Investigating Mechanisms of β-glucan-induced Trained Immunity in the Bone Marrow

Alexandre Grant
Supervisor: Dr. Maziar Divangahi

Faculty of Medicine and Health Sciences
Department of Microbiology and Immunology
Meakins-Christie Laboratories, RI-MUHC
McGill University, Montréal, Québec, Canada

April 2022

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master of Science (MSc)

Table of Contents

1. Abstract

Innate immune memory, or trained immunity, is characterized by the epigenetic and metabolic reprogramming of innate immune cells upon primary stimulation, leading to enhanced responsiveness or protection upon secondary stimulation. Bacille Calmette-Guérin (BCG), the live-attenuated vaccine against *Mycobacterium tuberculosis* (*Mtb*), and β-glucan, a glucose polymer found in the cell wall of fungi, are known to induce trained immunity. Our group has previously shown that BCG and β-glucan reprogram bone marrow (BM) hematopoietic stem cells (HSC) to induce trained immunity against *Mtb* infection. Interestingly, the virulent strain of *Mtb* impairs HSC-mediated trained immunity. However, it remains unclear how HSC reprogramming can be transmitted to progeny immune cells to generate trained immunity. We hypothesize that β-glucan can only induce trained immunity in HSC and not in fully differentiated myeloid cells (e.g., macrophages), and that β-glucan-induced reprogramming of HSC is epigenetically transmitted to trained myeloid cells. To test this hypothesis, we aimed to determine (i) at what stage of myelopoiesis β-glucan induces trained immunity; and (ii) how the reprogramming of BM HSC is transmitted downstream to myeloid cells using the tamoxifen-inducible reporter mice $Fgd5^{ZsGreen\cdot CreERT2}$ x Ai9 ($Fgd5^{ZsGreen\cdot CreERT2}$/tdTomato). Our data showed that exposure of BM HSC to β-glucan led to the generation of trained bone marrow-derived macrophages (BMDM) with an increased capacity to produce TNF-α and IL-6 upon LPS challenge *in vitro*. However, the direct exposure of fully differentiated BMDM to β-glucan did not generate trained immunity in macrophages *in vitro*. We also found that a single injection of β-glucan in mice leads to HSC and progenitor expansion in the BM, enhancing granulopoiesis and neutrophil production. Finally, using our *in vivo* tdTomato fate mapping model allowed us to track the contribution of long-term HSC (LT-HSC) to hematopoiesis in the BM following β-glucan administration. Collectively, these

results suggest that the plasticity of HSC is critical for the epigenetic imprinting and induction of trained immunity, and that fully differentiated macrophages lack this plasticity. Combining transcriptomic and epigenomic approaches with our fate mapping model of β-glucan-mediated trained immunity will help to elucidate the transmission of key trained immunity epigenetic signatures from BM HSC to progenitor and immune cells.

2. Résumé

La mémoire immunitaire innée, ou immunité entraînée, est caractérisée par la reprogrammation épigénétique et métabolique des cellules immunitaires innées par un premier stimulus, engendrant une réactivité ou une protection accrue à la suite d'un deuxième stimulus. Le Bacille Calmette-Guérin (BCG), le vaccin atténué contre le *Mycobacterium tuberculosis* (*Mtb*), et le β-glucane, un polymère de glucose présent dans la paroi cellulaire des champignons, sont connus pour leur capacité à induire l'immunité entraînée. Notre groupe a précédemment démontré que le BCG et le β-glucane reprogramment les cellules souches hématopoïétiques (HSC) de la moelle osseuse (BM) pour induire l'immunité entraînée contre l'infection par *Mtb*. De plus, la souche virulente de *Mtb* inhibe l'immunité entraînée au niveau des HSC. Cependant, on ne sait toujours pas comment la reprogrammation des HSC est transmise aux cellules immunitaires descendante pour générer l'immunité entraînée. Nous émettons l'hypothèse que le β-glucane ne peut induire une immunité entraînée que dans les HSC et non dans les cellules myéloïdes entièrement différenciées (par exemple, les macrophages), et que la reprogrammation induite par le β-glucane des HSC est transmise épigénétiquement aux cellules myéloïdes entraînées. Pour tester cette hypothèse, nous avons cherché à déterminer (i) à quel stade de la myélopoïèse le β-glucane induit l'immunité entraînée ; et (ii) comment la reprogrammation de HSC dans la BM est transmise aux cellules myéloïdes descendantes à l'aide de souris transgéniques présentant une expression inductible par le tamoxifène nommées Fgd5$^{ZsGreen\cdot CreERT2}$ x Ai9 (Fgd5$^{ZsGreen\cdot CreERT2}$/tdTomato). Nos données ont démontré que l'exposition de HSC dans la BM au β-glucane génère des macrophages dérivés de la moelle osseuse (BMDM) ayant une capacité accrue à produire du TNF-α et de l'IL-6 lors d'une stimulation *in vitro* avec du LPS. Cependant, l'exposition directe de BMDM entièrement différenciés au β-glucane n'a pas généré d'immunité entraînée dans les macrophages *in vitro*. Nous

avons également constaté qu'une seule injection de β-glucane chez la souris entraîne une expansion des HSC et des progéniteurs dans la BM, améliorant ainsi la granulopoïèse et la production de neutrophiles. Enfin, l'utilisation de notre modèle de souris transgéniques tdTomato nous a permis de suivre la contribution des HSC à long terme à l'hématopoïèse dans le BM après l'administration de β-glucane. Collectivement, ces résultats suggèrent que la plasticité des HSC est essentielle pour permettre l'empreinte épigénétique et la reprogrammation de l'immunité entraînée, et que les macrophages entièrement différenciés manquent de cette plasticité. La combinaison d'approches transcriptomiques et épigénomiques avec notre modèle transgénique aidera à examiner la transmission des signatures épigénétiques de l'immunité entraînée par le β-glucane des HSC dans la BM aux progéniteurs et aux cellules immunitaires.

3. Author Contributions

MD conceived the study. MD and AG designed the experiments. AG performed experiments with technical assistance from EP, NK, ML, MS, and KT. AG performed data analysis. AG and MD wrote the thesis.

4. Acknowledgements

Thank you to my supervisor, Dr. Maziar Divangahi, for his mentorship and encouragement throughout my studies. Training under his guidance has made me a better scientist, presenter, and writer. I am also grateful for his support for my work outside the lab and for helping me achieve my academic goals.

Thank you to the laboratory mates who have made my time in this laboratory such an enriching and enjoyable experience. Thank you to Dr. Erwan Pernet for going above and beyond as a teacher, for sharing his knowledge with me, and for keeping the French language alive in the laboratory. Thank you to Dr. Nargis Khan for all her insight and for her positive outlook. Thank you to Kim Tran and Mina Sadeghi for their help, support, and impeccable sense of humor. Thank you to everyone who has come and gone during my time in the lab for having made this such a memorable experience.

Thank you to my parents, Linda and Philippe, and my siblings, Jonathan and Emilie, who have never doubted me, loved me unconditionally, and encouraged me to pursue my passions. Thank you to Ricky for the laughs and scrumptious meals that have sustained me throughout my degree. Thank you to my dear friends Camille, Spencer, Jess, Jillian, Ida, Tania, Léanne, and Zoë. To my family and friends, thank you for having been there when I needed you most. I owe everything to you.

5. List of Abbreviations

BCG	-Bacille Calmette-Guérin
Mtb	-*Mycobacterium tuberculosis*
BM	-bone marrow
HSC	-hematopoietic stem cell
BMDM	-bone marrow-derived macrophage
TNF	-tumor necrosis factor
IL	-interleukin
PAMP	-pathogen-associated molecular pattern
PRR	-pattern recognition receptor
TLR	-Toll-like receptor
IFN	-interferon
NK	-Natural Killer
LPS	-lipopolysaccharide
H3K4me1/2/3	-histone 3 lysine 4 mono/di/trimethylation
H3K4Ac	-histone 3 lysine 4 acetylation
DC	-dendritic cell
WT	-wild-type

MPP	-multipotent progenitor
LKS	-lineage^{-} c-Kit^{+} Sca-1^{+}
HSPC	-hematopoietic stem and progenitor cells
GM-CSF	-granulocyte-macrophage colony-stimulating factor
DNA	-deoxyribonucleic acid
RNA	-ribonucleic acid
LT-HSC	-long-term hematopoietic stem cell
ST-HSC	-short-term hematopoietic stem cell
MPP3	-multipotent progenitor 3
MPP4	-multipotent progenitor 4
CMP	-common myeloid progenitor
CLP	-common lymphoid progenitor
GMP	-granulocyte-macrophage progenitor
MEP	-megakaryocyte-erythroid progenitor
GP	-granulocyte progenitor
LDH	-lactate dehydrogenase
LPM	-large peritoneal macrophages
SPM	-small peritoneal macrophages

TF -transcription factor

6. Chapter 1: Introduction

6.1. Trained Immunity

6.1.1. Evolutionary and Epidemiological Evidence for Trained Immunity

The mammalian immune system has traditionally been divided into the innate system, which provides non-specific protection, and the adaptive system, which provides antigen-specific protection and immunological memory(*1*). However, recent evidence has demonstrated that the innate immune system can in fact acquire non-specific memory upon primary stimulation, leading to increased responsiveness or protection upon a secondary stimulation or infection. This innate immune memory has been termed trained immunity(*1*).

It has long been recognized that plants and invertebrates can maintain nonspecific immune memory despite lacking a conventional adaptive immune system. Namely, plants rely on systemic acquired resistance to generate whole-plant immunity and immune memory following a localized infection(*2*). This is at least partially mediated through mobile immune signals that generate long-lasting changes in methylation and acetylation patterns of DNA and histones that prime the plant for future infection(*2*). In juvenile *Crassostrea gigas* oysters, polyinosinic-polycytidylic acid injections conferred long-lasting anti-viral immunity for at least 126 days(*3*). Together, these findings indicate that the capacity for immune memory is an evolutionarily conserved trait, which is not limited to adaptive immunity.

In humans, the implementation of widespread vaccination programs led to the finding that certain vaccines could confer nonspecific protective effects against unrelated pathogens(*4*). The development and implementation of the Bacille Calmette-Guérin (BCG) vaccine was initiated a century ago(*5*). BCG is a live attenuated strain of *Mycobacterium bovis* that remains the only available vaccine against *Mtb*, the causative agent of tuberculosis(*5*). BCG appears to

protect children from the early manifestations of tuberculosis, while the protective efficacy against pulmonary tuberculosis in adults ranges from 0-80%(*6*). However, in 1927, BCG vaccination was first observed to provide broad, nonspecific protection to young children by reducing the mortality rate nearly by threefold(*7*). In the 1940s and 1950s, controlled trials in the United States and United Kingdom also found a reduction in overall mortality in children who had received the BCG vaccine(*8*). In 2003, a BCG scar and positive tuberculin reaction were associated with a reduction in child mortality in West Africa that could not be attributed to protection against tuberculosis(*9*). Other studies also found that the live attenuated vaccines for measles and polio may reduce mortality against other infectious diseases(*4*). Collectively, these studies support the idea that BCG can provide cross-protection against several pathogens.

6.1.2. Peripheral Trained Immunity

Unlike adaptive immunity which relies on somatic diversification of T and B cell responses, trained immunity relies on the epigenetic and metabolic reprogramming of innate immune cells (**Figure 1A**). Following a primary stimulus or infection and a return to steady state, trained immunity leads to a greater or faster immune response and protection following a secondary unrelated stimulus or infection(*10, 11*). This training program depends on the recognition of pathogen-associated molecular patterns (PAMP) by pattern recognition receptors (PRR), such as Toll-like receptors (TLR), C-type lectin receptor, and NOD-like receptors, or by host factors such as interferon (IFN)-γ(*11*).

Despite being one of the most administered vaccines in the world, the protective mechanisms of BCG remain incompletely understood, as vaccine-induced adaptive immunity does not entirely account for the protective effects of BCG vaccination against heterologous pathogens(*12, 13*). This has led to the investigation of BCG as an inducer of trained immunity

in innate cells, including monocytes, macrophages, natural killer (NK) cells, and neutrophils(*13-15*). For instance, peripheral blood mononuclear cells isolated from individuals 3 months following BCG vaccination produced more of pro-inflammatory cytokines, like tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 after stimulation with *Mtb* lysate and heterologous pathogens such as *Staphylococcus aureus* and *Candida albicans*(*12*). This was associated with increased expression of TLR-4 and CD11b and with distinct epigenetic alterations in CD14+ monocytes(*12*). In severe combined immunodeficient mice (lacking T and B cells), BCG vaccination provides protection against lethal *C. albicans* infection(*12*). Similarly, NK cells isolated from BCG-vaccinated individuals produced more pro-inflammatory cytokines in response to homologous and heterologous infectious stimuli(*15*). BCG-vaccinated mice were also protected against disseminated candidiasis partially through NK cells(*15*). In a yellow fever vaccine model in humans, BCG vaccination reduced viremia by epigenetically reprogramming monocytes(*16*).

In addition to providing protection against homologous and heterologous infectious agents, BCG vaccination has been shown to be protective in cancer. In non-muscle-invasive bladder cancer, the most common type of bladder cancer, BCG immunotherapy remains the gold-standard treatment for preventing disease recurrence and progression(*17*). BCG instillation into the bladder leads to both localized and systemic immune responses that encompass both innate and adaptive immune responses(*17*). Interestingly, repeated BCG instillations can increase the intensity of the innate response throughout the therapy, a "prime-boost" phenomenon that may be explained by BCG-induced training of innate cells(*17, 18*).

In 1986, Bistoni *et al.* demonstrated that previous infection with an agerminative *C. albicans* strain conferred protection against virulent *C. albicans*, but also against *S. aureus*

infection in a T and B cell-independent manner(*19*). In 2012, Quintin *et al.* recapitulated the finding that nonlethal candidiasis conferred protection against lethal candidiasis independently of T and B cells(*20*). This protection was dependent on the functional reprogramming of monocytes and macrophages by exposure to purified β-glucan specifically, a main component of the cell wall of yeast and fungi that comprises approximately 60% of the *C. albicans* cell wall(*20, 21*). β-glucan-trained monocytes produced more proinflammatory cytokines (e.g., IL-6 and TNF-α) upon secondary stimulation with lipopolysaccharide (LPS), an effect that lasted at least two weeks after the initial training program(*20*). β-glucan-induced training of monocytes is dependent on the Dectin-1 receptor and is associated with stable epigenetic and transcriptomic alterations(*20, 22*). β-glucan-mediated training of monocytes induces a distinct metabolic signature, including increased glycolysis, glutaminolysis, and cholesterol synthesis(*23, 24*). Both BCG- and β-glucan-trained monocytes and macrophages are epigenetically imprinted via the trimethylation of histone 3 lysine 4 (H3K4me3) and acetylation of histone 3 lysine 4 (H3K4Ac) of promoter and enhancer elements, as well as at sites of key proinflammatory genes such as those encoding TNF and IL-6(*25-27*). β-glucan training of human monocytes also leads to transcriptomic alterations associate with increased production of TNF-α and IL-6 against *Mtb*(*28*).

Like BCG, β-glucan has been shown to possess antitumor activity in various tumor models, including in an inflammatory monocyte-dependent metastatic melanoma model and in a neutrophil-dependent Lewis lung carcinoma and melanoma model(*29, 30*). Dendritic cells (DC) have also been shown to be capable of acquiring a memory-like capacity through exposure to fungi, although a specific role for β-glucan has not been established(*31*). Previous fungal infection with a *Cryptococcus neoformans* strain engineered to express murine IFN-γ

provided long lasting protection against infection with wild-type (WT) *C. neoformans* via the functional reprogramming of DCs that was abrogated by specific histone modification inhibitors(*31*).

6.1.3. *Central Trained Immunity*

Trained immunity was first described as the reprogramming of mature innate immune cells(*1*). However, this definition fails to encompass the short-lived nature of these cells relative to the long-lasting protective effects of trained immunity(*1*). In contrast, hematopoietic stem cells (HSC) are long-lived self-renewing cells residing in the bone marrow (BM), where they generate multipotent progenitors (MPP) and lineage-committed progenitors that differentiate into all cells of the hematopoietic system, including mature innate immune cells(*32*). It was recently found that access of BCG to the BM of mice leads to the expansion of hematopoietic lineage$^-$ c-Kit$^+$ Sca-1$^+$ (LKS) progenitors, enhanced myelopoiesis, and significant transcriptomic alterations of HSC in an IFN-γ-dependent manner(*32*). These reprogrammed HSC gave rise to epigenetically and transcriptionally altered BM-derived macrophages (BMDM) that were more protective against virulent *Mtb* infection(*32*). Interestingly, virulent *Mtb* has also evolved immune evasion mechanisms that impair trained immunity in the BM by

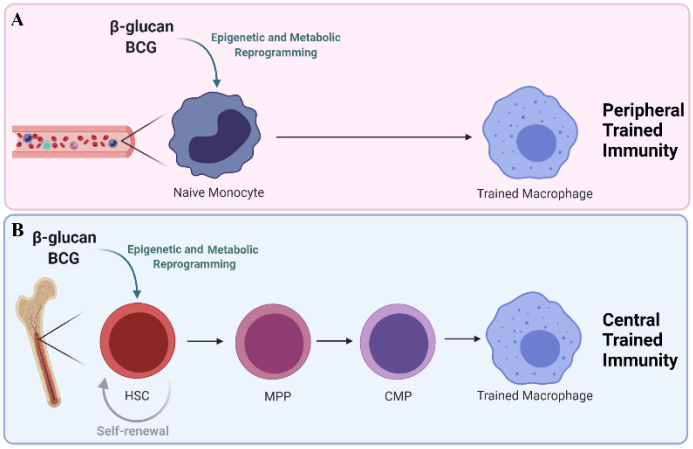

Figure 1. Central and peripheral trained immunity. **A.** Peripheral trained immunity relies on the epigenetic and metabolic reprogramming of differentiated innate immune cells. **B.** Central trained immunity relies on the epigenetic and metabolic reprogramming of HSC.

reprogramming HSC to limit myelopoiesis and inducing cell death in myeloid progenitors via type I IFN(*33*).

β-glucan is also a potent inducer of trained immunity at the level of hematopoietic stem and progenitor cells (HSPC), which contain multipotent stem cells and lineage-committed progenitors, in the BM. β-glucan induces the expansion of myeloid-biased hematopoietic stem and progenitor cells (HSPC) through granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-1β(*28, 34*). Specifically, β-glucan administration in mice enhances myelopoiesis and alters HSPC metabolism, including glycolysis and cholesterol biosynthesis(*34*). These changes in the BM were associated with long-term protection against LPS-induced DNA damage in HSPC, chemotherapy-induced myeloablation, and *Mtb* infection(*28, 34*). These studies suggest a key role for HSPC in trained immunity, although it remains unclear how β-glucan-induced changes in HSPC are transmitted to mature myeloid cells (**Figure 1B**).

6.2. β-glucans

6.2.1. β-glucans in Nature

For centuries, mushrooms have been consumed across civilizations for their apparent health benefits(*35*). Namely, many fungal and plant-derived bioactive polysaccharides used in Traditional Chinese Medicine have been investigated for their ability to activate the immune system, stimulate hematopoiesis, as well as protect against cancer and infection(*36*). In fungi, including mushrooms and yeast, β-glucans have been identified as a key bioactive component of capable of modulating the immune system(*35*). The immunogenicity of β-glucans varies depending on the source due to differences in their molecular structure and branching patterns, and higher degrees of structural complexity are associated with increased protective antitumor and antimicrobial effects(*37*).

Glucans are an incredibly diverse class of polysaccharides composed of D-glucose monomers that are found all throughout nature, from cereals, seaweeds, and mushrooms to yeasts, fungi, and bacteria(*38*). Glucans vary in their molecular structure depending on their length, molecular weight, branching patterns, and α or β isomers, all of which differ depending on their source(*38, 39*). In turn, these impact their physical and biological properties, including their solubility and ability to modulate the immune system(*39*). Cellulose, an essential component of plants and the most abundant organic polymer, is composed of linear β-1,4-glucan(*40*). Cereals possess mixed linear β-1,3- and β-1,4-glucan that has the distinct physiological properties of reducing plasma cholesterol by binding to bile acid and improving control over glucose and insulin responses following a meal(*38, 41*). In addition to being used as dietary fiber supplements in the food industry, cereal β-glucans are also used to modify the physical properties of certain foods by increasing their viscosity or as a low-calorie fat mimetic(*38, 42*). Yeast and fungi possess mixed linear β-1,3-glucan with β-1,6-glucan branches and have been studied for their immunomodulatory effects, particularly β-1,3-glucan from the yeast *Saccharomyces cerevisiae* (henceforth referred to as β-glucan) (*38, 39*).

6.2.2. β-Glucan and the Immune System

β-glucan is integral to the cell wall of pathogenic fungi and is therefore an important PAMP that is recognized by the immune system during fungal infections, activating the host immune system in both vertebrates and invertebrates(*43, 44*). β-glucan from yeast such as *S. cerevisiae* is mainly composed of β-1,3-glucan and is primarily recognized by Dectin-1, a C-type lectin PRR expressed by myeloid cells (monocytes, macrophages, DC, and neutrophils) and a subset of hematopoietic progenitor cells(*20, 45-48*). However, other PRR have been implicated in the recognition of β-glucan and in the orchestration of the immune response. For instance,

recognition of β-glucan by Dectin-1 alone is sufficient to induce phagocytosis, reactive oxygen species (ROS) generation, and microbial killing(*49*). Yet, signaling between Dectin-1, TLR2, and TLR4 is required for a robust induction of cytokine production(*50*). In addition, immune cells may be able to distinguish between particulate and soluble β-glucan through the clustering and activation of Dectin-1, leading to the formation of a so-called "phagocytic synapse". This function appears to be important in distinguishing between direct contact with fungal pathogens and fungal fragments, thus increasing the effectiveness of the phagocytic and ROS response and limiting excessive inflammation(*48*). While Dectin-1 is the major fungal PRR in macrophages, it is complement receptor 3 (CR3) that primarily mediates the immunomodulatory effects of β-glucan in neutrophils by triggering phagocytosis and degranulation in response to iC3b-opsonized yeast(*49, 51, 52*). β-glucan binding to CR3 on phagocytes enhances cytotoxicity against iC3b-opsonized cells, including tumors(*53, 54*). Indeed, upon oral administration, intestinal macrophages phagocytose β-1,3-glucan via Dectin-1 and traffic to lymphoid tissues, including the spleen, lymph nodes, and BM, where they degrade large β-1,3-glucan particles into soluble β-glucan fragments that are taken up by granulocytes via CR3(*55*).

6.3. Hematopoiesis

6.3.1. Hematopoiesis at Steady State

Hematopoiesis is the lifelong process by which short-lived circulating cells of the hematopoietic system are replenished by long-lived HSPC found primarily in the BM niche(*56*). Hematopoiesis is a process dependent on the capacity of LT-HSC to self-renew, generate multipotent and lineage-committed progenitors, and differentiate into all mature cell lineages that mediate essential homeostatic functions such as organ oxygenation, clotting, and

immunity(*57, 58*). At homeostasis, quiescent LT-HSC undergo symmetric division to self-renew or asymmetric division and differentiation to produce activated short-term HSC (ST-HSC) with a low self-renewal capacity. ST-HSC further differentiate into myeloid-biased multipotent progenitors (MPP3) or lymphoid-biased multipotent progenitors (MPP4)(*32, 59*). These multipotent progenitors (MPP) then give rise to differentiated progenitors that are increasingly committed to a lineage, such as common myeloid progenitors (CMP) that can differentiate into megakaryocyte-erythroid progenitors (MEP), which give rise to red blood cells and platelets, or granulocyte-macrophage progenitors (GMP), which give rise to innate immune cells(*32*). On the other hand, common lymphoid progenitors (CLP) give rise to adaptive immune cells (**Figure 2**)(*32*).

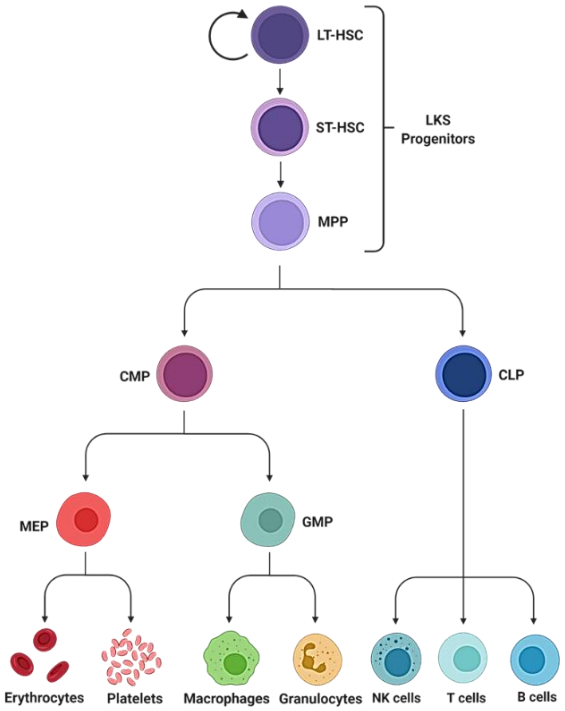

Figure 2. LT-HSC are the foundation of hematopoiesis at steady state. Long-lived, self-renewing LT-HSC produce all multipotent and lineage-committed progenitors and mature cells of the hematopoietic system in the BM.

To produce sufficient hematopoietic cells throughout the lifetime of an individual, HSPC function is closely regulated by autocrine signaling, but also by juxtacrine and paracrine signaling from stromal, neuronal, and hematopoietic cells within the BM niche(*56, 60*). Fibroblasts, endothelial cells, and osteoblasts provide signals to HSPC via hematopoietic growth factors, adhesion molecules, cytokines, and chemokines(*57*). In addition, macrophages residing in the BM have also been shown to promote the retention of HSPC within the BM(*60*).

On the other hand, circadian adrenergic signals from BM sympathetic nerves regulate the cyclical egress of HSC into the bloodstream(*61*). Nonmyelinating Schwann cells wrapped around these sympathetic nerves have also been found to maintain HSC quiescence(*62*).

6.3.2. Hematopoiesis and Stress

In addition to maintaining the pool of circulating hematopoietic cells, HSPC respond rapidly to stressors such as hypoxia, inflammation, and infection to meet the increased demand for blood and immune cells(*56*). These hematopoietic responses must be equally well regulated to prevent hematopoietic malignancies or HSPC aging and exhaustion(*63-65*). For instance, hypoxia induces the release of erythropoietin, leading to an increased production of erythrocytes to meet organs' oxygenation demands(*66*). During acute systemic bacterial infections, secreted host factors such as chemokines, cytokines, and growth factors stimulate HSPC to induce emergency myelopoiesis to produce immune cells that fight infection(*67, 68*). Cytokines, including type I and II IFN and TNF-α, orchestrate HSC activation and proliferation during infection(*68*). In trained immunity, BCG-induced myelopoiesis is dependent on type II IFN and β-glucan-induced myelopoiesis is dependent on IL-1β, while *Mtb* inhibits trained immunity and myelopoiesis via type I IFN(*28, 32, 33*). Thus, a unique set of cytokine networking can either promote or prevent trained immunity in the BM.

In addition to cytokines, HSPC also express PRR, including TLRs and Dectin-1, which allow them to directly sense and respond to PAMP and endogenous danger-associated molecular patterns (DAMPs) in the BM leading to cell cycle entry and myeloid differentiation(*67*). For instance, TLR2 is required for the expansion of HSPC in response to *C. albicans* infection, while Dectin-1 stimulation of HSPC promotes the differentiation of macrophages *in vitro*(*69, 70*). Interestingly, orally administered β-1,3-glucan can be taken up

by macrophages and transported to the BM where soluble β-1,3-glucan fragments are released(*55*). This finding is particularly relevant for the role of β-glucan in inducing trained immunity at the level of the BM, as BCG access to the BM is required for the reprogramming of HSPC in trained immunity. However, the impact of β-glucan on the reprogramming of BM HSPC remains incompletely understood, and it remains unclear whether β-glucan-induced trained immunity is dependent upon β-glucan access into the BM.

6.4. Macrophages

6.4.1. Macrophage Ontogeny

Macrophages are mononuclear phagocytes and are essential to tissue homeostasis and immunity, carrying out tissue-specific functions and protecting the host against infection(*71*). Macrophages vary in their tissue localization, function, and ontogeny(*71*). During early embryogenesis, the first macrophages arise through primitive hematopoiesis, generating yolk sac-derived primitive erythroblasts, megakaryocytes, and macrophages that seed the central nervous system to become microglia without going through a monocyte intermediate(*71-74*). Primitive hematopoiesis is quickly followed by transient hematopoiesis, during which erythro-myeloid precursors migrate into the fetal liver, from which the first fetal monocytes circulate and become tissue-resident macrophages by colonizing every tissue, except the brain(*75, 76*). Subsequently, HSC-dependent hematopoiesis, or definitive hematopoiesis, generates fetal liver-derived monocytes that continue to seed various tissues to become tissue-resident macrophages, namely alveolar macrophages in the lung, Langerhans cells in the epidermis, and Kupffer cells in the liver(*75, 77, 78*). At steady state during adulthood, tissue-resident macrophage populations are imprinted by their local environment and are maintained independently of circulating cells by relying on longevity or self-renewal(*79*). However, BM

HSC can contribute to the pool of tissue-resident macrophages, either through gradual replacement with aging or by recruitment to sites of inflammation where they can permanently contribute to the tissue macrophage population(*71, 79*).

6.4.2. Macrophage Function

At steady state, tissue-resident macrophages perform essential developmental and homeostatic functions. During embryogenesis, tissue-resident macrophages regulate neurogenesis, angiogenesis, osteogenesis and bone remodeling, and clearance of cellular debris during limb morphogenesis(*80-83*). In adulthood, resident macrophages are imprinted by their local environment to perform tissue-specific functions. For instance, Kupffer cells reside along sinusoidal endothelial cells in the liver, where they clear pathogens originating from the gut, contribute to iron, cholesterol, and bilirubin metabolism, and promote tissue repair(*78*). Alveolar macrophages occupy the alveolar space within the airway lumen, clearing surfactant and orchestrating the initiation and resolution of inflammation in response to pathogens and pollutants in the airway(*84*). In the peritoneal cavity, embryonically seeded large peritoneal macrophages (LPM) phagocytose cellular debris and bacteria, regulate the production of IgA by peritoneal B-1 cells, and participate in inflammatory responses(*85*). On the other hand, small peritoneal macrophages (SPM), which are constantly replenished by circulating monocytes, primarily mediate inflammatory responses in the peritoneal cavity(*85*).

Despite being imprinted by the tissues they occupy, macrophages also require plasticity to initiate, participate in, and resolve tissue inflammation(*86*). Traditionally, the ability of macrophages to adapt to changes in the tissue niche has been broadly categorized using the classically activated M1 macrophage or alternatively activated M2 macrophage dichotomy(*86*). M1 macrophages are generated via the recognition of LPS or by

proinflammatory cytokines such as IFN-γ, leading to a switch to glycolytic metabolism, the expression of inducible nitric oxide synthase, and the production of pro-inflammatory cytokines(*87-90*). M1 macrophages play a role in pathogen killing and tumor resistance(*91*). On the other hand, M2 macrophages are generated in response to anti-inflammatory signals such as IL-4 or IL-13, leading to a switch to oxidative phosphorylation, the expression of arginase-1, and the production of anti-inflammatory cytokines(*88, 92, 93*). M2 macrophages participate in the resolution of inflammation and tissue repair(*93*). Interestingly, macrophages that have polarized towards either M1 or M2 activation retain the plasticity to switch from one type to another, and this polarization should not be viewed as a strict binary but rather as a functional spectrum(*94, 95*). Macrophages are therefore capable of specializing depending on their localization and on the transient needs of the tissue they occupy(*96*).

6.4.3. Epigenetic and Transcriptomic Regulation of Macrophages

Macrophage specialization is dependent on ontogeny and local signals that induce epigenetic and transcriptional signatures that govern gene expression(*96*). Epigenetics refers to the DNA methylation and hydroxymethylation, post-translational histone modifications (methylation, acetylation, and phosphorylation), and non-coding RNA that govern the expression of genes without altering the genome(*95*). The epigenetic landscape of cells is dynamic to allow cells to differentiate and respond to stimuli, but remains stable enough to impart a specific cellular identity and memory to cells(*95*). For instance, monocyte-derived intestinal macrophages share similar H3K4me1-and H3K4me2-marked enhancers to circulating monocytes(*96*). At the same time, monocyte-derived SPM and embryonically-derived LPM share similar expression patterns that are imprinted by their shared tissue environment(*96*). Epigenetic signatures regulate the expression of specific genes by altering

the binding accessibility and localization of transcription factors (TF) in response to stimuli(*95*). In macrophages, the pioneer TF PU.1 occupies most genomic enhancers marked by H3K4me1, thus inducing and maintaining macrophage identity by controlling the entire genomic regulatory landscape of macrophages(*97*). In turn, the recruitment of stimulus-triggered TF to these "poised" enhancers and promoters regulate the expression and chromatin remodeling that govern tissue-specific functions and responses. For instance, heme induces TF that are important for red pulp macrophages to degrade senescent erythrocytes and recycle iron in the spleen(*98, 99*). In the peritoneal cavity, omental adipose tissue produces retinoic acid that stimulates the expression of GATA6, a TF that regulates LPM(*100*). M1 and M2 polarization is also controlled by various TF such as STAT1, STAT6, and PPARγ(*101*). Epigenetic and transcriptomic signatures are therefore essential in dynamically shaping macrophage identity, function, and responses(*96*). In trained immunity, epigenetic reprogramming of monocytes confers enhanced protection against infection, demonstrating that these signatures also provide cellular memory to innate immune cells via the persistence of epigenetic modifications at the site of latent or de novo enhancers after a training stimulus(*102*). Upon restimulation, these enhancers can undergo a stronger activation, thus allowing for a better secondary response to stimulation or infection(*102*).

6.5. Central Hypothesis and Aims

The central hypothesis of the current study is that **the induction and long-term maintenance of β-glucan-induced trained immunity is mediated by the epigenetic and transcriptomic reprogramming of HSC that generate trained myeloid cells.** To test this hypothesis, we have designed the following aims:

i. Determine at what stage of myelopoiesis β-glucan induces trained immunity.

ii. Determine how β-glucan-mediated reprogramming is transmitted from hematopoietic stem cells to myeloid cells.

7. Chapter 2: Materials and Methods

7.1 Mice

Six- to ten-week-old C57BL/6 mice (Jackson Lab) were bred in-house, and males were used for all experiments. Fgd5$^{ZsGreen\cdot CreERT2}$, Ai9, and Fgd5$^{ZsGreen\cdot CreERT2}$/tdTomato (Jackson Lab) mice were bred in-house, and males were used for all experiments except for Fgd5$^{ZsGreen\cdot CreERT2}$/tdTomato blood immunophenotyping following β-glucan administration. Given the low frequency of HSPC in the BM, male mice were used to ensure sufficient cell numbers for experiments, as female mice have approximately half the number of total BM cells. All experiments were conducted in accordance with the guidelines of the Animal Research Ethics Board of McGill University.

7.2 Extraction of BM Cells

Femora and tibiae were harvested from mice by cutting ends off bones and flushing with a total of 20ml RPMI using a syringe and needle. For the generation of BMDM, all manipulations were done aseptically. Extracted BM was homogenized by repeated aspiration with a syringe and needle, centrifuged at 1500rpm for 5 min, and resuspended in 1ml ACK lysis buffer for 2 minutes at room temperature. BM cells washed with 20ml PBS, centrifuged at 1500rpm for 5min, and resuspended in 1ml RPMI for counting. For the generation of BMDM, BM cells were not resuspended in ACK lysis buffer and were instead resuspended in 1ml BMDM medium.

7.3 Generation of BMDM

BMDM were prepared using aseptically extracted BM cells seeded in 10ml BMDM medium (RPMI supplemented with 10% FBS, 2 mM L-glutamine, 1% MEM, 1% NEAA, 1 mM sodium pyruvate, 2% HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin Gibco,

Invitrogen) containing 30% L929 supernatant in petri dishes. BM cells were allowed to differentiate for 6 days, and 10 ml of fresh BMDM medium containing 30% L929 supernatant was added to each petri dish at day 3. From day 7 onwards, BMDM medium was used. Purity was previously assessed at >99% by flow cytometry.

7.4 *In Vitro* Training of HSPC and BMDM

β-glucan (Sigma-Aldrich #G5011) was suspended in PBS at a concentration of 1mg/ml and suspended by repeated aspiration using a syringe and needle.

For HSPC training, BM cells were aseptically extracted and seeded in 10ml BMDM medium containing 30% L929 supernatant with β-glucan (Sigma-Aldrich #G5011) at various concentrations. After 24h hours, supernatant was collected and petri dishes were washed with 10ml PBS 3 times, and PBS was added to supernatant. The supernatant with PBS was then centrifuged at 1500rpm for 5 min and washed in 10ml PBS, and this was repeated twice. BM cells were reseeded into their original petri dishes with 10ml BMDM medium with 30% L929 for 5 days, with fresh BMDM medium containing 30% L929 supernatant added after 2 days. From day 7 onwards, BMDM medium was used, and cells were rested for a total of 4 days before being stimulated. A day before the stimulation, BMDM were collected using 4ml Cellstripper (Corning), resuspended in 1ml BMDM medium for cell counting, and seeded in a 96-well plate ($1x10^5$ cells per well).

For BMDM training, BMDM were generated as previously described. On day 7, macrophages were incubated with BMDM medium with β-glucan at various concentrations. After 24 hours, petri dishes were washed with 10ml PBS 3 times and cells were allowed to rest in BMDM medium for 3 days before being stimulated. A day before the stimulation, BMDM

were collected using 4ml Cellstripper (Corning), resuspended in 1ml BMDM medium for cell counting, and seeded in a 96-well plate ($1x10^5$ cells per well).

The lactate dehydrogenase (LDH) release assay (Thermo Fisher Scientific #C20302) was used to assess cytotoxicity following β-glucan treatment in HSPC and BMDM training according to the manufacturer's instructions.

7.5 Detection of Cytokines

Supernatant from BMDM was collected at various time points following stimulation and stored at -20°C. The secretion of IL-6 and TNF-α in the supernatant was measured using commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems) according to the manufacturer's instructions.

7.6 *In Vivo* Training

β-glucan (Sigma-Aldrich #G5011) was suspended in PBS at a concentration of 10mg/ml and suspended by repeated aspiration using a syringe and needle. At day 0, mice were intraperitoneally injected with 1mg β-glucan in 100µl PBS or PBS control. Organs were collected at various time points following the injection.

7.7 Fate Mapping of LT-HSC

Tamoxifen (Sigma-Aldrich #T5648) was dissolved in sterile corn oil in 10% ethanol at a concentration of 12.5mg/ml and stored at -20°C. $Fgd5^{ZsGreen\cdot CreERT2}$ /tdTomato male and female mice were intraperitoneally injected with 1.25mg tamoxifen in 100µl corn oil with 10% ethanol or vehicle once a day for 5 days and allowed to rest for 2 days. Saphenous vein blood was collected from female mice at various timepoints using heparinized capillaries. BM cells were

collected from male mice at various timepoints as previously described. All samples were protected from light immediately after being harvested.

7.8 Flow Cytometry

For BM staining, $3x10^6$ cells from each sample were stained in 96-well v-bottom plates following ACK lysis. For staining of LKS and progenitor cells from WT mice, cells were stained with fixable viability dye eFluor506 (eBioscience) at a dilution of 1:1000 in PBS for 30 minutes at 4°C. Cells were then washed with FACS buffer (0.5% BSA (Wisent) in PBS) and incubated with biotin-conjugated lineage markers (biotin anti-Ter119, biotin anti-CD11b, biotin anti-CD5, biotin anti-CD4, biotin anti-CD8, biotin anti-CD45R, and biotin anti-Ly6G/C) at a dilution of 1:100 in FACS buffer for 30 minutes at 4°C. Cells were then washed in FACS buffer and incubated with the following antibodies at a dilution of 1:100 in FACS buffer for 30 minutes at 4°C: streptavidin APC-efluor780, anti-cKit APC, anti-Sca-1 PE-Cy7, anti-CD150 V450, anti-CD48 BUV737 (1:50), anti-CD34 FITC (1:50), anti-Flt3 PE, anti-CD16/32 PerCP-eFluor 710, and anti-CD127 BV786.

For staining of LKS cells from $Fgd5^{ZsGreen\cdot CreERT2}$/tdTomato mice, cells were not stained with fixable viability dye, were stained with the same biotin-conjugated lineage markers as WT mice, and with the following antibodies: streptavidin APC-efluor780, anti-cKit APC, anti-Sca-1 BUV395, anti-CD150 V450, anti-CD48 BUV737 (1:50), anti-CD34 FITC (1:50), and anti-Flt3 PerCP-eFluor 710. For staining of progenitor cells from $Fgd5^{ZsGreen\cdot CreERT2}$/tdTomato mice, cells were not stained with fixable viability dye, were stained with the same biotin-conjugated lineage markers as WT mice, and with the following antibodies: streptavidin APC-efluor780, anti-cKit APC, anti-Sca-1 BUV395, anti-CD34 FITC (1:50), anti-CD16/32 PerCP-eFluor 710, and anti-CD127 BV786.

For staining of BM and peritoneal innate immune cells in WT mice, cells ($3x10^6$ cells for the BM and $0.5\text{-}1x10^6$ cells for the peritoneal cavity) were stained with fixable viability dye eFluor506 (eBioscience) as previously described and with the following antibodies at a dilution of 1:200: anti-CD45.2 APC, anti-CD11b BUV395, anti-CD11c PE-Cy7, anti-Ly6C FITC, anti-Ly6G PerCP-eFluor 710, anti-Siglec-F BV786, anti-NK1.1 BV650, anti-F4/80 APC-efluor780, and anti-CD16/32. For intracellular GATA6 staining, the Transcription Factor Fixation/Permeabilization Concentrate and Diluent kit (eBioscience # 00-5521-00) was used according to the manufacturer's instructions.

For blood staining of innate immune cells in $Fgd5^{ZsGreen\cdot CreERT2}$/tdTomato mice, 25µl of saphenous vein blood was stained in 25µl FACS buffer with the following antibodies at a dilution of 1:100 for 30 minutes at 4°C: anti-CD45.2 APC, anti-CD11b BUV395, anti-CD11c PE-Cy7, anti-Ly6C FITC, anti-Ly6G PerCP-eFluor 710, anti-Siglec-F BV786, and anti-NK1.1 BV650. For blood staining of adaptive immune cells in $Fgd5^{ZsGreen\cdot CreERT2}$/tdTomato mice, the following antibodies were used: anti-CD45.2 APC, anti-CD3 FITC, anti-CD4 V500, anti-CD8 AlexFluor700, anti-CD19 BUV395, and anti-CD49b V450. Samples were then suspended in 1ml ACK lysis buffer for 1 minute and washed in PBS.

Following staining, all samples were washed with FACS buffer and fixed and stored in 1% PFA at 4°C for a maximum of 72 hours. All antibodies used were from BD or eBioscience. All samples were acquired using a BD LSRFortessa X-20 flow cytometer with FACSDiva software (BD). Flow cytometry data were analyzed using FlowJo v10 (BD).

7.9 Statistical Analysis

Statistical analysis and visualization of flow cytometry data was performed using GraphPad Prism version 9.3.1 (GraphPad Software, San Diego, California, USA).

8. Chapter 3: Results

8.1. β-glucan-induced Trained Immunity *In Vitro*.

It has previously been shown that monocytes can be trained through direct exposure to β-glucan *in vitro*, leading to enhanced production of the proinflammatory cytokines TNF-α and IL-6 in response to a secondary stimulation (*1, 27, 28*). However, it remains unclear whether fully differentiated macrophages can be trained through direct exposure to β-glucan. We therefore generated BMDM from naïve WT C57BL/6J mice using 30% L929-conditioned media for six days. These BMDM were then treated with β-glucan at various concentrations (0.1, 1, 5, 10μg/ml) for 24 hours, washed, and allowed to rest for three days (**Figure 3A**). Across different β-glucan concentrations, no difference was observed in the number of BMDM or in the β-glucan-induced cytotoxicity as measured through the LDH release assay (**Figure 3B-C**). Upon stimulation with 100ng/ml LPS, no differences were observed in the secretion of TNF-α and IL-6 (**Figure 3D-E**). To test whether BMDM could be trained at an earlier stage of differentiation, whole BM, which contains heterogenous populations of innate and adaptive immune cells, structural cells, and HSPC, was treated with β-glucan at various concentrations (0.1, 1, 5, 10μg/ml) for 24 hours, washed, and used to generate BMDM (**Figure 3F**) (*70*). We found a significant decrease in the number of BMDM generated and a trend towards cytotoxicity at the highest β-glucan concentration (**Figure 3G-H**). To remain within the physiological range, the group treated with 10μg/ml β-glucan was excluded from future experiments. Upon stimulation with 100ng/ml LPS, BMDM generated from HSPC treated with 1 and 5μg/ml β-glucan secreted significantly more TNF-α and IL-6 at various time points, a signature of trained immunity (Figure 2I-J).

8.2. β-glucan-induced Trained Immunity *In Vivo*.

We have previously investigated the protective capacity of β-glucan in mice and found that two intraperitoneal (IP) injections led to LKS expansion and enhanced myelopoiesis in the BM, conferring significant protection against *Mtb* infection. However, it remains unknown how a single β-glucan injection strategy may induce trained immunity in mice. To this end, PBS or β-glucan (1mg) was administered IP and the HSPC response was assessed by flow cytometry at 1-, 2-, 4-, 5-, 7-, and 14-days post treatment (**Figure 4A**). Our results demonstrate a significant LKS expansion at 2- and 4-days post treatment. Although there was a trend towards increased frequency and number of LT-HSC (LKS+CD150+CD48-), the LKS expansion was primarily attributable to significant expansions in ST-HSC (LKS+CD150+CD48+), myeloid-biased MPP3 (LKS+CD150-CD48+CD34+Flt3-), and lymphoid-biased MPP4 (LKS+CD150-CD48+CD34+Flt3+). Interestingly, no expansion was found in the GMP (Lineage-CD127-Sca1-cKit+CD34+CD16/32+) as previously described in the two-dose β-glucan injection strategy(*28*). Instead, we found a significant decrease in the number of in the CMP (Lineage-CD127-Sca1-cKit+CD34+CD16/32-) and MEP (Lineage-CD127-Sca1-cKit+CD34-CD16/32-), and no differences were seen in the common lymphoid progenitors (CLP; Lineage-CD127+Sca1^{int}cKit+) (**Figure 4B-J**).

β-glucan-induced LKS expansion and myelopoiesis is dependent on IL-1β and GM-CSF signaling(*28, 34*). However, it is not known whether these effects are dependent on β-glucan accessing the BM. It has previously been shown that ingested β-glucan can be phagocytosed and shuttled to the BM by macrophages(*55*). To determine whether β-glucan may access the BM in our model, we immunophenotyped the peritoneal lavage fluid and BM of WT mice at 1- and 3-days following IP administration of PBS or β-glucan. Following β-glucan treatment,

we found a near-complete loss of LPM (CD45.2+NK1.1-CD11c-CD11b+Ly6G-F4/80^{hi}Sig-F+) and significant increase in SPM (CD45.2+NK1.1-CD11c-CD11b+Ly6G-F4/80^{lo}Sig-F-) in the peritoneal cavity at 1- and 3-days post treatment (**Figure 5A-C**). This finding was coupled with a significant increase in BM macrophages (CD45.2+NK1.1-CD11c-CD11b+Ly6G-F4/80+Sig-F-) at 1- and 3-days post treatment (**Figure 5D**). LPM are tissue-resident, self-renewing macrophages that can be identified by their expression of the transcription factor GATA6(*103*). To determine whether LPM egressed from the peritoneal cavity and entered the BM, potentially carrying phagocytosed β-glucan, we examined the presence of GATA6+ macrophages in these compartments. Our results showed a significant loss of GATA6+ macrophages (CD45.2+NK1.1-CD11c-CD11b+Ly6G-F4/80+Sig-F-GATA6+) in the peritoneal cavity following β-glucan treatment, but no changes in GATA6+ macrophages were observed in the BM (**Figure 5E-H**).

8.3. LT-HSC Fate Mapping

While β-glucan administration induces LKS expansion and enhances myelopoiesis, it is unknown if and how training-associated epigenetic alterations in LT-HSC may be maintained and passed down to downstream progenitor and innate immune cells such as monocytes, macrophages, and neutrophils. To this end, we have developed an *in vivo* fate mapping model by crossing Fgd5$^{ZsGr•CreERT2}$ and Ai9 mice (Jackson Lab) to generate Fgd5$^{ZsGr•CreERT2}$/tdTomato mice. In Fgd5$^{ZsGr•CreERT2}$/tdTomato mice, a subset of LT-HSC selectively expresses the embryonic development gene *Fgd5* which drives the expression of ZsGreen and the tamoxifen-inducible expression of Cre recombinase. Following tamoxifen administration, Cre recombinase can excise a loxP-flanked STOP sequence upstream of the CAG promoter-driven *tdTomato* allele, inducing the expression of tdTomato. After tamoxifen administration in

Fgd5$^{ZsGr•CreERT2}$/tdTomato mice, a subset of LT-HSC and their subsequent progeny cells become fluorescently tagged with tdTomato (tdTom+) (**Figure 6A**)(*104*). Thus. inducing the expression of tdTomato immediately prior to β-glucan administration enables the study of progenitor and effector immune cells that have been exclusively generated from LT-HSC exposed to β-glucan. To establish our model at homeostasis, we generated Fgd5$^{ZsGr•CreERT2}$/tdTomato mice, administered tamoxifen, and assessed the presence of tdTom+ immune cells in the blood at 7-, 14- ,21- ,28-, 42-, and 56-days post treatment (**Figure 6B**). Our results showed a gradually increasing proportion of tdTom+ immune cells in the blood, demonstrating that our model tracks the contribution and trafficking of progeny cells from LT-HSC in the periphery (**Figure 6C**).

We then aimed to assess how β-glucan administration may affect the contribution of LT-HSC to LKS expansion and myelopoiesis and to track downstream progeny cells generated from LT-HSC exposed to β-glucan. To this end, we treated Fgd5$^{ZsGr•CreERT2}$/tdTomato mice with tamoxifen to induce the expression of tdTomato in LT-HSC, followed by β-glucan and assessment of HSPC by flow cytometry at 0-, 3-, 7-, and 14-days post β-glucan treatment (**Figure 7A**). Our findings indicated that both tdTom+ and tdTom- LKS progenitors expanded at day 3 post treatment, and that this expansion was largely attributable to transient increases in ST-HSC and MPP3, and trending increases in MPP4 (**Figure 7B-J**). Our Fgd5$^{ZsGr•CreERT2}$/tdTomato model thus recapitulate our previous findings of LKS expansion and enhanced myelopoiesis found in WT mice.

9. Chapter 4: Discussion

Innate immune memory, or trained immunity, is currently defined by the long-term epigenetic and metabolic reprogramming of innate immune cells(*1*). However, these circulating cells are short-lived, a conundrum that has led to the investigation of long-lived, self-renewing HSC as key mediators of long-term trained immunity through the maintenance of training-associated epigenetic signatures and transmission to mature immune cells(*1*). Here, we have found that treating fully differentiated BMDM with β-glucan does not enhance their production of pro-inflammatory cytokines upon secondary stimulation *in vitro*. Yet, treating BM cells containing HSPC with β-glucan leads to the generation of trained BMDM with an enhanced capacity to produce IL-6 and TNF-α upon stimulation with LPS *in vitro*, a hallmark of trained immunity. Taken together, these findings suggest that fully differentiated macrophages may lack the plasticity to acquire training-associated epigenetic signatures and to become trained by direct exposure to β-glucan, but that HSPC can be trained to generate trained macrophages.

Monocytes are circulating precursors to macrophages and DC with an ability to specialize into functionally distinct subsets at steady state and in response to inflammation(*105, 106*). As intermediate cells between HSPC and monocyte-derived macrophages, circulating monocytes therefore possess the plasticity to acquire different functional programs(*22*). Previous studies have demonstrated that direct exposure of monocytes to β-glucan *in vitro* can epigenetically reprogram monocytes to become trained(*22, 23, 28, 107*). On the other hand, exposure to endotoxins such as LPS can induce tolerance, a functionally refractory state that can also be mediated by epigenetic alterations(*22, 108*). Interestingly, that epigenetic signature of LPS-tolerized monocytes can be reversed by early exposure to β-glucan, a clear demonstration of

the epigenetic plasticity of monocytes(*107*). However, it has not be determined whether fully differentiated macrophages are capable of such epigenetic plasticity to become trained upon direct exposure to β-glucan.

Macrophages have traditionally been considered to be highly plastic cells capable of switching from their tissue-specific homeostatic functions to an inflammatory state in response to infection(*109, 110*). Monocyte-derived macrophages must also adapt to their local tissue environment, a process that has been suggested could restrict the plasticity of macrophages within tissues(*110*). However, the role of epigenetic alterations in reprogramming tissue-specific macrophages remains unclear, as tissue-specific signals appear to supersede developmental origin in generating resident macrophages(*110, 111*).

Previous work has demonstrated that monocytes possess the epigenetic plasticity to become trained by direct exposure to β-glucan. Our *in vitro* results support the idea that epigenetically imprinted trained immunity must be induced at earlier stages of myeloid differentiation, and that this can occur as early as at the stage of HSPC in the BM. However, our *in vitro* approach does have some limitations. First, our HSPC treatment occurs in the presence of a heterogenous mix of cell populations, including mature innate and adaptive cells, that is not present in our BMDM treatment. It may therefore be possible that training is dependent on the presence of other cell populations. On the other hand, previous studies have demonstrated that monocytes alone are capable of being trained upon direct exposure to β-glucan *in vitro.* Second, our results show that β-glucan induces some cell death in our HSPC treatment, but not in BMDM training *in vitro*. It may be possible that cell death seen in HSPC treatment, but lacking in BMDM treatment, is required to the induction of trained immunity through cytokines such as IL-1β, given its established role in both β-glucan-induced trained

immunity and in caspase-1-dependent cell death(*112*). Despite the role of cell death in regulating hematopoiesis and immune cell function, it is unknown whether cell death can play a role in inducing trained immunity under specific conditions. Yet, *Mtb* has evolved the ability to impair training at the level of the BM by inducing cell death directly in myeloid progenitors through type I IFN(*33*).

Here, we have demonstrated that a single β-glucan injection strategy induces LKS expansion and enhanced myelopoiesis in the BM, similar to previously published work using a two-dose strategy. However, we did not find expansion in the CMP and GMP populations in this single-dose model. Recent work within our group has investigated whether the progenitor response may bypass the CMP and GMP to expand downstream progenitors within our single-dose injection model. We found a significant expansion in granulocyte progenitors at day 2 that was associated with an increased number of neutrophils in the BM at days 4 and 7 post treatment (Khan et al., unpublished). Together, these findings suggest that a single injection of β-glucan leads to LKS expansion and specifically enhances granulopoiesis and neutrophil production in the BM. However, it remains unclear whether these neutrophils are epigenetically or functionally altered to provide enhanced protection, and we are therefore currently investigating this question.

BCG access to the BM is essential for LKS expansion and training-associated reprogramming of HSPC(*32*). To better understand how β-glucan might mediate its effects on BM HSPC, we investigated whether LPM, embryonically seeded macrophages that make up the largest population of peritoneal macrophages at steady state, transported β-glucan from the peritoneal cavity to the BM(*85, 113*). Indeed, LPM have been found to respond to sterile inflammation by egressing from the peritoneal cavity and invading surrounding tissues, a

phenomenon termed macrophage disappearance reaction(*100, 114, 115*). Using the expression of GATA6 as a marker for LPM, we found that β-glucan led to a loss of GATA6+ macrophages in the peritoneal cavity. However, the increase in BM macrophages was not associated with an increased number of GATA6+ macrophages in the BM. These results suggest that LPM may not transport β-glucan to the BM, but that they may egress into other tissues such as the omentum. It may also be possible that these LPM lose their expression of GATA6 when migrating to other tissues due to a loss of niche-specific signals, or that they undergo cell death. However, this does not rule out the possibility that β-glucan reaches the BM, or that other phagocytes may take on the role of transporting β-glucan to the BM. It may be possible to utilize radiolabeled or fluorescent β-glucan, or other β-glucan detection assays such as the Limulus test to ascertain the presence of β-glucan within the BM.

Our findings demonstrate an acute HSPC response in the BM following β-glucan treatment. To better understand how long-lived HSPC may transmit training-associated epigenetic signatures to trained progeny cells such as short-lived monocytes, macrophages, and neutrophils, we have developed the Fgd5$^{ZsGr•CreERT2}$/tdTomato LT-HSC fate mapping model. Our results show that following tamoxifen administration in Fgd5$^{ZsGr•CreERT2}$/tdTomato mice, the ratio of tdTom+ circulating blood cells increased at steady state, demonstrating that we could track LT-HSC contribution to hematopoiesis in the periphery. Following β-glucan administration, we also observed that the ratio of tdTom+ HSPC in the BM increased with time and, importantly, that the HSPC response to β-glucan in WT mice was recapitulated in our fate mapping model. Here, we were able to track the contribution of LT-HSC to hematopoiesis in the BM up to 14 days after β-glucan treatment. We therefore plan to use this model to investigate how epigenetic and transcriptomic alterations in LT-HSC may be transmitted to

progeny cells to generate trained immunity. After treating Fgd5$^{ZsGr•CreERT2}$/tdTomato mice with tamoxifen and β-glucan as previously described, we plan to FACS sort tdTom^{+} HSC, MPP, CMP, and GMP, as well as monocytes, macrophages, and neutrophils. We then plan to assess their epigenomic profile using Assay for Transposase-Accessible Chromatin with high-throughput sequencing and their transcriptomic profile using single-cell RNA sequencing. Collectively, these experiments will allow us to determine whether the epigenetic landscape of trained LT-HSC is maintained and transmitted to downstream progenitor and myeloid cells.

However, there are certain limitations to this model. *Fgd5*, the embryonic development gene driving the expression of Cre recombinase in our model, is only expressed in a subset of LT-HSC, meaning that only a subset of LT-HSC and progeny cells become tdTom+ following tamoxifen administration(*104*). In our current model, only approximately 40% of LT-HSC become tdTom+ 14 days after tamoxifen administration, a similar ratio to what has been previously reported(*104*). In addition, it remains unclear whether *Fgd5* marks a specific subset of LT-HSC that respond differently to stimuli, including PAMPs such as β-glucan. Indeed, increasing evidence has suggested that HSC are in fact not a homogenous population, but rather a heterogenous population with distinctive proliferative potential and lineage bias(*116*). However, the function of Fgd5 has never been studied in HSC, and it remains unclear whether functional differences exist between Fgd5- and Fgd5+ HSC(*117*). For instance, Fgd5+ LT-HSC may have a distinct proliferative or differentiation capacity or may generate differentiated hematopoietic cells with unique functional characteristics. It is therefore essential that these implications be carefully assessed in our Fgd5$^{ZsGr•CreERT2}$/tdTomato model and in other LT-HSC fate mapping models.

10. Chapter 5: Conclusion

Trained immunity has been defined by the epigenetic reprogramming of innate immune cells, a process often demonstrated by the training of monocytes through direct exposure to fungal β-glucan. However, we have shown that β-glucan can train HSPC, but not fully differentiated BMDM to generate trained macrophages. We propose that the epigenetic reprogramming in trained immunity relies on the plasticity of progenitors, such as HSPC and monocytes, that may be lacking in fully differentiated cells such as macrophages. We also demonstrate that a single β-glucan injection induces LKS expansion and promotes granulopoiesis to increase neutrophil production *in vivo*. Yet, it remains unclear if and how training-associated epigenetic alterations in HSPC are transmitted to generate trained innate immune cells. Despite its limitations, our Fgd5$^{ZsGr•CreERT2}$/tdTomato fate mapping model serves as an important tool in elucidating the fundamental mechanisms that underlie β-glucan-induced trained immunity in the BM and beyond. Improving our understanding of trained immunity may contribute to the development of new immunization and therapeutic strategies against important pathogens such as *Mtb*.

11. Figures and Figure Legends

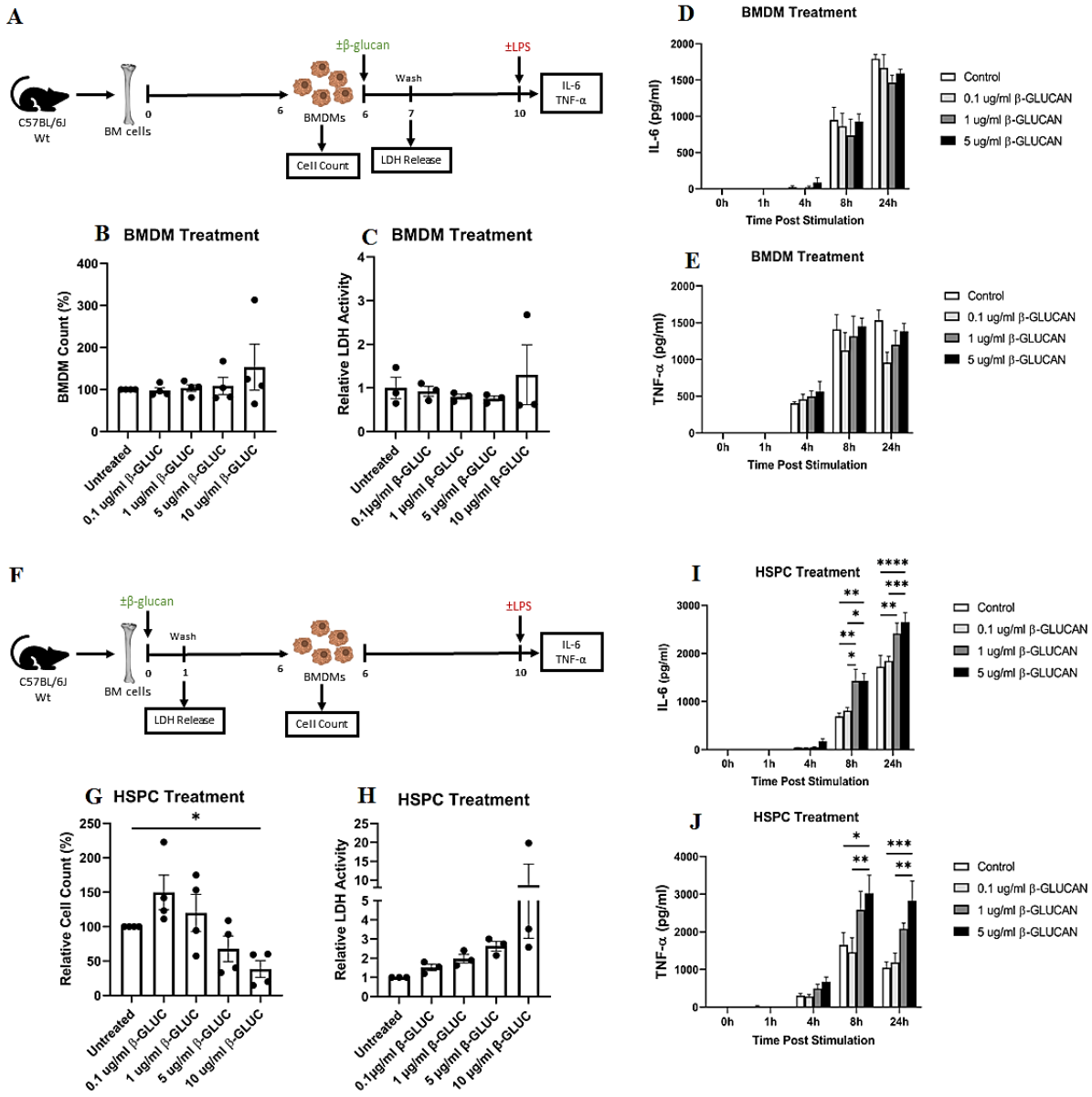

Figure 3. β-glucan induces trained immunity in HSPC *in vitro*.
A. *In vitro* BMDM treatment protocol. **B-C.** BMDM cell count and LDH release assay following BMDM treatment with β-glucan. **D-E.** BMDM secretion of IL-6 and TNF-α following stimulation with 100ng/ml LPS. **F.** *In vitro* HSPC treatment protocol. **G-H.** BMDM cell count and LDH release assay following HSPC treatment with β-glucan. **I-J.** BMDM secretion of IL-6 and TNF-α following stimulation with 100ng/ml LPS. All means displayed as ±SEM. **B-C, G-H.** One-way ANOVA. **D-E, I-J.** N=3. Two-way ANOVA with Tukey's multiple comparison test. (*<0.05, **<0.01, ***<0.001, ****<0.0001)

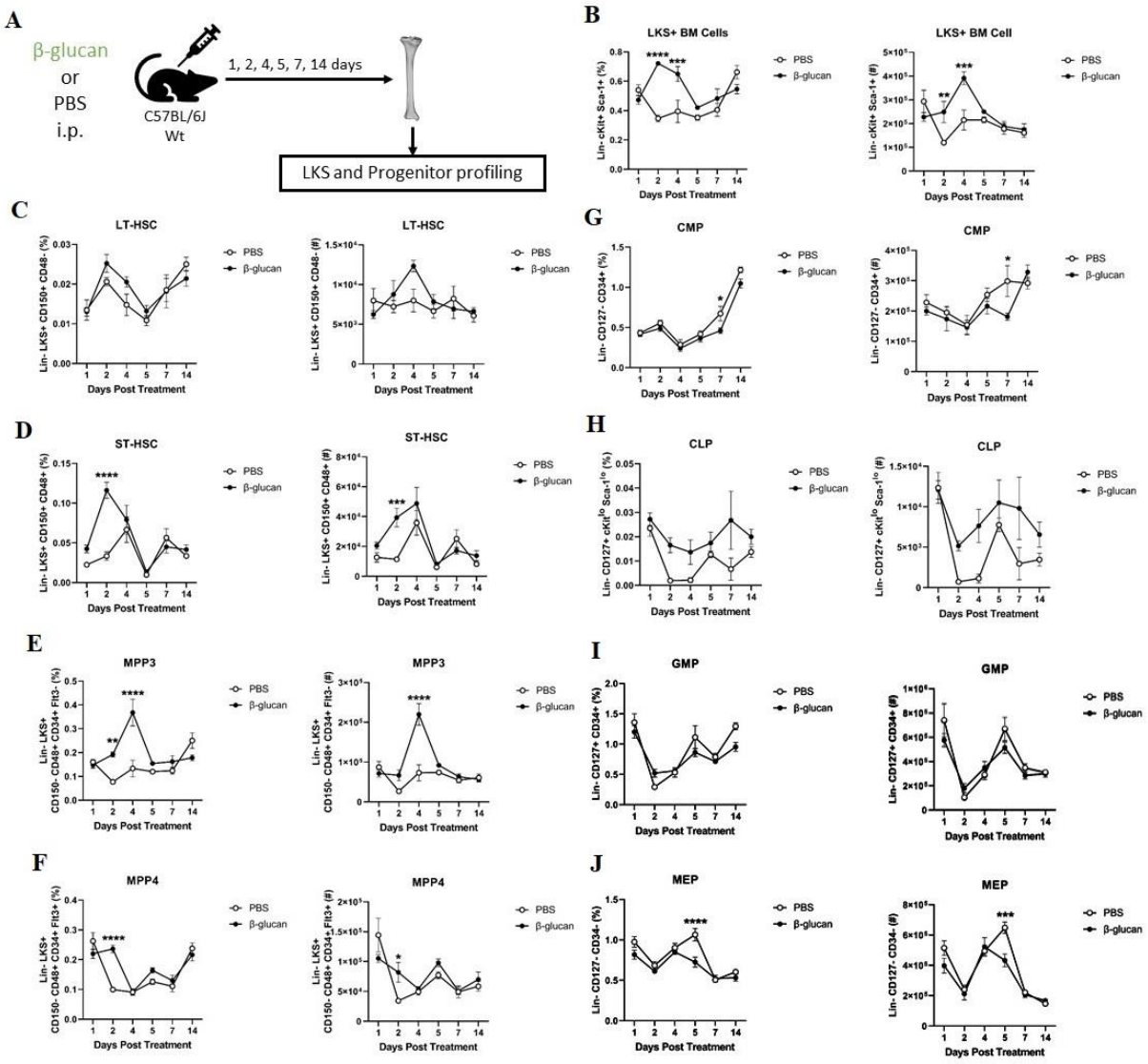

Figure 4. Single-dose β-glucan treatment induces LKS expansion *in vivo*.
A. Single-dose β-glucan treatment protocol. **B.-J.** Kinetics of hematopoietic stem and progenitor cells in the BM following β-glucan treatment. Means displayed as ±SEM. N=4-5. Two-way ANOVA with Sidak's multiple comparison test. (*<0.05, **<0.01, ***<0.001, ****<0.0001)

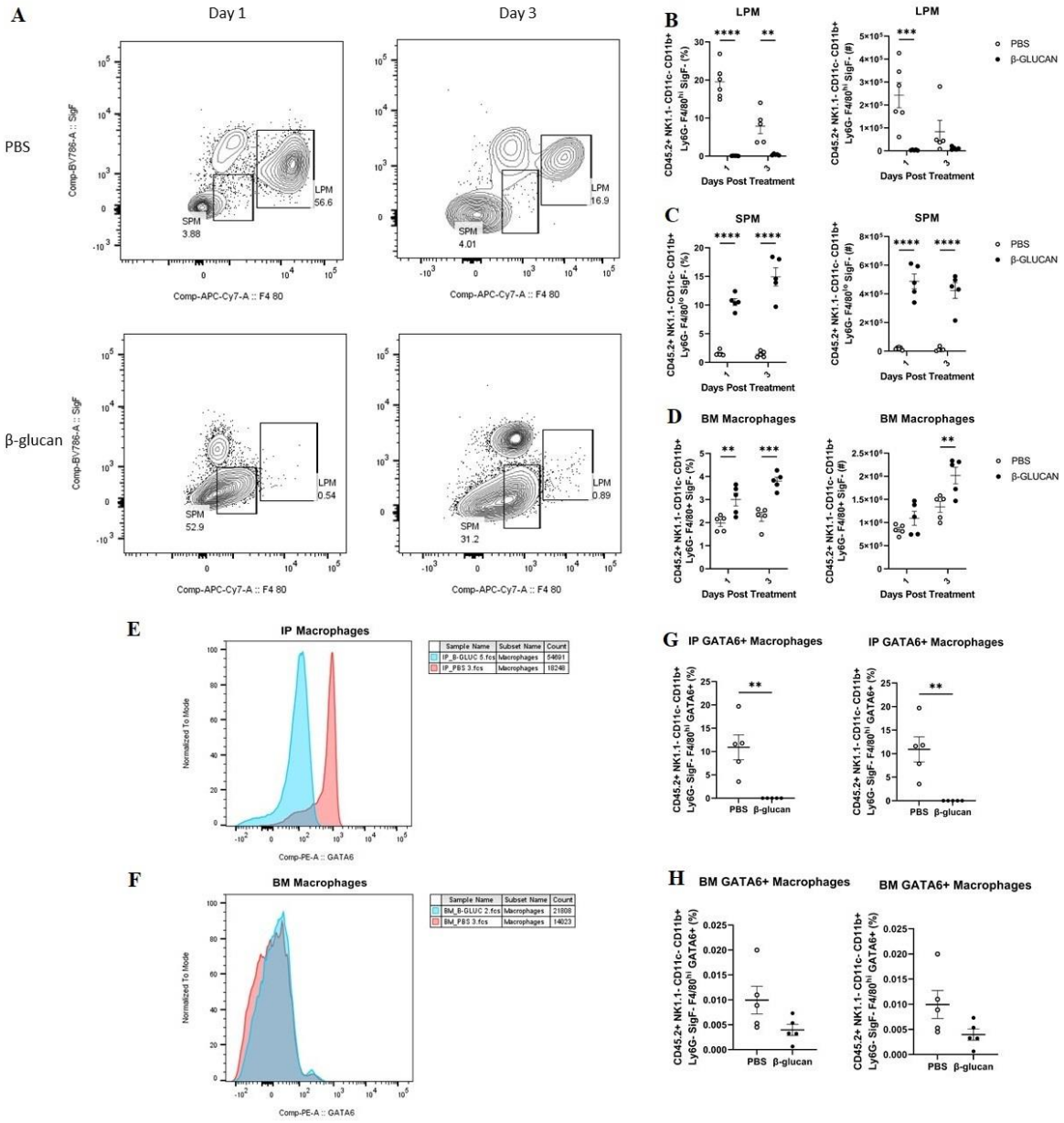

Figure 5. GATA6+ LPM do not egress to BM following single-dose β-glucan treatment.
A.-C. Representative FACS plots and quantification of small peritoneal macrophages (SPM) and large peritoneal macrophages (LPM) following β-glucan treatment. **D.** BM macrophages following β-glucan treatment. **E.-F.** GATA6 expression in peritoneal (IP) and BM macrophages 3 days following β-glucan treatment. **G.-H.** GATA6+ IP and BM macrophages 3 days following β-glucan treatment. Means displayed as ±SEM. **B.-D.**Two-way ANOVA with Sidak's multiple comparison test**. G.-H.** Unpaired t test. (*<0.05, **<0.01, ***<0.001, ****<0.0001).

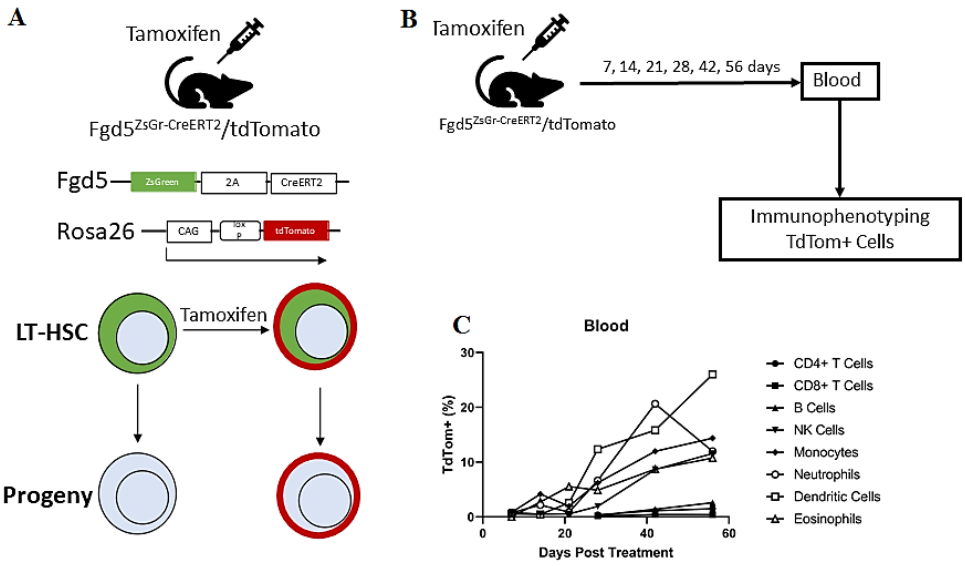

Figure 6. Fate mapping tracks LT-HSC contribution to circulating blood cells at steady state. **A.** Fgd5$^{ZsGr\text{-}CreERT2}$/tdTomato mice express tdTomato in LT-HSC and subsequent progeny cells following tamoxifen administration. **B.** Tamoxifen treatment and blood collection protocol. **C.** LT-HSC contribute to immune cells in the blood at steady state. N=2.

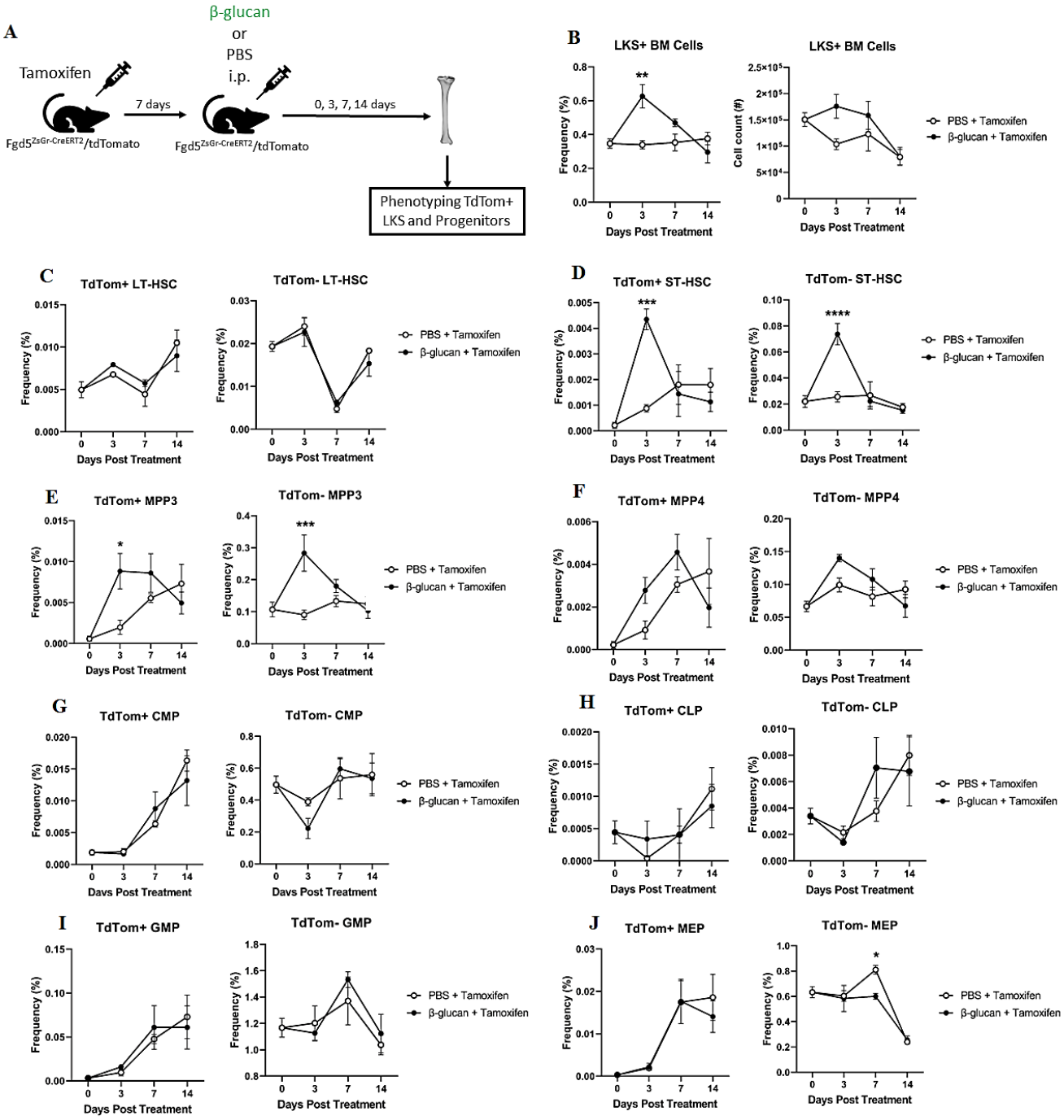

Figure 7. Fate mapping tracks LT-HSC contribution and LKS expansion following β-glucan treatment.

A. Tamoxifen and β-glucan treatment protocol of Fgd5$^{ZsGr\text{-}CreERT2}$/tdTomato mice. **B-J.** Kinetics of tdTom+ and tdTom- hematopoietic stem and progenitor cells in the BM following tamoxifen and β-glucan treatment. Means displayed as ±SEM. N=3. Two-way ANOVA with Sidak's multiple comparison test (*<0.05, **<0.01, ***<0.001, ****<0.0001).

12. References

1. M. G. Netea *et al.*, Defining trained immunity and its role in health and disease. *Nature Reviews Immunology* **20**, 375-388 (2020).
2. S. H. Spoel, X. Dong, How do plants achieve immunity? Defence without specialized immune cells. *Nature Reviews Immunology* **12**, 89-100 (2012).
3. M. Lafont *et al.*, Long-lasting antiviral innate immune priming in the Lophotrochozoan Pacific oyster, Crassostrea gigas. *Scientific Reports* **7**, 13143 (2017).
4. L. C. J. de Bree *et al.*, Non-specific effects of vaccines: Current evidence and potential implications. *Seminars in Immunology* **39**, 35-43 (2018).
5. V. Tran, J. Liu, M. A. Behr, BCG Vaccines. *Microbiol Spectr* **2**, Mgm2-0028-2013 (2014).
6. P. Andersen, T. M. Doherty, The success and failure of BCG — implications for a novel tuberculosis vaccine. *Nature Reviews Microbiology* **3**, 656-662 (2005).
7. P. Aaby, C. S. Benn, Saving lives by training innate immunity with bacille Calmette-Guérin vaccine. *Proceedings of the National Academy of Sciences* **109**, 17317 (2012).
8. F. Shann, The non-specific effects of vaccines. *Archives of Disease in Childhood* **95**, 662-667 (2010).
9. M.-L. Garly *et al.*, BCG scar and positive tuberculin reaction associated with reduced child mortality in West Africa: A non-specific beneficial effect of BCG? *Vaccine* **21**, 2782-2790 (2003).
10. M. Divangahi *et al.*, Trained immunity, tolerance, priming and differentiation: distinct immunological processes. *Nat Immunol* **22**, 2-6 (2021).
11. Mihai G. Netea, J. Quintin, Jos W. M. van der Meer, Trained Immunity: A Memory for Innate Host Defense. *Cell Host & Microbe* **9**, 355-361 (2011).
12. J. Kleinnijenhuis *et al.*, Bacille Calmette-Guerin induces NOD2-dependent nonspecific protection from reinfection via epigenetic reprogramming of monocytes. *Proc Natl Acad Sci U S A* **109**, 17537-17542 (2012).
13. M. Foster *et al.*, BCG-induced protection against Mycobacterium tuberculosis infection: Evidence, mechanisms, and implications for next-generation vaccines. *Immunol Rev* **301**, 122-144 (2021).
14. S. J. C. F. M. Moorlag *et al.*, BCG Vaccination Induces Long-Term Functional Reprogramming of Human Neutrophils. *Cell Reports* **33**, 108387 (2020).
15. J. Kleinnijenhuis *et al.*, BCG-induced trained immunity in NK cells: Role for non-specific protection to infection. *Clin Immunol* **155**, 213-219 (2014).
16. R. J. W. Arts *et al.*, BCG Vaccination Protects against Experimental Viral Infection in Humans through the Induction of Cytokines Associated with Trained Immunity. *Cell Host & Microbe* **23**, 89-100.e105 (2018).
17. C. Pettenati, M. A. Ingersoll, Mechanisms of BCG immunotherapy and its outlook for bladder cancer. *Nature Reviews Urology* **15**, 615-625 (2018).
18. A. Bisiaux *et al.*, Molecular analyte profiling of the early events and tissue conditioning following intravesical bacillus calmette-guerin therapy in patients with superficial bladder cancer. *J Urol* **181**, 1571-1580 (2009).
19. F. Bistoni *et al.*, Evidence for macrophage-mediated protection against lethal Candida albicans infection. *Infect Immun* **51**, 668-674 (1986).
20. J. Quintin *et al.*, Candida albicans infection affords protection against reinfection via functional reprogramming of monocytes. *Cell host & microbe* **12**, 223-232 (2012).
21. M. G. Netea, G. D. Brown, B. J. Kullberg, N. A. R. Gow, An integrated model of the recognition of Candida albicans by the innate immune system. *Nature Reviews Microbiology* **6**, 67-78 (2008).

22. S. Saeed *et al.*, Epigenetic programming of monocyte-to-macrophage differentiation and trained innate immunity. *Science* **345**, 1251086 (2014).
23. S. C. Cheng *et al.*, mTOR- and HIF-1α-mediated aerobic glycolysis as metabolic basis for trained immunity. *Science* **345**, 1250684 (2014).
24. R. J. Arts *et al.*, Glutaminolysis and Fumarate Accumulation Integrate Immunometabolic and Epigenetic Programs in Trained Immunity. *Cell Metab* **24**, 807-819 (2016).
25. R. J. W. Arts *et al.*, Long-term in vitro and in vivo effects of γ-irradiated BCG on innate and adaptive immunity. *Journal of leukocyte biology* **98**, 995-1001 (2015).
26. R. J. W. Arts *et al.*, Immunometabolic Pathways in BCG-Induced Trained Immunity. *Cell Rep* **17**, 2562-2571 (2016).
27. J. Quintin *et al.*, Candida albicans infection affords protection against reinfection via functional reprogramming of monocytes. *Cell Host Microbe* **12**, 223-232 (2012).
28. S. J. C. F. M. Moorlag *et al.*, β-Glucan Induces Protective Trained Immunity against Mycobacterium tuberculosis Infection: A Key Role for IL-1. *Cell Reports* **31**, 107634 (2020).
29. L. Kalafati *et al.*, Innate Immune Training of Granulopoiesis Promotes Anti-tumor Activity. *Cell* **183**, 771-785.e712 (2020).
30. M. P. Alexander, S. N. Fiering, G. R. Ostroff, R. A. Cramer, D. W. Mullins, Beta-glucan-induced inflammatory monocytes mediate antitumor efficacy in the murine lung. *Cancer Immunology, Immunotherapy* **67**, 1731-1742 (2018).
31. C. R. Hole *et al.*, Induction of memory-like dendritic cell responses in vivo. *Nature Communications* **10**, 2955 (2019).
32. E. Kaufmann *et al.*, BCG Educates Hematopoietic Stem Cells to Generate Protective Innate Immunity against Tuberculosis. *Cell* **172**, 176-190.e119 (2018).
33. N. Khan *et al.*, M. tuberculosis Reprograms Hematopoietic Stem Cells to Limit Myelopoiesis and Impair Trained Immunity. *Cell* **183**, 752-770.e722 (2020).
34. I. Mitroulis *et al.*, Modulation of Myelopoiesis Progenitors Is an Integral Component of Trained Immunity. *Cell* **172**, 147-161.e112 (2018).
35. V. Vetvicka, L. Vannucci, P. Sima, J. Richter, Beta Glucan: Supplement or Drug? From Laboratory to Clinical Trials. *Molecules* **24**, 1251 (2019).
36. R. Chang, Bioactive polysaccharides from traditional Chinese medicine herbs as anticancer adjuvants. *J Altern Complement Med* **8**, 559-565 (2002).
37. J. Chen, R. Seviour, Medicinal importance of fungal beta-(1-->3), (1-->6)-glucans. *Mycol Res* **111**, 635-652 (2007).
38. B. Du, M. Meenu, H. Liu, B. Xu, A Concise Review on the Molecular Structure and Function Relationship of β-Glucan. *Int J Mol Sci* **20**, (2019).
39. H. S. Goodridge, A. J. Wolf, D. M. Underhill, Beta-glucan recognition by the innate immune system. *Immunol Rev* **230**, 38-50 (2009).
40. D. Klemm, B. Heublein, H.-P. Fink, A. Bohn, Cellulose: Fascinating Biopolymer and Sustainable Raw Material. *Angewandte Chemie International Edition* **44**, 3358-3393 (2005).
41. M. S. I. Costas G. Biliaderis, *Functional Food Carbohydrates*. Cereal-Glucans: Structures, Physical Properties, and Physiological Functions (CRC Press, 2006).
42. P. Volikakis, C. G. Biliaderis, C. Vamvakas, G. K. Zerfiridis, Effects of a commercial oat-β-glucan concentrate on the chemical, physico-chemical and sensory attributes of a low-fat white-brined cheese product. *Food Research International* **37**, 83-94 (2004).
43. G. D. Brown, S. Gordon, Immune recognition of fungal beta-glucans. *Cell Microbiol* **7**, 471-479 (2005).
44. S. Soltanian, E. Stuyven, E. Cox, P. Sorgeloos, P. Bossier, Beta-glucans as immunostimulant in vertebrates and invertebrates. *Crit Rev Microbiol* **35**, 109-138 (2009).

45. A. R. V. Pedro *et al.*, Dectin-1-Mediated Production of Pro-Inflammatory Cytokines Induced by Yeast β-Glucans in Bovine Monocytes. *Front Immunol* **12**, 689879 (2021).
46. P. R. Taylor *et al.*, Dectin-1 is required for beta-glucan recognition and control of fungal infection. *Nat Immunol* **8**, 31-38 (2007).
47. A. Yáñez, J. Megías, J.-E. O'Connor, D. Gozalbo, M. L. Gil, Candida albicans Induces Selective Development of Macrophages and Monocyte Derived Dendritic Cells by a TLR2 Dependent Signalling. *PLOS ONE* **6**, e24761 (2011).
48. H. S. Goodridge *et al.*, Activation of the innate immune receptor Dectin-1 upon formation of a 'phagocytic synapse'. *Nature* **472**, 471-475 (2011).
49. G. Camilli, G. Tabouret, J. Quintin, The Complexity of Fungal β-Glucan in Health and Disease: Effects on the Mononuclear Phagocyte System. *Frontiers in immunology* **9**, 673-673 (2018).
50. G. Ferwerda, F. Meyer-Wentrup, B. J. Kullberg, M. G. Netea, G. J. Adema, Dectin-1 synergizes with TLR2 and TLR4 for cytokine production in human primary monocytes and macrophages. *Cell Microbiol* **10**, 2058-2066 (2008).
51. R. van Bruggen *et al.*, Complement receptor 3, not Dectin-1, is the major receptor on human neutrophils for beta-glucan-bearing particles. *Mol Immunol* **47**, 575-581 (2009).
52. G. D. Brown *et al.*, Dectin-1 is a major beta-glucan receptor on macrophages. *J Exp Med* **196**, 407-412 (2002).
53. V. Vetvicka, B. P. Thornton, G. D. Ross, Soluble beta-glucan polysaccharide binding to the lectin site of neutrophil or natural killer cell complement receptor type 3 (CD11b/CD18) generates a primed state of the receptor capable of mediating cytotoxicity of iC3b-opsonized target cells. *J Clin Invest* **98**, 50-61 (1996).
54. G. D. Ross, V. Vetvicka, J. Yan, Y. Xia, J. Vetvicková, Therapeutic intervention with complement and beta-glucan in cancer. *Immunopharmacology* **42**, 61-74 (1999).
55. F. Hong *et al.*, Mechanism by Which Orally Administered β-1,3-Glucans Enhance the Tumoricidal Activity of Antitumor Monoclonal Antibodies in Murine Tumor Models. *The Journal of Immunology* **173**, 797-806 (2004).
56. A. Batsivari *et al.*, Dynamic responses of the haematopoietic stem cell niche to diverse stresses. *Nature Cell Biology* **22**, 7-17 (2020).
57. S. N. Hurwitz, S. K. Jung, P. Kurre, Hematopoietic stem and progenitor cell signaling in the niche. *Leukemia* **34**, 3136-3148 (2020).
58. N. Cabezas-Wallscheid *et al.*, Identification of Regulatory Networks in HSCs and Their Immediate Progeny via Integrated Proteome, Transcriptome, and DNA Methylome Analysis. *Cell Stem Cell* **15**, 507-522 (2014).
59. E. Laurenti, B. Göttgens, From haematopoietic stem cells to complex differentiation landscapes. *Nature* **553**, 418-426 (2018).
60. A. Chow *et al.*, Bone marrow CD169+ macrophages promote the retention of hematopoietic stem and progenitor cells in the mesenchymal stem cell niche. *Journal of Experimental Medicine* **208**, 261-271 (2011).
61. S. Méndez-Ferrer, D. Lucas, M. Battista, P. S. Frenette, Haematopoietic stem cell release is regulated by circadian oscillations. *Nature* **452**, 442-447 (2008).
62. S. Yamazaki *et al.*, Nonmyelinating Schwann Cells Maintain Hematopoietic Stem Cell Hibernation in the Bone Marrow Niche. *Cell* **147**, 1146-1158 (2011).
63. H. Luo *et al.*, Mitochondrial Stress-Initiated Aberrant Activation of the NLRP3 Inflammasome Regulates the Functional Deterioration of Hematopoietic Stem Cell Aging. *Cell Rep* **26**, 945-954.e944 (2019).
64. S. Jaiswal, Clonal hematopoiesis and nonhematologic disorders. *Blood* **136**, 1606-1614 (2020).

65. F. Lussana, A. Rambaldi, Inflammation and myeloproliferative neoplasms. *J Autoimmun* **85**, 58-63 (2017).
66. R. N. Foley, Emerging erythropoiesis-stimulating agents. *Nature Reviews Nephrology* **6**, 218-223 (2010).
67. H. Takizawa, S. Boettcher, M. G. Manz, Demand-adapted regulation of early hematopoiesis in infection and inflammation. *Blood* **119**, 2991-3002 (2012).
68. A. Yáñez, H. S. Goodridge, D. Gozalbo, M. L. Gil, TLRs control hematopoiesis during infection. *Eur J Immunol* **43**, 2526-2533 (2013).
69. A. Yáñez, J. Megías, J.-E. O'Connor, D. Gozalbo, M. L. Gil, Candida albicans induces selective development of macrophages and monocyte derived dendritic cells by a TLR2 dependent signalling. *PloS one* **6**, e24761-e24761 (2011).
70. C. Bono *et al.*, Dectin-1 Stimulation of Hematopoietic Stem and Progenitor Cells Occurs In Vivo and Promotes Differentiation Toward Trained Macrophages via an Indirect Cell-Autonomous Mechanism. *mBio* **11**, (2020).
71. F. Ginhoux, S. Jung, Monocytes and macrophages: developmental pathways and tissue homeostasis. *Nature Reviews Immunology* **14**, 392-404 (2014).
72. M. Naito, F. Yamamura, S. Nishikawa, K. Takahashi, Development, differentiation, and maturation of fetal mouse yolk sac macrophages in cultures. *J Leukoc Biol* **46**, 1-10 (1989).
73. K. Takahashi, F. Yamamura, M. Naito, Differentiation, maturation, and proliferation of macrophages in the mouse yolk sac: a light-microscopic, enzyme-cytochemical, immunohistochemical, and ultrastructural study. *J Leukoc Biol* **45**, 87-96 (1989).
74. F. Ginhoux *et al.*, Fate mapping analysis reveals that adult microglia derive from primitive macrophages. *Science (New York, N.Y.)* **330**, 841-845 (2010).
75. Y. Wu, K. K. Hirschi, Tissue-Resident Macrophage Development and Function. *Front Cell Dev Biol* **8**, 617879-617879 (2021).
76. G. Hoeffel, F. Ginhoux, Fetal monocytes and the origins of tissue-resident macrophages. *Cellular Immunology* **330**, 5-15 (2018).
77. E. Evren, E. Ringqvist, T. Willinger, Origin and ontogeny of lung macrophages: from mice to humans. *Immunology* **160**, 126-138 (2020).
78. O. Krenkel, F. Tacke, Liver macrophages in tissue homeostasis and disease. *Nature Reviews Immunology* **17**, 306-321 (2017).
79. Y. Lavin, A. Mortha, A. Rahman, M. Merad, Regulation of macrophage development and function in peripheral tissues. *Nature Reviews Immunology* **15**, 731-744 (2015).
80. P. Squarzoni *et al.*, Microglia modulate wiring of the embryonic forebrain. *Cell Rep* **8**, 1271-1279 (2014).
81. T. DeFalco, I. Bhattacharya, A. V. Williams, D. M. Sams, B. Capel, Yolk-sac-derived macrophages regulate fetal testis vascularization and morphogenesis. *Proc Natl Acad Sci U S A* **111**, E2384-E2393 (2014).
82. J. Hopkinson-Woolley, D. Hughes, S. Gordon, P. Martin, Macrophage recruitment during limb development and wound healing in the embryonic and foetal mouse. *J Cell Sci* **107 (Pt 5)**, 1159-1167 (1994).
83. Y. Yahara *et al.*, Erythromyeloid progenitors give rise to a population of osteoclasts that contribute to bone homeostasis and repair. *Nature cell biology* **22**, 49-59 (2020).
84. N. Joshi, J. M. Walter, A. V. Misharin, Alveolar Macrophages. *Cellular Immunology* **330**, 86-90 (2018).
85. A. d. A. Cassado, M. R. D'Império Lima, K. R. Bortoluci, Revisiting Mouse Peritoneal Macrophages: Heterogeneity, Development, and Function. *Frontiers in Immunology* **6**, (2015).

86. T. Hussell, T. J. Bell, Alveolar macrophages: plasticity in a tissue-specific context. *Nature Reviews Immunology* **14**, 81-93 (2014).
87. C. F. Nathan, H. W. Murray, M. E. Wiebe, B. Y. Rubin, Identification of interferon-gamma as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity. *J Exp Med* **158**, 670-689 (1983).
88. C. D. Mills, K. Kincaid, J. M. Alt, M. J. Heilman, A. M. Hill, M-1/M-2 macrophages and the Th1/Th2 paradigm. *J Immunol* **164**, 6166-6173 (2000).
89. P. J. Murray *et al.*, Macrophage activation and polarization: nomenclature and experimental guidelines. *Immunity* **41**, 14-20 (2014).
90. G. M. Tannahill *et al.*, Succinate is an inflammatory signal that induces IL-1β through HIF-1α. *Nature* **496**, 238-242 (2013).
91. F. O. Martinez, S. Gordon, The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000Prime Rep* **6**, 13-13 (2014).
92. M. Stein, S. Keshav, N. Harris, S. Gordon, Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. *J Exp Med* **176**, 287-292 (1992).
93. D. Vats *et al.*, Oxidative metabolism and PGC-1β attenuate macrophage-mediated inflammation. *Cell Metabolism* **4**, 13-24 (2006).
94. L. He *et al.*, Global characterization of macrophage polarization mechanisms and identification of M2-type polarization inhibitors. *Cell Reports* **37**, (2021).
95. L. B. Ivashkiv, Epigenetic regulation of macrophage polarization and function. *Trends in immunology* **34**, 216-223 (2013).
96. Y. Lavin *et al.*, Tissue-Resident Macrophage Enhancer Landscapes Are Shaped by the Local Microenvironment. *Cell* **159**, 1312-1326 (2014).
97. G. Natoli, Maintaining cell identity through global control of genomic organization. *Immunity* **33**, 12-24 (2010).
98. M. Haldar *et al.*, Heme-Mediated SPI-C Induction Promotes Monocyte Differentiation into Iron-Recycling Macrophages. *Cell* **156**, 1223-1234 (2014).
99. M. Kohyama *et al.*, Role for Spi-C in the development of red pulp macrophages and splenic iron homeostasis. *Nature* **457**, 318-321 (2009).
100. Y. Okabe, R. Medzhitov, Tissue-Specific Signals Control Reversible Program of Localization and Functional Polarization of Macrophages. *Cell* **157**, 832-844 (2014).
101. T. Lawrence, G. Natoli, Transcriptional regulation of macrophage polarization: enabling diversity with identity. *Nature Reviews Immunology* **11**, 750-761 (2011).
102. M. G. Netea *et al.*, Trained immunity: A program of innate immune memory in health and disease. *Science* **352**, aaf1098 (2016).
103. M. Rosas *et al.*, The transcription factor Gata6 links tissue macrophage phenotype and proliferative renewal. *Science (New York, N.Y.)* **344**, 645-648 (2014).
104. R. H. Chapple *et al.*, Lineage tracing of murine adult hematopoietic stem cells reveals active contribution to steady-state hematopoiesis. *Blood Adv* **2**, 1220-1228 (2018).
105. C. Auffray, M. H. Sieweke, F. Geissmann, Blood monocytes: development, heterogeneity, and relationship with dendritic cells. *Annu Rev Immunol* **27**, 669-692 (2009).
106. M. Guilliams, A. Mildner, S. Yona, Developmental and Functional Heterogeneity of Monocytes. *Immunity* **49**, 595-613 (2018).
107. B. Novakovic *et al.*, β-Glucan Reverses the Epigenetic State of LPS-Induced Immunological Tolerance. *Cell* **167**, 1354-1368.e1314 (2016).
108. S. K. Biswas, E. Lopez-Collazo, Endotoxin tolerance: new mechanisms, molecules and clinical significance. *Trends in Immunology* **30**, 475-487 (2009).

109. T. A. Wynn, A. Chawla, J. W. Pollard, Macrophage biology in development, homeostasis and disease. *Nature* **496**, 445-455 (2013).
110. M. Guilliams, F. R. Svedberg, Does tissue imprinting restrict macrophage plasticity? *Nature Immunology* **22**, 118-127 (2021).
111. I. Amit, D. R. Winter, S. Jung, The role of the local environment and epigenetics in shaping macrophage identity and their effect on tissue homeostasis. *Nature Immunology* **17**, 18-25 (2016).
112. D. Brough, N. J. Rothwell, Caspase-1-dependent processing of pro-interleukin-1beta is cytosolic and precedes cell death. *J Cell Sci* **120**, 772-781 (2007).
113. M. Liu, A. Silva-Sanchez, T. D. Randall, S. Meza-Perez, Specialized immune responses in the peritoneal cavity and omentum. *Journal of Leukocyte Biology* **109**, 717-729 (2021).
114. M. Honda, M. Kadohisa, D. Yoshii, Y. Komohara, T. Hibi, Directly recruited GATA6 + peritoneal cavity macrophages contribute to the repair of intestinal serosal injury. *Nature Communications* **12**, 7294 (2021).
115. P. A. Louwe *et al.*, Recruited macrophages that colonize the post-inflammatory peritoneal niche convert into functionally divergent resident cells. *Nature Communications* **12**, 1770 (2021).
116. K. Minton, HSC function determined by epigenetic memory. *Nature Reviews Immunology* **17**, 5-5 (2017).
117. R. Gazit *et al.*, Fgd5 identifies hematopoietic stem cells in the murine bone marrow. *J Exp Med* **211**, 1315-1331 (2014).