Title: Toll-like Receptor Signaling in Hematopoietic Stem and Progenitor Cells

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Abstract

Purpose of review- The innate immune system is essential in the protection against microbial infection and facilitating tissue repair mechanisms. During these stresses, the maintenance of innate immune cell numbers through stress-induced or emergency hematopoiesis is key for our survival. One major mechanism to recognize danger signals is through the activation of toll-like receptors on the surface of hematopoietic cells, including hematopoietic stem (HSC) and progenitor (HPC) cells, and non-hematopoietic cells, which recognize pathogen-derived or damaged-induced compounds and can influence the emergency hematopoietic response. This review explores how direct pathogen-sensing by HSC/HPC regulates hematopoiesis, and the positive and negative consequences of these signals.

Recent findings- Recent studies have highlighted new roles for toll-like receptors in regulating HSC and HPC differentiation to innate immune cells of both myeloid and lymphoid origin and augmenting HSC and HPC migration capabilities. Most interestingly, new insights as to how acute versus chronic stimulation of toll-like receptor signaling regulates HSC and HPC function has been explored.

Summary- Recent evidence suggests that toll-like receptors may play an important role in many inflammation-associated diseases. This suggests a possible use for toll-like receptor agonists or antagonists as potential therapeutics. Understanding the direct effects of toll-like receptor signaling by HSC and HPC may help regulate inflammatory/danger signal-driven emergency hematopoiesis.

Keywords: Hematopoiesis, Inflammation, Infection, TLR
Introduction

Hematopoietic stem cells (HSCs) are in low frequency within the bone marrow (BM) and have multi-lineage differentiation potential as well as the ability for life-long self-renewal potential [1, 2]. Long-term (LT)-HSCs are retained in a BM microenvironment that promotes their survival and maintains them in a non-cycling state [3, 4]. Thus, when there is a demand for more mature immune cells following infection, blood loss or tissue repair, the BM has the required pool of HSCs and hematopoietic progenitor cells (HPC) needed to ramp up production of replacement mature hematopoietic cells. This stress-induced increase in production of mature immune cells has been dubbed emergency hematopoiesis. How the hematopoietic system recognizes when to switch gears from normal, steady-state production to emergency hematopoiesis is driven by many factors such as pathogen-sensing (either directly by the HSC/HPC or indirectly by mature hematopoietic or other non-hematopoietic cells), cytokine/growth factor production, or other ‘danger’ signals (e.g. ROS production) and can vary greatly depending on the type of infection, site of injury, and duration of inflammatory response. How mature hematopoietic and non-hematopoietic cells regulate emergency hematopoiesis by external cues such as cytokine/growth factor production has been extensively reviewed [5**, 6]. Therefore, in this review the focus is on how immune cell numbers are regulated under emergency conditions via direct pathogen-sensing by HSCs and HPCs, and positive and negative consequences of these signals.

Recognizing danger and/or infection

In order to induce emergency hematopoiesis the hematopoietic system must first recognize that a stress event has taken place. Toll-like receptors (TLRs; a type of pattern recognition receptor) recognize pathogen-associated molecular patterns (PAMPs) [7, 8]. TLRs are expressed on cells of hematopoietic origin (e.g. dendritic cells, macrophages, lymphocytes, HSCs, and HPCs) as well as non-hematopoietic cells (e.g. endothelial and mesenchymal stem
cells) [9-14]. Some TLRs are localized to the plasma membrane (e.g. TLR1, TLR2, TLR4, TLR5, TLR6, and TLR11) while others are found in endosomes (e.g. TLR3, TLR7, TLR8, and TLR9) with most acting as homodimers with the exception of TLR2 which heterodimerizes with TLR1 or TLR6 [11, 15, 16]. The ligands for TLRs, PAMPs, are unique to groups of related microorganisms, e.g. lipopolysaccharide (LPS) found in bacteria cell walls or viral single-stranded RNA, that are not associated with normal host cells. In addition to recognizing pathogen-associated danger signals, TLRs can also recognize injury or tissue repair danger-associated molecular patterns (DAMPs) [17]. DAMPs (e.g. HMGB1 and ATP) are cell-derived molecules that are normally expressed by cells undergoing necrosis that can trigger an innate immune response.

Once TLRs recognize PAMPs or DAMPS, a signaling cascade involving myeloid differentiation primary response gene 88 (MyD88) recruitment is followed by complex formation with the serine-threonine kinase (IRAK) family members, leading to nuclear factor k-light chain-enhancer of activated B cells (NF-κB) and mitogen-activated protein kinase (MAPK) pathway activation resulting in pro-inflammatory cytokine (e.g. IL-1, IL-6, IL-8 and TNFα) production [11, 18-20]. However, there are exceptions such as TLR3 signaling which is MyD88-independent, instead depending on the TIR-domain-containing adaptor inducing interferon-B (TRIF) pathway which activates interferon regulatory factor-3 (IRF3) leading to production of type 1 interferons [21-23]. TRIF signaling can also lead to MyD88-independent activation of NF-κB and MAPK. TLR4, one of the most thoroughly investigated TLRs, utilizes both MyD88- and TRIF-dependent signaling pathways [24].

Direct recognition of danger by hematopoietic stem and progenitor cells

Evidence that danger signals can trigger emergency hematopoiesis in vivo is strong as demonstrated by the fact that direct injection of PAMPs and DAMPs (e.g. LPS) and/or infecting
mice with pathogens (e.g. *Candida albicans* or *Staphylococcus aureus*) can increase HSC proliferation and skew differentiation toward increased myeloid cell (e.g. neutrophil and macrophage) production in a TLR-dependent manner often at the expense of lymphopoiesis and erythropoiesis [25-30]. However, administration of systemic TLR agonists or the use of infection models such as those stated above do not address the direct role of TLRs within HSC and HPC populations. To test if TLR agonists can directly stimulate HSCs/HPCs, lineage marker negative, stem cell antigen positive-1, c-kit positive BM cells from wild type mice were transplanted into lethally irradiated TLR2, TLR4 or MyD88 knockout mice and then immediately exposed *in vivo* to soluble TLR ligands Pam3CSK4 (a TLR2 agonist), LPS (a TLR4 agonist) or CpG oligodeoxynucleotide (ODN; a TLR9 agonists that is dependent on MyD88 signaling) respectively. Transplanted HSCs and HPCs in response to these TLR ligands differentiated preferentially to macrophages demonstrating that TLR signaling directly from the HSC/HPC compartment can regulate specific blood cell production [29].

TLRs are expressed on HSCs and HPCs both in mice and humans and have been shown both *in vitro* and *in vivo* to affect HSC/HPC functions [12, 31-34]. Both highly phenotypically-defined LT-HSC populations and more broadly-defined HSC/HPC populations express both TLR2 and TLR4. When purified mouse LT-HSCs (sorted using SLAM markers) were stimulated directly with LPS, the TLR4/NF-κB axis was activated [35]. *In vitro* stimulation of less strictly defined HSC/HPC populations with Pam3CSK4 or LPS has also been shown to drive myeloid differentiation in a MyD88-dependent manner [13, 25-29, 33, 35-38]. Further studies utilizing HSCs/HPCs demonstrated that TLR4 signaling can also occur through a TRIF-dependent pathway, ultimately leading to activation of NF-κB and/or IRF transcription factors [8]. Stimulation of human CD34⁺ BM cells with small interfering RNAs and R848 (specific ligands for TLR7 and TLR8) resulted in increased IL-1β, IL-6, IL-8, TNF-α, and GM-CSF production as well as inducing these cells to differentiate into macrophages and monocytic
dendritic precursors that expressed CD13, CD14 and/or CD11c markers [34] suggesting that TLR7 and TLR8 may also directly influence HSCs and/or HPCs. Supporting these findings, TLR7-mediated stimulation of common myeloid progenitors (CMPs), synergistically with type 1 interferons, promoted monocyte/macrophage differentiation. Interestingly, this was through an mTOR/PI3K-dependent pathway [39]. These findings suggest that HSCs and HPCs can directly signal through TLRs driving myeloid differentiation, indicating that HSCs/HPCs themselves can act as pathogen sensors.

However, these findings do not necessarily indicate whether the triggering of emergency hematopoiesis (especially skewing towards increased production of mature myeloid cells) is through the direct regulation of transcription factors that lead to the differentiation of these mature cell populations (an autocrine effect), by the production of cytokines and/or growth factors that promote these pathways (a paracrine effect), or both. Further work to differentiate the importance of the autocrine and paracrine TLR signaling effects on HSCs and HPCs needs to be performed. It is becoming clearer, however, that HSC/HPC populations may be very important contributors to the pro-inflammatory cytokine and growth factor milieu following TLR stimulation. The stimulation of HSCs/HPCs, specifically short-term (ST)-HSCs and multipotent progenitors (MPPs), with various bacterial products resulted in production of a diverse cytokine milieu through NF-κB signaling [35]. In particular HSCs/HPCs produced large quantities of IL-6 which is known to drive myeloid differentiation and HSC/HPC proliferation. Interestingly, cytokine production by ST-HSC and MPP following TLR-stimulation often trumped mature immune cell production of cytokines following stimulation with the same doses of TLR agonist [35].

Stimulation of TLRs using PAMPs has also been demonstrated to change chemokine receptor expression on the surface of HSC/HPC and the ability of these cells to be retained within the BM [12, 40, 41]. Following a stress event, the appearance of immature myeloid precursor cells and other earlier progenitor cells often occurs in the peripheral blood (PB). This
is referred to as a PB left shift [5**]. Upon stimulation of TLRs, common dendritic cell progenitors (CDPs) down-regulate expression of CXCR4 (a required chemokine receptor for CDP retention within the BM) and up-regulate the expression of CCR7 (a chemokine receptor required for CDPs to enter preferentially into inflamed lymph nodes) [12]. These findings suggest that TLR signaling in dendritic cell progenitors allows for dendritic cell homeostasis at sites of inflammation. Recently it was found that injecting a low dose of zymosan (a TLR2 agonist) into the peritoneal cavity of mice resulted in increased numbers of innate lymphoid cells, a phenomena dependent on the in situ differentiation of BM-derived HPCs and innate lymphoid cell precursors [42**]. This suggests that TLR signaling may play a role in the migration and differentiation of innate lymphoid cells and their progenitors/precursors. It is important to note, however, that induction of HSC/HPC migration by TLR signaling is not straight forward. Interestingly, MyD88 knockout mice injected with LPS and zymosan demonstrated normal HSC/HPC migration into the PB, similar to that of wild type controls, suggesting that another factor may influence the migration of these populations into the blood [40].

**Complicated interactions between granulocyte-colony stimulating factor (G-CSF) and TLRs in HSCs/HPCs**

G-CSF is thought to be one of the principle cytokines involved in regulating granulopoiesis under steady-state conditions and following many different stress events including infection [43-45]. One way G-CSF regulates granulopoiesis is by inducing commitment of MPPs down the myeloid lineage [46]. G-CSF and G-CSF receptor-deficient animals exhibit severe neutropenia (a condition when there is reduced neutrophil numbers in the blood) [47, 48] which correlates with the fact that mice that are deficient in G-CSF receptor have increased numbers of common lymphoid progenitors and decreased CMPs thus influencing granulopoiesis [46]. Following a stress event, changes in pro-inflammatory
cytokines such as TNF-α, IL-1, and IL-17 (amongst others) can increase G-CSF expression [49, 50]. The enhanced induction of G-CSF expression following pro-inflammatory cytokine exposure, often induced by TLR stimulation, also contributes to the PB left shift phenomenon, as G-CSF induces release of BM HSCs/HPCs and myeloid precursors into the blood by regulating surface expression of the chemokine receptor CXCR4 and production of its ligand CXCL12 (also known as stromal cell-derived factor-1 or SDF-1) [51-54]. The fact that G-CSF turns off the HSC/HPC retention signal is used in the clinic to mobilize HSCs/HPCs to the PB for the use in transplantations [51-54].

Interestingly, even though TLR signaling produces a pro-inflammatory microenvironment that regulates G-CSF production, utilizing G-CSF as a mobilizer in MyD88 knockout mice resulted in enhanced HSC/HPC migration into the PB [40]. Transplantation studies revealed that this enhancement in HSC/HPC migration was due to cells of hematopoietic origin. Since TLR activation crosstalks with activation of the complement cascade associated with innate immune responses and augments production of pro-inflammatory regulators [40, 55-57], it was proposed that one of these factors may be mechanistically involved in the altered mobilization of HSCs and HPCs following G-CSF administration. MyD88 knockout BM cells, in particular, stem cell antigen-1 positive cells, demonstrated greatly reduced expression of heme oxygenase (HO)-1 (a negative regulator of the complement cascade activation and a negative regulator of HSC/HPC mobilization) suggesting that TLR signaling enhances expression of HO-1 in hematopoietic cells, especially within HSC/HPC populations, resulting in the reduced migration of HSCs and HPCs from the BM to the blood [40, 55, 58]. This observation suggests that utilizing TLR antagonists may aid in enhancing G-CSF-mediated HSC/HPC mobilization.

G-CSF plays a role in regulating hematopoiesis. G-CSF treatment over a prolonged period of time in vivo can result in an initial increase in phenotyped HSC numbers. However, this is followed by an increase in HSC quiescence associated with a marked decrease in HSC repopulating capacity [59]. Reduction of HSC function after prolonged exposure to G-CSF is
associated with increased TLR expression and signaling as the G-CSF-mediated alteration of HSC function is greatly reduced in mice lacking TLR2, TLR4 or the TLR signaling adaptor MyD88. These findings are further supported by the fact that prolonged exposure to G-CSF also can regulate TLR expression (e.g. TLR-1) and induce TLR signaling without the added presence of additional TLR ligands [59]. Interestingly, mice maintained under germ-free conditions and mice given antibiotics to suppress intestinal flora manifest reduced HSC dysregulation following prolonged exposure to G-CSF, suggesting that TLR stimulation is most likely a result of TLR ligands produced by the animal’s own microbiome [59]. Taken together, G-CSF treatment appears to act as a stress itself, altering TLR signaling that results not only in changes to G-CSF treatment-induced mobilization of HSCs and HPCs, but also to HSC function. The role of this pharmacologically induced stress in reduction of HSC/HPC mobilization by mature hematopoietic components of the BM (associated with the increased expression of DAMPs, ROS, and proteolytic and lipolytic enzymes) has recently been reviewed [60**] and will not be discussed here. However, this complicated interaction between G-CSF therapy and activation of TLR signaling poses a new and promising potential therapeutic target that deserves to be carefully explored.

**Chronic exposure to TLR ligand effects HSC/HPC function**

Prolonged exposure to TLR stimulation is associated with impaired hematopoiesis [5**, 6, 11, 38, 59]. Repeated, prolonged exposure of mice to LPS leads to increased HSC proliferation and an increase skewing towards myeloid cell differentiation, but is associated with decreased HSC repopulating capability following transplantation [36, 38]. Similarly, chronic exposure of mice to PAM3CSK4 led to increased HSC numbers, both in BM and spleen, but resulted in decreased HSC function [61]. Effects of prolonged exposure to either LPS or PAM3CSK4 on HSC function, however, do not appear to be linked to direct effects on HSCs or HPCs, but rather to increased production of pro-inflammatory stimuli such as TNF-α and G-CSF
Negative effects to HSC function by prolonged exposure to TLR stimuli is further supported by the finding that prolonged exposure to cytokines/growth factors, such as the pharmacological administration of G-CSF, creating an environment constantly exposed to low doses of TLR ligands, had similar effects on HSC function [59]. However, short-term or single exposure to TLR agonists, as mentioned previously, resulted in increased HSC activity even though increased pro-inflammatory cytokine production was present [30]. These findings suggest that the specific effects of TLR agonists on HSC and HPC function are dependent on dose or duration of exposure (Figure 1). Why short-term and long-term exposure to TLR agonists both resulted in increased cytokine production but have opposite effects on HSC function remains unclear but may represent a possible direct HSC autocrine TLR signaling mechanism or differential cytokine/chemokine protein or receptor production/expression. Understanding how this phenomenon occurs requires further investigation.

Accumulating evidence also suggests a role for TLRs in development of hematopoietic malignancies and BM failure, possibly shedding light as to how chronic stimulation of TLRs or aberrant TLR signaling may have detrimental effects on hematopoiesis. TLRs have been associated with myelodysplastic syndromes, acute myeloid leukemia, multiple myeloma, chronic lymphocytic leukemia, non-Hodgkin’s lymphoma, mantle cell lymphoma, and activated B-cell diffuse large B-cell lymphoma amongst other hematological disease [11]. Aberrant TLR signaling (e.g. overexpression of the TLR effector TRAF6 and the chronic activation of epigenetic regulators such as KDM6B) has been linked to serval lymphoproliferative disorders and myelodysplastic syndromes that possess a high risk of transformation to leukemia [11, 62*, 63*]. Whether this is due to autocrine effects on HSCs and/or HPCs or the release of pro-inflammatory cytokines that could create a microenvironment conducive to promoting tumorigenesis has been recently reviewed extensively [11].

**Conclusions**
There is still a lot unknown about how TLR signaling influences HSC and HPC cycling, differentiation and mobilization via both autocrine and paracrine mechanisms. However, as more and more studies find a role for TLRs in disease progression (e.g. cancer, autoimmune disease, graft-versus-host disease, etc.) where targeting TLR signaling pathways through the use of TLR agonist or antagonist is a possible therapeutic approach, utilizing cytokines/growth factors as therapeutics (e.g. G-CSF) that can activate TLR signaling, or utilizing TLR agonist and antagonist to mitigate emergency hematopoiesis itself, it has become increasingly essential that we understand how these treatments might regulate HSC/HPC function [11, 40, 59, 60, 64*, 65*, 66*, 67*, 68*]. It is possible that with a better understanding as to how TLR stimulation effects HSC and HPC function directly, we may be able to create better therapeutics to regulate hematopoiesis or alter disease progression by manipulating the hematopoietic emergency response to danger signals.

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Conflict of Interests

The author declares no conflict of interest
Key Points

- TLR stimulation of HSC/HPC populations can directly affect their function both in an autocrine and paracrine manner leading to increased innate immune cell production of both myeloid and lymphoid cells.
- Prolonged, therapeutic G-CSF therapy and other inducers of chronic TLR signaling negatively influence HSC function.
- Chronic inflammatory stress and aberrant TLR signaling is associated with malignant hematopoietic transformation.
References


This review goes over in detail the importance of pro-inflammatory molecules in regulating emergency hematopoiesis and the influence of both mature hematopoietic and non-hematopoietic cells on this process.


*The paper showed that the production of innate immune cells of lymphoid as well as myeloid lineage can occur following TLR-mediated HSC/HPC differentiation.*


**60. Ratajczak MZ, Adamiak M, Plonka M, et al. Mobilization of hematopoietic stem cells as a result of innate immunity-mediated sterile inflammation in the BM microenvironment-the involvement of extracellular nucleotides and purinergic signaling. Leukemia. 2018;32(5):1116-23. This is a great review to understand the role of DAMPs in regulating hematopoiesis.


*65. Lee YK, Kang M, Choi EY. TLR/MyD88-mediated Innate Immunity in Intestinal Graft-versus-Host Disease. Immune Netw. 2017;17(3):144-51. TLRs are being shown to play a big role in Graft-versus-Host disease leading many people to propose the use of TLR agonists or antagonists to regulate disease progression and/or severity.

*66. Ranganathan P, N'gakue A, Zitzer NC, et al. Serum miR-29a Is Upregulated in Acute Graft-versus-Host Disease and Activates Dendritic Cells through TLR Binding. J Immunol. 2017;198(6):2500-12. TLRs are being shown to play a big role in Graft-versus-Host disease leading many people to propose the use of TLR agonists or antagonists to regulate disease progression and/or severity.

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