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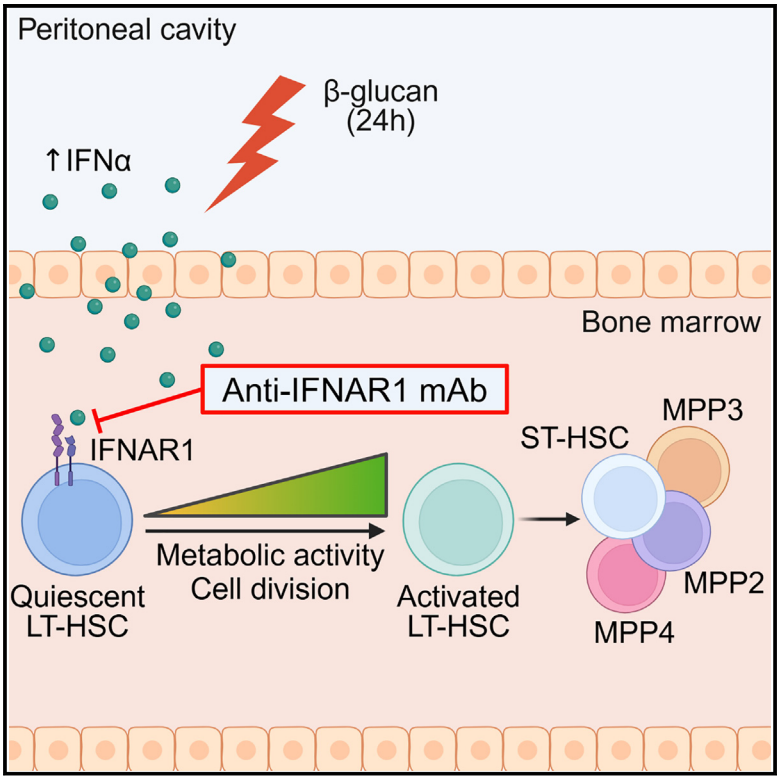
Type I interferon signaling controls the early hematopoietic expansion in response to β-glucan

Graphical abstract



Highlights

- β-glucan induces rapid expansion and metabolic activation in HSPCs
- IFNα accumulates in the bone marrow and peritoneal cavity post-β-glucan treatment
- IFNAR1 levels increase on LT-HSCs alongside a type I gene signature post-β-glucan
- IFNAR1 blockade impairs β-glucan-induced expansion/metabolic activation of LT-HSC

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In brief

Immunology; Immune response; Cell biology; Functional aspects of cell biology

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Type I interferon signaling controls the early hematopoietic expansion in response to β-glucan

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SUMMARY

Rapid hematopoietic adaptations are important for building and sustaining the biological response to β-glucan. The signals involved in these early events have not yet been fully explored. Given that type I interferons are produced in response to β-glucan and can profoundly impact hematopoietic stem cell (HSC) function, we hypothesized that this pathway may be involved in the early bone marrow response to β-glucan. *In vivo* administration of β-glucan led to local interferon-α production in the peritoneal cavity and bone marrow, up-regulation of its receptor, IFNAR1, specifically on long-term hematopoietic stem cells (LT-HSCs), and broad expansion of downstream progenitor subpopulations. We demonstrate that intact type I interferon signaling is critical for β-glucan-mediated LT-HSC proliferation, mitochondrial activity, and glycolytic commitment. By determining that type I interferon signaling is important for LT-HSCs, which sit at the apex of the hematopoietic hierarchy, we uncover an important component of the early inflammatory response to β-glucan.

INTRODUCTION

The processes that maintain appropriate blood cell production via hematopoiesis in response to acute inflammation or chronic disease across an individual's lifetime are not fully understood. Hematopoietic stem cells (HSCs), also known as long-term (LT)-HSCs, sit at the apex of the hematopoietic hierarchy and possess self-renewal and multilineage differentiation capacity. These abilities allow them to maintain the HSC pool while also enabling the generation of all mature blood cell lineages.[1] HSCs differentiate into multipotent progenitors (MPPs) and, together, these cells are classified as hematopoietic stem and progenitor cells (HSPCs).[2] During homeostasis, most adult HSCs remain in a quiescent state, residing in the G_0 phase of the cell-cycle with low energy and nutrient needs.[3] However, when HSCs sense environmental signals and exit dormancy, their energy demands increase and are met by elevated rates of glycolysis and oxidative phosphorylation (OXPHOS).[4] This coordinated process is an important component of balanced hematopoietic outcomes.[5] Evidence has now emerged that HSCs may retain 'memory' of past inflammatory events, which can have a lasting impact on hematopoietic output and on the function of mature progeny cells.[6–8]

β-glucan is one such signal that has been shown to functionally alter HSPCs, favoring the sustained over-production of myeloid cells.[9–11] Similar hematopoietic alterations have also been demonstrated in response to the Bacillus Calmette-Guerin (BCG) vaccine,[12] systemic lupus erythematosus (SLE),[13] and a high-fat diet.[14] Disordered hematopoiesis and progenitor cell activation are also a feature of diabetes, periodontal disease, and stroke, linking these conditions to comorbidities, such as cardiovascular disease.[15–17] β-glucan binds primarily to Dectin-1 but may also interact with complement receptor 3 (CR3) or certain integrin receptors.[18] Although Dectin-1 is not expressed in HSPCs,[19] the resulting inflammatory signals that contribute to these hematopoietic changes are varied. Notably, interleukin-1β (IL-1β) appears to be an important mediator driving myelopoiesis in response to β-glucan[9,10] or a high-fat diet.[14] In these instances, perhaps counterintuitively given its ability to block IL-1β production,[20] type I interferons (IFNs) have also been found to play a role in the reprogramming of myeloid progenitor cells.[9,10,14]

Type I IFNs activate dormant HSCs by briefly relaxing the protective program of quiescence[21,22] whereas chronic exposure to type I IFNs, via repeated TLR3 stimulation, progressively degrades HSC function and diminishes lymphoid cell production.[6] Despite evidence that type I IFNs are important for the anti-tumor phenotype adopted by neutrophils from β-glucan-treated mice,[10] it is currently unknown what influence type I IFNs may have on more primitive HSC/HSPC populations in the context

of β-glucan activation. The current study aimed to characterize the impact of type I IFN signaling on the major HSC/HSPC subsets during the early hematopoietic response to β-glucan. Using multiple *in vivo* approaches, we identified a role for type I IFN (IFNα) in the proliferation and metabolic activation of LT-HSCs associated with β-glucan.

RESULTS

β-glucan administration promotes expansion and metabolic alterations in LSK subsets

To first explore the early hematopoietic response to β-glucan, mice were administered a single intraperitoneal (i.p.) injection of β-glucan. Following 24 h, bone marrow cells were harvested to quantify the frequency and absolute number of LT-HSCs ($Flt3^-CD48^-CD150^+LSK$), ST-HSCs ($Flt3^-CD48^-CD150^-LSK$), MPP 2 ($Flt3^-CD48^+CD150^+LSK$), MPP3 ($Flt3^-CD48^+CD150^-$ LSK), and MPP4 ($Flt3^+CD48^+CD150^-LSK$), which together comprise the major hematopoietic progenitor (HSPCs or LSKs; $Lin^-cKit^+Sca1^+$) subsets.

β-glucan treatment led to an expansion in both the frequency and number of LSK cells (Figures 1A–1C), which was due to an increase in all MPP subsets (i.e., MPP2-4; Figures 1B and 1C). No difference was found in LT-HSCs, while a decrease in the frequency and number of ST-HSCs were measured following β-glucan. MPP2 (erythroid-biased), MPP3 (myeloid-biased), and MPP4 (lymphoid-biased) subsets have distinct lineage-skewing potential.[23] We also noted that β-glucan increased the frequency of $CD41^+$ LT-HSCs (Figure 1D), a subset that becomes more prevalent with age and during inflammation.[24,25] To further investigate the impact of β-glucan, we stained for phosphorylated-H2AX (γ-H2AX), a marker of replication stress, and found a significant increase in LSKs (Figure 1E). This effect was accompanied by elevated glucose uptake (Figure 1F) and lactate production (Figure 1G) in LSKs following β-glucan administration. Proliferation and differentiation are closely linked to changes in cellular metabolism. To explore metabolic adaptations more closely, we probed all major LSK subpopulations for cell-cycle progression (Ki-67/DAPI), mitochondrial membrane potential (TMRE) as a marker of mitochondrial respiration,[26] superoxide production (MitoSOX), and glucose uptake (2-NBDG) using flow cytometry. Cell-cycle analysis found that β-glucan boosted the percentage of LT-HSC, ST-HSC, MPP2, MPP3, and MPP4 in the S-G_2-M phase (Figure 1H), suggesting a broad proliferation/differentiation response elicited by β-glucan. HSPCs upregulate OXPHOS as they proliferate and exit quiescence,[4] which can be traced by measuring changes in mitochondrial membrane potential with the cationic fluorescent probe TMRE. Consistent with our data showing progress through the cell-cycle, we found that TMRE staining was higher across all LSK subsets following β-glucan treatment (Figure 1H), implying increased mitochondrial/electron transport chain (ETC) activity. The higher mitochondrial membrane potential in LSKs was not coupled to changes in mitochondrial superoxide production, as MitoSOX levels were unchanged in all cell subsets post-β-glucan (Figure 1H). Using 2-NBDG to monitor glucose uptake, we found that LT-HSC and MPP2-4 isolated from mice administered β-glucan had significantly increased glucose uptake (Figure 1H). Together, these data demonstrate that β-glucan drives a rapid and broad proliferation/differentiation signal across LSK subsets, in turn boosting mitochondrial activity, glucose uptake, and glycolysis.

IFNα production and type I interferon signaling control LSK expansion following β-glucan administration

To better document and characterize the early hematopoietic changes following β-glucan treatment, we sought to determine the local inflammatory response to β-glucan. β-glucan particles interact with Dectin-1 and the phagocytic synapse on responding cells.[27] Dectin-1 is not expressed in HSPCs.[19] However, it is detected on resident bone marrow macrophages, plasmacytoid dendritic cells (pDCs), and classical DCs (cDCs) (Figure 2A). To a lesser extent, $Ly6C^{lo}$ monocytes also express Dectin-1. In the peritoneal cavity, Dectin-1 expression is highest on small peritoneal macrophages (SPM) compared to large peritoneal macrophages (LPM) and is also present on resident dendritic cells (DCs) (Figure 2A). To interrogate whether β-glucan traffics to the bone marrow compartment (from the peritoneal cavity) to elicit a local inflammatory response, mice were injected (i.p.) with fluorescently labeled β-glucan (β-glucan-DTAF).[28] After 24h, we collected peritoneal exudate cells, blood cells, and bone marrow cells to assess for the presence of β-glucan-DTAF in each site by flow cytometry. However, there was minimal β-glucan-DTAF signal detected in either the blood or the bone marrow, and only residual β-glucan-DTAF in the peritoneal cavity (Figure 2B), likely due to the rapid loss of resident peritoneal macrophages via the "macrophage disappearance reaction".[29,30] We then measured the abundance of key hematopoietic-related cytokines in the bone marrow exudate fluid (BMEF) 24h after β-glucan treatment and detected elevated levels of IFNα, while IL-1β, IL-6, TNFα, G-CSF, and IFNβ remained unchanged (Figure 2C). Intracellular cytokine staining showed that the frequency of $IFN\alpha^+$ bone marrow-resident pDCs, but not macrophages, increased following β-glucan treatment (Figure 2D). IFNα levels were also elevated in the peritoneal exudate fluid (PEF) of mice treated with β-glucan, accompanied by a trend toward increased plasma IFNα levels (Figure 2E). We also detected a significant increase in $IFNAR1^+$ LT-HSCs (Figure 2F) in the bone marrow, suggesting that these primitive HSCs may be uniquely responsive to the systemic IFNα produced in response to β-glucan.

A hallmark of trained immunity is elevated inflammatory cytokine production.[31] Thus, we used the classic secondary LPS challenge[32,33] as an *in vivo* model to determine whether blocking type I IFN signaling during the early β-glucan 'training' phase affects cytokine production. To do this, mice were administered a single dose of β-glucan along with either a neutralizing anti-IFNAR1 mAb or an isotype control mAb. Seven days later, the mice were challenged with LPS (3h; i.p.) and plasma cytokine levels were measured. Plasma TNFα levels were increased in β-glucan-treated animals compared to control mice, with a trend toward elevated IL-6. IL-1β levels were comparable between β-glucan and control mice (Figure 2G). Blockade of type I IFN signaling had no observable impact on LPS-induced cytokine release, suggesting that IFNα produced early in response to β-glucan does not play a major role in the specific secondary

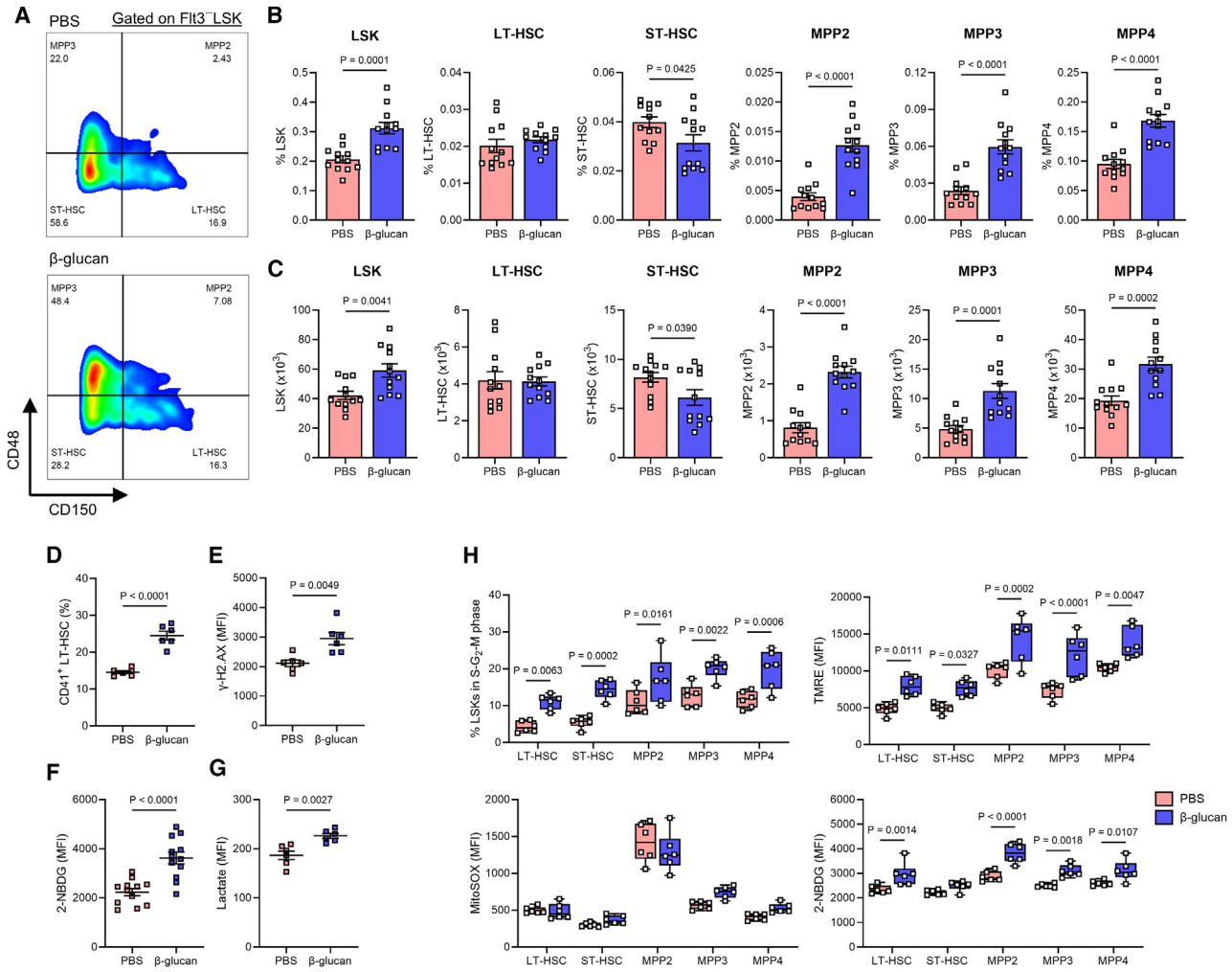

Figure 1. β-glucan administration promotes expansion and metabolic alterations in LSK subsets

Wild-type mice were administered β-glucan or PBS, and BM analysis was performed after 24 h.

(A) Representative fluorescence-activated cell sorting (FACS) plots for the identification of hematopoietic progenitor cells. After gating for Lin^- cells, LSK cells were characterized as $cKit^+Sca1^+$ cells. After gating on $Flt3^-$LSK, LT-HSC ($CD48^-CD150^+$LSK), ST-HSC ($CD48^-CD150^-$LSK), MPP2 ($Flt3^-CD48^+CD150^+$LSK), and MPP3 ($Flt3^-CD48^+CD150^-$LSK) were characterized. After gating on $Flt3^+$LSK, MPP4 ($Flt3^+CD48^+CD150^-$LSK) were characterized (gating not depicted).

(B and C) Cell frequency (B) (% of viable cells) and absolute number (C) of LSK, LT-HSC, ST-HSC, MPP2, MPP3, and MPP4 in the BM of mice after treatment with PBS or β-glucan (n = 12 mice/group).

(D) Frequency of $CD41^+$ LT-HSCs (in total LT-HSCs) in the BM of mice after treatment with PBS or β-glucan (n = 6 mice/group).

(E–G) Median fluorescence intensity (MFI) of phosphorylated γ-H2AX, 2-NBDG, and lactate in total LSKs in the BM of mice after treatment with PBS or β-glucan (n = 6 mice/group).

(H) Boxplots of the frequency of LSK subsets in the S-G_2-M phase and the MFI of TMRE, MitoSox, and 2-NBDG in LSK subsets in the BM of mice after treatment with PBS or β-glucan (n = 6 mice/group).

Data are presented as mean ± SEM. Data points represent individual mice. Significance was determined by two-tailed Student's t test (B, C, D, E, F, G, and H).

cytokine response to LPS. This prompted us to investigate the role of type I IFN signaling during the early β-glucan training phase, with a particular focus on the hematopoietic response. To do this, mice were again administered PBS or β-glucan in the presence of neutralizing anti-IFNAR1 mAb or isotype control mAb, and analysis of bone marrow progenitor cells was performed after 24 h. Antibody-mediated blockade of type I IFN signaling in β-glucan-treated mice significantly reduced the frequency of LSKs and the number/frequency of the constituent MPP2, MPP3, and MPP4 subpopulations (Figures 2H and 2I) relative to isotype control-treated animals. Neutralizing anti-IFNAR mAb had no observable impact on the frequency of LT-HSCs or ST-HSCs. Type I IFNs are known to promote Sca-1 expression.[34] To confirm that the observed anti-IFNAR1 mAb blockade of β-glucan-mediated LSK expansion was genuine and not an artifact from the loss of surface Sca-1 levels on LSKs, we next tested the purity of the $cKit^+$ ($Lin^-cKit^+Sca1^-$) compartment using alternative markers. This was achieved by employing ESAM, a marker strongly expressed on HSCs, MPP2, and MPP3 cells (Figure 2J),[22] to detect whether LSK

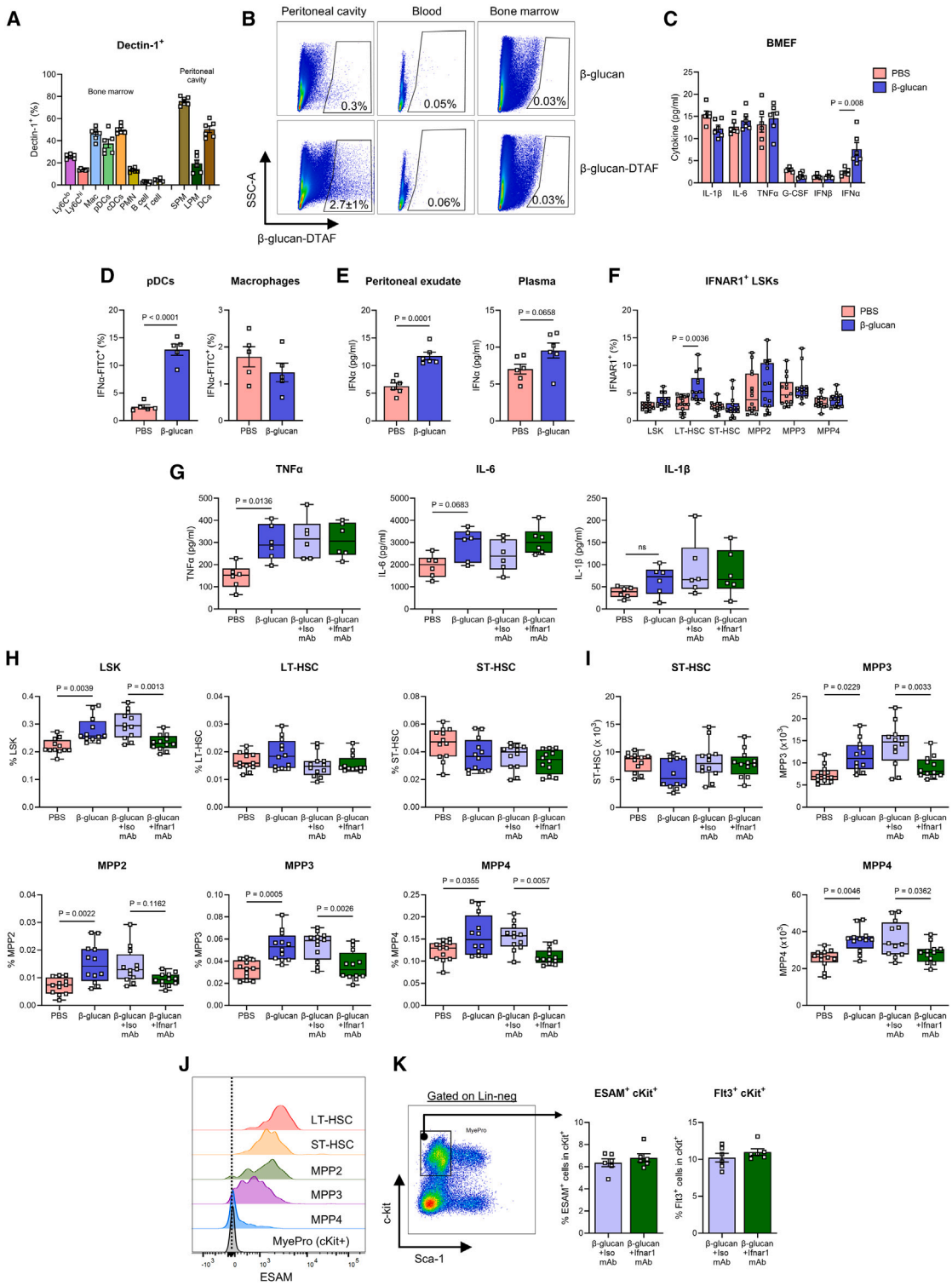

Figure 2. IFNα production and type I interferon signaling control LSK expansion following β-glucan administration

(A) Frequency of Dectin-1$^+$ leukocytes in the BM or peritoneal cavity of wild-type mice (n = 5–6 mice/group).

(B) Wild-type mice were administered DTAF-labelled β-glucan and 24 h later different tissues (n = 3) were harvested and assessed for the presence of DTAF-β-glucan by FACS. As shown, DTAF$^+$ gates were set using mice administered unlabeled β-glucan for each tissue. Representative dot plots and percentage positive are shown.

(C) Levels of cytokines/type I IFNs in the BM extracellular fluid (BMEF) of mice 24 h after PBS or β-glucan administration (n = 6 mice/group).

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subsets were present in the cKit$^+$ fraction in mice treated with β-glucan in the presence of anti-IFNAR1 mAb. Note: Flt3 was used to identify potential MPP4 contamination in the cKit$^+$ compartment as these cells only express low levels of ESAM (Figure 2J). Importantly, we found that there was no difference in the frequency of ESAM$^+$ or Flt3$^+$ cells in the cKit$^+$ compartment (Figure 2K) in mice treated with β-glucan in the presence of the anti-IFNAR1 mAb or the isotype control mAb. Collectively, these findings support the notion that IFNα generation (in the peritoneal cavity and/or bone marrow) and subsequent type I IFN signaling are important drivers of the rapid hematopoietic expansion observed following β-glucan administration.

Type I interferon signaling promotes LT-HSC proliferation and metabolic adaptations following β-glucan administration

LT-HSCs are sensitive to type I IFN signaling, which awakens them from dormancy and enables progression through the cell-cycle.[21,22] Considering β-glucan increased IFNAR1$^+$ LT-HSCs (Figure 2F) and led to a broad expansion of all downstream MPP subsets (Figures 1B and 1C), we investigated whether type I IFN signaling controls the proliferation of LT-HSCs, as well as the associated metabolic adaptations in response to β-glucan. To test for β-glucan-mediated type I IFN signaling in LT-HSCs, we first analyzed a publicly available RNA-seq dataset (GEO: GSE95617) and found that genes associated with the type I IFN signaling pathway were enriched in LT-HSCs from β-glucan-treated mice compared to PBS-treated animals (Figure 3A).[9] We also found a broad suppression of quiescence-enforcing genes, including *Cdkn1b* (p27), *Foxo1*, *Foxo3*, and *Notch2,* in LT-HSCs isolated from β-glucan-treated animals (Figure 3A). These data are reminiscent of the relaxation of the quiescence program observed in LT-HSCs following activation with the type I IFN-inducing agent poly I:C.[22] Analysis of cell-cycle progression found that 24 h after β-glucan treatment, the proportion of quiescent (G_0 phase) LT-HSCs decreased whilst the frequency of those actively dividing (S-G_2-M phase) increased (Figure 3B), consistent with the elevated numbers of progeny MPP2-4 cells (Figures 1B and 1C) and aligned with the transcriptional changes seen in LT-HSCs following β-glucan activation. Type I IFN signaling blockade, via anti-IFNAR1 mAb, reduced the frequency of LT-HSCs from β-glucan-treated mice in the S-G_2-M phase (Figure 3B). Interestingly, when the other discrete LSK subsets were analyzed, we found that the anti-IFNAR1 mAb had no impact on cell-cycle progression in ST-HSC, MPP2, MPP3, or MPP4 subpopulations after β-glucan treatment (Figure 3C). This conforms with β-glucan increasing IFNAR1 expression levels solely on LT-HSCs (Figure 2F). The increase in frequency of CD41$^+$ LT-HSC following β-glucan administration (Figure 1D) was partially blunted by co-treatment with anti-IFNAR1 mAb (Figure 3D), consistent with reports that poly I:C-induced inflammation boosts CD41$^+$ LT-HSCs.[25] This data confirms that type I IFN signaling is critical for the early proliferative response of LT-HSCs to β-glucan-induced inflammation.

To investigate the connection between cellular metabolism, β-glucan-induced proliferation, and type I IFN, we assessed mitochondrial activity in LT-HSCs. OXPHOS and ETC genes were enriched in LT-HSCs isolated from β-glucan-treated versus PBS-treated mice (Figure 3E). Consistent with this, β-glucan increased mitochondrial activity in LT-HSCs (as before, see Figure 1H), which was reduced by co-treatment with anti-IFNAR1 mAb (Figure 3F). As observed in our analysis of cell-cycle progression across the various LSK subsets (Figure 3C), blockade of type I IFN signaling did not impact the β-glucan-mediated increase in mitochondrial activity in ST-HSCs or MPP2-4 subsets (Figure 3G). The β-glucan increase in glucose uptake was reduced by anti-IFNAR1 mAb in LT-HSCs (Figure 3H) and MPP2 (Figure 3I) with no impact on the other LSK subsets. Further implicating a unique role for type I IFN signaling in LT-HSCs, we found that IFNAR1 blockade lowered γ-H2AX staining and reduced lactate production in response to β-glucan (Figure 3J). These latter findings indicate an increased reliance on glycolysis to fuel LT-HSC activation/proliferation, as has been suggested by previous studies.[9] Together, these data highlight the impact of β-glucan on LT-HSCs and underscore the central role of type I IFN signaling in the control of cell-cycle progression, mitochondrial activity, and glycolysis.

DISCUSSION

The dynamic process of hematopoietic activation in response to systemic inflammatory signals, chronic disease states, or sterile injury is an area of considerable interest. With regard to β-glucan, these adaptations are central to the sustained myelopoiesis and durable innate immune cell phenotypes it can generate.[31] HSCs/HSPCs are more sensitive than traditionally thought to inflammatory cytokines, microbial products, and metabolic cues.[8] Agents that can activate HSPCs and cause lasting changes to hematopoietic outcomes are under intense investigation for their clinical utility.[35] We have previously demonstrated the importance of type I IFN signaling in regulating macrophage metabolism.[36,37] Here, our study reveals a central role for type I IFN signaling in

(D) Intracellular staining of IFNα in plasmacytoid DCs or macrophages in the BM of mice 24 h after PBS or β-glucan administration (n = 5 mice/group).
(E) IFNα levels in peritoneal exudates and plasma of mice 24 h after PBS or β-glucan administration (n = 6 mice/group).
(F) Boxplot of the frequency of IFNAR1$^+$ LSK subsets in the BM of mice 24 h after PBS or β-glucan treatment (n = 12 mice/group).
(G) Plasma levels of TNFα, IL-6, and IL-1β from mice challenged with LPS stimulation (10μg, 3h) 7 days after administration of PBS, or β-glucan ± anti-IFNAR1 mAb or isotype control mAb (n = 6 mice/group).
(H and I) Wild-type mice were administered with PBS, or β-glucan in the presence of anti-IFNAR1 mAb or isotype control mAb, and analysis of BM was performed after 24 h. Frequency of LSK subsets (H) (% of viable cells) and absolute number (I) of ST-HSC, MPP3 and MPP4 in the BM after indicated treatments (n = 12 mice/group).
(J and K) Representative FACS plots for ESAM expression (J) on LSK subsets and identification of cKit$^+$ (c-kit$^+$Sca1$^-$) cells that are ESAM$^+$ or Flt3$^+$ (K) in mice treated with β-glucan in the presence of anti-IFNAR1 mAb or isotype control mAb (n = 6 mice/group).
Data are presented as mean ± SEM. Data points represent individual mice. Significance was determined by two-tailed Student's t-tests (C,D,E), one-way ANOVA (G,H,I), or multiple t-tests (F).

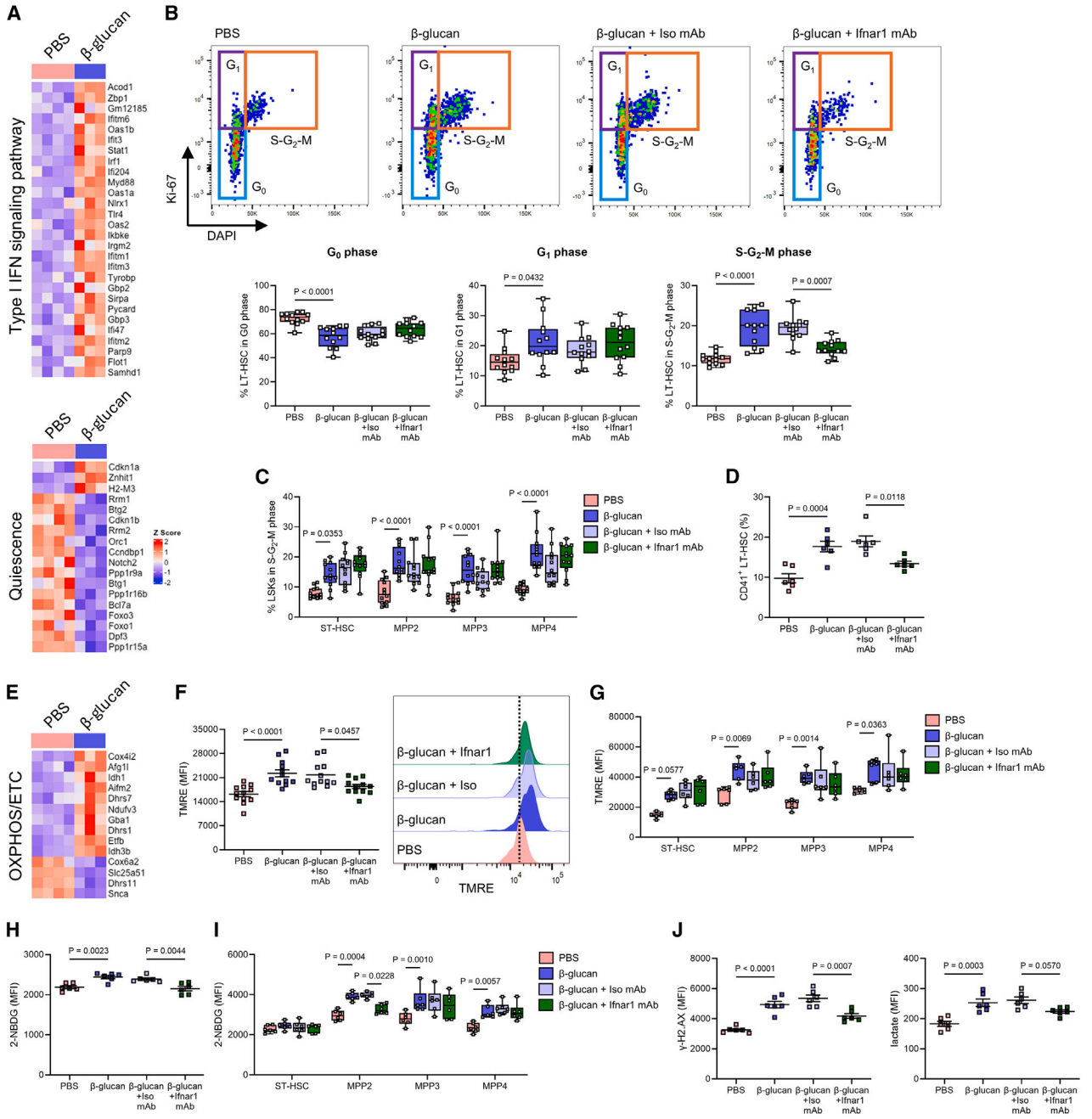

Figure 3. Type I interferon signaling promotes LT-HSC proliferation and metabolic adaptations following β-glucan administration

Wild-type mice were administered PBS, or β-glucan in the presence of anti-IFNAR1 mAb or isotype control mAb, and analysis of BM performed after 24 h.

(A) Heatmap of genes involved in the type I IFN signaling pathway and HSC quiescence in LT-HSCs from β-glucan-treated mice compared to PBS-treated mice. Gene expression values are *Z* score standardized. RNA-seq dataset (GEO: GSE95617).

(B) Representative FACS plots for cell-cycle analysis of LT-HSCs (Ki-67/DAPI) and boxplots of the frequency of LT-HSCs in G_0, G_1, or S-G_2-M phases (n = 12 mice/group).

(C) Boxplot of the frequency of different LSK subsets in the S-G_2-M phase in the BM of mice 24 h after administration with PBS, or β-glucan in the presence of anti-IFNAR1 mAb or isotype control mAb (n = 12 mice/group).

(D) Frequency of $CD41^+$ LT-HSCs (in total LT-HSCs) in the BM of mice 24 h after administration with PBS, or β-glucan in the presence of anti-IFNAR1 mAb or isotype control mAb (n = 6 mice/group).

(E) Heatmap of genes involved in OXPHOS/electron transport chain pathways in LT-HSCs from β-glucan-treated mice compared to PBS-treated mice. Gene expression values are *Z* score standardized. RNA-seq dataset (GEO: GSE95617).

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the early hematopoietic response to β-glucan. We report that IFNα is produced in the bone marrow accompanied by a specific increase in IFNAR1 surface expression in LT-HSCs following acute β-glucan stimulation. A broad expansion across all multipotent progenitor (i.e., MPP2-4) subsets was observed after β-glucan, which was blocked by neutralizing anti-IFNAR1 mAb. Subsequently, we found that type I IFN signaling controls LT-HSC cell-cycle progression and metabolic activation in response to β-glucan. Thus, not only does type I IFN signaling contribute to exaggerated myeloid cell function,[10,14] but it may also initiate the acute proliferative response of HSCs to β-glucan.

The importance of inflammatory mediators, such as IL-1β, to the expansion of HSPCs following β-glucan activation has been demonstrated.[9,11] In these reports, β-glucan induced rapid IL-1β release in the bone marrow and increased the early expansion of the myeloid-biased MPP3 subset, an effect that was abrogated when IL-1 signaling was blocked by IL-1Ra.[9] This is consistent with the myeloid gene program that IL-1 signaling promotes.[38] By way of comparison, IFNγ signaling is essential for BCG-mediated HSPC proliferation and myelopoiesis.[12] Unlike these earlier reports, we found that β-glucan specifically induced IFNα production and promoted a broad expansion of all MPP subsets, an effect that was blocked by anti-IFNAR1 mAb. These discrepancies may be due to the different β-glucan preparations used and the unique immune responses they elicit depending on their source, solubility, and purity.[27] The increased pool of CD41$^+$ LT-HSCs detected, in our view, represents a response to β-glucan-induced inflammation (which includes a key role for type I IFN) rather than the development of a myeloid-skewing subset of LT-HSCs. The observed expansion we find across MPP subsets is consistent with the ability of type I IFN to relax the expression of the quiescence-enforcing genes, such as cyclin-dependent kinase inhibitor (*Cdkn1b*) and transcription factors (*Foxo1, Foxo3*), in primitive LT-HSCs.[22] Our data likely reflects the specific action of type I IFN signaling on LT-HSCs, as evidenced by the ability of anti-IFNAR1 mAb to impair β-glucan-induced cell-cycle progression, glucose uptake, and mitochondrial activity exclusively in LT-HSCs. The kinetics of β-glucan-mediated inflammation remain unclear, as IFNα-producing pDCs and macrophages were detected in the bone marrow 7 days after β-glucan administration.[10] The hematopoietic response to β-glucan most probably involves the coordinated action of multiple inflammatory pathways (e.g., IFNα, IL-1β, G-CSF) acting across distinct progenitor populations in a temporal and overlapping manner. Indeed, β-glucan-induced type I IFN signaling may contribute to the priming of HSCs, promoting the expression of critical innate immune signaling pathways (e.g., *Tlr4, Myd88;* Figure 3A) that enable their ability to sense and respond to inflammatory challenges.[39]

Blockade of type I IFN signaling, with a single dose of anti-IFNAR1 mAb during the initial β-glucan treatment period had no impact on subsequent LPS-induced cytokine responses at day 7. This suggests that, at least in the context of inflammatory responses to LPS, the early metabolic and proliferative events driven by type I IFN in LT-HSCs, are not coupled. Whether systemic LPS stimulation, widely used to confirm the generation of trained immunity,[9,32,33] is the ideal context for exploring the role of type I IFN in innate immune training may be debatable. Recent elegant work has uncovered a role for β-glucan in reprograming hematopoiesis toward the formation of regulatory neutrophils that promote disease tolerance in the setting of lung infection.[40] In this particular study, type I IFN signaling played a crucial role in promoting granulopoiesis and a distinct program of neutrophil differentiation.[40] These findings suggest that innate immune training is not limited solely to enhanced effector function and that it may operate across diverse physiological contexts. Given that HSCs retain a 'memory' of past inflammatory events[6,7] and that type I IFNs can modulate the cellular epigenome,[37,41] further examination of the long-term impact and kinetics of type I IFN signaling/inflammation on HSC function is warranted.

Recently, i.p-administered β-glucan was found to traffic to the pancreas, carried there by resident Dectin-1$^+$ peritoneal macrophages,[28] while an earlier report suggested that it reached the peripheral blood and bone marrow.[42] Here, we found that resident SPM, LPM, and DC populations express Dectin-1. These Dectin-1$^+$ populations likely initiate the local inflammatory response triggered by β-glucan and release IFNα (and many other mediators) into the peritoneal cavity. Dectin-1 activation is known to induce type I IFN release from DCs via Syk, CARD9, and IRF5 in response to fungal challenges, where it plays a role in building antifungal defenses.[43,44] The β-glucan-mediated signaling pathways that lead to the production of type I IFNs and their involvement in trained immunity deserve further investigation. We found no evidence that β-glucan trafficked to the bone marrow compartment. β-glucan recognition in the bone marrow is possible, as resident myeloid and DC populations were found to express Dectin-1, and in the case of pDCs, were identified as a major cellular source of IFNα. However, evidence for the direct detection of β-glucan by pDCs in the bone marrow is lacking. Again, the different sources of β-glucan and their unique modes of presentation to the immune system likely impact their ability to traffic to distal sites. β-glucan may also exert its influence on hematopoiesis via 'spillover' of inflammatory mediators, such as IFNα, from the peritoneal cavity into the circulation. In agreement with this notion, a trend toward elevated plasma IFNα levels was found in β-glucan treated animals. Regardless of the site(s) of

(F and G) MFI of TMRE in LT-HSCs (F) and LSK subsets (G) in the BM of mice 24 h after administration with PBS, or β-glucan in the presence of anti-IFNAR1 mAb or isotype control mAb (n = 6–12 mice/group).
(H and I) MFI of 2-NBDG in LT-HSCs (H) and LSK subsets (I) in the BM of mice 24 h after administration with PBS, or β-glucan in the presence of anti-IFNAR1 mAb or isotype control mAb (*n* = 6 mice/group).
(J) MFI in LT-HSCs of phosphorylated γ-H2AX and lactate in the BM of mice administered with PBS, or β-glucan in the presence of anti-IFNAR1 mAb or isotype control mAb (*n* = 6 mice/group).
Data are presented as mean ± SEM. Data points represent individual mice. Significance was determined by one-way ANOVA (B,D,F,H,J), or by separate one-way ANOVA (C,G,I).

β-glucan action, our study found that IFNα production in the bone marrow was linked to rapid hematopoietic activation in response to β-glucan and suggests that it impacts primitive HSCs. Limiting lysosomal activity is another major factor in preserving HSC quiescence and self-renewal capacity.[4] Beyond its ability to relax HSC quiescence,[22] type I IFNs may induce lysosomal activation in HSCs, hastening their activation, as has been shown in other cell types, namely DCs and epithelial cells.[45,46] Our work illuminates the specific role of type I IFN signaling in HSC proliferation and metabolic activation in the context of β-glucan. Whether HSCs retain epigenetic and metabolic memory, resulting in significant and durable type I IFN-mediated effects in downstream hematopoietic and immune cell populations, remains to be determined. Similarly, the dependence on LT-HSCs and the relative contribution of more mature progenitors to the induction of trained immunity remain unclear.

There is considerable individual variance in type I IFN production, affected by factors such as age, sex, immunodeficiency, obesity, and disease.[47,48] These differences may ultimately dictate or predict the responsiveness of HSCs to agents like β-glucan, which are used to trigger an immune response. This could be particularly important for ongoing efforts to modulate trained immunity as a strategy for developing new vaccines.[35] Proper consideration of the peripheral[49,50] and central (i.e., bone marrow)[9,10,14] roles of type I IFNs in building innate immune memory is warranted. This is certainly true given the ability of pDCs in the bone marrow to sense systemic insults and alter hematopoietic outcomes, via IFNα release.[51]

Limitations of the study

Our study suggests an important role for type I IFN in the early hematopoietic responses to β-glucan. As our focus was primarily on the acute impact of β-glucan on primitive HSCs, we did not comprehensively assess type I IFNs contribution to the generation of trained mature myeloid cells or the formation of any 'memory' in these cells. As discussed, evidence for type I IFN in the building of innate immune memory in mature immune cells has been demonstrated.[10,14,40] It is also worth noting that our study used β-glucan derived from barley, and it remains unclear whether our findings are applicable to other β-glucan preparations. For example, we observed a broad expansion of all MPP subsets, whereas others have reported a distinct myeloid bias characterized by an increase in MPP3 following treatment with fungal-derived β-glucan.[9] A recent comparison of different sources and routes of administration (i.p. vs. oral) for β-glucan provides useful insights.[27] It should also be noted that all our studies were carried out in male mice.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Andrew Fleetwood (andrew.fleetwood@baker.edu.au).

Materials availability

This study did not generate any unique reagents.

Data and code availability

- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

Conceptualization, A.J.F.; Methodology, A.J.F., Y.X., N.A.D., Z.F., M.K.S.L., C.B.V., and D.D.; Validation, A.J.F. and Y.X.; Resources, N.A.D., P.J.H., and A.J.M.; Writing, A.J.F., A.J.M., Z.F., D.S., and Y.X.

DECLARATION OF INTERESTS

The authors declare no conflict of interests.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-IFNAR1 mAb (MAR1-5A3)	BioXCell	#BP0241
IgG1 isotype control (MOPC-21)	BioXCell	#BE0083
anti-mouse CD3-FITC	eBioscience	Cat #11-0032-82;RRID: AB_2572431
anti-mouse CD4-FITC	eBioscience	Cat #11-0041-82;RRID: AB_464892
anti-mouse CD8-FITC	eBioscience	Cat #11-0081-82;RRID: AB_464915
anti-mouse CD11b-FITC	eBioscience	Cat #12-0112-82;RRID: AB_464935
anti-mouse CD19-FITC	eBioscience	Cat #12-0199-42;RRID: AB_10669461
anti-mouse Gr-1-FITC	eBioscience	Cat #11-5931-82;RRID: AB_465314
anti-mouse Ter119-FITC	eBioscience	Cat #11-5921-82;RRID: AB_465311
anti-mouse c-kit-APC/Cy7	eBioscience	Cat #A15423;RRID: AB_2534436
anti-mouse Sca1-Pacific Blue	BioLegend	122520;RRID: AB_2143237
anti-mouse CD48-BUV395	BD Biosciences	740236;RRID:AB_2739984
anti-mouse CD150-PE or BV605 or PE-Cy7	BioLegend	115904;RRID: AB_313682 or 115927;RRID: AB_11204248 or 115914;RRID: AB_439796
anti-mouse CD135-APC	BioLegend	135310;RRID: AB_1953264
anti-mouse CD41-BV605	BioLegend	133921;RRID: AB_2563933
anti-mouse IFNAR1-PE	eBioscience	Cat #12-5945-82;RRID: AB_2572646
anti-mouse ESAM-PE	BioLegend	136204;RRID: AB_1953300
anti-mouse Ki-67-BV605	BD Biosciences	567122
anti-H2A.X-AF594	BioLegend	600204;RRID: AB_2734498
anti-IFNα-FITC	PBL Assay Science	22100-3
anti-Dectin-1-PE	BioLegend	144304;RRID: AB_2561500
anti-CD45	Biolegend	103132;RRID: AB_893340
anti-NK1.1	Biolegend	108729:RRID: AB_2074426
anti-CD11c	Biolegend	117310;RRID: AB_313778
anti-CD11b	Biolegend	101216; RRID: AB_312798
anti-B220	Biolegend	103207;RRID: AB_312992
ant-BST2/CD317	Biolegend	127023;RRID: AB_2687109
anti-CD169	Biolegend	142417;RRID: AB_2565640
anti-CD115	Biolegend	135523;RRID: AB_2566459
anti-F40/80	Biolegend	123116;RRID: AB_893481
anti-CD90.2	Biolegend	105319;RRID: AB_493724
anti-mouse Gr-1	BD Biosciences	562709;RRID: AB_2737736
anti-MHC II	BD Biosciences	742894;RRID: AB_2734759
Chemicals, peptides, and recombinant proteins		
β-glucan (barley)	Sigma	G6513
Lipopolysaccharide	InvivoGen	tlrl-3pelps
5-([4,6-Dichlorotriazin-2-yl]amino)fluorescein hydrochloride (DTAF)	Sigma	D0531
Tetramethylrhodamine (TMRE)	Thermo Fisher	T669
Verapamil	Sigma	V4629
2-NBDG (2-(*N*-(7-Nitrobenz-2-oxa-1, 3-diazol-4-yl)Amino)-2-Deoxyglucose)	Thermo Fisher	N13195

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
DAPI	Thermo Fisher	62248
Fixable Viability Stain 700	BD Biosciences	564997
Critical commercial assays		
Mouse TNF alpha ELISA Kit	Invitrogen	Cat #BMS607-3
Mouse IL-6 ELISA Kit	Invitrogen	Cat #88-7064-88
Mouse IL-1b ELISA Kit	Invitrogen	Cat #BMS6002
Mouse G-CSF ELISA Kit	Invitrogen	Cat #EMCSF3
Mouse IFN-a (high sensitivity)	PBL Assay Science	42115-1
Mouse IFN-beta (high sensitivity)	PBL Assay Science	42410-1
Glycolysis Cell-Based Assay Kit	Cayman Chemicals	600450
MitoSOX Red	Thermo Fisher	M36008
eBioscience Transcription Factor Staining Buffer Set	Thermo Fisher	00-5523-00
UltraComp eBeads™ Compensation Beads	Invitrogen	01-2222-42
Deposited data		
RNA sequencing data	Mitroulis et al.[9]	GEO: GSE95617
Experimental models: Organisms/strains		
C57/BL6	AMREP Animal Service Centre	In-house colony
Software and algorithms		
FlowJo	BD LifeSciences	v10.8
Prism v10.4.0	GraphPad	https://www.graphpad.com/
R studio	R Studio 2020 Team	http://www.rstudio.com/

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Experimental model

Mice

Male C57Bl/6 mice (8-10 weeks of age, weights 18-22 grams) were housed under specific pathogen-free conditions on a standard 12/12h light/dark cycle in the AMREP Animal Service Centre. Food and water were provided *ad libitum*. All experimental procedures were approved by the Institutional Animal Ethics Committee in accordance with the Australian NHMRC guidelines (Ref: E/2067/2021/B).

In vivo *administration of β-glucan*

Mice were injected intraperitoneally (i.p.) with 1mg of β-glucan (G-6513; Sigma) in 300μl of pyrogen-free phosphate-buffered saline (PBS) or PBS alone (as a control). To assess the role of type I IFN signaling, mice challenged with β-glucan were also treated i.p. with 0.5mg/mouse of anti-IFNAR1 mAb (clone MAR1-5A3) or anti-IgG1 isotype control (clone MOPC-21). Mice were euthanized 24hrs later. Tibias and femurs were cleansed of muscles and connective tissue and bone marrow cells were flushed out in PBS with a 25-gauge needle through a 40μM filter to remove any tissue. Cells were centrifuged at 3000 rpm for 5 minutes at 4°C to collect bone marrow cell pellets. Pellets were resuspended with 1mL RBC lysis buffer and incubated on ice for 5 minutes. The samples were then processed for flow cytometry. Peritoneal lavage fluid was collected by flushing the peritoneal cavity with 3 ml ice cold PBS. The samples were centrifuged, and the supernatant was used for ELISA or cell pellets prepared for flow cytometry. To assess secondary inflammatory responses, mice (treated as detailed above) were injected i.p. on day 7 with 10μg of lipopolysaccharide (LPS; InvivoGen) in 200μl of PBS. Plasma was collected by cardiac puncture from these animals 3h following LPS injection and samples were stored at -20°C for subsequent cytokine analysis.

METHOD DETAILS

Flow cytometry of hematopoietic stem cells

Cell suspensions were incubated with the following antibodies: lineage cocktail consisting of multilineage monoclonal antibodies, containing CD3/4/8 (lymphocytes), CD11b (leukocytes), CD19 (B cells), Gr-1 (granulocytes), TER119 (erythroid cells). HSC/HSPC subsets were identified by staining with Sca-1, cKit, CD48, CD150, CD135 (Flt3), CD41, and ESAM at 1:400 dilution for 45 min in the dark on ice in FACS buffer (Hanks' Balanced Salt Solution; ThermoFisher Scientific, containing 2% FBS). All samples were stained with viability dye to exclude dead cells. For cell-cycle analysis, cells were first stained for surface markers (as above), fixed and permeabilized using fixation/permeabilization buffer (TF Buffer Set; eBioscience) and stained with anti–Ki-67 (BD Biosciences). After

washing, cells were stained with DAPI (ThermoFisher Scientific) and analyzed by flow cytometry. For analysis of phospholylated-γ-H2AX, cells were first stained for surface markers (as above), fixed and permeabilized. Cell analysis was performed on a LSRFortessa X-20 flow cytometer (BD, Heidelberg, Germany). The final analysis, including the generation of FACS plots and histograms, was performed using FlowJo software v.10.9 (BD).

Intracellular IFNα staining and Dectin-1 measurement

To measure the levels of IFNα in bone marrow dendritic cells and macrophages, single-cell suspensions were stained for cell surface markers, fixed, permeabilized and stained for IFNα. Staining for pDCs ($CD45^+NK1.1^-CD90.2^-CD19^-CD11c^+CD11b^-B220^+BST2^+$) and $CD169^+$ macrophages ($CD45^+Gr\text{-}1^-CD115^{int}F4/80^+SSC^{lo}CD169^+$) was performed. For measurement of Dectin-1 on mature bone marrow leukocytes, cells were incubated with FACS buffer containing antibodies (at 1:400 dilution) against Dectin-1, CD45, CD115, Gr1 (Ly6C/G), CD19, CD3, NK1.1, CD90.2, CD11c, B220, BST2, and CD169. To measure Dectin-1 on peritoneal leukocytes, cells were incubated with FACS buffer containing antibodies against F4/80, MHC Class II, CD11c, and CD11b.[52]

Mitochondrial membrane potential and superoxide production

After cell surface staining (as above), cells were washed and incubated for 30 min at 37°C in FACS buffer with mitochondrial dyes: TMRE (25nM, ThermoFisher) or MitoSOX Red Mitochondrial Superoxide Indicator (5μM, ThermoFisher) according to manufacturer's instructions, together with verapamil (50uM). Cells were washed twice (400xg, 5 min) and analyzed by flow cytometry.

Glucose uptake assay and lactate production

For measurement of glucose uptake, freshly collected whole bone marrow cells were cultured immediately in 100μL of glucose-free medium containing 200μM of 2-(n-(7-nitrobenz-2-oxa-1,3-diazol-4-ylamino)-2-deoxyglucose (2-NBD-Glucose, 2NBDG) at 37°C. After 2hrs, cells were washed and processed for surface staining (as above) before analysis by flow cytometry. Quantification of 2NBDG uptake was measured by MFI. Cellular lactate content was assessed by adapting the spectrophotometric glycolysis cell-based assay kit (Cayman Chemical) for analysis by flow cytometry. Briefly, isolated bone marrow cells were resuspended in the glycolysis assay mixture according to the manufacturer's instructions. The cells were incubated in the dark at 37°C for 1hr before being washed twice in FACS buffer and stained with the HSC/HSPC surface markers (as above). Cell populations were identified based on their surface marker expression as shown in the general methods. Cellular lactate content within these populations was measured as the MFI of the fluorescent product of the glycolysis assay, formazan, which has a spectral profile similar to PerCP-Cy5.5.

β-glucan labeling

DTAF (Sigma-Aldrich) at 2 mg/mL was mixed with a suspension of 20 mg/mL β-glucan in borate buffer (pH 10.8) then incubated at room temperature for 8hrs with continuous mixing.[28] Following incubation, the β-glucan-DTAF was centrifuged and washed with cold sterile endotoxin-free DPBS (Sigma-Aldrich) five times or until the supernatant no longer contained visible DTAF. The concentration was adjusted to 10 mg/mL in the endotoxin-free DPBS for storage. 1 mg of the DTAF-WGP was injected (i.p.) into C57BL/6 mice. Organs were harvested 24hrs later.

ELISA

To collect bone marrow extracellular fluid (BMEF), femur and tibia bones were centrifuged (500g for 3 min) in 200μl of ice-cold PBS and the resulting supernatant was collected for ELISA. IFNα (PBL Assay Science, #42155-1), IFNβ (PBL Assay Science, #42410-1), TNFα, IL-6, IL-1β and G-CSF (Invitrogen) in BMEF, peritoneal exudates, or plasma was measured by ELISA as per the manufacturer's instructions on a Varioskan Lux Plate Reader (Thermo Fisher).

RNAseq analysis

To investigate the effect of β-glucan on LT-HSCs, we utilized a publicly available RNA-seq dataset (GEO: GSE95617), comprising transcriptome data of LT-HSCs isolated from mice administered β-glucan. As detailed in[9], genes with an adjusted p value (padj) < 0.05 and counts > 50 were considered as differentially expressed. This yielded 1,683 differentially expressed genes (false discovery rate [FDR = 0.05]) in cells from the β-glucan-injected group, compared to those from PBS-treated mice. To generate heatmaps of genes involved in the type I IFN pathway, quiescence, and OXPHOS/ETC, we used the KEGG database.

QUANTIFICATION AND STATISTICAL ANALYSIS

The specific tests used to analyze experiments and sample sizes are indicated in the figure legends, where n, represents the number of animals. Data are presented as mean ± SEM. Pairwise comparisons were performed using an unpaired two-tailed Student's t-test, and multivariate comparisons were performed using one-way ANOVA (Kruskal-Wallis test) with a Tukey's multiple comparisons test or a two-way ANOVA with a Sidak's multiple comparisons test for grouped analyses. All statistical analysis was performed using Prism software (GraphPad Software 10.3.1) and considered significant if $p < 0.05$.