

Early and Delayed STAT1-Dependent Responses Drive Local Trained Immunity of Macrophages in the Spleen

Reviewed Preprint

v1 • October 7, 2024

Not revised

Aryeh Solomon, Noa Bossel Ben-Moshe, Dotan Hoffman, Sebastien Trzebanski, Dror Yehezkel, Leia Vainman, Mihai Netea, Roi Avraham 

Department of Immunology and Regenerative Biology, Weizmann Institute of Science, 7610001 Rehovot, Israel • Department of Internal Medicine and Radboud Center for Infectious Diseases, Radboud University Medical Center, Nijmegen, Netherlands • Department of Immunology and Metabolism, Life and Medical Sciences Institute, University of Bonn, Bonn, Germany

 https://en.wikipedia.org/wiki/Open_access

 Copyright information

Abstract

Trained immunity (TI) is the process wherein innate immune cells gain functional memory upon exposure to specific ligands or pathogens, leading to augmented inflammatory responses and pathogen clearance upon secondary exposure. While the differentiation of hematopoietic stem cells (HSCs) and reprogramming of bone marrow (BM) progenitors are well-established mechanisms underpinning durable TI protection, remodeling of the cellular architecture within the tissue during TI remains underexplored. Here, we study the effects of peritoneal *Bacillus Calmette–Guérin* (BCG) administration to find TI-mediated protection in the spleen against a subsequent heterologous infection by the Gram-negative pathogen *Salmonella Typhimurium* (S.Tm). Utilizing single cell RNA-sequencing and flow cytometry, we discerned STAT1-regulated genes in TI-associated resident and recruited splenic myeloid populations. The temporal dynamics of TI were further elucidated, revealing both early and delayed myeloid subsets with time-dependent, cell type-specific STAT1 signatures. Using lineage tracing, we find that tissue-resident red pulp macrophages (RPM), initially depleted by BCG exposure, are restored from both tissue-trained, self-renewing macrophages and from bone marrow-derived progenitors, fostering long lasting local defense. Early inhibition of STAT1 activation, using specific JAK-STAT inhibitors, reduces both RPM loss and recruitment of trained monocytes. Our study suggests a temporal window soon after BCG vaccination, in which STAT1-dependent activation of long-lived resident cells in the tissue mediates localized protection.

eLife Assessment

This **important** work advances our understanding of trained immunity, especially in the context of Bacillus Calmette-Guérin (BCG) administration and host-pathogen interactions. The evidence supporting the conclusions are **convincing**, based on a combination of state-of-the-art omics techniques such as bulk and single-cell RNA sequencing with the use of JAK/STAT signaling inhibitors. The work will be of broad interest to immunologists and infection biologists.

<https://doi.org/10.7554/eLife.100922.1.sa2>

Introduction

Trained immunity (TI) is defined as the capacity of innate immune cells to recall and modulate their subsequent responsiveness following prior exposure to a diverse array of stimuli^{1,2}. Unlike the adaptive immune system, these changes are antigen-agnostic and are more reflective of sustained alterations in cellular and systemic states. TI has been demonstrated to directly reprogram the metabolic, transcriptional, and epigenetic state of monocytes and macrophages, generating heightened inflammatory capabilities in-vitro^{1,2,3}. As the transient lifespans of these cells are weeks only, this alone is insufficient to explain the sustained protection generated by TI that can last from months to years. To address this limitation, current research has been focused on long-lived multipotent progenitor immune cells.

BCG vaccine (Bacillus Calmette-Guérin), an attenuated strain of *Mycobacterium bovis*, is a potent inducer of TI, which facilitate nonspecific protection against heterologous pathogens in both mice, primates, and humans^{3,4}. The effects of intravenous (i.v.) BCG vaccination, where bacilli persist within the bone marrow (BM), drive the expansion of LSK⁺ hematopoietic stem cells (HSCs) and multipotent progenitors (MPPs)⁴. In conjunction, MPPs undergo biased differentiation towards myelopoiesis, increasing the proliferation of myeloid cells. These effects are driven primarily by interferon activation, particularly that of interferon-gamma (IFN γ) and its downstream transcription factors, STAT1 and IRF1, in MPPs and short-term HSCs, resulting in a transcriptional program of cell cycling (Cdk1, Cdk4, Ccnf) and histone modifications. Furthermore, these newly differentiated circulating cells have enhanced microbicidal properties^{5,6}. Upon infection, in order to elicit protection against invading agents, trained progenitor immune cells from the BM interact within local immune cell compartments in the tissue. The nature of these interactions and how TI translates within the tissue and the local cellular architectures is not completely understood.

Most tissues harbor long-lived resident macrophages capable of self-renewal, independent of BM-derived precursors. These resident populations, including pulmonary alveolar macrophages, Kupffer cells in the liver and red pulp macrophages in the spleen, originate from embryonic erythro-myeloid progenitors⁵. Antimicrobial activity of resident populations control early stages of invasion by pathogens but are then depleted, and the niche is repopulated either by self-renewal⁶ or by progenitors derived from the BM to maintain local functions⁷. Intriguingly, resident alveolar macrophages (AM) have been shown to undergo in-situ training, maintaining an altered phenotype over an extended period^{10,11,12}. One mechanism driving this training in-situ in AMs involves CX3CR1⁺ T cells, which have been implicated in local reprogramming via IFN γ release. This process has been demonstrated to occur around three weeks after initial BCG vaccination, after which interferon-driven responses occur across the organism, including lung

and BM, resulting in viral and mycobacterial protection. It remains unclear however, how during TI, a bridge is formed between peripheral, resident cells and BM-derived recruitment of trained myeloid cells.

In this study, we set out to investigate the interplay of circulating and tissue-level immune cells that mediate protection and remodeling during TI. To induce tissue specific TI, we used a model of intraperitoneal (i.p.) injection of BCG, which rapidly delivers the bacteria to target lymphatic organs (e.g., spleen), and measured protection against subsequent *Salmonella* Typhimurium (S.Tm) challenge. Within the spleen's unique structure and cellular composition, we characterized STAT1-mediated, cell type-specific TI signatures. We demonstrate that an initial depletion of resident red-pulp macrophages is followed by replenishment by recruited trained monocytes and a local self-renewing population, contributing to the maintenance of STAT1 signatures and long-lasting protection within the tissue.

Results

Intraperitoneal BCG results in heterologous S.Tm protection and a distinct myeloid subsets with signatures driven by STAT1

To establish an in-vivo BCG training model targeted to the splenic tissue, and assess the extent of cross pathogen tissue protection conferred, we administered BCG-Pasteur⁸ (5×10^6 colony forming units (CFU)) or PBS as a control via i.p injection to 8-week-old female C57BL/6J mice. Following a two-week training period, mice were challenged with S.Tm (5×10^5 CFU) through i.p inoculation (**Fig. 1A**). Mice were sacrificed after twenty-four hours, and spleen homogenates were cultured on LB agar medium to quantify S.Tm load using CFU (**Fig. 1B**). Relative to the control group, mice that received BCG exhibited enhanced protection against S.Tm, with a four- to five-fold decrease in CFU, indicating TI-mediated splenic protection.

For identification of transcriptional changes in splenic myeloid populations related to TI, we isolated splenocytes and performed staining with CD11b, Ly6C, and F4/80 gating for myeloid mononuclear phagocytes (MPs), including classical monocytes (CM) ($CD11b^+Ly6C^+$), and $CD11b^+Ly6C^-$ MPs comprising non-classical monocytes (NCM) and conventional dendritic cells (cDC), and also resident red pulp macrophages (RPM) ($CD11b^-F4/80^+$) (**Fig. 1C**, **Figure 1 — figure supplement 1A**). We observed an overall expansion of the myeloid compartment due to training, with an increase in the $CD11b^+$ subset, and a pronounced reduction of resident RPM (**Fig. 1D**). This observed loss of RPM is common during infection and inflammation and has been previously described as the resident macrophage disappearance reaction⁹.

We next determined transcriptional alterations caused by TI across the myeloid compartment. $CD11b^+$ cells from trained and naïve mice were isolated by sorting and processed for single-cell RNA-sequencing (scRNA-seq)¹⁰. K-nearest neighbor (K-NN) clustering differentiated six distinct cell types, designated as CM, NCM, immature DC2 (cDC2), mature DC2 (mDC2), neutrophils, and NK cells (**Fig. 1E**). The identity of each cell type cluster was based on established transcriptional markers (**Fig. 1F**). Additional analysis was performed to identify differentially expressed genes (DEG) unique to training (**Fig. 1G-I**; **Table S1**). While most populations derived from control or trained mice clustered together by cell type, we identified a subset of trained CM (CM-T) particular to BCG (**Fig. 1G**). These cells uniquely express a set of inflammatory genes regulated by STAT1 activity, as part of the IFN γ response (e.g., *Gbp2*, *Ly6a*, *Cxcl9*, and *Irg1*) (**Fig. 1J**). Notably, these STAT1 activation genes are known to drive maturation and activation of monocytes and macrophages, enhancing their antimicrobial response¹¹. GBP2 (guanylate binding protein 2), part of the broader GTPase family, is highly activated during IFN γ stimulation and can bias macrophages to an inflammatory M1 state¹², as well as inhibit gram-negative intracellular bacterial growth via disruption of the pathogen containing vacuole and pyroptosis induction¹³.

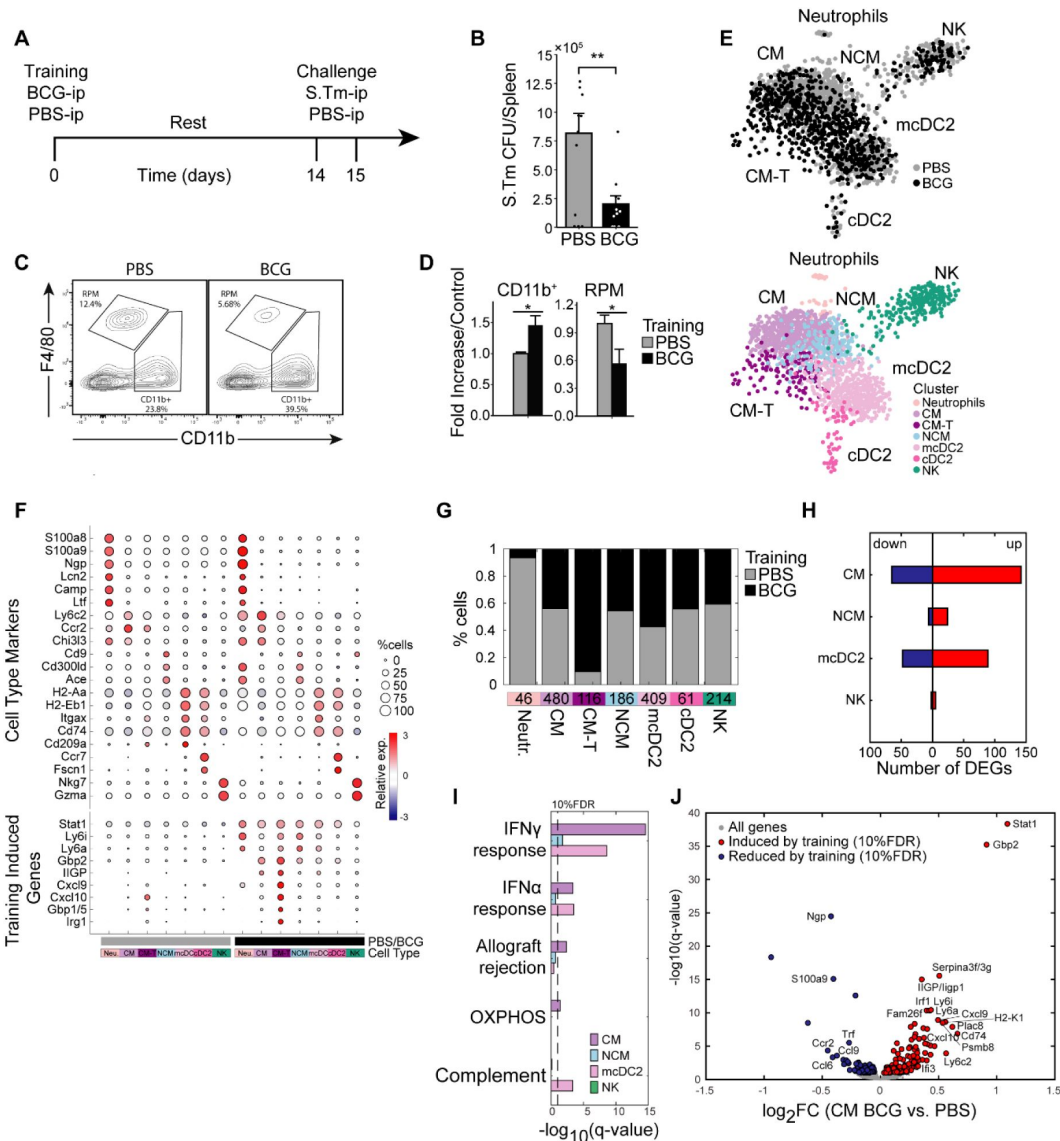


Figure 1.

Intraperitoneal BCG results in heterologous *S.Tm* protection and a distinct myeloid subsets with signatures driven by STAT1.

(A) Experimental schema of in-vivo training. (B) Splenic *S.Tm* CFU 24 hours post infection between control ($n=11$) and trained mice ($n=11$). (C-D) Flow cytometry plots of myeloid populations (C) and mean percentage fold change of BCG over control for each given gated population percent from the Lin⁺ population (D). (E) K-nearest neighbors (KNN) plot for total CD11b⁺ single cells sorted from control and BCG mice. Color is based on conditions, or cluster identity. (F) Cell markers and training induced genes for each subset. Size and color intensity indicates percentage of cells within a given cluster expressing the gene and average expression. (G) Proportions of monocyte subsets based on classifications in E. (H-I) Number of DEGs in each cell subset (H), and their corresponding gene set enrichment analysis (I). (J) Volcano plot of DEGs in CM-T subset. Data in bar graphs are presented as mean $\pm$ SEM, with each individual point in B a biological repeat. Two-tailed *t*-test used for data in B and D (* $P<0.05$, ** $P<0.01$).

CXCL9 (chemokine ligand 9) is known to be involved in T cell and monocyte recruitment to infection sites and their subsequent activation¹⁴, and its role in trained immunity has been previously suggested, as it is a key player in the formation of a pro-inflammatory milieu that facilitates rapid and efficient pathogen clearance¹⁵. IRG1, an enzyme that catalyzes the production of itaconate, is a metabolite that triggers metabolic reprogramming of MPs with both antimicrobial and immunomodulatory properties¹⁶. The enhanced production of itaconate may further contribute to the protective effects of TI by directly inhibiting pathogen growth and fine-tuning the immune response to prevent excessive inflammation¹⁷. However, the role of IRG1 in training is context dependent, as monocyte exposure to β -glucan exposure, a common training ligand, has been demonstrated to block its activity and the subsequent accumulation of itaconate¹⁸.

Stat1 and its downstream targets (e.g., *Gbp2*, *Irf1*, and *Ly6a*), exhibited a pronounced elevation also in other myeloid cells (**Figure 1 — figure supplement 1B**), aligning with an earlier report that BCG induces BM and HSC remodeling via interferon signaling, resulting in STAT1 upregulation in progenitors¹⁹. However, not all populations were similarly activated in conjunction with STAT1-regulated genes, with gene set enrichment analysis (GSEA) demonstrating differential pathway upregulation across cell types (**Fig. 1I**). CM and mcDC2 primarily activated the IFN γ response, while complement associated genes were enriched in mcDC2. NK cells alone showed minimal activation due to training across all detected pathways (**Figure 1 — figure supplement 1B**).

We also observed increased SCA-1 (*Ly6a/e*) expression across the monocyte subsets and mcDC2, most notably in NCM (**Fig. 1I**; **Figure 1 — figure supplement 1B**). SCA-1 (stem cell antigen-1) is a glycosylphosphatidylinositol-anchored cell surface protein commonly used as a marker for murine hematopoietic stem and progenitor cells within the BM. The STAT1 signaling pathway has been shown to be involved in the regulation of SCA-1 expression²⁰, suggesting a role for STAT1 in the modulation of SCA-1 during development of TI. Recent studies have also demonstrated a link between SCA-1 and inflammation, with its expression found to be upregulated on a specific subset of Ly6C⁺ monocytes during infection²¹. These SCA-1⁺ monocytes exhibited a pro-inflammatory phenotype, characterized by the production of inflammatory cytokines and chemokines, and were implicated in the amplification of these responses. For Ly6C⁺ NCM, the role of SCA-1 has not yet been studied.

In addition to the upregulation of inflammatory and antimicrobial genes, we also observed a significant downregulation of specific genes in CM-T as a result of training, including *Ccr2*, *S100A8/9*, and *Ngp*. CCR2, a chemokine receptor crucial for monocyte recruitment, was found to be downregulated in response to IFN γ . This reduction in CCR2 expression is mediated by IFN γ -induced mRNA instability, potentially serving to retain monocytes at the site of recruitment and dampen a positive feedback loop²². Similarly, BCG has been shown in other contexts to reduce the expression of S100A8 and S100A9, two calcium-binding proteins that can heterodimerize and stimulate IFN γ production in CD4⁺ T-cells via an IL-10 dependent mechanism²³. By suppressing their activity, runaway signaling and exhaustion is avoided. Lastly, NGP (neutrophilic granule protein), has been implicated in the regulation of inflammation through its ability to block NF- κ B signaling²⁴. The downregulation of these genes suggests that trained immunity not only enhances pro-inflammatory responses but also modulates the expression of key regulators to maintain a balanced immune response and prevent excessive inflammation.

Dynamics of TI-associated subsets and signatures indicates early and delayed kinetics

To gain insight into dynamic processes underlying splenic cell type-specific TI, we conducted a two-month experiment, sacrificing mice at days 3, 14, 30, 45, and 60 post-vaccination with BCG (**Fig. 2A**). We assessed BCG growth in BM and spleen, resistance to *S.Tm* infection, flow cytometry analysis with cell type-specific training markers (CXCL9 and SCA-1), and bulk RNA-seq.

Notably, protection against *S.Tm* persisted for two months post-vaccination, albeit with waning resistance over time (**Fig. 2B**). While intravenous (i.v) BCG delivery and localization in BM were crucial for robust training^{19,25}, our results indicate that during i.p administration, direct BCG-immune cell interactions in the BM may not be required, as no BCG bacteria within the BM could be detected (**Fig. 2C**). Conversely, in the spleen, we isolated BCG at all time points, with CFU declining sharply by day 14, reaching the limit of detection by day 30 with minimal bacterium remaining (**Fig. 2C**).

Flow cytometry analysis revealed a dynamic process during trained immunity (**Figure 2—figure supplement 1A**). BCG exposure triggers a dramatic decline in RPMs at day 14, that subsides thereafter (**Fig. 2D**, **Figure 2—figure supplement 1B**). At the same time, we observed rapid recruitment of CMs in the spleen, starting already at day three after BCG, peaking at day 14 before returning to baseline (**Fig. 2D**). Early recruited CM expressed CXCL9, a marker gene of STAT1-mediated TI, which we observed was most elevated at day 14 (**Figure 2—figure supplement 1C**). In a separate experiment to assess early kinetics of CXCL9⁺ CM, we measured a significant increase of this subset already at five days post BCG (**Figure 2—figure supplement 1D**), indicating an early recruitment of these cells due to the initial response to BCG. NCMs exhibit a different TI signature and kinetic pattern. While their ratio initially declines, they return to steady-state levels by day 30 (**Fig. 2D**). NCMs express SCA-1 (*Ly6a*), another STAT1-regulated gene, which persists and remains highly elevated at all subsequent time points (**Figure 2—figure supplement 1C**). When challenged with a subsequent *S.Tm* infection at day 14, CXCL9 expression in CMs was increased, but these differences were entirely lost by day 60 (**Figure 2—figure supplement 1E**). In contrast, SCA-1 expression in NCMs remained upregulated (**Figure 2—figure supplement 1E**).

Notably, the expression of STAT1-mediated TI markers is not solely restricted to BM-derived myeloid cells but also occurs in tissue-resident RPM. RPM loss is evident after 14 days of training (**Fig. 2D**), together with increased expression of the STAT1-regulated CXCL9 (**Figure 2—figure supplement 1C, D**). SCA-1⁺ RPM levels fluctuate, elevating at day 14 and increasing again at day 60. Given their pivotal role in curbing the early replication of intracellular pathogens²⁶, such as *S.Tm*, and their long life span, as opposed to CM and NCM²⁷, trained RPM could be an additional determinant in generating long-lasting, localized tissue protection.

From the splenocytes collected during the kinetics experiment, we generated and analyzed bulk RNA-seq data. Principal component analysis (PCA) displayed a substantial shift along PC1 due to BCG training (**Figure 2—figure supplement 1F**). Examination of all differentially expressed genes (DEGs) between BCG and PBS across these time points revealed the greatest transcriptional changes at day 14 and 30 (**Fig. 2E**; **Table S2**). To investigate the biological processes represented at these time points, GSEA was performed. At day 14, we detected enrichment for IFN γ response, IFN α response, and IL6-JAK-STAT signaling, with many genes shared across these pathways. In contrast, day 30 was characterized by enrichment of E2F targets, G2M checkpoint, and heme metabolism (**Fig. 2E, F**). Within these terms, especially for the IFN γ response, we noted that associated genes have differential expression patterns. For example, *Irg1* upregulation is limited to day 14 (**Figure 2—figure supplement 1G**) returning to control values for all subsequent time points. This may reflect that IRG1 induction requires bacterial phagocytosis and activation of the TLR-2/MYD88/NF κ B axis²⁸. Conversely, an increase in *Cxcl9* expression is already observed by day 3, plateauing at day 14, and decreasing by day 30, mirroring its induction in CM-T in the flow cytometry analysis. Curiously, despite its loss in CM, the bulk transcriptional expression of *Cxcl9* is still sustained after 2 weeks, though at reduced levels. This may be attributed to its activity in other immune cells or regulatory mechanisms preventing further translation. *Stat1* and its downstream target *Ly6a* (SCA-1) maintain high expression 2 months post vaccination (**Figure 2—figure supplement 1G**). Several other interferon-regulated genes were also upregulated at day 14, including the *Gbp* gene family, *Ifitm3*, and *Socs3* (**Fig. 2G**). GBPs are guanylate-binding proteins involved in antimicrobial activity and inflammasome activation²⁹, while IFITM3 is an interferon-induced transmembrane protein that plays a role in the antiviral response³⁰. SOCS3,

a suppressor of cytokine signaling, particularly via IL-6/STAT3, is necessary component for mycobacterial infection control. By blocking IL-6 signaling, SOCS3 allows for TNF and IL-12 signaling to occur, initiating CD4-dependent IFN γ release³¹. Importantly, while the IFN γ response was reduced after 2 weeks, we detected sustained upregulation of these genes for all subsequent time points (**Fig. 2H**), strengthening its role and that of the JAK-STAT pathway in mediating the effects of TI.

Our findings also revealed that day 30 was enriched for cell cycling and heme metabolism (**Fig. 2F**). Interestingly, RPM play a crucial role in iron homeostasis, recycling and storing iron from senescent erythrocytes, preventing accumulation of free iron that can lead to ROS production, oxidative damage, and pathogen utilization³². Given the dynamics of RPM loss by day 14, it is tempting to speculate that their subsequent replenishment is reflected by these processes.

We then wanted to determine whether TI signatures in the spleen are due to recruitment of progenitors from the BM or sustained local signals in the tissue. BCG or PBS were injected i.p into mice with either a CD45.1 or CD45.2 background, respectively (**Figure 2—figure supplement 1H**). After two weeks, BM was harvested from both, HSCs mixed 1:1, and injected into irradiated mice for BM transplant. Six weeks post transfer, mice were sacrificed and myeloid cells in the spleen were assessed (**Figure 2—figure supplement 1I**). Within each mouse, we found in the spleen a greater fraction of NCM expressed SCA-1 from trained donors relative to the naïve control (**Figure 2—figure supplement 1J**). CXCL9, which we had previously observed in CM, was undetectable in the populations, possibly because its expression is early and transient, as seen in the kinetics data.

RPM niche is replenished by recruited trained monocytes and by local training of tissue-resident populations

We observed a substantial reduction in RPM numbers upon BCG, followed by the expression of STAT1-mediated TI markers in RPMs. We hypothesized that upon BCG, two possible scenarios for training and replenishment of open niche are possible. The first is self-renewal by the remaining local tissue-resident macrophages who are trained within the tissue, while the other is that BM-derived trained monocytes differentiate and repopulate the open niche. While BM-derived macrophages may adopt signatures and function of their local counterparts, they may also retain aspects of their origin, particularly enhanced inflammatory capacities (**Fig. 3A**). To investigate this, we employed MS4A3^{TdTomato};CX3CR1^{GFP} reporter mice³³ with a knock-in flox-cre system to selectively label BM-derived monocytes with TdTomato fluorescence. As MS4A3 is distinctly expressed in granulocyte-monocyte progenitors (GMP), only this lineage will be TdTomato positive. These mice were administered BCG or PBS-i.p following the training protocol and sacrificed two weeks later (**Fig. 3B**). Effective labeling was determined by flow cytometry, measuring the fraction of MP expressing TdTomato and/or CX3CR1 (**Figure 3—figure supplement 1A**). As expected, the monocyte population were primarily double positive (**Figure 3—figure supplement 1B**). Intriguingly, training increased the percentage of labeled cells across monocytes, suggesting a potential lineage bias towards granulocyte-monocyte progenitors (GMPs). This observation is consistent with prior studies demonstrating that various microbial components can induce short-term differentiation biases in monocytes derived from GMPs or MDPs (monocyte-dendritic progenitors), endowing them with neutrophil- or dendritic cell-like properties³⁴.

For the majority of RPM, we expected minimal TdTomato expression, representative of self-replenishment during homeostasis. However, already within the control we observed that ~16% of the population were labeled (**Fig. 3C**). When this population was depleted during training, an even greater fraction was positively labeled. Accordingly, even under steady-state conditions, BM-derived cells contribute to the resident niche, but when sufficiently diminished post vaccination, active replenishment from the BM does occur.

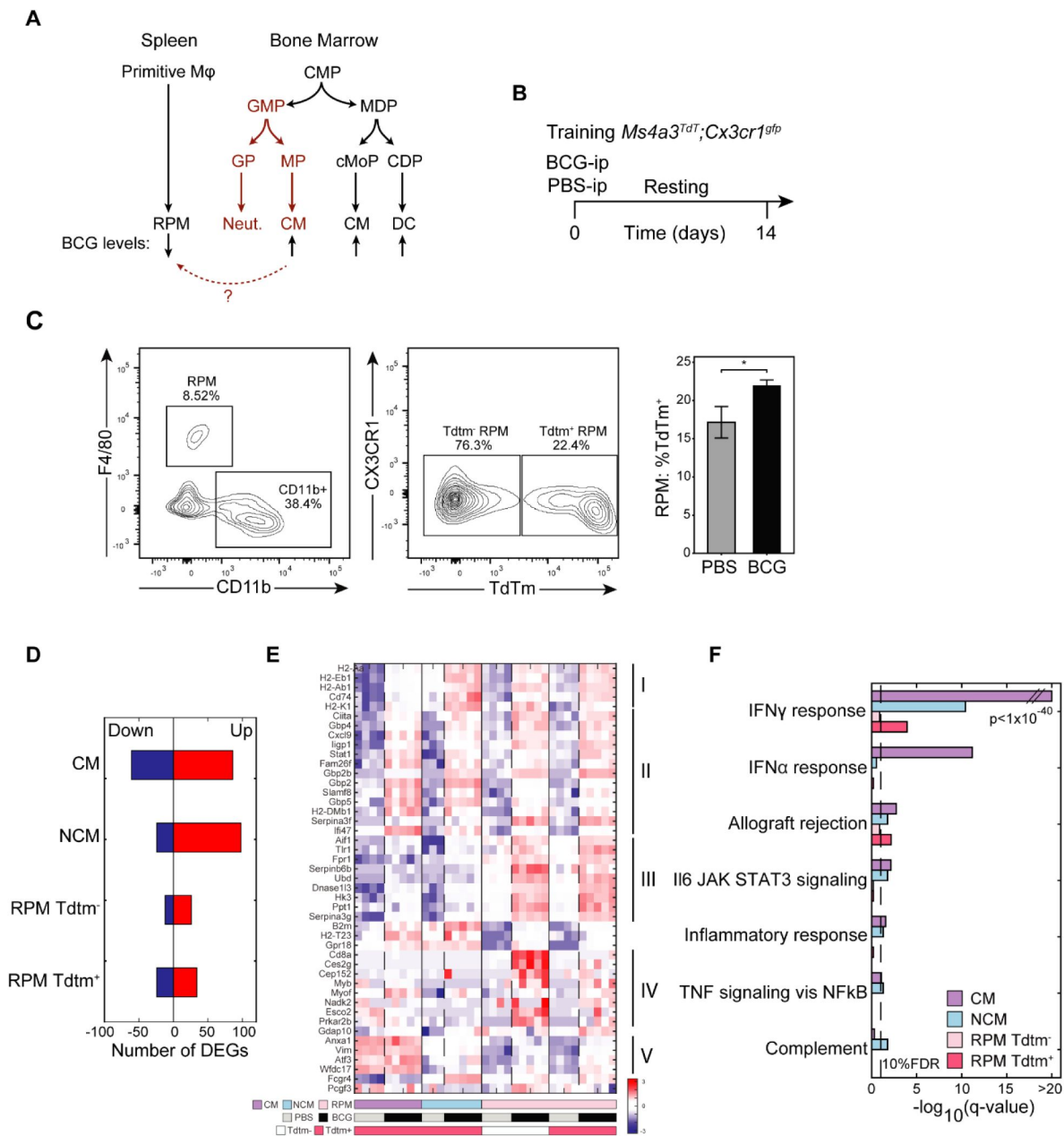


Figure 3.

RPM niche is replenished by recruited trained monocytes and by local training of tissue-resident populations.

(A) Scheme representing known myeloid differentiation pathways and potential trans-differentiation of trained CM to RPM. (B) Experimental schema for lineage tracing of TI-associated MP populations. (C-D) Flow cytometry analysis of Tdtm⁺ or Tdtm⁻ Ly6C⁺ MPs and RPM and quantification of RPM Tdtm⁺ subset (n=3). (D-F) Number of DEGs of each sorted population (D), heatmap of normalized log₂ expression from TI-associated DEGs specific to trained RPM populations and gene set enrichment analysis of DEGs in each sorted population (F). Data in bar graphs are presented as mean±SEM. Heatmap rows in E indicate biological replicates. Two-tailed *t*-test used for data in C (**P*<0.05).

To determine whether Tdtm⁺ BM-derived RPM acquired a distinct transcriptional profile due to training compared to the native Tdtm⁻ population, we sorted Tdtm⁺ RPMs, Tdtm⁻ RPMs, and Tdtm⁺ CM and NCM, from both trained and naïve conditions. These sorted cell populations were then subjected to bulk RNA-seq and subsequent analysis. Crucially, training associated genes were differentially expressed across all sorted cell types (**Fig. 3D** [↗](#); **Figure 3—figure supplement 1C**; **Table S3**). When evaluating specific DEGs upregulated across trained RPM, we identified five gene clusters that varied between CM, NCM, and RPM, as well as genes primarily upregulated in Tdtm⁺ or Tdtm⁻ RPM (**Fig. 3E** [↗](#)). Cluster I is composed of MHCII-associated genes (*H2-Aa*, *H2-Eb1*, *CD72*, etc.), and is enriched in NCM and RPM cells, indicating enhanced antigen presentation that may facilitate the activation of humoral immunity. Cluster II consists of the STAT-1 regulated TI hallmark genes, including *Cxcl9*, *Gbp2* and *Stat1* and is upregulated in all trained subsets. Genes in Cluster III, such as *Aif1*, *Fpr1*, and *Hk3*, are primarily observed in trained RPM and are involved in response to tissue damage/disruption and immune infiltration. AIF1 is an established marker of macrophage activation and is functionally involved in phagocytosis and membrane ruffling³⁵ [↗](#). Similarly, FPR1, a formyl peptide receptor, induces chemotaxis, phagocytic uptake, and reactive oxygen species (ROS) production³⁶ [↗](#). Cluster IV is upregulated in BCG Tdtm⁻ RPM, though the role of most genes detected in this cluster remains unknown in regard to their tissue-specific function and activation. Interestingly, *CD8A* expression, typically relegated to lymphocytes, was observed in this cluster. In monocytes, CD8A can co-engage with FcR, resulting in TNF release³⁷ [↗](#). Lastly, cluster V, which is primarily activated in CM regardless of training, was also upregulated in RPM due to BCG. Three of the genes within the cluster, *Anxa1*, *Vim*, and *Wfdc17*, have all been shown to dampen excess inflammatory responses through various mechanisms, including suppressing oxidative stress and promoting local resolution^{38–40} [↗](#).

GSEA performed across all upregulated genes revealed that the IFN γ response was enriched, followed by IFN α response, allograft rejection, and JAK-STAT signaling (**Fig. 3F** [↗](#)). Although the IFN γ signature was most prominent in CM, it was also observed in NCM and in the Tdtm⁺ RPM. This finding suggests that engrafted monocytes differentiating within the niche may maintain a more inflammatory phenotype and a heightened sensitivity to IFN γ activation. However, the BM-derived RPM are not the sole population responsive to training, as the local fraction also upregulates the same genes in clusters I-III. Notably, RPM, as a whole, demonstrate a greater capacity to upregulate the expression of many interferon genes, including *Cxcl9* and *Stat1* (**Figure 3—figure supplement 1D**). Taken together, our results indicate that BCG can reprogram populations and generate training via two separate routes. First, the recruitment of trained progenitors and monocytes within the spleen, which differentiate within a vacant niche, retaining their trained identity. Second, activation directly within the spleen in the context of native tissue-resident RPM, generating tissue-specific protection.

Transient IFN γ -STAT1 inhibition prevents TI signatures and splenic infection resistance

We observed that STAT1 signaling holds a critical role in training, with its regulated gene expression elevated across the myeloid population. However, BCG is an intact attenuated bacterium that can activate numerous PRRs. To evaluate whether STAT1 is necessary for TI signatures and protection, we vaccinated STAT1-KO mice⁴¹ [↗](#) with either PBS or BCG-i.p (**Figure 4—figure supplement 1A**). After a two-week period, we assessed the myeloid population and STAT1 regulated genes. We observed a complete absence of expression for both CXCL9 and SCA-1 across myeloid populations (**Figure 4—figure supplement 1B**). This aligns with previous findings that demonstrated compromised acquisition of trained immunity in IFN γ R^{-/-} mice¹⁹ [↗](#). Interestingly, RPM, typically depleted following BCG inoculation, remain preserved in STAT1-KO mice (**Figure 4—figure supplement 1C**), suggesting that STAT1-mediated pathways are involved in triggering the cellular death processes that occur during BCG interaction and/or engulfment.

There is however a significant limitation in this mouse model, as STAT1^{-/-} mice are highly susceptible to infection due to a severe compromise of immune homeostasis, limiting our ability to assess TI-mediated protection upon *S.Tm* challenge. In light of our findings that STAT1 signaling is activated shortly after BCG administration, we sought to transiently restrict STAT1 activity at these early time points, also enabling us to investigate the effect of inhibition of STAT1 signaling on training and protection, without affecting STAT1 activation during a secondary *S.Tm* challenge. To accomplish this, we used Fedratinib, a specific inhibitor of JAK2 activation of STAT1 through IFN γ signaling³⁴, and Deucravacitinib, a specific inhibitor of TYK2 activation of STAT1/STAT2 through IFN α/β signaling⁴². First, we administered DMSO (control), Deucravacitinib, and Fedratinib i.p followed by BCG or PBS-i.p four hours after. These inhibitors were then injected daily via i.p for the following four days, followed by a subsequent nine-day rest period (**Figure 4—figure supplement 1D**). At the two-week mark, splenocytes were extracted from all mice and analyzed by bulk RNA-seq. Only Fedratinib, not Deucravacitinib, resulted in inhibition of STAT1-mediated TI signatures (**Figure 4—figure supplement 1E**; **Table S4**).

We then repeated this experiment, focusing on training phenotypes in the spleen and BM. At the two-week mark, splenocytes were extracted from all mice for flow cytometry analysis, with CXCL9 utilized as a marker for STAT1-mediated TI signature in monocytes. As observed in the transcriptional response, only Fedratinib ablated expression of CXCL9 in CM (**Figure 4—figure supplement 1F**). We also sought to determine if these effects were localized solely to the tissue, or if they extended to progenitors in the BM, which expand upon BCG exposure¹⁹. To ascertain this, we isolated BM from the femur, measuring the percent of LSK⁺ HSCs (**Figure 4—figure supplement 1G**). Here too, only Fedratinib resulted in suppressing their expansion to levels comparable to the control (**Figure 4—figure supplement 1H**). Conversely, perturbation of IFN α/β signaling with Deucravacitinib lead to no observable changes on trained subsets, suggesting that it is not involved in our BCG-i.p model. Type-I interferon has been established as a training signaling pathway in other contexts, as observed with β -glucan⁴³, *Candida Albicans*⁴⁴ and LPS⁴⁵.

In order to prove that early STAT1 inhibition is sufficient to block the TI protective phenotype, and not just downstream markers, we repeated the inhibitor regime with control or trained mice receiving either Fedratinib or DMSO, with or without *S.Tm* infection after 2 weeks (**Fig. 4A**). We then extracted spleens to measure splenic expansion, splenocyte population levels and marker expression with flow cytometry, bulk splenocyte transcription, and *S.Tm* susceptibility. Importantly, while we observed no differences in CFU between control mice with or without Fedratinib, trained mice receiving Fedratinib were significantly more susceptible to infection (**Fig. 4B**). Treatment with Fedratinib resulted in diminished recruitment and splenocyte expansion, causing an appreciable reduction in spleen size comparable to the control (**Figure 4—figure supplement 1I**). Accordingly, the balance of monocyte ratios, particularly NCM, was shifted to levels similar to those observed in the DMSO control (**Fig. 4C**). In conjunction, the expression of CXCL9, an early training marker, was reduced in both CM and RPM (**Fig. 4D**). Finally, RPM, which typically undergo depletion after training, exhibited significantly enhanced survival, similar to the observations in STAT1-KO mice.

To probe the effects of Fedratinib inhibition beyond myeloid expansion and marker acquisition, we conducted bulk RNA sequencing on total splenocytes isolated from all experimental conditions. PCA of the resulting data revealed two major axes of divergence among the populations: PC1, associated with training, and PC2, associated with the response to *S.Tm* (**Figure 4—figure supplement 1J**). While the PBS-i.p mice treated with either DMSO or Fedratinib were grouped together, the BCG-i.p samples treated with Fedratinib clustered distinctly, shifting closer to the PBS control group. Analysis of downregulated DEGs identified the IFN γ response as the most significantly affected pathway due to JAK2 inhibition by Fedratinib. This included its downstream effector *Stat1*, and other key STAT1-regulated trained immunity genes, such as *Cxcl9/10*, *Irf1*, *Gbp2*, and *Irg1*, across all treated mice (**Fig. 4E, F**; **Table S5**). To further validate our findings and ensure that the loss of the trained immunity signature was not solely a result of blocking other

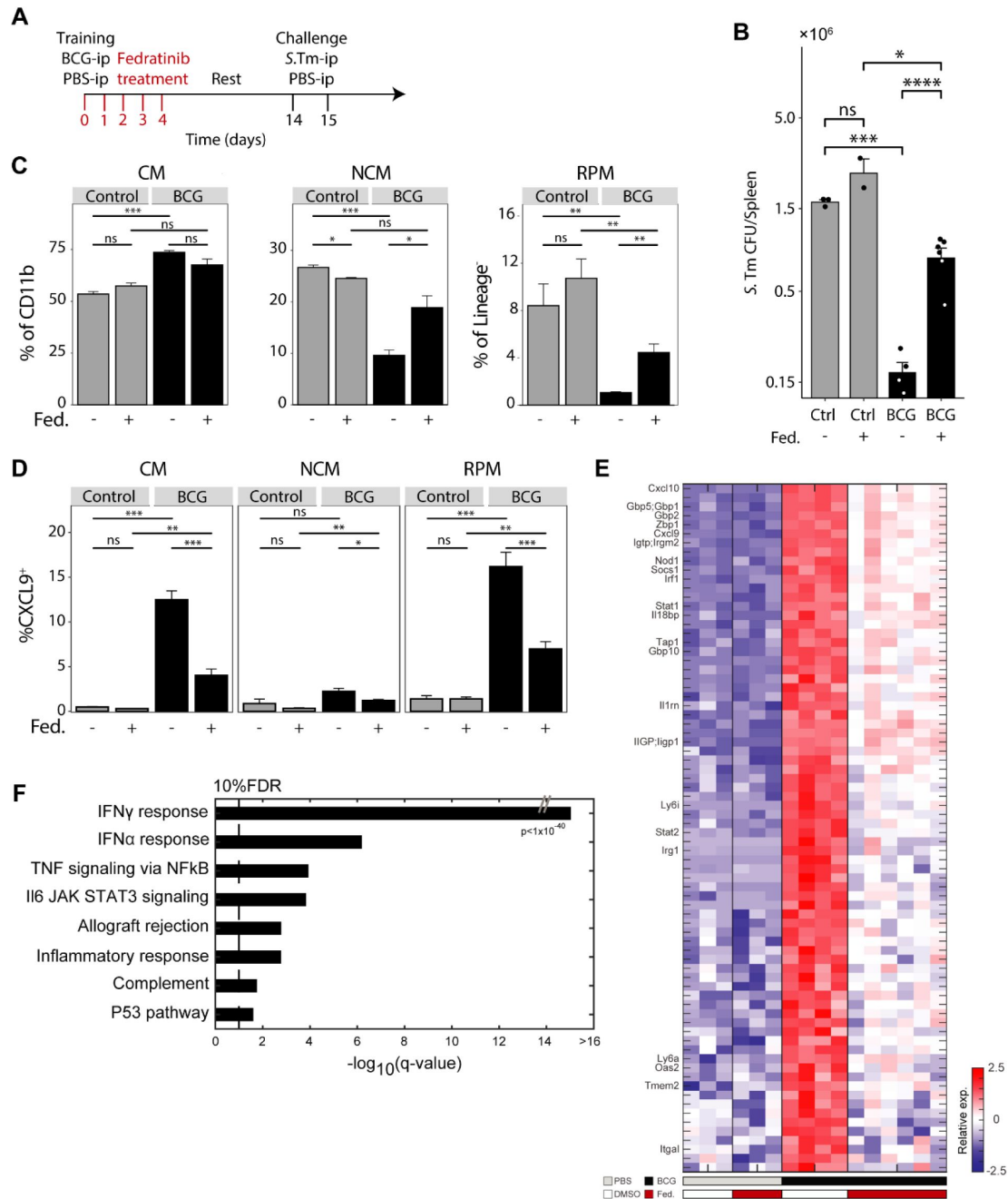


Figure 4.

Transient IFN γ -STAT1 inhibition prevents TI signatures and splenic infection resistance.

(A) Experimental schema of training with early treatment of Fedratinib inhibitor. (B) Splenic *S. Tm* CFU for control and BCG mice, with and without Fedratinib inhibitor, 24h post infection (n=2-6). (C) MP populations from control (gray) and BCG (black) mice, with or without Fedratinib inhibition. Percentage of CM and NCM cells calculated from CD11b⁺ population. Percentage of RPM calculated from Lin⁺ population. (D) Percentage of CM, NCM, and RPM populations expressing CXCL9 from control (gray) and BCG (black) mice, with or without Fedratinib inhibition (control: n=3, BCG: n=4, control+Fedratinib: n=3, BCG+Fedratinib: n=6). (E) Heatmap of normalized log₂ expression of DEGs across naïve and training conditions. (F) Gene set enrichment analysis of DEGs from E. Data in bar graphs are presented as mean±SEM. For bar graph B each individual point is a biological repeat. Heatmap rows in E indicate biological replicates. Two-tailed *t*-test used for data in B, C, and D (**P*<0.05, ***P*<0.01, ****P*<0.005, *****P*<0.001).

JAK2-associated pathways, we repeated the inhibitor experiment using recombinant α -IFN γ . Mice were given either α -IFN γ or an isotype control after BCG or PBS vaccination, with injections on days 0, 2, and 4, and assayed two weeks post-vaccination. Upon sorting and sequencing CM and RPM from these treated mice, we found that the STAT1 regulated TI signatures were completely ablated with early inhibition (**Figure 4—figure supplement 1K-L**). Importantly, when we plated spleen homogenates at the two-week interval, we still detected viable BCG, demonstrating that training can be blocked even when BCG remain in the tissue (**Figure 4—figure supplement 1M**).

Thus, the impact of Fedratinib and α -IFN γ treatment on the TI phenotype further underscores the pivotal role of IFN γ and the JAK2-STAT1 axis in orchestrating the early programs and signatures required for long-term local and recruited myeloid populations within the tissue. When compromised, remodeling is suppressed and so too protection. Crucially, even though its administration was early and removed after only five days administration, inhibition occurred. This indicates that the acquisition of TI in the tissue occurs within a critically narrow temporal window.

Discussion

TI, or innate immune memory, embodies the capacity of innate immune cells to remember past encounters and modify their subsequent responses, triggered by diverse conditions and stimuli. In-vivo these processes are sustained in progenitor cells within the BM to provide long term TI, with differentiated cells inheriting this memory^{19,46}. In this study, we set out to demonstrate how long-term protection events in the BM affords training within the tissue through local interactions with resident populations. We demonstrated how the immune effects of BCG vaccination are differentially imparted across recruited trained monocytes from the BM and diverse MP populations within the spleen, the temporal dynamics governing these processes, and the mechanisms necessary for them to be sustained long-term.

We verified that BCG-i.p inoculation stimulates an expansion of myeloid MP, notably CM, within the spleen. Our dissection of single-cell training signatures uncovered a fundamental program highlighting a signature of IFN γ response and STAT1 regulated genes across diverse populations. By monitoring the kinetics of training over an extended duration, we observed that the initial surge in CM recruitment is confined to the first two weeks of training, potentially signaling inflammatory recruitment triggered by BCG exposure and RPM loss. Concurrently, the presence CXCL9⁺ CM-Ts starts to diminish at a similar rate and is eventually lost, suggesting that these cells may be differentiated to trained MPs in the tissue.

The depletion of RPMs at 14 days post-vaccination sets in motion a series of complex cellular responses. Notably, we observed an enrichment of heme metabolism at day 30, which may indicate an ongoing process of RPM replenishment. This process typically involves two key mechanisms: the local expansion and repopulation by native populations, and the recruitment of erythroid and myeloid progenitors, including classical and non-classical monocytes, to the organ⁴⁷. These recruited cells, particularly monocytes, can differentiate within the niche, transitioning through a pre-RPM state via heme-mediated pathways⁴⁸. Interestingly, heme metabolism has also been implicated in the control and pathogenicity of mycobacterial species, particularly *Mycobacterium tuberculosis* (MtB). During systemic infection, MtB activates type I interferon (IFN-I) signaling, which, along with its own virulence factors, disrupts iron transport and uptake²⁵.

The presence of TI markers not only in BM-derived myeloid cells such as CM and NCM, but also in RPM, prompted us to uncover how these cells acquired this phenotype. Utilizing Ms4a3^{TdM} labeled mice, we investigated whether trained RPM were being derived from cells originating in the BM. Unlike CM and NCM, which are predominantly TdM⁺, RPM express this lineage tracing

marker at steady state around 16%, which was further increased with training. Probing the transcriptional profile of these cells also revealed differences due to origin, with Tdtn⁺ RPM demonstrating a heightened response to IFN γ stimulation and elevated expression of STAT1. However, both populations exhibit significant upregulation of TI-associated genes post-vaccination. These findings indicate that TI in RPM is not solely due to BM-derived precursors filling a depleted niche but suggests a capacity for both resident and BM-derived RPM to undergo training within the tissue itself. Considering the importance of these cells in restricting early infection events²⁶, their capacity for training reveals an additional factor contributing to the TI phenotype. While our examination was limited to a singular tissue-resident macrophage subset, other splenic macrophages like marginal zone macrophages (MZMs) and marginal metallophilic macrophages (MMMs), known to have immunological roles, may similarly exhibit a capacity for being trained. This research can be further broadened to include other resident myeloid populations, to explore how BCG can bestow localized protection independent of central HSC reprogramming.

When investigating the longevity of the trained transcriptional response within the spleen, we noted that *Stat-1* and other interferon response genes remained upregulated even two months after vaccination. We found one such gene SCA-1 (*Ly6A*), was continuously expressed in NCM due to training, and was still identifiable after a BM transplant. Although SCA-1 is traditionally a marker for upstream progenitors, recent studies link its expression to inflammation, with upregulation noted in B/T cells during *Mycobacterium Tuberculosis* (Mtb) infection⁴⁹. Further exploration is warranted to investigate the mechanisms that enable such enduring expression, particularly in this cell type. Also, while *Ly6a* (SCA-1) is unique to mice, a recent study identified a human equivalent termed *Ly6s*, primarily expressed in splenic NCM and regulated by interferon signaling, that was linked to an inflammatory cell phenotype with resistance to viral infections⁵⁰.

Prior publications have demonstrated the importance of BCG localization in generating local vs. systemic changes^{19,51}, and that BCG's initial presence within the BM is associated with HSC reprogramming and long-term TI. Despite this, while we were able to isolate BCG from splenic tissue, not BM, from all sampled time points, we could still achieve training and protection, including LSK⁺ subset expansion in the BM. This leads us to hypothesize that the signaling initiated due to BCG can act in trans, affecting system wide changes, and is not solely acquired due to local pathogen interactions in the BM. Supporting this, recent findings suggest that training in alveolar macrophages can also occur through subcutaneous BCG administration, potentially acting via the gut-lung axis⁵².

Finally, given STAT1's ubiquity among all trained cells, we hypothesized that this transcription factor and its upstream activation via interferon signaling were instrumental in driving BCG-induced trained immunity. We observed that early transient inhibition, specifically targeting IFN γ -mediated STAT1 activation, effectively negated the hallmarks of trained immunity. These include myeloid recruitment, disappearance of RPM, the expression of training markers, LSK⁺ expansion, transcriptional alterations, and, crucially, heterologous *S. Tm* protection. Furthermore, while it is generally known that BCG can reside within the tissue for weeks after inoculation, our findings suggest that even brief STAT1 and IFN γ inhibition is sufficient to disrupt the development of trained immunity, regardless of the pathogen's presence. It is plausible that during the initial stages of BCG exposure and inflammation, IFN γ -secreting cells such as T/NKs initiate the immunological remodeling needed for training. Should these processes be obstructed at this critical juncture, the opportunity for subsequent training is lost. While our inhibitor protocol was applied for a period of five days, the minimal duration required for effective inhibition and comparable outcomes may be even shorter.

In summary, our study emphasizes that examining the tissue, specifically the spleen, as a tissue for probing TI offers valuable insights into the temporal dynamics and signaling cascades that instigate and sustain TI locally, in parallel with established systemic effects. Central to these processes is the IFN γ -STAT1 pathway, which we identified as a key driver in establishing TI, by replenishment of resident naïve MPs with trained recruited and local immune populations. We further delineated that during intraperitoneal vaccination the resultant immune interactions limit BCG dissemination, while still effectively eliciting training. Our findings open new avenues to harness STAT1 pathway induction for optimized training and heterologous protection.

Methods

Experimental Methods

Mice and bacteria strains

C57BL/6J mice, 7-9 weeks old, were purchased from ENVIGO, housed at the Weizmann Institute pathogen-free facility, and provided with standard food and water ad libitum. The Nr4a1 super-enhancer sub-domain E2-KO (E2^{-/-} or C57BL/6-Rr39^{em1Ched/J}) mice were purchased from The Jackson Laboratory (#030204)⁵³. The mice strains below were kindly provided by the following investigators:

- STAT1-KO mice by Prof. Dr. Mathias Müller⁴¹.
- Ms4a3Cre^{TdTomato}-CX3CR1^{GFP} and CD45.1 mice by Prof. Steffen Jung.

All experiments were performed in accordance with the guidelines outlined by the Weizmann Institute Committee on Animal Care.

For in-vivo training, BCG-Pasteur, generously donated by Dr. Daniel Barkan, was utilized. The *Salmonella enterica* serovar Typhimurium strain SL1344 was used exclusively for all infection challenge experiments.

Mice training and infection

BCG were grown in Middlebrook 7H9 media (BD) supplemented with Middlebrook OADC (BD) at 37°C for 1 week to stationary phase. Bacterial aliquots of 1mL were dispensed to 2mL cryotubes (Simport) and frozen at -80C for long term storage. Prior to inoculation, tubes were thawed, centrifuged (10,000g, 2 min, RT), with pellet resuspended in 1 mL phosphate-buffered saline (PBS) (Sartorius). Bacterial concentration was calculated based on optical density at 600nm (OD₆₀₀) assuming a concentration of 5×10⁸ CFU/OD, with BCG diluted to 25×10⁶ CFU/mL in PBS. Mice were injected intraperitoneally (i.p) with 200μl containing 5×10⁶ CFU or PBS (as controls). At given time points, mice were euthanized by CO₂, spleens and/or BM from the femur were harvested, and CFU numbers were evaluated by plating serial 10-100-fold dilutions of homogenized spleens or BM suspension on selective 7H9-Middlebrook agar plates.

For the initial challenge as observed in **Fig. 1A**, cultures of *S.Tm* were grown in Luria-Bertani (LB) medium (BD) at 37°C for 16 hours to stationary phase. For all subsequent experiments, *S.Tm* were grown at 37°C for 16 hours to stationary phase in SPI-2 inducing media⁵⁴; MgMES media (170 mM 2-(N-morpholino) ethanesulfonic acid (MES) at pH 5.0, 5 mM KCl, 7.5 mM (NH₄)₂SO₄, 0.5 mM K₂SO₄, 1 mM KH₂PO₄, 8 mM MgCl₂, 38 mM glycerol, and 0.1% casamino acids. Cultures were centrifuged (10,000g, 2 min, RT), with pellet resuspended in PBS. Bacteria were diluted 10-fold in PBS and concentration calculated based on optical density at 600nm (OD₆₀₀). Assuming a concentration of 1×10⁹ CFU/OD, *S.Tm* was diluted to 2.5×10⁶ CFU/mL in PBS. Mice were injected

intraperitoneally (i.p) with 200 μ l containing 1×10^5 CFU of bacteria or PBS (as controls). Injected bacterial load was verified by CFU. 24 hours post infection, mice were euthanized by CO₂, spleens were harvested, and CFU numbers were evaluated by plating serial 10-100-fold dilutions of homogenized spleens on streptomycin LB agar plates.

BCG CFU

Spleens or BM were homogenized and serially diluted in PBS + 0.1% Triton on 7H9 Middlebrook media + OADC with Zeocin and Kanamycin. Plates were incubated for three weeks in a humidified 5% CO₂ incubator, with CFU determined using an automated colony counter.

In-vivo interferon inhibition

JAK-STAT inhibitors Fedratinib (cat #202893), and Deucravacitinib (cat #555349) (MedKoo Biosciences) were resuspended in DMSO, aliquoted, and stored at -80C for later use. For injection, a mixture of PEG-300 (Sigma):Tween-80 (Sigma) was prepared at a ratio of 18:1 and filtered using a 0.22 μ m filter. For each injection, 10 μ l of DMSO with or without the inhibitor was added to 105 μ l of the PEG:Tween mix, followed by 180 μ l of PBS for a total of 30 μ l and injected i.p. Inhibitor concentrations are 1mg and 0.5mg per mouse for Fedratinib and Deucravacitinib respectively. Four hours post inhibitor, BCG or PBS-i.p was injected for training. Daily repeat injections of the inhibitors were repeated for four additional days.

For the antibody inhibition experiment, 1mg of monoclonal α IFN γ antibody (clone: XMG1.2) or isotype control (clone: Rat IgG1) was injected i.p. four hours pre BCG vaccination (day 0), then every other day (day 2 and 4).

Splenocytes and BM isolation and flow cytometry preparation

Spleens and BM from femurs were extracted and stored in cooled FACS Buffer (PBS, 10 mM EDTA, 2% FBS) until further extraction.

For BM extraction, femurs were cut at both ends and placed in 0.5mL microfuge tube with a small hole in the bottom cut out using an 18G needle. This tube was then placed in a 1.5mL microfuge tube and centrifuged (3 min, 500g, 4°C). The pellet was resuspended in red blood cell (RBC) lysis buffer for 4 minutes at room temperature, centrifuged (3 min, 500g, 4°C) and re-suspended with FACS buffer. For CFU determination, suspension is used directly for serial dilution. For further flow cytometry processing, FACS buffer containing CD16/CD32 blocking antibodies (BioLegend) is added for a 15-minute incubation on ice. All subsequent processing is identical to splenocytes.

The spleens were dissected, mashed against a 70 μ m cell strainer (Falcon) and washed with 5mL of cold FACS buffer. 1mL of splenocytes were aliquoted to microfuge tubes and centrifuged twice (3 min, 500g, 4°C). Pellets were re-suspended with RBC lysis buffer (Sigma), incubated for 4 minutes at room temperature, centrifuged (3 min, 500g, 4°C) and re-suspended with FACS buffer containing CD16/CD32 blocking antibodies (BioLegend) for 15 minutes on ice. Cells were centrifuged once more, and pellets were transferred to wells of a 96-well low attachment plate for multi-sample preparation. Subsequently, fluorophore-conjugated antibodies cocktails (listed below) in Brilliant Stain Buffer (BD) were used to resuspend pellets, followed by a 30-minute incubation on ice. Cells were washed, re-suspended with 500-1000 μ l FACS buffer and passed through a 35 μ m cell strainer (Falcon). For absolute quantification of cell populations, 10-50 μ l of Precision Count beads (BioLegend) were added to the samples.

Antibodies used in this study for splenocyte and BM staining

F4/80	PE	BM8	BioLegend
F4/80	Brilliant Violet 421™	BM8	BioLegend
I-A/I-E (MHC-II)	Brilliant Violet 605™	M5/114/15.2	BioLegend
Ly-6A/E (Sca-1)	PE/Cyanine7	E13-161.7	BioLegend
CD11c	PerCP/Cyanine5.5	N418	BioLegend
Ter-119	FITC	TER-119	BioLegend
CD4	FITC	GK1.5	BioLegend
CD8a	FITC	53-6.7	BioLegend
Gr-1	FITC	RB6-8C5	BioLegend
CD45R/B220	FITC	RA3-6B2	BioLegend
CD11b	FITC	M1/70	BioLegend
CD117/c-Kit	APC	2B8	BioLegend
Ly-6A/E (Sca-1)	PE-Vio 770™	REA422	Miltenyi

Flow cytometry and sorting for RNA sequencing

Flow cytometry and sorting was performed using the BD FACSAria III (BD). Single cells were sorted into 384-well plates (Eppendorf) containing 2µl of a solution containing barcoded poly-T primers for reverse transcription (Sigma, Israel) according to the MARS-seq v2.0 protocol⁵⁵. For bulk cell capture, 5×10^3 cells from each population were sorted into tubes containing 300µl RLT buffer (Qiagen) with β-mercaptoethanol (BME). Immediately after sorting, plates or tubes were spun down, flash-frozen in a mixture of dry ice and ethanol and stored in -80C until processing.

Single-cell RNA-seq library preparation

Single-cell libraries were prepared as described⁵⁵. Briefly, mRNA from cells was converted to cDNA alongside barcoding and UMI addition. The cDNA of each plate was pooled followed by second DNA strand synthesis and T7 in vitro transcription. Amplified RNA was fragmented, followed by ligation of partial P5 Illumina sequence, and converted to cDNA. Full sequence of barcoded P5 and P7 of P5 were added by PCR for a sequence ready library. Final libraries were quantified for peak size and concentration using the Agilent TapeStation and Qubit HS DNA Assay kit (Invitrogen), respectively.

Bulk RNA-seq library preparation

RNA was extracted and cleaned using the RNeasy mini kit (Qiagen) with DNaseI digestion. Libraries were then prepared according to an in-house MARS-seq or CEL-seq protocol optimized for bulk RNA samples. Final libraries were quantified for peak size and concentration using the TapeStation 4200 (Agilent) and Qubit HS DNA Assay kit (Invitrogen), respectively.

Library Sequencing

Bulk and single cell libraries were diluted to a concentration of 1.8pM and run on the NextSeq platform (Illumina) according to Illumina guidelines, with 75 reads for read1, and 15 reads for read2. A mean of 6M reads per library for the kinetics data; a mean of 12M reads per library for

IFN γ inhibitor data; and a mean of 3M reads per library for the MS4a3^{Td τ} bulk sorted population data.

CD45.1/CD45.2 adoptive transfer

C57BL/6J mice, expressing CD45.1 or CD45.2, were trained according to our standard protocol using BCG or PBS, respectively. After two weeks, BM was isolated from the femur from both mice, resuspended in PBS, and mixed in a 1:1 ratio. Recipient mice (WT C57/BL6J) were irradiated with a single dose of 950 cGy using an XRAD 320 machine (Precision X-Ray [PXI]) and reconstituted the next day via retro-orbital injection of 5×10^6 mixed donor BM cells/mouse in 200 μ l PBS. Mice were given 6 weeks to allow for reconstitution and repopulation of the hematopoietic system.

Flow cytometry, CFU, and spleen size analysis and quantification

Flow cytometry data was analyzed using the FlowJo software.

For size quantification, spleens were imaged against a contrasting background, and two-dimensional area was calculated using the ImageJ software.

All graphs quantifying the results from flow cytometry and CFU results were performed using R on RStudio with the Tidyverse package⁵⁶[56](#).

Bioinformatics Analysis

scRNA-seq data analysis

Data preprocessing

MARS-seq pipeline⁵⁵[55](#) was used for demultiplexing, alignment to the genome (mm9), and gene counting by unique molecular identifier (UMI). Overall, we sequenced 1536 cells (768 from control mouse and 768 from trained mouse), with 1474 median UMI count per cell and 668 median genes per cell.

Data normalization and gene filtration

Only genes with at least one UMI count detected in at least one cell were used. Data was normalized to a library size factor. Factors were calculated by dividing total UMI counts in each cell to the median of the total UMI counts across all cells. Data was transformed to log₁₀ scale ($\log_{10}(\text{UMI count} + 1)$). Cells with less than 200 UMIs were excluded due to low coverage (24 cells, 9 from naïve mouse and 15 from trained mouse). We filtered out cell cycle and ribosomal genes and selected the top 425 most variable genes for further analysis. Variable genes were selected based on fitting of the data to a simple noise model based on the genes mean expression and dispersion (coefficient of variance).

Data clustering and annotations

Principal component analysis (PCA) was performed on the variable genes, and the first 40 PCs were used for downstream analysis for k-nearest neighbor (KNN)-graph, based on Euclidian distance in PC space. Clustering was performed using Louvain community detection on the KNN-graph ($k=20$). Overall, we obtained 7 clusters. Cluster identity was inferred using cluster-specific and manually selected genes based on cell classification literature.

Bulk RNA-seq data processing and normalization

MARS-seq pipeline was used for samples demultiplexing, alignment to the genome (mm9), and gene counting. Data was normalized to a library size factor. Factors were calculated by dividing the total number of reads from each sample to the median total number of reads across all samples. These procedures were done for each dataset alone.

Kinetics data

Data was transformed to log2 scale, and minimal expression threshold was set to 3. Replicate samples of each condition were averaged, except for 1 sample that was excluded due to low coverage (<100k reads; BCG 30d replicate 3). Preceding PCA analysis genes were centered and normalized to a mean of 0 and a standard deviation of 1. To identify genes that were up-regulated due to training we calculated the differences between the integrals of each gene in BCG relative to PBS along time. The differences across all genes were approximately normally distributed, with a mean of 0.2 and a standard deviation of 10.11. Genes with more than 3 standard deviations above the mean were defined as up-regulated due to training.

Bulk inhibitor data

Data was transformed to log2 scale, and minimal expression threshold was set to 3. Two Fedratinib samples were excluded from analysis due to technical issues during injection that resulted in a lack of inhibition. Heatmap was generated using DEGs calculated by ANOVA (5% FDR and a minimal 2-fold; 167 genes).

Fedratinib inhibitor data

Data was transformed to log2 scale, and minimal expression threshold was set to 4. One sample was excluded due to low coverage (<100k reads; PBS + S.Tm + Fedratinib replicate 3). Preceding PCA analysis genes were centered and normalized to a mean of 0 and a standard deviation of 1. PCA analysis was performed on DEGs calculated using two-sided t-tests between all relevant conditions: control vs. trained samples, trained with or without inhibitor, control with or without inhibitor, uninfected vs. infected, infected with or without training, infected vs. infected with training with inhibitor, and infected with training vs. infected with training with inhibitor (5% FDR; 453 genes). Heatmap was generated using two-sample t-test between control and BCG-trained samples (1% FDR).

α IFN γ inhibitor data

Data was transformed to log2 scale, and minimal expression threshold was set to 3. Two sample was excluded due to low coverage (<250k reads CM BCG + Isotype replicate 1 and RPM BCG + Isotype replicate 4). Heatmap genes were selected from cluster I and II from the lineage tracing experiment representing shared interferon/STAT-1 upregulated genes.

MS4A3^{Tdtm} bulk population data

Data was transformed to log2 scale, and minimal expression threshold was set to 3. One sample was excluded due to low coverage (<150k reads; control NCM-Tdtm⁺ replicate 4). Preceding PCA analysis genes were centered and normalized to a mean of 0 and a standard deviation of 1. DEGs between control and trained mice were calculated using two-sided t-test between all control samples versus all trained samples (from all sorted populations together; 10% FDR, 382 genes).

Acknowledgements

This research was supported by the Israel Science Foundation (ISF grant No. 1289/22), the Minerva Foundation with funding from the Federal Ministry for Education and Research, the Dr. Barry Sherman Institute for Medicinal Chemistry, and the Shimon and Golde Picker Weizmann Annual Grant.

References

1. Arts R. J. W., et al. (2015) **Long-term in vitro and in vivo effects of γ -irradiated BCG on innate and adaptive immunity** *Journal of Leukocyte Biology* **98**:995–1001
2. Saeed S., et al. (2014) **Epigenetic programming of monocyte-to-macrophage differentiation and trained innate immunity** *Science* **345**
3. Darrah P. A., et al. (2020) **Prevention of tuberculosis in macaques after intravenous BCG immunization** *Nature* **577**:95–102
4. Moorlag S. J. C. F. M., Arts R. J. W., van Crevel R., Netea M. G (2019) **Non-specific effects of BCG vaccine on viral infections** *Clinical Microbiology and Infection* **25**:1473–1478
5. Kurotaki D., Uede T., Tamura T (2015) **Functions and development of red pulp macrophages** *Microbiology and Immunology* **59**:55–62
6. Hoeffel G., et al. (2015) **C-Myb(+) erythro-myeloid progenitor-derived fetal monocytes give rise to adult tissue-resident macrophages** *Immunity* **42**:665–678
7. Lai S. M., et al. (2018) **Organ-Specific Fate, Recruitment, and Refilling Dynamics of Tissue-Resident Macrophages during Blood-Stage Malaria** *Cell Reports* **25**:3099–3109
8. Zhang L., et al. (2016) **Variable Virulence and Efficacy of BCG Vaccine Strains in Mice and Correlation With Genome Polymorphisms** *Molecular Therapy* **24**:398–405
9. Barth M. W., Hendrzak J. A., Melnicoff M. J., Morahan P. S (1995) **Review of the macrophage disappearance reaction** *Journal of Leukocyte Biology* **57**:361–367
10. Keren-Shaul H., et al. (2019) **MARS-seq2.0: an experimental and analytical pipeline for indexed sorting combined with single-cell RNA sequencing** *Nat Protoc* **14**:1841–1862
11. Hu X., Ivashkiv L. B (2009) **Cross-regulation of Signaling and Immune Responses by IFN- γ and STAT1** *Immunity* **31**:539–550
12. Li X., et al. (2023) **GBP2 promotes M1 macrophage polarization by activating the notch1 signaling pathway in diabetic nephropathy** *Front. Immunol* **14**
13. Meunier E., et al. (2014) **Caspase-11 activation requires lysis of pathogen-containing vacuoles by IFN-induced GTPases** *Nature* **509**:366–370
14. Groom J. R., Luster A. D (2011) **CXCR3 ligands: redundant, collaborative and antagonistic functions** *Immunol Cell Biol* **89**:207–215
15. Joosten S. A., et al. (2018) **Mycobacterial growth inhibition is associated with trained innate immunity** *The Journal of Clinical Investigation* **128**
16. Michelucci A., et al. (2013) **Immune-responsive gene 1 protein links metabolism to immunity by catalyzing itaconic acid production** *Proc Natl Acad Sci U S A* **110**:7820–7825

17. Lampropoulou V., et al. (2016) **Itaconate Links Inhibition of Succinate Dehydrogenase with Macrophage Metabolic Remodeling and Regulation of Inflammation** *Cell Metab* **24**:158–166
18. Domínguez-Andrés J., et al. (2019) **The Itaconate Pathway Is a Central Regulatory Node Linking Innate Immune Tolerance and Trained Immunity** *Cell Metabolism* **29**:211–220
19. Kaufmann E., et al. (2018) **BCG Educates Hematopoietic Stem Cells to Generate Protective Innate Immunity against Tuberculosis** *Cell* **172**:176–190
20. Yang S.-H., et al. (2017) **IFN- γ -STAT1-iNOS Induces Myeloid Progenitors to Acquire Immunosuppressive Activity** *Frontiers in Immunology* **8**
21. Biram A., et al. (2022) **Bacterial infection disrupts established germinal center reactions through monocyte recruitment and impaired metabolic adaptation** *Immunity* **55**:442–458
22. Penton-Rol G., et al. (1998) **Selective Inhibition of Expression of the Chemokine Receptor CCR2 in Human Monocytes by IFN- γ 1** *The Journal of Immunology* **160**:3869–3873
23. Wang Y., et al. (2023) **Bacillus Calmette–Guérin-induced interleukin-10 inhibits S100A8/A9 production and hinders development of T helper type 1 memory in mice** *European Journal of Immunology* **53**
24. Liu K., et al. (2020) **Neutrophilic granule protein (NGP) attenuates lipopolysaccharide-induced inflammatory responses and enhances phagocytosis of bacteria by macrophages** *Cytokine* **128**
25. Khan N., et al. (2020) **M. tuberculosis Reprograms Hematopoietic Stem Cells to Limit Myelopoiesis and Impair Trained Immunity** *Cell* **183**:752–770
26. Hoffman D., et al. (2021) **A non-classical monocyte-derived macrophage subset provides a splenic replication niche for intracellular Salmonella** *Immunity* **54**:2712–2723
27. Yona S., et al. (2013) **Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis** *Immunity* **38**:79–91
28. Bomfim C. C. B., et al. (2022) **Mycobacterium tuberculosis Induces Irg1 in Murine Macrophages by a Pathway Involving Both TLR-2 and STING/IFNAR Signaling and Requiring Bacterial Phagocytosis** *Front Cell Infect Microbiol* **12**
29. Tretina K., Park E.-S., Maminska A., MacMicking J. D (2019) **Interferon-induced guanylate-binding proteins: Guardians of host defense in health and disease** *J Exp Med* **216**:482–500
30. Clement M., et al. (2022) **IFITM3 restricts virus-induced inflammatory cytokine production by limiting Nogo-B mediated TLR responses** *Nat Commun* **13**
31. Carow B., et al. (2013) **Critical and independent role for SOCS3 in either myeloid or T cells in resistance to Mycobacterium tuberculosis** *PLoS Pathog* **9**
32. Vogt A.-C. S., et al. (2021) **On Iron Metabolism and Its Regulation** *International Journal of Molecular Sciences* **22**

33. Liu Z., et al. (2019) **Fate Mapping via Ms4a3-Expression History Traces Monocyte-Derived Cells** *Cell* **178**:1509–1525
34. Yáñez A., et al. (2017) **Granulocyte-Monocyte Progenitors and Monocyte-Dendritic Cell Progenitors Independently Produce Functionally Distinct Monocytes** *Immunity* **47**:890–902
35. De Leon-Oliva D., et al. (2023) **AIF1: Function and Connection with Inflammatory Diseases** *Biology (Basel)* **12**
36. Prevete N., Liotti F., Marone G., Melillo R. M., de Paulis A (2015) **Formyl peptide receptors at the interface of inflammation, angiogenesis and tumor growth** *Pharmacol Res* **102**:184–191
37. Gibbings D. J., Marcet-Palacios M., Sekar Y., Ng M. C. Y., Befus A. D (2007) **CD8 alpha is expressed by human monocytes and enhances Fc gamma R-dependent responses** *BMC Immunol* **8**
38. Sugimoto M. A., Vago J. P., Teixeira M. M., Sousa L. P (2016) **Annexin A1 and the Resolution of Inflammation: Modulation of Neutrophil Recruitment, Apoptosis, and Clearance** *J Immunol Res* **2016**
39. Håversen L., et al. (2018) **Vimentin deficiency in macrophages induces increased oxidative stress and vascular inflammation but attenuates atherosclerosis in mice** *Sci Rep* **8**
40. Karlstetter M., et al. (2010) **The Novel Activated Microglia/Macrophage WAP Domain Protein, AMWAP, Acts as a Counter-Regulator of Proinflammatory Response** *The Journal of Immunology* **185**:3379–3390
41. Kernbauer E., et al. (2012) **Conditional Stat1 Ablation Reveals the Importance of Interferon Signaling for Immunity to Listeria monocytogenes Infection** *PLoS Pathog* **8**
42. Lé A. M., Puig L., Torres T (2022) **Deucravacitinib for the Treatment of Psoriatic Disease** *Am J Clin Dermatol* **23**:813–822
43. Kalafati L., et al. (2020) **Innate Immune Training of Granulopoiesis Promotes Anti-tumor Activity** *Cell* **183**:771–785
44. Huijser E., et al. (2022) **Trained Immunity in Primary Sjögren's Syndrome: Linking Type I Interferons to a Pro-Atherogenic Phenotype** *Frontiers in Immunology* **13**
45. Zahalka S., et al. (2022) **Trained immunity of alveolar macrophages requires metabolic rewiring and type 1 interferon signaling** *Mucosal Immunol* **15**:896–907
46. Moorlag S. J. C. F. M., et al. (2020) **β -Glucan Induces Protective Trained Immunity against Mycobacterium tuberculosis Infection: A Key Role for IL-1** *Cell Reports* **31**
47. Liao C., Prabhu K. S., Paulson R. F (2018) **Monocyte-derived macrophages expand the murine stress erythropoietic niche during the recovery from anemia** *Blood* **132**:2580–2593
48. Haldar M., et al. (2014) **Heme-mediated SPI-C induction promotes monocyte differentiation into iron-recycling macrophages** *Cell* **156**:1223–1234

49. Akter S., et al. (2022) **Mycobacterium tuberculosis infection drives a type I IFN signature in lung lymphocytes** *Cell Reports* **39**
50. Shmerling M., et al. (2022) **LY6S, a New IFN-Inducible Human Member of the Ly6a Subfamily Expressed by Spleen Cells and Associated with Inflammation and Viral Resistance** *ImmunoHorizons* **6**:253–272
51. Vierboom M. P. M., et al. (2021) **Stronger induction of trained immunity by mucosal BCG or MTBVAC vaccination compared to standard intradermal vaccination** *Cell Rep Med* **2**
52. Jeyanathan M., et al. (2022) **Parenteral BCG vaccine induces lung-resident memory macrophages and trained immunity via the gut-lung axis** *Nat Immunol* **23**:1687–1702
53. Thomas G. D., et al. (2016) **Deleting an Nr4a1 Super-Enhancer Subdomain Ablates Ly6Clow Monocytes while Preserving Macrophage Gene Function** *Immunity* **45**:975–987
54. Stapels D. A. C., et al. (2018) **Salmonella persists undermine host immune defenses during antibiotic treatment** *Science* **362**:1156–1160
55. Keren-Shaul H., et al. (2019) **MARS-seq2.0: an experimental and analytical pipeline for indexed sorting combined with single-cell RNA sequencing** *Nat Protoc* **14**:1841–1862
56. Wickham H., et al. (2019) **Welcome to the Tidyverse** *Journal of Open Source Software* **4**

Editors

Reviewing Editor

Bryan Bryson

Massachusetts Institute of Technology, Cambridge, United States of America

Senior Editor

Carla Rothlin

Yale University, New Haven, United States of America

Reviewer #1 (Public review):

Summary:

In the submitted manuscript, Solomon et al carefully detail shifts in tissue-specific myeloid populations associated with trained immunity using intraperitoneal BCG injection as a model for induction. They define the kinetics of shifts in myeloid populations within the spleen and the transcriptional response associated with IP BCG exposure. In lineage tracing experiments, they demonstrate that tissue-resident macrophages, red-pulp macrophages (RPM) that are rapidly depleted after BCG exposure, are replenished from recruited monocytes and expansion of tissue-resident cells; they use transcriptional profiling to characterize those cells. In contrast to previous descriptions of BCG-driven immune training, they do not find BCG in the bone marrow in their model, suggesting that there is not direct training of myeloid precursor populations in the bone marrow. They then link the observed trained immunity phenotype (restriction of heterologous infection with ST) with early activation of STAT1 through IFN- γ .

Strengths:

The work includes careful detailing of shifts and origins of myeloid populations within tissue associated with trained immunity and is a meaningful advance for the field. Given that the temporality of exposure relative to trained immunity phenotypes is a major focus of the work, there are some additional experiments that would make the work stronger.

Weaknesses:

(1) The contribution of persistent BCG in spleen to the observed trained immunity phenotypes is not clear: The trained immunity phenotypes are interpreted as being driven by the early (within days) response to BCG exposure. While the fedratinib data generally support this interpretation, the authors show that BCG remains present in spleen albeit at low levels all the way out to 60 days post exposure. Given that the focus in the paper is on tissue-specific immune training, it would be helpful to know whether the ongoing presence of BCG at low levels in the profiled tissue contributes to the trained immunity phenotypes observed.

(2) Unclear temporality of STAT1/IFN- γ requirement for the trained immunity phenotype: The data demonstrate that STAT1/IFN- γ are required at the earliest time points post-BCG exposure for trained immunity to be initiated. Related to the point about BCG above, it would be helpful to understand whether this is a specifically time-limited requirement when trained immunity is first induced, or whether ongoing signaling through this axis is required for maintenance of the observed trained immunity phenotypes.

<https://doi.org/10.7554/eLife.100922.1.sa1>

Reviewer #2 (Public review):

Summary:

In this study, Solomon and colleagues demonstrate that trained immunity induced by BCG can reprogram myeloid cells within localised tissue, which can sustain prolonged protective effects. The authors further demonstrate an activation of STAT1-dependent pathways.

Strengths:

The main strength of this paper is the in-depth investigation of cell populations affected by BCG training, and how their transcriptome changes at different time points post-training. Through use of flow cytometry and sequencing methods, the authors identify a new cell population derived from classical monocytes. They also show that long-term trained immunity protection in the spleen is dependent on resident cells. Through sequencing, drug and recombinant inhibition of IFN γ pathways, the authors reveal STAT1-dependent responses are required for changes in the myeloid population upon training, and recruitment of trained monocytes.

Weaknesses:

A significant amount of work has already been performed for this study. No significant weaknesses found.

<https://doi.org/10.7554/eLife.100922.1.sa0>