ whole bone marrow from WT (CD45.2) mice and transplanted into lethally irradiated (CD45.2) recipients. Peripheral blood chimerism of myeloid (Mac1+), B-lineage (B220+) and T-lineage (CD3+) cells was analyzed by flow cytometry over successive 4 week intervals. (A, C) n = 12 mice per condition from 2 experiments. *p<0.05 by t-test. (B, D) n= 9-10 mice per condition from two experiments. *p<0.05 **p<0.05 by Two way ANOVA versus recipients of PBS cells at each time point. Error bars represent SEM.

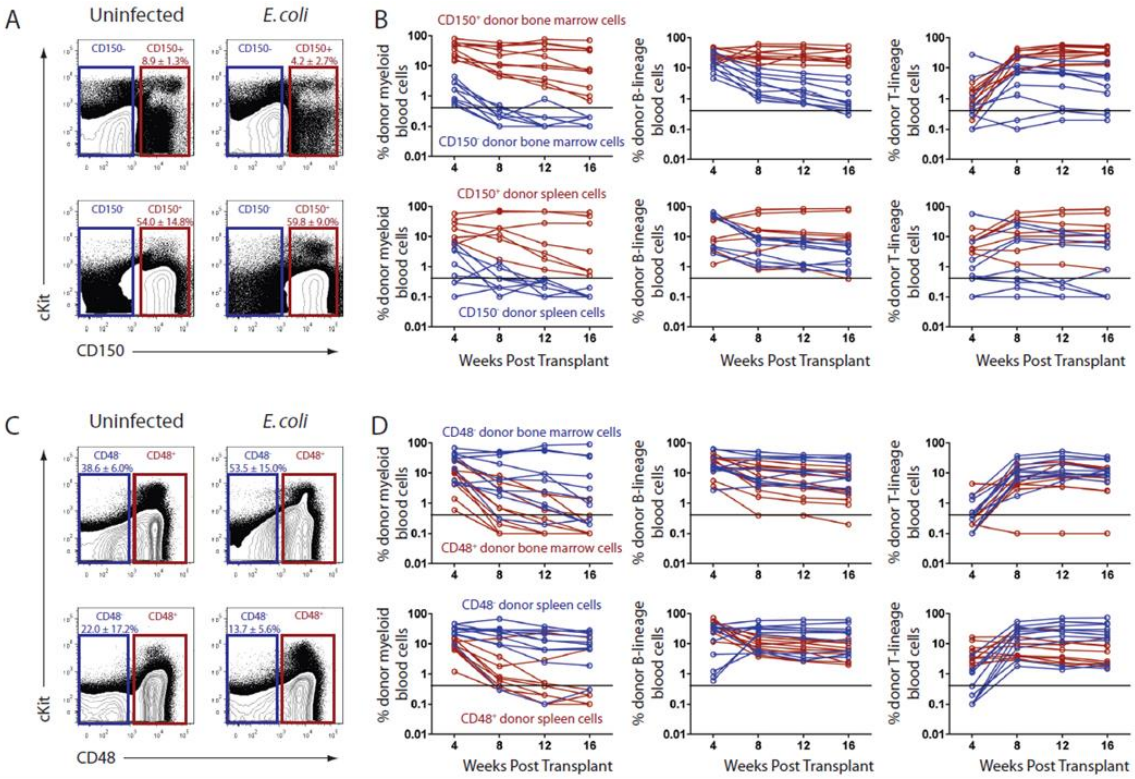

Figure 3.2 | The majority of HSC activity exists in the CD150+ CD48- fraction of bone marrow and spleen during infection

(A-D) Wild type (CD45.1) mice were injected with ~1x10^8 E. coli K12 i.p and after six days, (top) bone marrow or (bottom) spleen cells were sorted and resorted based on positive or negative expression of **(A, B)** CD150 or **(C, D)** CD48. **(B, D)** Positive or negative fractions from (top) bone marrow or (bottom) spleen were mixed with 3x10^5 whole bone marrow from WT (CD45.2) mice and transplanted into lethally irradiated (CD45.2) recipients. Peripheral blood chimerism of myeloid (Mac1+), B-lineage (B220+) and T-lineage (CD3+) cells were analyzed by flow cytometry. Connected data points represent individual mice bled over successive four week intervals. Data are from two experiments for each condition. The black line represents the background threshold of 0.3% below which we could not detect chimerism.

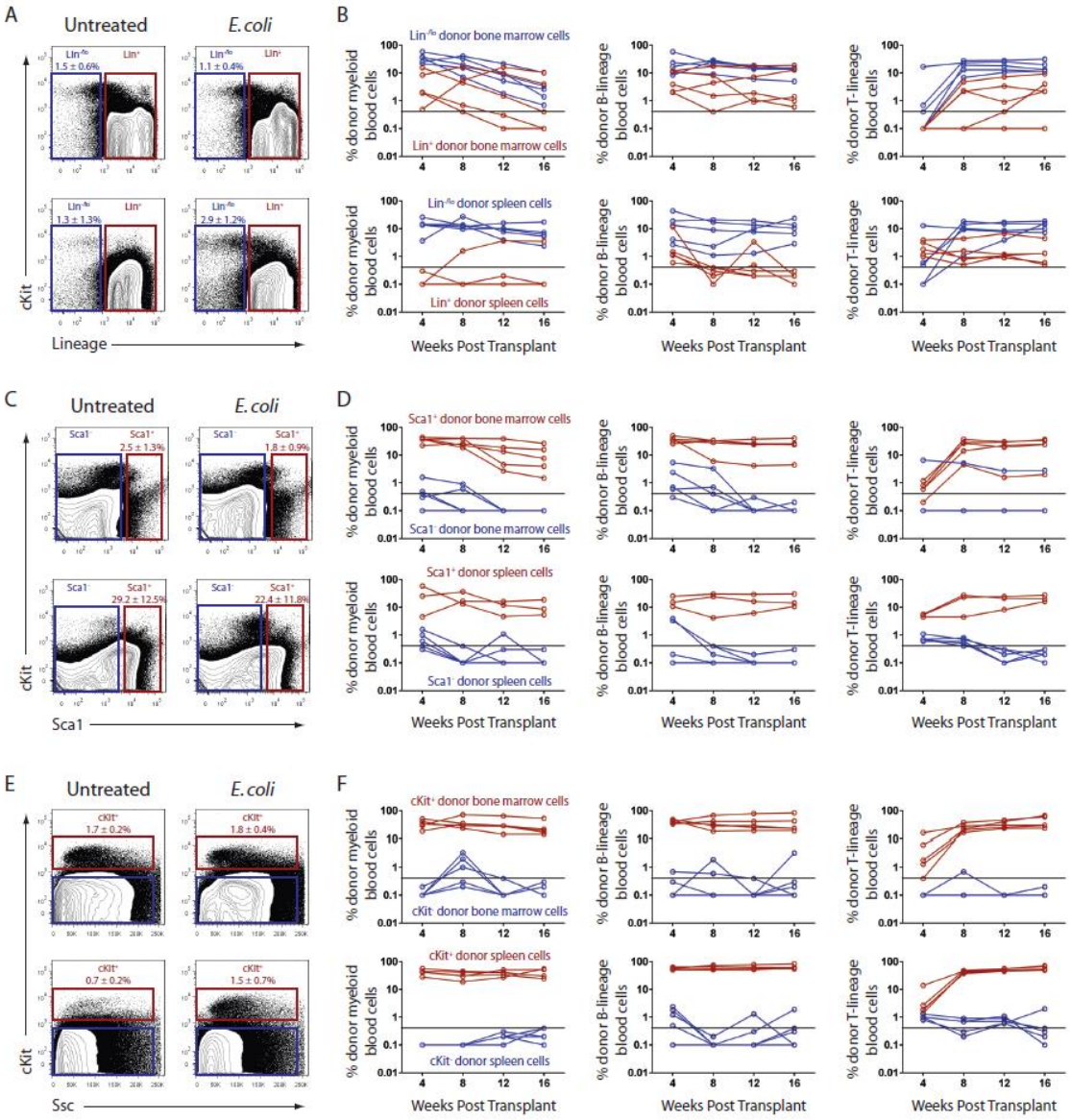

Figure 3.3 | **All HSC activity exists in the cKit$^+$ Sca1$^+$ fraction of bone marrow and spleen after infection**

(A-F) Wild type (CD45.1) mice were injected with ~1x10^8 E. coli K12 i.p and after 6 days, (top) bone marrow or (bottom) spleen cells were sorted and resorted based on positive or negative expression of **(A, B)** Lineage (CD3, CD4, CD8, B220, Ter119, Gr-1, Mac1) **(C, D)** Sca1 or **(E,F)** cKit. **(B, D, F)** Positive or negative fractions from (top) bone marrow or (bottom) spleen were mixed with 3x10^5 whole bone marrow from WT (CD45.2) mice and transplanted into lethally irradiated (CD45.2) recipients. Peripheral blood chimerism of Myeloid (Mac1+), B-lineage (B220+) and T-lineage (CD3+) cells were analyzed by flow cytometry. Connected data points represent individual mice bled over successive 4 week intervals.

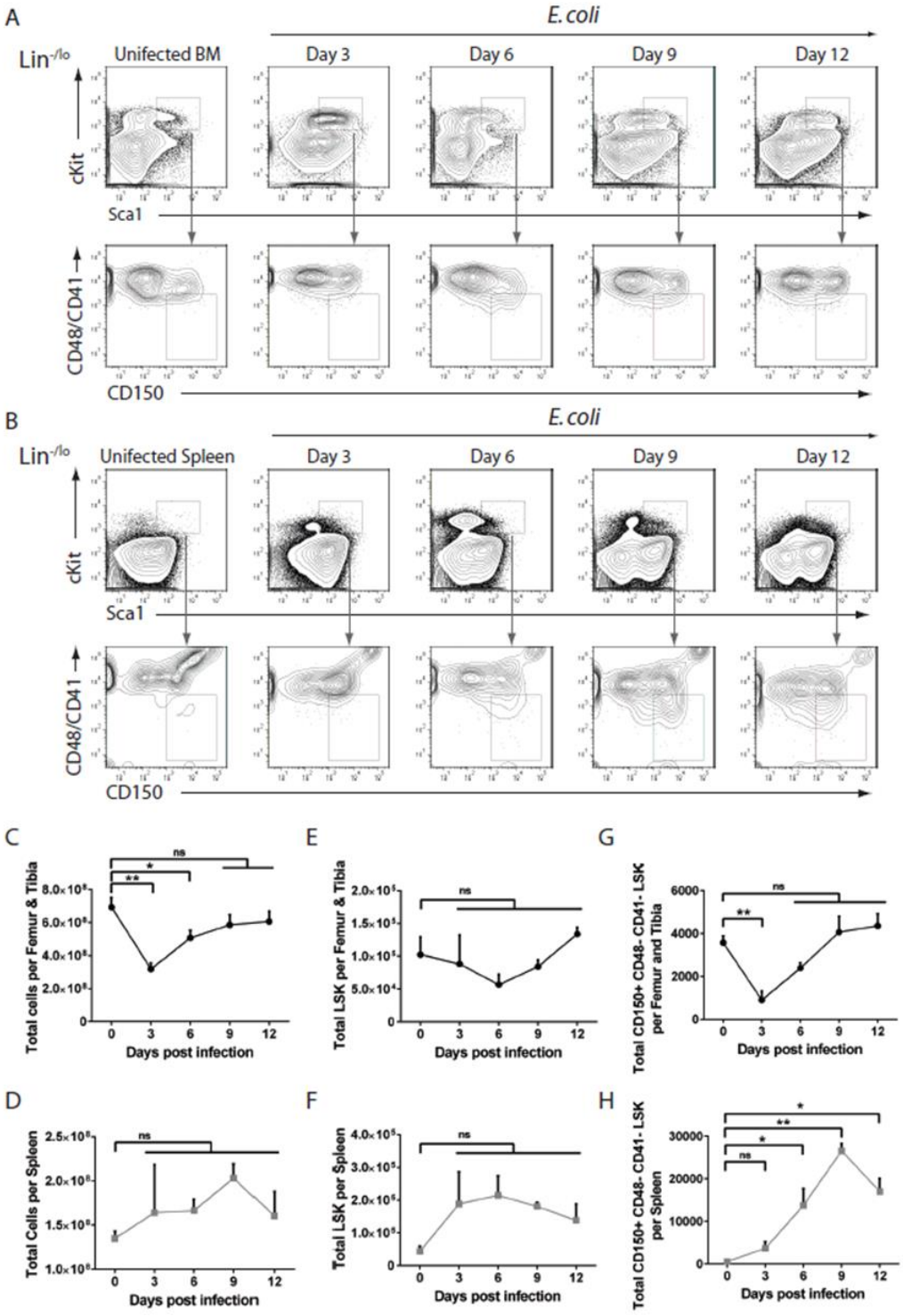

Figure 3.4 | **Kinetics of hematopoietic stem and progenitors during systemic E. coli infection**
(A-H) Wild type mice were injected with ~1x10^8 E. coli K12 i.p and femur and tibia or spleens were isolated at indicated time points. Shown are representative flow plots from **(A)** bone marrow (BM) or **(B)** spleen and quantification of **(C)** total BM cells, **(D)** total splenocytes, **(E)** total BM Lineage$^{-/low}$ Sca1$^+$ cKit$^+$ (LSK), **(F)** total splenic LSK, **(G)** total BM HSC (CD150+ CD48- CD41- LSK) and **(H)** total splenic HSC. n = 3-8 mice per time point. Error bars indicate SEM. *p<0.05 **p<0.01 by One way ANOVA. (ns) not significant.

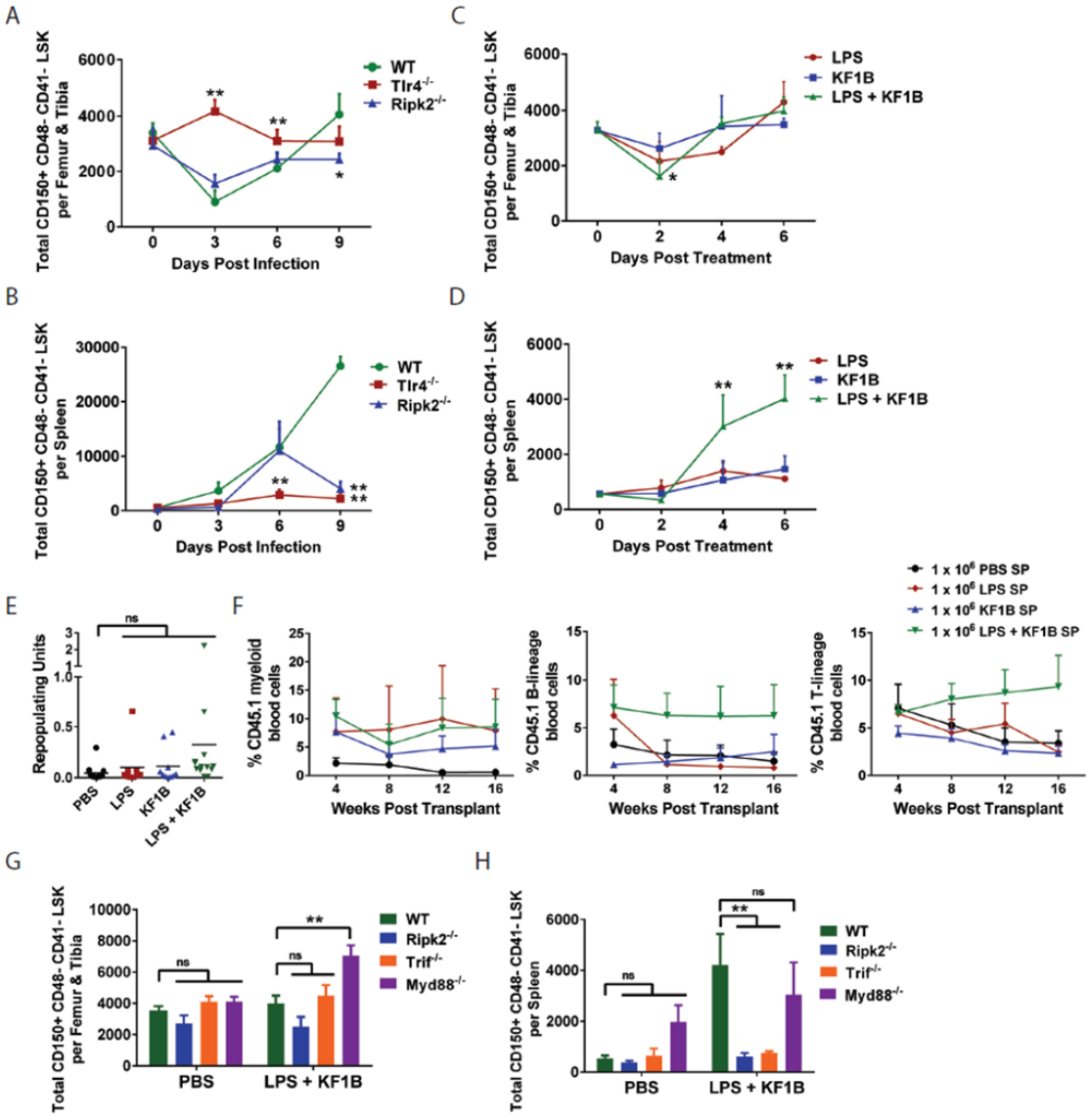

Figure 3.5 | Combined signaling of NOD-like Receptors and Toll-like Receptors is important for HSC expansion in spleen during infection

(A, B) Wild type, *Tlr4*$^{-/-}$ or *Ripk2*$^{-/-}$ mice were infected with ~1x10^{8} E. coli CFU i.p. and femur and tibia or spleen were isolated at indicated time points. Total HSC (CD150^{+} CD48^{-} CD41^{-} LSK) were enumerated in **(A)** bone marrow and **(B)** spleen. n = 3-8 mice per time point. *p<0.05 **p<0.01 by Two way ANOVA versus WT mice at same time point. **(C, D)** Wild type were injected with PBS, the TLR4 agonist LPS (100 ug), the NOD1 agonist KF1B (50 ug) or both i.p. and HSC were quantified in **(C)** bone marrow and **(D)** spleen at indicated time points. n = 3-6 mice per time point. *p<0.05 **p<0.01 by Two way ANOVA versus PBS treated mice. **(E, F)** Six days after injection of compounds, 1x10^{6} splenocytes from WT (CD45.1) mice were mixed with 3x10^{5} (CD45.2) WBM and transplanted into lethally irradiated (CD45.2) recipients and peripheral blood chimerism was analyzed every four weeks. n = 9-12 mice per condition from 2-3 experiments. **(E)** Each point represents one mouse. **(F)** Error bars represent SEM. **(G, H)** Wild type, *Ripk2*$^{-/-}$, *Trif*$^{-/-}$ or *Myd88*$^{-/-}$ mice were injected with PBS or LPS + KF1B and HSC (CD150^{+} CD48^{-} CD41^{-} LSK) were quantified in **(G)** bone marrow and **(H)** spleen after six days. n = 3-6 mice per condition. **p<0.01 by One way ANOVA.

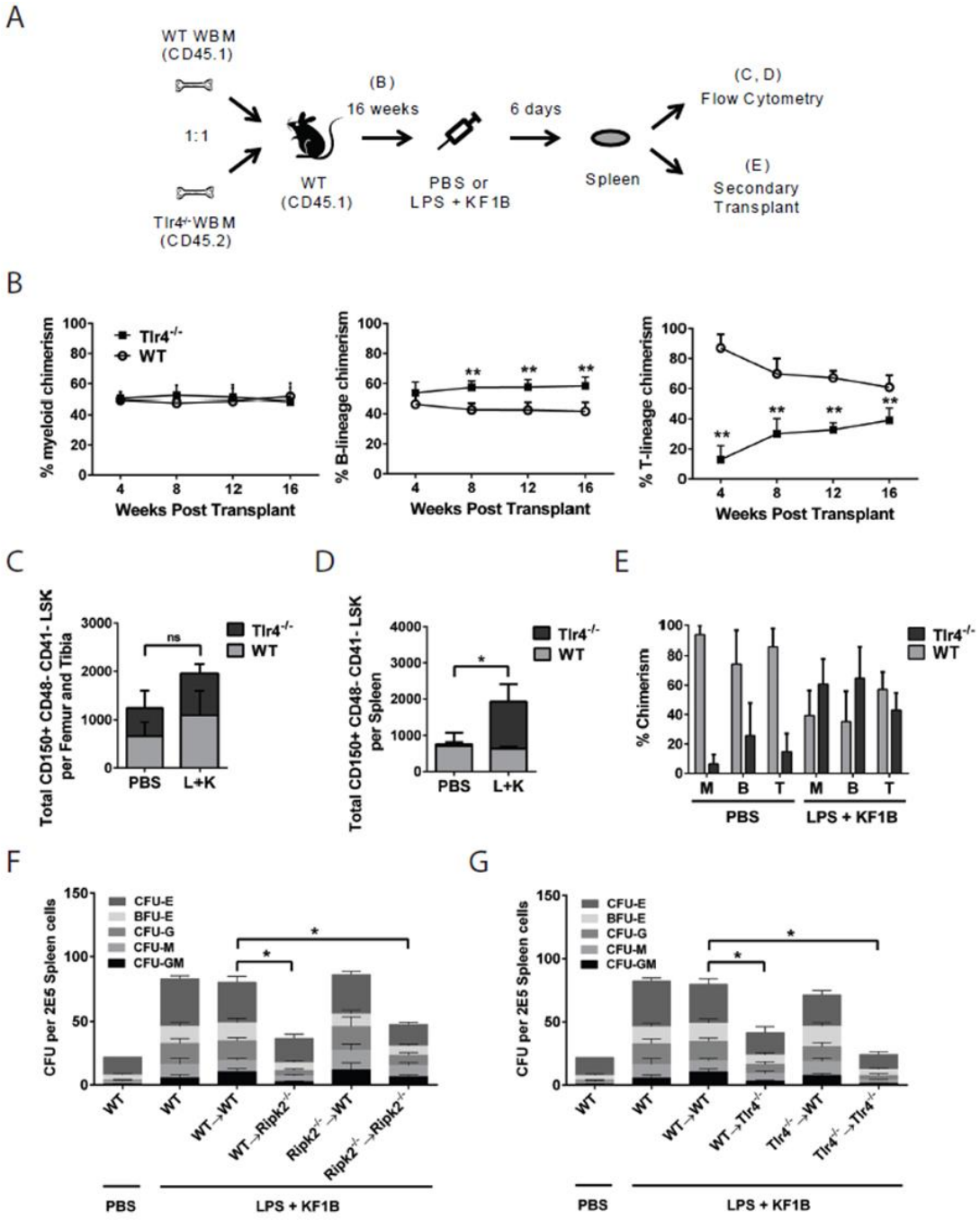

Figure 3.6 | Radio-resistant cells are important for NOD1 and TLR4 mediated HSC expansion in spleen
(A) Schematic of experiments in (B-E). **(B)** Peripheral blood chimerism of (CD45.1) recipient mice transplanted with a 1:1 mixture of (CD45.1) WT and (CD45.2) *Tlr4*$^{-/-}$ WBM and bled every 4 weeks. n = 7 mice **p<0.01 by Two way ANOVA. After 16 weeks, chimeric mice were injected with PBS or LPS + KF1B and six days later, the absolute number of HSC (CD150^{+} CD48^{-} CD41^{-} LSK) in **(C)** bone marrow or **(D)** spleen were enumerated. n = 3-4 mice per condition. *p<0.05 by One way ANOVA. **(E)** 3x10^{7} splenocytes from mice in (B-D) were transplanted non-competitively into lethally irradiated (CD45.1) recipients and peripheral blood chimerism measured 16 weeks after transplant. **(F, G)** Chimeras were generated as described in text. Mice were injected with PBS or LPS + KF1B and after 6 days, splenocytes were plated in Methocult 3434 (Stem Cell Technologies) for 8-12 days. n = 3-7 mice per condition. *p<0.05 by One way ANOVA. Error bars represent SEM.

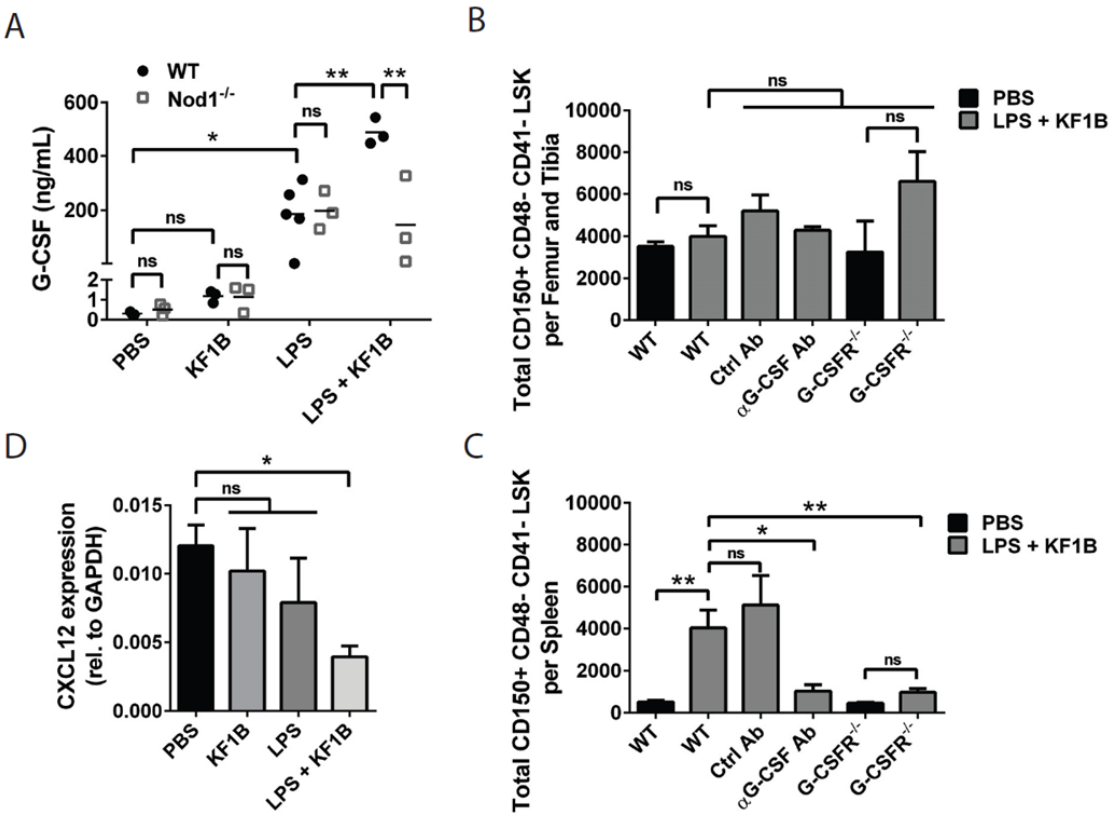

Figure 3.7 | **NOD1 and TLR4 induced HSC expansion in spleen is dependent on G-CSF**
(A) ELISA of serum G-CSF levels four hours after i.p. injection of indicated compounds. Each data point represents one mouse. **(B, C)** Quantification of HSC ($CD150^+$ $CD48^-$ $CD41^-$ LSK) in **(B)** bone marrow or **(C)** spleen six days after administration of compounds. Where indicated, wild type mice received 250 ug of isotype control or G-CSF-blocking antibody (Ab) i.p. 1 hour before administration of compounds. n = 3-5 mice per condition. **(D)** Real time RT-PCR of CXCL12 expression in WBM two days after administration of compounds. n = 3-5 mice per condition. * $p < 0.05$ ** $p < 0.01$ by One-way ANOVA for all graphs.

Table 3.1 Antibodies used in flow cytometry

Target	Clone	Company	Conjugate(s)	Dilution	Time
CD3e	145-2C11	BioLegend	FITC, PE	1:200	25 mins
CD4	GK1.5	eBiosciences	FITC	1:200	25 mins
CD8	53-6.7	eBiosciences	FITC	1:200	25 mins
B220	RA3-6B2	eBiosciences	FITC, PE-Cy5	1:200	25 mins
Gr-1	RB6-8C5	BioLegend	FITC, PE-Cy7	1:200, 1:600	25 mins
Mac1 (CD11b)	M1/70	eBiosciences	FITC, APC	1:200, 1:400	25 mins
TER119	TER119	BioLegend	FITC	1:200	25 mins
Lineage panel	(CD3e, B220, Mac1, Gr-1, TER119)	eBiosciences	Biotin	1:200	20 mins, 1.5 hrs
CD4	GK1.5	BioLegend	Biotin	1:400	20 mins, 1.5 hrs
CD8	53-6.7	BioLegend	Biotin	1:400	20 mins, 1.5 hrs
CD150	TC15-12F123.2	BioLegend	PE	1:200	20 mins, 1.5 hrs
CD48	HM48-1	BioLegend	FITC, PE-Cy7	1:200	20 mins, 1.5 hrs
CD41	MWReg	BioLegend	FITC, PE-Cy7	1:200	20 mins, 1.5 hrs
Sca1	E13-161.7	BioLegend	PE-Cy7, APC	1:200	20 mins, 1.5 hrs
cKit	2B8	BioLegend	APC-Cy7	1:200	20 mins, 1.5 hrs
CD34	RAM34	eBiosciences	FITC	1:50	1.5 hrs
CD45.1	A20	BioLegend	APC-Cy7	1:200	25 mins
CD45.2	104	BioLegend	FITC	1:400	25 mins
Streptavidin	-	Invitrogen, Biolegend	Pacific Orange, APC	1:400	20 mins

Table 3.2 Summary of cells injected for Figures 3.2 and 3.3

	Avg. % within infected tissue				Number of cells injected			
	Bone marrow		Spleen		Bone marrow		Spleen	
Population	+	-	+	-	+	-	+	-
CD150	4	94	60	36	1×10^4	2.8×10^5	1.2×10^6	7×10^5
CD48	33	54	81	14	1×10^5	1.6×10^5	1.6×10^6	3×10^5
Lineage	98	1.1	96	3	2.9×10^5	3×10^3	1.9×10^6	6×10^6
Sca1	1.8	78	22	53	5×10^3	2×10^5	4×10^5	1×10^6
cKit	1.8	96	1.5	98	5×10^3	2.9×10^5	3×10^4	1.9×10^6

Percentages represent the average of each gated population in bone marrow or spleen of mice that were infected six days prior with *E. coli*. The number of cells to inject (along with $3x10^5$ congenic WBM) was calculated by multiplying the percentage of the gated population by $3x10^5$ for bone marrow and by $2x10^6$ for spleen.

Table 3.3 Summary of peripheral blood reconstitution in Figure 3.2

Population	Donor Tissue	Week	LTMR (>0.3% ea.)	My (Avg. %)	BC (Avg. %)	TC (Avg. %)
CD150+	Bone Marrow	4	8/10	41	29	1
		8	10/10	24	29	23
		12	10/10	27	30	34
		16	10/10	20	27	33
	Spleen	4	7/7	23	20	7
		8	7/7	28	26	24
		12	7/7	25	28	28
		16	7/7	21	28	28
CD150-	Bone Marrow	4	6/9	2	17	4
		8	2/9	0.2	4	7
		12	1/9	0.2	2	5
		16	0/9	0.1	1	4
	Spleen	4	5/9	2	31	8
		8	3/9	0.5	5	8
		12	2/9	0.2	4	5
		16	0/9	0.1	3	4
CD48+	Bone Marrow	4	3/10	11	24	0.8
		8	5/10	2	10	6
		12	3/10	0.6	7	10
		16	2/10	0.2	5	7
	Spleen	4	10/10	10	39	6
		8	7/10	2	10	10
		12	5/10	3	8	8
		16	2/10	2	7	7
CD48-	Bone Marrow	4	5/10	28	25	0.5
		8	9/10	18	20	14
		12	9/10	23	18	19
		16	7/10	18	16	16
	Spleen	4	5/10	30	18	1
		8	9/10	24	26	23
		12	9/10	16	24	28
		16	9/10	13	24	27

Table 3.4 Summary of peripheral blood reconstitution in Figure 3.3

Population	Donor Tissue	Week	LTMR (>0.3% ea.)	My (Avg. %)	BC (Avg. %)	TC (Avg. %)
Lineage+	Bone Marrow	4	0/5	6	6	0
		8	3/5	6	6	2
		12	3/5	6	6	2
		16	3/5	3	7	4
	Spleen	4	0/5	0.2	3	2
		8	1/5	0.4	0.3	2
		12	0/5	0.8	0.9	2
		16	0/5	0.8	0.2	2
Lineage-/lo	Bone Marrow	4	3/5	36	23	4
		8	5/5	23	21	18
		12	5/5	9	14	19
		16	5/5	4	12	18
	Spleen	4	3/5	14	17	3
		8	5/5	15	10	11
		12	5/5	9	10	10
		16	5/5	8	11	13
Sca1+	Bone Marrow	4	4/5	36	36	0.7
		8	5/5	29	27	22
		12	5/5	16	23	21
		16	5/5	11	24	24
	Spleen	4	3/3	29	17	5
		8	3/3	22	19	18
		12	3/3	11	17	18
		16	3/3	11	18	21
Sca1-	Bone Marrow	4	0/5	0.6	2	1
		8	1/5	0.3	0.9	1
		12	0/5	0	0.1	0.5
		16	0/5	0	0.1	0.6
	Spleen	4	2/5	0.8	2	0.8
		8	1/5	0.1	0.2	0.6
		12	0/5	0.3	0.1	0.2
		16	0/5	0.1	0.1	0.2
cKit+	Bone Marrow	4	5/5	34	41	5
		8	5/5	38	38	25
		12	5/5	33	38	33
		16	5/5	25	38	42
	Spleen	4	5/5	43	57	4
		8	5/5	33	62	43
		12	5/5	38	61	51
		16	5/5	38	63	58
cKit-	Bone Marrow	4	0/5	0.1	0.2	0
		8	1/5	1	0.5	0.2
		12	0/5	0.2	0.1	0
		16	0/5	0.2	0.8	0.1
	Spleen	4	0/5	0.1	1	1
		8	0/5	0.1	0.1	0.6
		12	0/5	0.2	0.3	0.8
		16	0/5	0.3	0.5	0.6

CHAPTER IV

Conclusions and Discussion

Summary

Sensing of foreign entities by pattern recognition receptors is a crucial component of the innate immune system. Recognizing infectious stimuli is essential for mounting appropriate immune responses, yet deregulation of PRR signaling can result in autoimmunity, such as NOD2 mutations predisposing to Crohn's disease. How aberrations in NOD2 signaling lead to CD requires further investigation. Intriguingly, PRRs have been implicated in the regulation of hematopoietic progenitors, including hematopoietic stem cells, following exposure to infectious agents. Given the necessity for cell production during infection, it follows that stem cell activity may be altered to meet the increasing demands, yet mechanisms underlying the response of HSCs to infection remain elusive. This thesis takes both a global approach to identify novel networks of genes involved in regulation of the NOD2 signaling pathway as well as a targeted approach to understand how PRR signaling influences cells at the apex of the hematopoietic hierarchy. Together, these results shed light on the diversity of pathways that regulate and are regulated by PRR signaling and offer insight into mechanisms that promote extramedullary hematopoiesis during infection.

Insights into the pathogenesis of Crohn's disease

The first definitive description of Crohn's disease (originally termed Regional Ileitis) was published in 1932 (Crohn et al., 1932). In that landmark article, the authors defined the features of disease to include isolated regions of intestinal inflammation primarily occurring in the terminal ileum that span the entire thickness of the digestive tube, which frequently contained granulomas and fistulas linking bowel sections and often resulted in abdominal pain, diarrhea, bowel obstruction and emaciation (Crohn et al., 1932). Though the physical parameters of disease were clear, the underlying cause was elusive. Crohn stated:

> *In a disease of this type, in which an attempt is being made to establish the etiology of the disease, we have naturally taken great pains to exclude every known etiologic factor. Histologic sections were made of the tissues and stained with various types of stains. Cultures were made. Ground material was injected into guinea-pigs and fowl. Various types of laboratory animals were used to eliminate any possible form of tuberculosis. Löwenstein cultures were made. Dr. Klemperer, the pathologist, exhausted all the known possible scientific methods of finding an etiologic factor. I can say that no etiologic factor was found.*

Since that seminal description, much has been learned regarding the pathogenesis of CD (Baumgart and Sandborn, 2012), but the full spectrum of contributing features remains to be uncovered.

Strong evidence for a genetic contribution to CD came from studies showing higher incidence of disease in monozygotic twins (which have essentially identical genomic makeup) versus dizygotic twins (which share on average half of their genetic makeup) as well as the tendency for disease to occur in multiple family members (Orholm et al., 1991; Tysk et al., 1988). Even before the identification of specific genes, the success of corticosteroids, immunomodulatory agents and broad spectrum antibiotics

in promoting remission of CD indicated that interactions between immune cells and the microbiota play an important role in CD pathogenesis (Fiocchi, 1998; Selby, 2000). The discoveries that (1) *NOD2* polymorphisms predispose to CD, that (2) NOD2 is important for sensing components of bacteria and activating pro-inflammatory signaling cascades and that (3) CD-associated NOD2 mutations render it inactive, all supported a central role for NLR signaling in balancing protection and autoimmunity at the intestinal interface (Hugot et al., 2001; Inohara et al., 2003; Ogura et al., 2001a). Accordingly, we hypothesized that a comprehensive understanding of genes that regulate NOD2 signaling would yield insight into the mechanisms underlying CD pathogenesis.

In **Chapter II**, I described the results of a genome-wide screen analyzing the influence of individually knocking down just over 18,000 genes on NOD2-induced NF-κB activation. The screen was performed using HEK293 cells stably expressing human NOD2 and a NF-κB luciferase reporter gene. By measuring viability using a fluorescence based assay and NF-κB luciferase in tandem, we were able to discriminate between genes that genuinely altered NOD2 signaling from those that negatively affected cell number. Using this strategy, we identified multiple core components of the NOD2/NF-κB signaling pathway including *NOD2*, *RIPK2*, *NEMO* and *RELA* as well as components of the proteasome, which is required for degradation of the inhibitory protein IκB that restricts NF-κB in the cytoplasm, and components of the nuclear pore, which is essential for nuclear translocation of NF-κB following its release. These findings verified that our screen was capable of uncovering genes important for many different stages of NOD2 signaling and supported roles for novel genes identified as positive or negative regulators of the pathway.

One of our original goals was to identify genes that were specifically required for NOD2 signaling. Having identified numerous putative NOD2 regulators in our primary screen, we proceeded to conduct a secondary counter screen using independent siRNA pools to control for off target effects. To distinguish between NOD2 specific regulators and general regulators of NF- κB, we stimulated our reporter cells with either MDP or TNFα, which activates NF-κB transcription independently of NOD2 (Aggarwal, 2003). Results from the counter screen revealed that nearly all the validated regulators influenced both MDP- and TNFα-induced NF-κB activation. We therefore concluded that the immune system largely relies on a few upstream sensors and adaptors that converge on common NF-κB transcriptional machinery. Because NOD2 mutations, but not TNFα or TLR mutations, are strongly linked to CD, it is likely that the cellular context in which the pro-inflammatory signaling pathways are activated play a large role in the effect of each regulatory gene on disease.

One example of the importance of context can be illustrated by Paneth cells, which express NOD2 (Ogura et al., 2003). Paneth cells are located in the crypts of Lieberkühn within the small intestine and contribute to intestinal barrier defense through the secretion of antimicrobial peptides (AMPs) (Baumgart and Sandborn, 2012; Clevers and Bevins, 2013). AMPs limit the translocation of bacteria from the gut lumen to the underlying mucosa by direct microbicidal activity and formation of nanonets (Chu et al., 2012; Ouellette, 2011; Wu et al., 2004). Disruption of Paneth cells and reduction of AMPs is a common feature in CD patients, especially those with inactivating NOD2 mutations (Wehkamp et al., 2004; Wehkamp et al., 2005). Since our screen was performed in an epithelial cell line, it is likely that a subset of genes which were

identified as NOD2 regulators also function in Paneth cells to regulate AMP secretion. The lack of Paneth cell-specific cell lines limits the ability to perform a complementary genome-wide screen and likely necessitates that any putative regulators of AMP production be verified *in vivo*. A reasonable strategy would be to compare our list of NOD2 regulators with a list of genes expressed in Paneth cells by micro-array thereby reducing the number of genes to focus on for time consuming and costly follow-up studies.

Our screen identified numerous genes that were previously associated with CD risk as being NOD2 regulators, supporting a role for members of the NOD2 signaling pathway, beyond just NOD2, in the pathogenesis of CD. This idea is further supported by the fact that a subset of CD patients exhibit impaired MDP responses in the absence of *NOD2* mutations (Seidelin et al., 2009). Furthermore, over 1000 CD susceptibility loci have been discovered, yet only 71 loci have been assigned to a specific gene (Barrett et al., 2008; Franke et al., 2010; Rivas et al., 2011), highlighting the need to define causative factors at these unassigned loci. One potential application of our data set is in overlapping our list of NOD2 regulators with genome-wide association studies and next-generation DNA sequencing to explore the genetic basis of CD. In risk loci containing multiple genes, our data set could assist in the selection of candidate genes for future hypothesis based studies. Moreover, it is likely that many unidentified risk loci are partially penetrant and only present in a small number of diseased individuals, making it difficult to obtain statistical significance on a genome-wide scale. By limiting the analysis to SNPs within exons or promoter regions of genes identified in our screen, we could substantially restrict the search space for potentially causative variants.

Consequently, the lack of association in current studies should not invariably exclude a role for a particular gene in CD pathogenesis.

One example of a gene not previously implicated in CD risk is neutrophil cytosolic factor 1 (NCF1), a member of the NADPH oxidase complex that functions to generate reactive oxygen species (ROS) in phagocytes (Bedard and Krause, 2007), which was identified and validated as a positive regulator of NOD2 and TNFα signaling in our screens. Although *NCF1* has not previously been linked to CD, other NADPH oxidase components including *NCF2* and *NCF4* have been associated with CD (Muise et al., 2012; Rioux et al., 2007). Interestingly, inactivating mutations in *Ncf1* sensitize rats and mice to experimental models of collagen-induced arthritis, while copy number variants of human *NCF1* are linked to rheumatoid arthritis (Hultqvist et al., 2004; Olofsson et al., 2003; Olsson et al., 2012). Furthermore, mutations in *NCF1* and other components of the NADPH oxidase complex are the etiologic agent in chronic granulomatous disease, a disorder in which phagocytes from affected patients are unable to kill ingested bacteria and which shares some pathological features with CD (Francke et al., 1990; Marks et al., 2009). Together, these findings indicate that components of the NADPH oxidase machinery play an important role in immunity and resistance to autoimmune disease.

A common criticism of genome-wide screens concerns the use of highly transfectable immortalized cell lines, such as HEK293, that may not represent the physiologic context of the signaling pathway being analyzed. To address this concern, we tested whether NCF1 was important for NOD2 signaling in bone marrow derived macrophages in which NOD2 is known to function *in vivo.* When WT and *Ncf1*$^{-/-}$ macrophages were stimulated with MDP in culture, we found that despite having similar

levels of phosphorylated IκB (p-IκB) in *Ncf1*$^{-/-}$ and WT cells, total IκB protein was not diminished in *Ncf1*$^{-/-}$ cells. Since phosphorylation of IκB typically precedes its degradation and subsequent release of NF-κB from negative regulation, this finding suggests that NCF1 influences the recognition and degradation of p-IκB. This regulation was specific to NF-κB, since MAPK signaling was unperturbed in *Ncf1*$^{-/-}$ macrophages stimulated with MDP. Multiple molecular mechanisms could explain these results and require further exploration. One hypothesis is that NCF1 may be important for trafficking of p-IκB to the proteasome. This could be tested using confocal microscopy of WT and *Ncf1*$^{-/-}$ macrophages stimulated with MDP to track the intracellular location of p-IκB in relation to the proteasome. Alternatively, NCF1 may promote the dissociation of p-IκB from NF-κB to facilitate IκB degradation. This could be assessed by stimulating WT and *Ncf1*$^{-/-}$ macrophages with MDP and immunoprecipitating p-IκB, followed by western blotting for NF-κB. Lastly, NCF1 could be essential for proteasome function itself. To address this, WT and *Ncf1*$^{-/-}$ macrophages could be stimulated with interferon gamma followed by western blotting to measure JAK2 degradation, which is also dependent on the proteasome (Ungureanu et al., 2002). Regardless of the mechanism, these results are consistent with a role for NCF1 in promoting the activation of NF-κB, but not MAPKs, downstream of NOD2 signaling.

How might a requirement for NCF1 in NOD2/NF-κB signaling affect CD susceptibility? Phagocytes deficient in ROS production are more likely to harbor viable intracellular bacteria (Jackson et al., 1995). If NCF1 is perturbed by mutation or inactivation, it may be advantageous to limit pro-survival signals emanating from NF-κB within infected cells while preserving secretion of pro-inflammatory cytokines and

chemokines downstream of MAPKs. Apart from their role in destroying bacteria, ROS are also important for suppressing immune responses through inactivation of pro-inflammatory molecules in the extracellular milieu (Harrison et al., 1999). Alteration of NCF1-mediated ROS production could therefore contribute to perpetuation of inflammatory conditions in CD patients. Of note, pharmacological induction of ROS was able to prevent disease in a model of arthritis driven by inactive NCF1 (Olofsson et al., 2003). Thus, drugs promoting NCF1 and ROS production may represent novel therapeutic strategies for treating CD and warrants further investigation.

Using an unbiased genome-wide screening approach, we have identified and validated over 200 positive and negative regulators of the NOD2/NF-κB signaling pathway. By overlapping these genes with publicly available protein-protein interaction databases, we were able to visualize novel networks of regulatory complexes. Many genes within these complexes had previously been linked to CD, reinforcing a critical role for NOD2 and NF-κB in susceptibility to this disease. In total, our results provide a platform to understand how aberrant NOD2 signaling predisposes to autoimmunity and helps to generate novel hypotheses to investigate the pathogenesis of CD.

A role for extramedullary hematopoietic stem cells during infection

Hematopoiesis is sustained throughout life by a small pool of self-renewing hematopoietic stem cells. Adult HSCs typically reside in specialized niches within the bone marrow where a complex milieu of supporting cells, extracellular matrix components, growth factors and retention signals contribute to their long term

persistence. Disruption of the niche through genetic ablation of essential genes, pharmacologic activation of niche cells or exogenous administration of mobilizing agents can indirectly influence the behavior and localization of HSCs. Cell intrinsic mechanisms such as regulation of the cell cycle and responsiveness to extracellular cues are also important for regulating HSC function. Investigating the physiologic signals that affect HSCs under homeostatic and stressful conditions is critical for understanding how HSCs contribute to disease states and for improving HSC utility in the clinic. As bacterial infection is a physiologically relevant stressor that can have profound effects on the hematopoietic system and is common in recipients of hematopoietic transplants in the clinic, it serves as a useful tool to investigate the mechanisms governing HSC responses when homeostasis is disrupted.

A central unresolved question in stem cell biology is whether the function of long term multilineage repopulating HSCs is altered in the context of infection. Previous reports have offered conflicting and often inconclusive results. Using a model of systemic LPS challenge followed by transplantation of bone marrow-derived LSK cells, one study found that HSC function was markedly reduced (Rodriguez et al., 2009), whereas another study found that HSC function was enhanced (Takizawa et al., 2011). Another group infected mice with *Mycobacterium avium* and transplanted bone marrow-derived LSK cells capable of excluding Hoechst dye and observed a massive reduction in HSC function compared to uninfected cells (Baldridge et al., 2010). The interpretation of these results is complicated by the possibility that surface markers or dye exclusion properties used to isolate HSCs under steady-state conditions may be altered following infection, which would result in either a dilution or enrichment of HSCs within the sorted

population. Other studies have circumvented this limitation by transplanting unfractionated bone marrow cells from mice treated with LPS and found that total HSC activity in bone marrow was reduced under those conditions (Chen et al., 2010; Esplin et al., 2011). Still, it is debatable whether the concentrations of LPS used in those studies accurately reflect the physiological context of live bacterial infection. Moreover, none of those studies tested whether HSC activity is altered in sites outside the bone marrow, which might shed light on some of the observed results. Thus, an unbiased functional characterization of HSC activity in the bone marrow and periphery following live bacterial infection was required to resolve this controversy.

In **Chapter III**, I described the functional characterization of murine HSCs following systemic infection with the gram negative bacterium *Escherichia coli* K12. We selected this bacterium because intraperitoneal administration did not result in lethality, supporting our goal to understand the physiologically relevant responses of HSCs to infection. Competitive transplantation of unfractionated bone marrow or splenocytes from mice that had been infected for six days revealed that total HSC activity, which we defined as myeloid and lymphoid reconstitution over sixteen weeks, was reduced by approximately 2-fold in the bone marrow, whereas total HSC activity in the spleen was increased nearly 40-fold. From this we concluded that infection with *E. coli* resulted in moderate reduction of HSC activity in the bone marrow coupled with robust expansion of extramedullary HSC activity.

The systemic dissociation of microbes can promote a condition termed sepsis, in which recognition of microbial products in the blood results in the overwhelming production of inflammatory cytokines that can lead to multiple organ failure (Cohen,

2002). Following the immediate systemic inflammatory response, a compensatory anti-inflammatory response develops, which is characterized by excessive production of anti-inflammatory cytokines, decreased numbers of immune cells and enhanced susceptibility to secondary infection (Ward et al., 2008). Indeed, patients that survive systemic infection exhibit increased risk of mortality for years beyond the initial septic insult (Perl et al., 1995). Given these lasting effects, it has been suggested that epigenetic alterations that diminish HSC function might contribute to perpetual immune deficiencies following sepsis (Carson et al., 2011). Additionally, it has been proposed that the reduction of HSC activity in the bone marrow following administration of LPS is a consequence of cellular damage induced either by LPS itself or inflammatory cytokines that are produced during infection (Chen et al., 2010; Esplin et al., 2011). It would be interesting to determine whether HSC activity in the bone marrow is irreparably altered following systemic infection or whether HSC activity is eventually restored, supporting alternative mechanisms for the observed immunodeficiencies.

One limitation of the competitive transplantation assay is that it measures total HSC *activity* within a test population, but does not distinguish between the *number* of HSCs within that population and the *function* of each HSC. Thus, the 2-fold reduction of HSC activity observed in bone marrow could represent a 2-fold reduction of total self-renewing HSCs or a 2-fold reduction in the ability of each HSC to give rise to progeny in an irradiated host, or some combination of these factors. The inverse could also be argued for the increased HSC activity we observed within infected spleen, although it seems unlikely that the robust expansion of extramedullary HSC activity following infection is due only to the enhanced function of resident splenic HSCs. To quantify the

number of HSCs in bone marrow and spleen after infection, limiting dilution assays could be performed in which three doses of unfractionated WBM or splenocytes are competitively transplanted into irradiated recipients. Using Poisson statistics it is possible to extrapolate the number of HSCs within each tissue from the fraction of recipient mice that are *not* reconstituted at each dose (Szilvassy et al., 1990). If the total number of HSCs in the bone marrow had not changed following systemic *E. coli* infection, this would support the hypothesis that HSCs are damaged following infection. An observation of fewer bone marrow HSCs and greater splenic HSCs after infection would support the hypothesis that HSCs are not damaged during infection and are merely relocated to sites other than the bone marrow.

A pitfall of the limiting dilution assay is that it cannot account for the number of HSCs that engraft a particular recipient mouse; it only determines whether a mouse was engrafted or not and therefore is uninformative for comparing the function of individual HSCs within the test population. HSCs from unmanipulated bone marrow and spleen have been shown to be functionally comparable by sorting and competitively transplanting individual cells based on their surface marker expression (Morita et al., 2011). To compare HSCs from the bone marrow and spleen of infected mice using this assay, it is essential to verify that the surface markers used to sort HSCs are not altered. Therefore, we rigorously tested whether functional HSCs are contained within established surface marker profiles by sorting and competitively transplanting bone marrow and splenocytes from infected mice based on positive or negative expression of CD150, CD48, Lineage, cKit and Sca1. We found that while all HSC activity was retained in the $CD150^{+}$, $cKit^{+}$ and $Sca1^{+}$ fractions of bone marrow and spleen and most

HSCs were $CD48^-$ and $Lineage^{-/lo}$, subsets of HSCs in the bone marrow expressed CD48 and Lineage markers and a subset of splenic HSCs expressed CD48. These findings illustrate the need to validate new surface markers that can be used to enrich for all functional HSCs in the context of infection. Since the majority of HSC activity was retained within the $CD150^+$ $CD48^-$ $Lineage^{-/lo}$ $Sca1^+$ $cKit^+$ population after infection, this combination of markers was still useful to interpret the dynamics of HSC localization.

To better understand how HSC populations changed during infection, we measured the number of $CD150^+$ $CD48^-$ $CD41^-$ LSK cells at various times after infection. CD41 was included in our staining cocktail to exclude contaminating megakaryocytes (Kiel et al., 2005). We observed a transient decrease of $CD150^+$ $CD48^-$ $CD41^-$ LSK cells in bone marrow that reached a nadir three days after infection along with an expansion of $CD150^+$ $CD48^-$ $CD41^-$ LSK cells in spleen that reached an apex nine days after infection. This kinetic could support a model in which bacterial infection drives the mobilization of HSCs from the bone marrow through the blood stream to the spleen, but given the presence of local splenic HSCs under steady-state conditions, these kinetics do not distinguish between mobilization and local proliferation. To unequivocally test whether HSCs are mobilized following infection, congenic mice could be linked by parabiosis and after a shared circulatory system is established one mouse could be challenged with *E. coli*. After six days of infection, the mice could be separated and allowed to recover for sixteen weeks. If HSCs from the infected mouse were mobilized to the blood stream, a proportion would have engrafted the connected mouse and given rise to differentiated progeny. If the expansion of splenic HSCs was entirely due to local proliferation, then the proportion of peripheral blood cells derived from the partner would be similar to that

of mice separated without infection. Furthermore, if the results suggest that mobilization had occurred, it would be interesting to measure the proportion of foreign HSC activity in the bone marrow and spleen of the previously conjoined mouse, which would offer insight into the long term residency of mobilized HSCs.

Infection results in the enhancement of extramedullary HSC activity, yet it is unclear whether peripheral HSCs play an important role in biology. One possibility is that mobilization of HSCs is merely a secondary consequence of an essential requirement to mobilize neutrophils in order to clear the systemic infection. Indeed, both neutrophils and HSCs are retained in the bone marrow by CXCL12/CXCR4 signaling and can be rapidly mobilized by systemic administration of the inflammatory cytokine IL-8 (Delano et al., 2011; Laterveer et al., 1996; Sugiyama et al., 2006). Moreover, intravenous injection of neutrophils is sufficient to mobilize hematopoietic progenitors to the blood and reduce CXCL12 in the bone marrow, while antibody mediated depletion of neutrophils reduces circulating HSCs and increases CXCL12 expression in bone marrow, indicating that trafficking of neutrophils and HSCs are tightly correlated (Casanova-Acebes et al., 2013). A reasonable experiment would be to test whether infection expands extramedullary HSC activity in the absence of neutrophils or in the presence of some factor that blocks neutrophil egress from the bone marrow.

Regarding a biological role for extramedullary HSCs, one group suggested that HSCs can localize to sites of inflammation and differentiate into type 2 macrophages to help resolve toxic liver injury (Si et al., 2010), while another group proposed that HSCs traffic through peripheral tissues in order to provide a local source of effector cells (Massberg et al., 2007). Given the strict requirement for niche-derived signals to

maintain HSCs long term, mobilization during infection may be an important mechanism to relocate these powerful cells to a specialized niche where alternative functions, such as symmetric self-renewal divisions that support lasting cell production, can be sustained. One prediction of this hypothesis is that the splenic HSC niche is expanded during infection. Alterations in the splenic HSC niche could be visualized by administering *E. coli* to mice in which a fluorescent protein is expressed under the endogenous promoter of SCF or CXCL12 (Ding and Morrison, 2013; Ding et al., 2012; Greenbaum et al., 2013). Moreover, one could sort HSCs from the spleen or bone marrow of *E. coli* infected mice and transplant them into a mouse challenged with a lethal model of sepsis, such as cecal ligation and puncture, to determine whether peripheral HSCs are better suited to protect the injured host and to maintain cell production beyond the resolution of infection.

Pattern recognition receptors promote extramedullary hematopoietic stem cell expansion indirectly

Escherichia coli contain LPS and peptidoglycan that are recognized by TLR4 and NOD1/NOD2, respectively. To test whether the expansion of extramedullary HSCs was dependent on PRR signaling, we infected *Tlr4*$^{-/-}$ and *Ripk2*$^{-/-}$ mice with *E. coli* and found that after nine days the accumulation of CD150^{+} CD48^{-} CD41^{-} LSK cells in the spleens of these mice was reduced compared to WT mice. Whereas administration of ultrapure LPS was sufficient to produce hundreds of nanograms of G-CSF in the serum, it was insufficient to expand HSC activity in the spleen. Administration of the NOD1 agonist

KF1B did not substantially increase G-CSF in the serum and was also insufficient to expand HSC activity in the spleen, but dual administration of LPS and KF1B synergistically enhanced G-CSF in the serum and HSC activity in the spleen. Using G-CSF-neutralizing antibodies and mice deficient for the G-CSFR, we show that G-CSF is required for extramedullary HSC expansion following LPS and KF1B administration. Furthermore, by challenging chimeric mice with LPS and KF1B we determined that PRR signaling in radio-resistant cells was important for promoting the accumulation of hematopoietic progenitors in the spleen. Together, these results demonstrate that recognition of bacterial components by TLRs and NLRs in radio-resistant cells are necessary and sufficient to expand extramedullary HSCs in the context of infection. Moreover, they suggest that contamination of LPS preparations with NLR-activating moieties likely contributed to the expansion of peripheral HSPCs that was described previously (Vos et al., 1972).

How do TLR4 and NOD1 cooperate to induce extramedullary hematopoiesis? One possibility is that the amplitude or duration of G-CSF exposure following LPS is too weak to trigger release of HSPCs from the bone marrow. The enhanced production of G-CSF induced by the presence of both LPS and KF1B may be necessary to exceed a threshold required for mobilization. This hypothesis is supported by the fact that repeated administration of G-CSF over 4-5 days is required to mobilize HSPCs (Petit et al., 2002). G-CSF-induced mobilization involves multiple mechanisms, one of which involves downregulation of CXCL12 in cells of the bone marrow niche (Petit et al., 2002). We found that CXCL12 expression was reduced in the bone marrow two days after dual stimulation of TLR4 and NOD1, but not with activation of either PRR alone. It

is unclear whether downregulation of CXCL12 is purely a consequence of reaching a threshold of G-CSF activation or whether TLR4 and NOD1 provide complimentary signals in CXCL12-expressing cells to regulate transcription of this gene. Intriguingly, G-CSF treatment has been associated with reduced clinical symptoms in some CD patients, which correlated with increased T regulatory cells and plasmacytoid dendritic cells at sites of inflammation (Guidi et al., 2008; Mannon et al., 2009). Since many CD patients harbor mutations in NOD2, an interesting possibility is that reduced NOD2 signaling could limit production of G-CSF, which may be important for mobilizing HSPCs to the site of infection where their progeny could contribute to clearance of bacteria and resolution of inflammation.

Reverse chimera experiments highlighted a role for TLR and NLR signaling in radio-resistant cells to promote extramedullary hematopoiesis, but these experiments did not determine the exact identity or location of cells responsible for mediating the observed effects. Thus, TLR4 and NOD1 could be acting either in the same cell or different cells, which could be present within the bone marrow or peripheral tissues. To dissect which cell type is responsible for mediating the expansion of peripheral HSCs following TLR4 and NOD1 stimulation, one would need to generate mice carrying floxed alleles of TLR4 and NOD1, which could be selectively deleted using cell type specific promoters to drive cre recombinase. Likely candidates to test are Col2.3 expressing osteoblasts, Tie2 expressing endothelial cells and Prx1 expressing mesenchymal progenitors that have been previously implicated in various aspects of the bone marrow niche, such as expression of CXCL12 (Ding and Morrison, 2013; Greenbaum et al., 2013).

If TLR4 and NOD1 signaling are important within the bone marrow niche to promote HSC mobilization, it would be interesting to understand how PRR signaling regulates CXCL12 expression. Classical NF-κB signaling has been shown to negatively regulate CXCL12 expression in cultured endothelial cells through inhibition of non-canonical NF-κB-dependent transcription (Madge and May, 2010). CXCL12 expression is enhanced under hypoxic conditions and this is dependent on Hif-1α (Ceradini et al., 2004). Since Hif-1α is an NF-κB target gene (Rius et al., 2008), it is possible that PRRs regulate CXCL12 expression through this pathway. This hypothesis could be tested by culturing primary CXCL12-expressing cells from the bone marrow in the presence of LPS, KF1B or both and measuring CXCL12 expression by quantitative PCR and Hif-1α occupancy on the CXCL12 promoter by chromatin immunoprecipitation. Another interesting possibility is that PRR signaling in the spleen is important for recruiting HSCs. Activation of TLR4 and subsequent production of G-CSF may be sufficient to mobilize HSCs from the bone marrow to the blood stream, but NOD1 signaling could be required to activate adhesion molecules on splenic endothelial cells to trap circulating HSCs. To support this mechanism, one would need to identify an adhesion molecule that is induced on splenic endothelial cells following NOD1 (or NOD1/TLR4) activation and block this interaction with an antibody to show that HSC recruitment to the spleen is diminished.

A major finding of our work was that TLR4 expression on HSCs is not required for their expansion in spleen following LPS and KF1B stimulation. A subtle, yet notable finding was that *Tlr4*$^{-/-}$ HSCs were not present in the spleens of irradiated mice that received equal numbers of WT and *Tlr4*$^{-/-}$ hematopoietic cells after 16 weeks. One

interpretation of this finding is that *Tlr4*$^{-/-}$ HSCs preferentially localize to the bone marrow or fail to localize to the spleen when transplanted into an irradiated host. These possibilities could be dissected by transplanting equal numbers of sorted WT and *Tlr4*$^{-/-}$ HSCs and measuring their numbers in bone marrow and spleen after a few hours. If WT and *Tlr4*$^{-/-}$ HSCs have similar capacity to localize to the spleen after transplantation, this would suggest that *Tlr4*$^{-/-}$ HSCs compete poorly with WT HSCs for limited niches within the spleen and transit back to the bone marrow over time. To directly test whether TLR4 deficient HSCs compete poorly with WT HSCs for splenic niches, one could transplant equal numbers of cells from WT and TLR4$^{fl/fl}$ mice into irradiated recipients and measure HSC residency in the spleen after inducible deletion of TLR4.

It has been suggested that *Tlr4*$^{-/-}$ HSCs have a competitive advantage compared to WT HSCs in transplantation assays (Ichii et al., 2010). We found that myeloid reconstitution was equivalent in WT and *Tlr4*$^{-/-}$ HSCs when competed in the same mouse, but *Tlr4*$^{-/-}$ HSCs gave rise to more B-lineage cells over 16 weeks, which may explain the previous group's findings. Although our reverse chimera experiments indicated that NOD-like receptor signaling in radio-resistant cells is important for promoting extramedullary hematopoiesis, they did not rule out the possibility that NOD1 or NOD2 may play a role within HSCs. To determine whether NLR signaling is important within HSCs, one could mix and transplant equal numbers of hematopoietic cells from congenic *Ripk2*$^{-/-}$ and WT mice and measure peripheral blood chimerism over 16 weeks. If NLR signaling affects HSC function, this could have implications for therapies in which pharmacological agents used to elicit responses in HSCs are purified from bacteria and contain residual NLR-activating peptidoglycan.

In total, the results presented in this thesis expand our knowledge of how PRR signaling is controlled and provide insight into how PRRs influence the activity of HSCs. Given our discoveries that NLRs and TLRs are important for expanding extramedullary HSCs in the context of infection and that this phenomenon is dependent on G-CSF, it is possible many of the genes identified as regulators of NOD2/NF-κB signaling in our genome-wide screen may influence HSC mobilization in concert with G-CSF. Thus, regulators of NOD2/NF-κB might serve as useful targets to improve mobilization therapies. While our findings establish a framework to understand the biology of PRR signaling, further investigation is required to understand their contribution to autoimmune disorders and to define mechanisms underlying the regulation of HSCs and their niche(s) during infection.

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