THE ROLE OF HEMATOPOIETIC PROGENITORS IN IMMUNE REGULATION AND MEMORY

EDITED BY: Flora Zavala, César Nombela Arrieta, Paolo Fiorina and Moufida Ben Nasr
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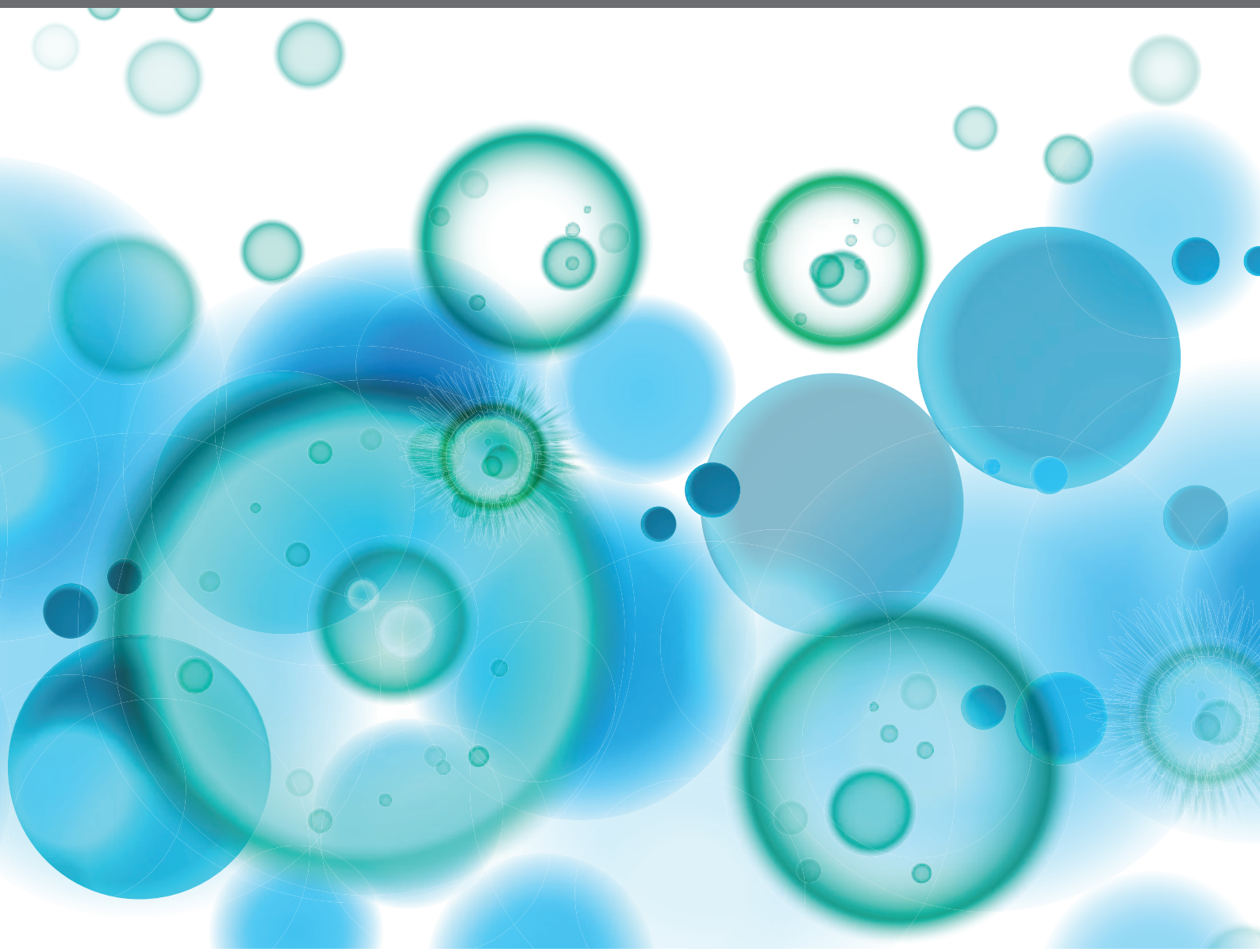

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THE ROLE OF HEMATOPOIETIC PROGENITORS IN IMMUNE REGULATION AND MEMORY

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Editorial: The Role of Hematopoietic Progenitors in Immune Regulation and Memory

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Editorial on the Research Topic

The Role of Hematopoietic Progenitors in Immune Regulation and Memory

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Hematopoietic stem cells and progenitors (HSPCs) represent an indispensable reservoir for the continuous replenishment of all immune and blood cells. HSPCs mostly reside within the bone marrow (BM) microenvironment in close interaction with a variety of stromal cell types that provide a regulatory infrastructure that controls quiescence or multilineage differentiation through the provision of instructive signals. The first part of this Research Topic focuses on the intricate nature of the cellular crosstalk between HSPCs and their niche, specifically, i) the functional and spatial complexity of hematopoietic niches, ii) the effects of infectious and inflammatory signals on the integrity of niches and hematopoiesis. The second set of articles explores the evidence suggesting that stimulation of HSPCs by various inflammatory or infectious signals can promote/enhance their trafficking and interaction with mature immune cells in peripheral tissues (1, 2), where they exert either an immune-enhancing effect or, conversely, an immunoregulatory effect on initiating or ongoing immune responses. Finally, several research papers characterize selective hematopoietic progenitor subsets with immunoregulatory properties *in vitro* as well as in experimental models of infection, autoimmune and alloimmune responses.

The complexity and overlapping roles of the hematopoietic and immune cell niches are reviewed in detail by Miao et al. The authors cast a special focus on the CXCR4/CXCL12 axis as a core pathway controlling quiescence and access of HSPCs to their niches and highlight the key functional roles of CXCL12-producing mesenchymal stromal cells (MSCs), in the replenishment of mature components of innate immunity in homeostasis as well as during stress. As the most prominent source of key cytokines instructing lineage specification, survival, and long-term maintenance of HSPCs, perturbations of their structural and functional integrity (3) underlie prototypical features of hematopoietic responses to infection and inflammation. As prime examples, the authors analyze the disruptive effects that WHIM syndrome, a combined immunodeficiency disease caused by a genetic mutation in the chemokine receptor gene CXCR4, myeloablative irradiation and leukemia, trigger in HSPC niches and the stromal infrastructure through the activation of a proinflammatory program. Such insults are known to cause defects in

hematopoietic cell development and recirculation, leading to immune deficiency and favoring malignant transformation.

In two related articles, Sezaki et al. as well as Johnson et al., describe how infectious challenges or, conversely, antibiotic treatments affect hematopoiesis and the BM microenvironment. A number of recent exciting findings are highlighted in this context. Among them i) the emerging role of microbiota in fine-tuning hematopoiesis through the effects of circulating microbial molecules on BM resident hematopoietic and stromal cells ii) evidence suggesting the contribution of emergency granulopoiesis to anti-tumoral immunity (4), iii) the potential role of infection-mediated mobilization of HSCs from the BM through pathways involving downregulation of CXCL12 to the replenishment of empty niches in distal bones, iv) the detrimental effects of infections of stromal cells, such as the observed depletion of osteoblasts during sepsis, which leads to inefficient lymphopoiesis because of insufficient IL-7 production. Of major interest is the discussion on breakthroughs in our understanding on how microbial-dependent inflammation educates HSCs, induces a bias towards the myeloid lineages, and leads to the generation of monocytes and macrophages, presenting a primed state of hyper-responsiveness that enhances their innate immune function upon subsequent challenge. This type of unspecific, innate memory, termed trained immunity, is imprinted at the epigenetic and metabolic level in HSPCs, and has attracted major attention in recent times (5–7). Conversely, these reviews also describe studies showing how immunoregulatory properties are instructed by a variety of innate signals and pharmacological agents on specific HSPC subsets. This phenomenon could be fundamentally exploited for the control of immune responses by resetting an aberrant autoreactive immune system to a *de novo* self-tolerant immune system (8, 9).

Pastore et al. review the defects in HSC characterizing the murine model of spontaneous type 1 diabetes in the Non-Obese Diabetic (NOD) mouse, in which high CXCL12 BM levels alter the trafficking of HSCs and Tregs and favor T1D onset. The mixed-chimerism induced by HSC infusion re-established autoreactive thymic T-cell deletion and delayed T1D onset. Moreover, infusion of ex vivo genetically engineered HSPCs, for instance, to express proinsulin or transgenically target MHC class II, successfully prevented T1D onset in NOD mice by reshaping the immune reservoir and facilitating tolerance towards auto-antigens. They further review clinical trials of HSCT in new-onset T1D patients (9, 10) that conferred insulin independence for 4-6 years after HSCT and particularly delineated a selective subgroup of patients with a different immune profile that may benefit the most from AHSCT. However, HSCT required myeloablation, which may limit its clinical application. The alternative infusion of pharmacologically modulated or genetically engineered HSPCs, avoiding myeloablation, is advocated (9, 11–14).

Finally, several papers describe selective immature progenitor subsets endowed with immunoregulatory properties.

Marinescu et al. describe a BM subset contaminating MSCs at initial culture passages that exhibits a phenotype of anti-inflammatory macrophages and inhibits T-cell proliferation *in vitro*, but, unlike BM MSCs, does not exert anti-tumoral effects *in vivo*.

Elahi et al. and Mashhouri et al. focus on $CD71^+$ erythroid progenitors, a prominent source of Reactive Oxygen Species (ROS) production in both mice and humans, more abundant in female than in male, with immunosuppressive properties that compromise immune response against systemic *Listeria monocytogenes* infection in neonatal mice. Their data underline the tight regulation of the immune system in newborns/infants.

Korniotis et al. and D'Aveni et al. focus on G-CSF mobilized multipotent MPP3 progenitors that display the property to selectively promote the proliferation of TCR-activated $Foxp3^+$ Tregs *in vitro* and *in vivo*. This underlines their capacity to reduce autoimmune encephalomyelitis (EAE), a murine model of multiple sclerosis. Additionally, sustained beneficial effects comprised prevention of the onset of T1D in NOD mice (15) and also a reduction of Graft-*versus*-Host Disease (GVHD), a deleterious complication of allogeneic HSC transplantation observed in patients with hematological malignancies. The human counterpart of this suppressive mobilized MPP subset is characterized.

Overall, the contributions in this Research Topic focus on the use of *ex vivo* conditioned HSPCs as a potentially safer therapy than AHSCT, minimizing/eliminating the toxic conditioning that infers unacceptable risks for autoimmune patients. HSPCs have successfully rendered patients suffering from autoimmune diseases, disease-free. In addition, they may as well reduce the severity of GVHD post allogeneic HSCT in patients with hematological malignancies.

AUTHOR CONTRIBUTIONS

All authors contributed equally to the redaction of the research focus. All authors have seen and agree with the final version.

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CD71$^+$ Erythroid Cells in Human Neonates Exhibit Immunosuppressive Properties and Compromise Immune Response Against Systemic Infection in Neonatal Mice

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Newborns are highly susceptible to infectious diseases. The underlying mechanism of neonatal infection susceptibility has generally been related to their under-developed immune system. Nevertheless, this notion has recently been challenged by the discovery of the physiological abundance of immunosuppressive erythroid precursors **C**D71$^+$ **e**rythroid **c**ell**s** (CECs) in newborn mice and human cord blood. Here, as proof of concept, we show that these cells are also abundant in the peripheral blood of human newborns. Although their frequency appears to be more variable compared to their counterparts in mice, they rapidly decline by 4 weeks of age. However, their proportion remains significantly higher in infants up to six months of age compared to older infants. We found CD45 expressing CECs, as erythroid progenitors, were the prominent source of reactive oxygen species (ROS) production in both humans and mice. Interestingly, a higher proportion of CD45$^+$CECs was observed in the spleen versus bone marrow of neonatal mice, which was associated with a higher ROS production by splenic CECs compared to their siblings in the bone marrow. CECs from human newborns suppressed cytokine production by CD14 monocytes and T cells, which was partially abrogated by apocynin *in vitro*. Moreover, the depletion of CECs in neonatal mice increased the number of activated effector immune cells in their spleen and liver, which rendered them more resistant to *Listeria monocytogenes* infection. This was evident by a significant reduction in the bacteria load in the spleen, liver and brain of treated-mice compared to the control

group, which enhanced their survival rate. Our finding highlights the immunoregulatory processes mediated by CECs in newborns. Thus, such tightly regulated immune system in newborns/infants may explain one potential mechanism for the asymptomatic or mild COVID-19 infection in this population.

Keywords: CD71+ erythroid cells, newborns, neonatal infections, immunosuppression, COVID-19

INTRODUCTION

Infectious disease is still a major global cause of childhood mortality (1, 2). Neonates are highly susceptible to a variety of infectious agents, which are often fatal and causing ~ 700,000 deaths per year (1–3). While infant mortality is < 5 per 1,000 live births in developed states, this rate is often > 30 times higher in resource-limited countries (4). Even in resource-rich countries, infections in young infants incur an enormous burden; approximately each infectious disease hospitalization for every 14 infants in the U.S. results in an annual cost of ~$700 Million (5). Of the serious infections in infants, sepsis, and meningitis carry the highest morbidity and mortality rates (6, 7). Considering the magnitude of this global health problem, even moderate efficient interventions can save millions of lives and billions of dollars. Enhancing the neonatal immune responses against pathogens through immune modulation/vaccination appears to be an attractive approach. However, this will not be possible unless we gain a better and deeper insight into developmental changes occurring in the neonatal immune system at the cellular and molecular levels.

There are clear qualitative and quantitative differences in both innate and adaptive immune systems between adults and neonates, which at least partially can explain the increased susceptibility to infection (8). The fetal immune system is biased towards an anti-inflammatory, T helper 2 (Th2) response, as is that of the pregnant woman. The fetus is antigenically different from its mother and could be compared to the immunological mismatch that can occur during transplantation with the risk of rejection (9–11). As such, the immune response during pregnancy appears to have evolved to prevent potentially damaging inflammation that otherwise may result in abortion or preterm delivery (12).

As beneficial as the tolerogenic state might be *in utero*, growing evidence suggests that this may predispose the newborn to severe infections and impairs their immune responses to vaccinations in postnatal life.

Another important event to consider is the sudden removal of the fetus from a highly privileged and protected environment that is aquatic, warm, and almost sterile, to a new, cold and dry environment full of pathogenic and/or non-pathogenic microorganisms and other antigens. The newborn faces many challenges in this new environment while growth and development are her/his top priority. Therefore, a highly dynamic and tolerant immune system is necessary for the newborn's survival. Initially, this tolerogenic phenomenon was related to the immune system immaturity in newborns (9, 12). However, this concept has recently been challenged and replaced by a novel concept (13). Neonatal infection susceptibility results from the temporal presence of physiologically enriched immunosuppressive erythroid precursors (13, 14). These erythroid precursors co-express the transferrin receptor CD71 and erythroid marker TER119 in mice, and CD71 and CD235a in humans (13, 14). $CD71^+$ erythroid cells (CECs) are abundant in the spleen of neonatal mice (13–15), placenta tissues and expand during pregnancy in the peripheral blood/spleen of allogeneic mice (16). Similarly, they are enriched in the human cord blood, placenta tissues, the peripheral blood during pregnancy (17–20) and in the liver of human neonates (21). CECs exhibit immunosuppressive functions regardless of their anatomical location (22). More importantly, CECs deplete L-arginine by the expression of arginase-II and subsequently impair immune activation in antigen-presenting cells and T cells *in vitro* (13, 14). In agreement with our observations, the expansion of immunosuppressive CECs in the spleen of mice in a tumor model of melanoma has also been reported that CECs inhibit antigen-specific $CD8^+$ T cell responses *via* the production of reactive oxygen species (ROS) (23). CECs from the mice placental tissue co-express PDL-1/PDL-2 and *via* interaction of these ligands with PD-1 on T cells suppress IFN-γ production *in vitro* (16). These observations demonstrate that CECs are a heterogeneous population of erythroid progenitors and precursors (22) and depending on their microenvironmental localization may utilize different mechanisms to suppress or modulate immune responses (8, 24). Moreover, splenic CECs express V-domain Ig Suppressor of T Cell Activation (VISTA) (25), a newly discovered inhibitory receptor and a transmembrane immunoglobulin superfamily also known as (PD-1H, vsir) (26–28). VISTA+CECs are the major source of TGF-β and *via* this cytokine promote the induction of regulatory T cells (Tregs), while $VISTA^-$CECs or CECs from VISTA-KO mice failed to promote the induction of Tregs (25). Overall, these pieces of evidence demonstrate a wide range of immunological properties for these less appreciated cells.

Although the presence of reticulocytes from the hematological perspective in human infancy has been reported (29), their biological properties have never been investigated. Therefore, in this study we investigated the frequency and functionality of CECs in the peripheral blood of human newborns/children from 1-day to 5-year and compared them with their counterparts in the neonatal mice. This study, for the very first time, provides a novel insight into the immunomodulatory capabilities of CECs in human newborns. Besides, we utilized a neonatal mouse model of *Listeria monocytogenes* (L.m) infection, as a significant cause of meningitis (30) in infants, to evaluate the role of CECs *in vivo*.

METHODS

Human Sample Collection and Processing

Blood was collected from healthy adult volunteers, and cord blood from full-term deliveries at the Grey Nuns Community Hospital, Edmonton. Thereafter, peripheral blood mononuclear cells (PBMCs) or cord blood mononuclear cells (CBMCs) were isolated over Ficoll-Hypaque gradients. For $CD71^+$ cell or mock depletion, cord blood samples were stained using anti-CD71 or isotype control biotin-conjugated antibody and fractioned using streptavidin linked magnetic beads (Miltenyi Biotec) according to our previous reports (13, 14, 17, 31). The appropriate Institutional Human Review Ethics Boards at the University of Alberta approved human studies with the ethics # Pro0046080 and Pro00063463. All study participants gave written informed consent to participate in this study. Studies related to human newborns were mainly performed in Mexico. The Ethics Committee of the Hospital de la Mujer (Women´s Hospital), the Mexican Ministry of Health approved the study (Reg. HM-INV/2018:02.09). In addition, some neonatal blood specimens were collected at the University of Alberta Hospital from infants who had elective operations. The appropriate Institutional Human Review Ethics Boards at the University of Alberta approved such studies (ethics # Pro00001408). Parents gave written informed consent form to participate in the neonatal related studies in Mexico and Canada.

Animals

Male and female BALB/c mice were purchased from the Charles River Institute. BALB/c mice were bred together, and pregnant mice were checked twice daily to establish birth timing. All animals were maintained and bred under pathogen free conditions within the animal care facility at the University of Alberta. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Canadian Council for Animal Care with animal ethics # AUP00001021.

The Experimental Model of Bacterial Infection

Listeria monocytogenes (L.m) was used as a disease model in newborn mice but the sex was not identifiable. For *in vivo* depletion of CECs, 80 μg purified anti-CD71 antibody (clone 8D3) was administered intraperitoneally (i.p.) at 4 and 5 days of age according to previous reports (13, 14). The same amount of IgG2a isotype control was injected i.p. into different neonatal mice to serve as controls. On day 6, mice were orally fed with ~10^7 colony forming units (CFUs) of L. m in 10 μl PBS. Two days later animals were euthanized for harvesting the spleen, liver, and brain. Bacterial counts were assessed by plating serial dilutions of the homogenates onto Luria broth (LB) plates and incubation at 37°C for 1–2 days. In parallel, some neonatal mice were fed with PBS to serve as controls. The similar infection approach was used for the survival study. Neonatal mice were monitored twice a day to record any mortality and kept up to 2 weeks post-infection.

Antibodies and Flow Cytometry

Fluorophore or biotin-conjugated antibodies with specificity to mouse cell surface antigens and cytokines were purchased from BD Biosciences or Thermo Fisher Scientific. Specifically, the following antibodies were used: anti-CD71 (R17217 and C2F2), anti-Ter119 (TER-119), anti-CD45 (30-F11), anti-VISTA (MIH64), anti-CD11b (M1/70), anti-CD11c (N418), anti-B220 (RA3-6B2), anti-CD40 (1C10), anti-CD80 (16-10A1), anti-CD86 (GL1), anti-CD3 (145-2C11), anti-CD4 (GK1.5), and anti-CD8a (53–6.7) for mice, For human studies, the following fluorophore or biotin-conjugated antibodies with specificity to surface markers or cytokines were used: anti-CD3 (HIT3a), anti-CD4 (RPA-T4), anti-CD8 (RPA-T8), anti-CD45 (H-130 or 2D1), anti-VISTA (B7H5DS8) and anti-IFN-γ (4S.B3), anti-CD71 (MA712), and anti-CD235A (HIR2). ROS staining (Sigma) was performed by flow cytometry per the manufacturer's protocols and our previous reports (17, 31). Live/dead fixable dead cell stains (ThermoFisher) were used to exclude dead cells in flow cytometry. Paraformaldehyde fixed cells were acquired by flow cytometry using a LSRFORTESSA flow cytometer (BD) and analyzed with FlowJo software.

Co-Culture and Stimulation

For *in vitro* intracellular cytokine staining, neonatal PBMCs were cultured and stimulated with anti-CD3/CD28 in RPMI media supplemented with 10% FBS for 24 h in the presence or absence of CECs according to our previous report (15). For co-culture, a fixed number (1 x 10^5) of PBMCs were seeded into 96 well round bottom plates individually or together with neonatal CECs at 1:1 ratio, Brefeldin A (10 μg/ml) was added 6 h prior to analysis. In some experiments, apocynin (1–2 mM) was added at the time of stimulation to abrogate the effects of ROS *in vitro* (31). For mice studies, splenocytes were harvested, and single-cell suspensions were made by grinding between sterile frosted glass slides in RBC lysis buffer and filtering through nylon mesh. Splenocytes were washed by centrifugation and used for subsequent *in vitro* studies.

Statistical Analysis

Statistical comparison between various groups was performed by the t-test using PRISM, Graph Pad software. Also, differences were evaluated using One-Way ANOVA followed by Tukey's test for multiple comparisons. Results are expressed as mean± SEM. P-value <0.05 was considered as statistically significant.

RESULTS

CECs Are Physiologically Abundant in Human Newborns but Their Frequency Declines With Age

We have previously shown that CECs were physiologically enriched in the spleen of 6–28 days old neonatal mice (13, 14). Here, we quantified the frequency of CECs in the spleen of day-3 to adult mice and found that the frequency of CECs was significantly lower in day-3 and day-5 compared to day-6.

These cells enriched to their maximum levels at days 6 to 9 before beginning to gradually disappear by 4 weeks of age (**Figures 1A, B**, **Supplementary Figure 1A**). When analyzing CECs in the spleen of neonatal mice, we noticed two subpopulations of CECs defined as CD71midTER119$^+$ and CD71highTER119$^+$ cells (**Figure 1C**). Our further investigations indicated that CD71highTER119$^+$ subpopulations were enriched with CD45$^+$CECs (**Figures 1D, E**). Although erythrocytes do not express CD45 (32), they are generated from CD45+ hematopoietic stem cells (HSC) and downstream erythroid progenitors through cytokine signaling such as erythropoietin (EPO) and stem cell factor (33). In agreement with previous

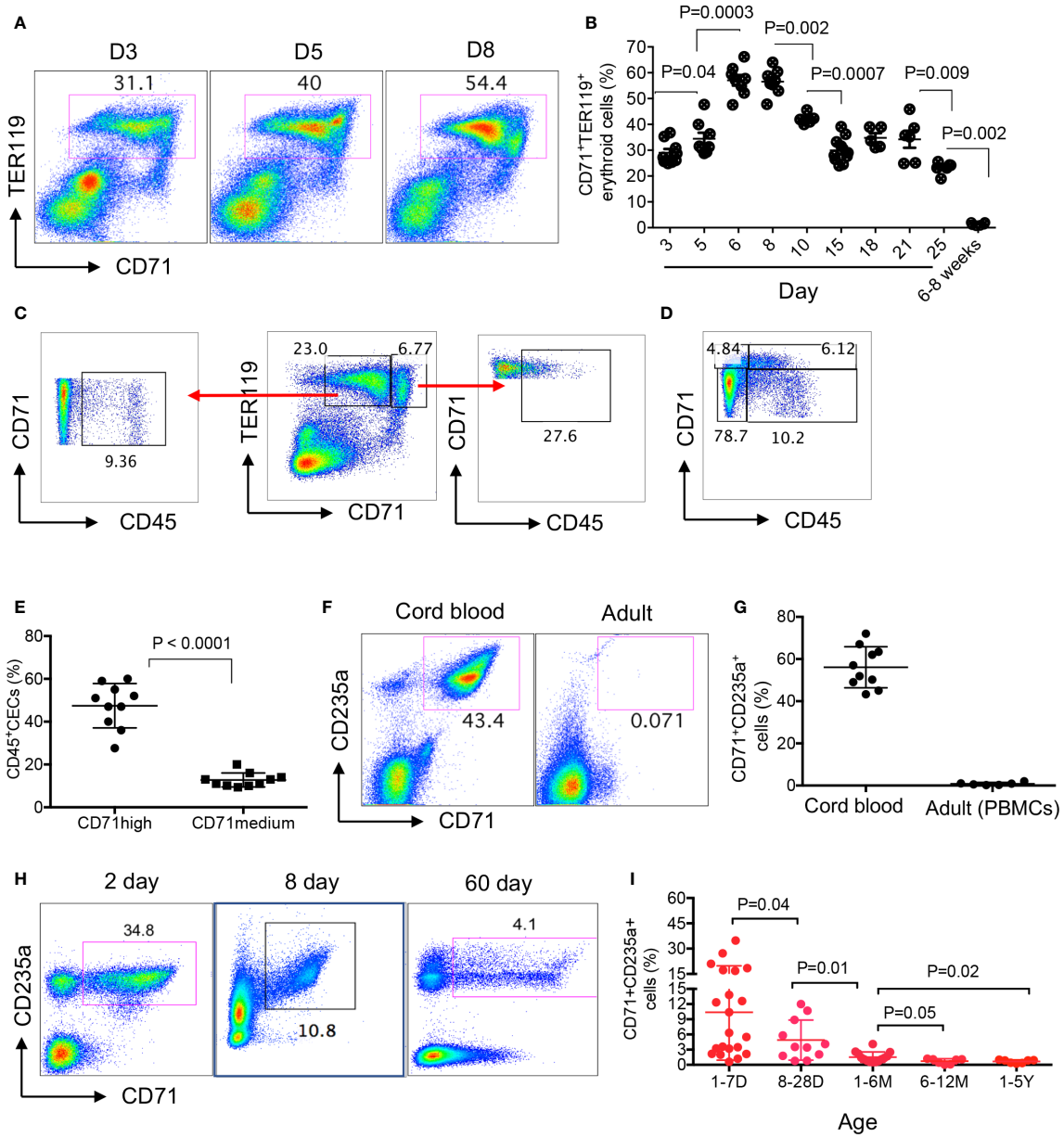

FIGURE 1 | Physiological abundance of CECs in newborns. **(A)** Representative flow cytometry plots of frequency of CECs in the spleen of days 3, 5, and 8 mice. **(B)** Cumulative data showing percentages of CECs in spleens of day-3 to adult (6–8 weeks) mice. **(C, D)** Representative plots showing percentages of CD45$^+$ in TER119$^+$CD71medium and TER119$^+$CD71high CECs. **(E)** Cumulative data showing percentages of CD45+ subpopulation of CECs in TER119$^+$CD71medium and TER119$^+$CD71high CECs in the spleen of neonatal mice. **(F)** Representative plots of frequency of CECs in the cord blood or the peripheral blood of human adults. **(G)** Cumulative data showing the percentages of CECs in the cord blood versus the peripheral blood of adult humans. **(H)** Representative plots of frequency of CECs in the peripheral blood of human newborns (days 2 and 8 and 2 months). **(I)** Cumulative data showing the frequency of CECs in the peripheral blood of human infants/children from day 1 to 5 years of age. Each point represents data from an individual mouse or human subject (adult, cord blood, or neonatal blood). Bar, mean ± one standard error.

reports, we found that the human cord blood was enriched with CECs while they were almost absent or at very low frequency in the peripheral blood of human adults (**Figures 1F, G**). For the very first time, to our knowledge, we found that CECs were abundant in the peripheral blood of human neonates prior to day-28. The frequency of CECs was significantly higher in the peripheral blood of 1–7 day old compared to 8- to 28-day-old neonates but significantly declined thereafter (**Figures 1H, I**, **Supplementary Figure 1B**). Despite their substantial reduction by 4 weeks, we found their frequency was still significantly higher in age group (1–6M) compared to those older groups (**Figure 1I**). These observations indicate that human newborns similar to mice newborns are physiologically enriched with CECs at the early stage of life.

CECs Are Heterogeneous and CD45$^+$CECs Are a Greater Source of ROS Than CD45$^-$ CECs

To better characterize CECs in human infants, we analyzed the expression of different regulatory molecules on their surface (e.g. PDL-1/PDL-2, galectins, and CD73/CD39), however, unlike their counterparts in mice we observed negligible levels of these molecules (data not shown). Then to gain a better insight into the neonatal CECs, we analyzed the expression of CD45. We found that almost one-third of CECs in human infants express CD45 from birth to age 5 years (**Figures 2A, B**). Recently, we reported that cord blood CECs express NOX-2 gene and *via* the production of ROS enhance HIV-infection and replication in CD4$^+$ T cells *in vitro* (31). Therefore, we decided to measure the production of ROS by CECs. Interestingly, we observed that CECs either from the human cord blood or peripheral blood of neonates expressed similar levels of ROS (**Figures 2C, D**). However, CD45$^+$CECs appeared to be the major source of ROS compared to their CD45$^-$ counterparts in human neonates (**Figures 2E–G**). Although we did not observe a significant difference in the frequency of CD45$^+$CECs in human neonates during the development (**Figure 2B**), we found a substantial increase in the abundance of CD45$^+$CECs after age 8-day in the spleen of neonatal mice which significantly increased at day 10 and remained constant until day 21, once again, the frequency of CD45$^+$CECs significantly increased at day 25 (**Figures 3A, B**). Next, CECs from newborn mice were subjected to ROS production analysis to determine whether they have similar capabilities in terms of ROS production to human CECs. Interestingly, we found that CD45$^+$CECs subpopulation in neonatal spleen were the most dominant source of ROS compared to their CD45$^-$ siblings (**Figures 3C–E**). Moreover, we compared CD45+ expression in the bone marrow-derived CECs compared to their counterparts in the spleen. CECs from the bone marrow had significantly lower levels of CD45 compared to their counterparts in the spleen (**Figures 3F, G**). Subsequently, CECs from the bone marrow had lower ROS expression compared to the splenic CECs (**Figures 3H, I**). These observations indicate that CD45$^+$CECs are to be the main source of ROS in both humans and mice. CD45, the receptor-like tyrosine phosphatase, is expressed on nucleated hematopoietic cells including erythroid progenitors. However, it gets downregulated as erythroid progenitors become mature (34). This implies that erythroid progenitors are the main source of ROS compared to their older siblings. Importantly, CECs in the bone marrow of mice express lower CD45 and have lower ROS production compared to those in the spleen.

Human Neonatal CECs Have Immunosuppressive Properties and Suppress Both Monocytes and T Cells *In Vitro*

We have previously shown that neonatal CECs in mice have immunosuppressive properties and suppress CD11b+ and CD11c+ cells (13, 14). In addition, we have reported that CECs suppress T cell activation and cytokine production *in vitro* and *in vivo* (13–15). This immunosuppression was mediated through the expression of arginase-II by CECs (13, 14). However, it was impossible to measure the arginase-II activity in human CECs. In contrast to mice, human neonatal CECs get lysed when exposed to the fixed/perm buffer for the intracellular staining. Despite this technical issue, we have already reported that human CECs express arginase-II mRNA (17). Thus, we believe human CECs similar to their counterparts in mice also express arginase-II. Since human CECs express NOX-2 gene and utilize ROS for their immunomodulatory functions (31), we decided to determine whether neonatal CECs vis ROS exhibit immunosuppressive properties. Therefore, neonatal PBMCs were cultured in the presence or absence of CECs and stimulated with LPS (2 μg/ml) and a Golgi blocker for 6 h. We found that CECs at 1:1 ratio significantly reduced TNF-α production by monocytes (CD14+ cells). These observations suggest that human neonatal CECs utilize ROS for their immunosuppressive properties as apocynin partially abrogated their inhibitory properties (**Figures 4A, B**). Similar effects were observed for IFN-γ production by both CD4$^+$ and CD8$^+$ T cells when stimulated with anti-CD3/CD28 antibodies in the presence/absence of CECs and a ROS scavenger (apocynin) (**Figures 4C–E**). Although we have shown that apocynin at 1 mM completely abrogated CECs-mediated enhanced HIV-1 infection/replication in CD4$^+$ T cells in the cord blood (31), it partially reversed the immunosuppressive properties of neonatal CECs even when 2 mM apocynin was used (**Figures 4A–E**). This suggests that human CECs may utilize another potential mechanism to mediate their immunosuppressive properties (e.g. arginase-II). As such, the removal of CECs unleashed cytokine production by both monocytes and T cells in PBMCs of human neonates which supports their immunosuppressive properties.

Neonatal CD45$^+$CECs in Humans and Mice Express High Levels of VISTA

We have previously shown that CECs in the spleen of neonatal mice express substantial levels of VISTA, which *via* TGF-β promote the induction of Tregs from naïve CD4$^+$ T cells (25). However, in that study, we reported that the cord blood CECs expressed a negligible amount of surface VISTA (25). In

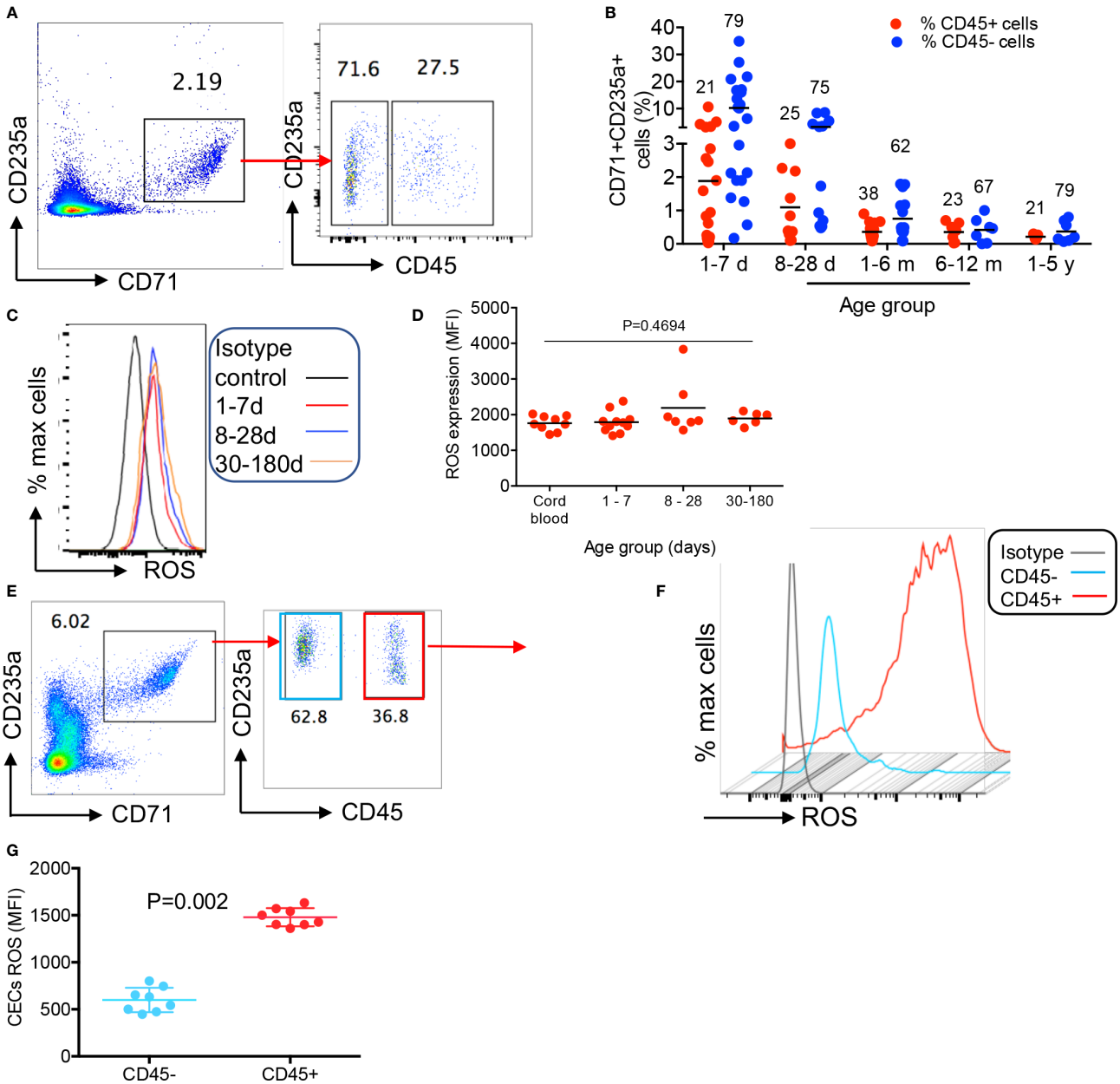

FIGURE 2 | Differential expression of ROS by CD45$^+$ versus CD45$^-$CECs in humans. **(A)** Representative plot showing differential expression of CD45 in CECs from a human infant. **(B)** Cumulative data showing percentages of CD45$^+$ and CD45$^-$ CECs in human infants/children from day-1 to 5 years of age. The numbers on red and blue symbols showing % CD45$^+$ or CD45$^-$CECs. **(C)** Representative histogram of ROS expression in CECs, and **(D)** cumulative data showing mean fluorescence intensity (MFI) of ROS in CECs from human cord blood and neonatal blood at shown ages. **(E)** Representative plots showing frequency of CD45$^+$ versus CD45$^-$ CECs in the peripheral blood of an 8-day-old human newborn. **(F)** Representative histogram of ROS expression in CD45$^+$ versus CD45$^-$CECs in an 8-day-old human newborn. **(G)** Cumulative data showing mean fluorescence intensity (MFI) of ROS in CD45$^+$/CD45$^-$CECs of human newborns.

agreement with our previous studies, we confirmed that CECs in the spleen of neonatal mice express VISTA (**Supplementary Figure 2A**). In contrast to CECs in the human cord blood, we found that CECs in the neonatal peripheral blood express significant levels of VISTA (**Supplementary Figure 2B**). More importantly, these observations confirmed that VISTA was mainly expressed by CD45$^+$CECs but not their CD45$^-$ siblings (**Supplementary Figure 2C and 2D**). These data provide additional insight into the phenotypical characterization of neonatal CECs in human. Whether VISTA on human CECs plays a regulatory role or promotes the induction of Tregs merits further investigations.

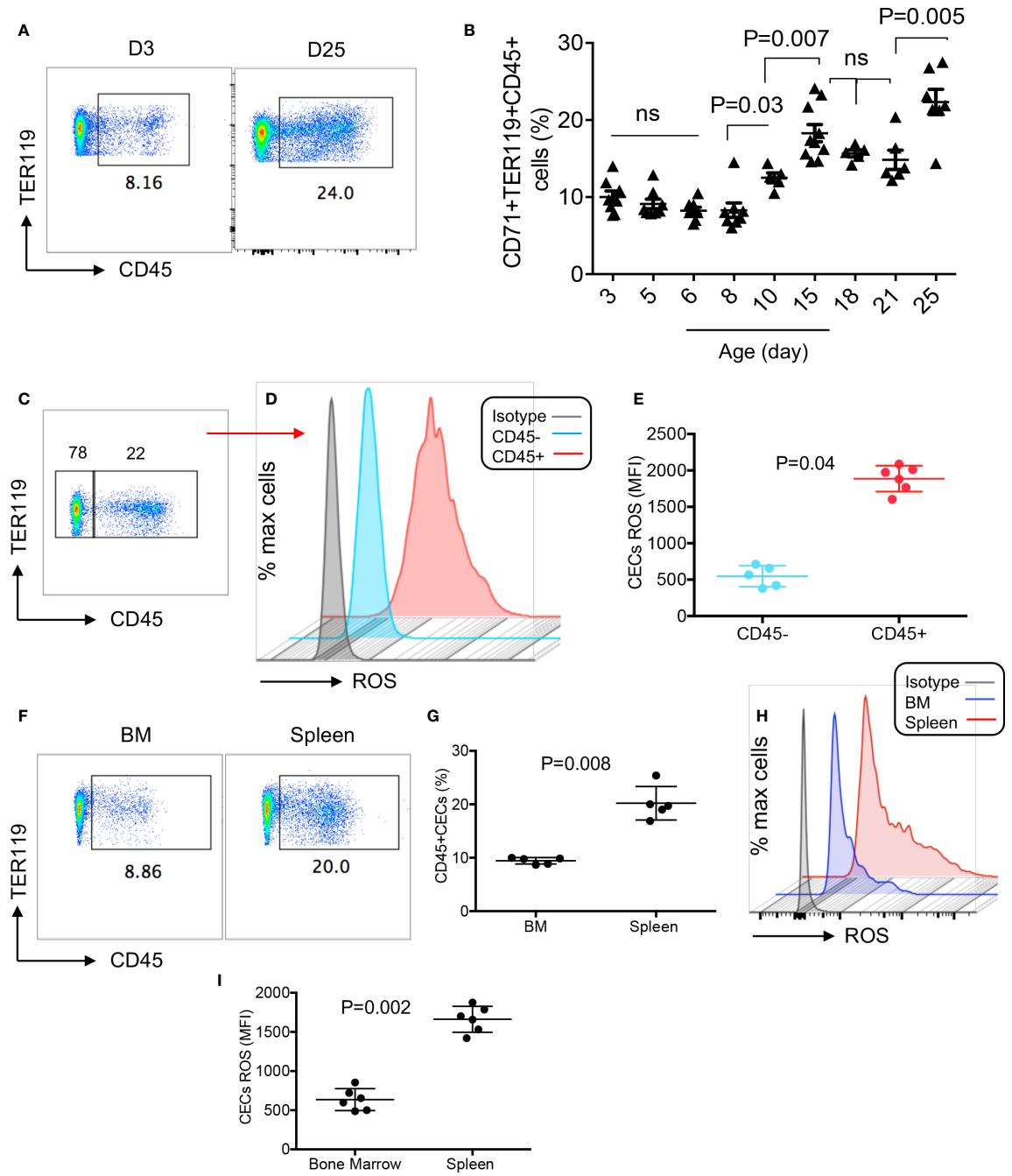

FIGURE 3 | Differential expression of ROS by CD45+ and CD45– CECs in mice. **(A)** Representative plots showing percentages of CD45+ subpopulation among mice splenic CECs at day-3 and day-25. **(B)** Cumulative data showing percentages of CD45+ CECs among splenic mice CECs from day 3 to day 25. **(C)** Representative plot of CD45 expressing CECs in splenic CECs of a 15-day-old mice. **(D)** Representative histogram of ROS expression in CD45+ versus $CD45^{-}$CECs from the spleen of a 15-day-old mouse. **(E)** Cumulative data showing mean fluorescence intensity (MFI) of ROS in $CD45^{+}/CD45^{-}$CECs of neonatal mice (day 15). **(F)** Representative plots of CD45 expression among splenic CECs versus their counterparts in the bone marrow (BM) of 15-day-old mice. **(G)** Cumulative data showing percentages of CD45+ CECs in the spleen versus bone marrow on neonatal mice. **(H)** Representative histogram of ROS expression in total CECs in the bone marrow compared to the spleen of a day-15 mouse. **(I)** Cumulative data showing mean fluorescence intensity (MFI) of ROS in the bone marrow versus spleen of neonatal mice.

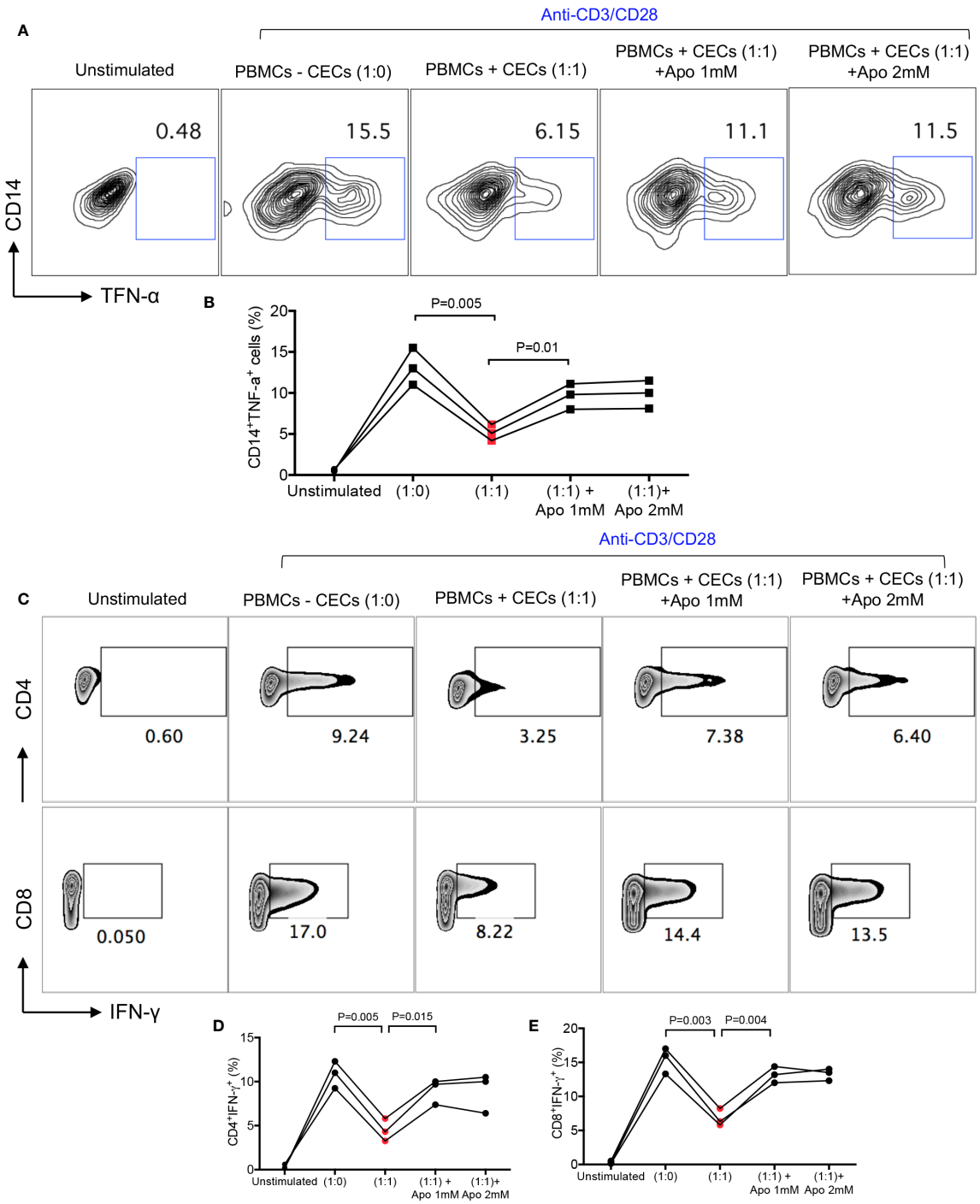

FIGURE 4 | CECs from human infants exhibit immunosuppressive properties *in vitro*. **(A)** Representative plots, and **(B)** cumulative data showing TNF-α production by CD14+ cells following stimulation with LPS (2 μg/ml) in the absence/presence of CECs (1:1) ratio and presence of apocynin at shown concentrations for 6 h with a Golgi blocker. Samples are from three infants aged 2–4 weeks. **(C)** Representative plots, and **(D)** cumulative data showing IFN-γ production by $CD4^+$ or **(E)** $CD8^+$ T cells following stimulation with anti-CD3/CD28 in the absence/presence of CECs (1:1) ratio and presence of apocynin at shown concentrations for 18 h with an additional 6 h in the presence of a Golgi blocker. Samples are from three infants age 2 to 4 weeks.

Pre-Emptive Depletion of CECs Protects Neonatal Mice Against Systemic Infection

We have previously shown that depletion of CECs resulted in increased protection against L.m. infection (13), however, in previous studies mice were infected *via* i.p. inoculation of bacteria. In the present study, to mimic the natural route of infection, we orally administered the bacteria to the neonatal mice. To determine whether the physiological abundance of CECs predisposes infants to systemic infection; first, we analyzed the effects of anti-CD71 neutralizing antibody (80 μg) on the frequency of CECs in the spleen and liver of treated mice versus controls. We observed this strategy significantly decreased the frequency of CECs in the spleen and liver of neonatal mice (**Figures 5A–C**). It is worth noting that as reported elsewhere (13), different anti-CD71 clones were used for the treatment (clone 8D3) versus flow cytometry staining (R17217 and C2F2). We administered the anti-CD71 antibody at day 4 and 5 followed by L.m infection (orally) at day 6. On day 8 (2 days post-infection), we quantified the number of bacterial colonies in the spleen, liver, and brain of neonatal treated mice compared to

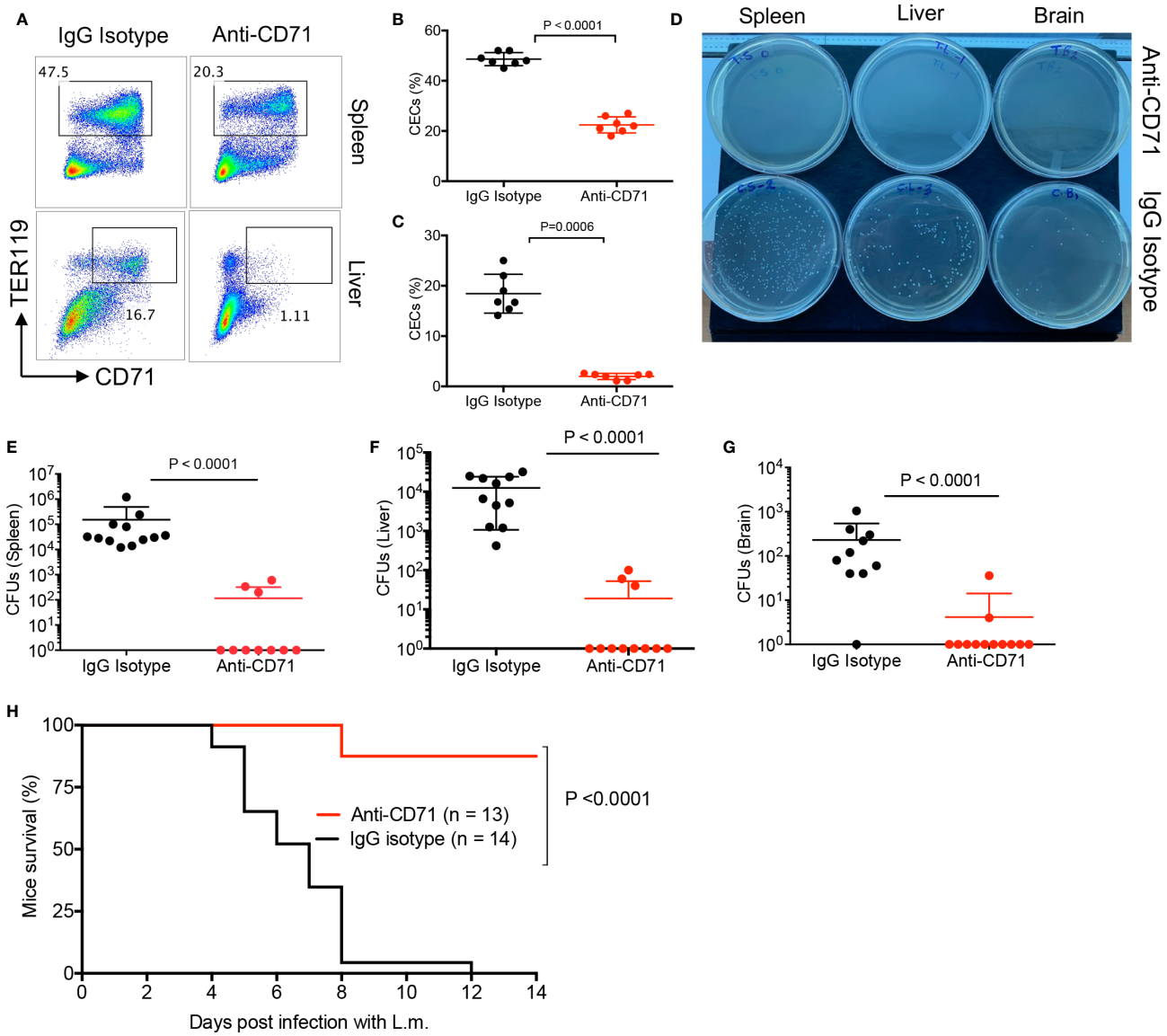

FIGURE 5 | The depletion of CECs reduces bacterial load in tissues. **(A)** Representative plots and **(B)** and **(C)** cumulative data showing quantification of CECs in the spleen and liver of mice following anti-CD71 or IgG2a isotype control administration (i.p.) at days 4 and 5, measured 2 days later. **(D)** Representative L.B plates comparing bacterial colonies in anti-CD71 treated versus IgG2a isotype control treated mice. **(E)** Cumulative data of *Listeria monocytogenes* colony forming units (CFUs) in the spleen, **(F)** liver, and **(G)** brain of mice either treated with anti-CD71 antibody or IgG2a isotype control. **(H)** Survival for neonate mice either treated with isotype control or anti-CD71 at days 4 and 5 then orally administered with 1×10^7 CFUs *Listeria monocytogenes* at day 6, and monitored over 2 weeks. Differences in mean values analyzed by two-tailed Student's t test; p value is shown; N > 7/group, from at least two experiments.

controls (isotype control). We observed that the depletion of CECs resulted in a significant decline in the number of L.m in the spleen, liver, and brain of neonatal mice (**Figures 5D–G**), which improved their survival rate (**Figure 5H**). Taken together, our data show that pre-emptive depletion of CECs significantly enhances the resistance of neonatal mice to L.m infection.

Depletion of CECs Enhances the Recruitment of Immune Cells in the Spleen and Liver

Considering the immunosuppressive properties of CECs (8, 13, 22, 24), we quantified the percentages of different immune cells after L.m oral infection. At 24 h after oral infection with L.m, depletion of CECs allowed for a surge in CD11b and CD11c cells in the spleen and liver of neonatal mice (**Figures 6A–C**). Importantly, these expanded CD11b/CD11c cells had an activated phenotype compared to those from the control group. This was evident by significantly higher expression of CD40, CD80, and CD86 levels on CD11b cells in the spleen (**Figures 6D–I**), and the liver (**Figures 6J–O**). A similar phenotype was observed for CD11c cells (data not shown). In addition, we found that depletion of CECs resulted in the recruitment of T cells in the spleen and liver of treated animals (**Supplementary Figures 3A–D**). Since T cells following activation/infection can upregulate CD71 (the transferrin receptor), we measured the surface expression of CD71 on splenic T cells. We observed although T cells is neonatal mice express about 10–15% CD71, the anti-CD71 antibody treatment did not have a significant effect on the expression of this molecule on both $CD4^+$ and $CD8^+$ T cells (**Supplementary Figures 3E, F**) despite its neutralizing effects on CECs (**Figure 5A**). These observations support a potential role for CECs in suppressing splenic and hepatic immune cells in the neonatal period.

DISCUSSION

In the present study, we investigated the frequency of CECs in the peripheral blood of human newborns from 1-day to 5 years of age. We found that CECs were highly abundant in the peripheral blood of human newborns at 1–7 days followed by 8–28 day but their frequency significantly declined afterward. This trend was opposite to what we observed in the spleen of neonatal mice, where CECs appeared to be significantly lower at days 3 and 5 but reached their maximum level by day 6–9. Based on our previous observations, CECs are a heterogeneous population of erythroid progenitors and precursors (22, 24). Therefore, we decided to phenotypically characterize human neonatal CECs by measuring the surface expression of PD-L, PDL-2, galectins (1, 3, and 9), and the ectoenzymes CD73/CD39. However, human neonatal CECs expressed none or a negligible amount of these molecules. To further characterize these cells, we investigated the expression of CD45, a transmembrane molecule shown on the membrane of all nucleated hematopoietic cells and their precursors (35). Erythrocytes do not express CD45 (32), however, they are generated from CD45+ hematopoietic stem cells (HSC) and downstream erythroid progenitors through cytokine signaling such as EPO and stem cell factor (33). Therefore, we analyzed the percentages of $CD45^+$CECs among PBMCs in human newborns/children in comparison with their counterparts in the spleen of neonatal mice. Interestingly, we found that the majority of human CECs lacked CD45 but about one-third of them had the surface expression of CD45 without substantial changes throughout childhood. In contrast, the expression of CD45 on the surface of splenic CECs significantly increased as mice aged. Another interesting observation was that CECs from the bone marrow had significantly lower CD45 expression compared to their counterparts in the spleen of neonatal mice. This may suggest the differential properties of extramedullary generated CECs versus those produced in the medullary spaces. To establish the association of CD45 with the functionality of CECs, we analyzed ROS expression in $CD45^+$CECs and $CD45^-$CECs. In agreement with a report on the predominant expression of ROS in $CD45^+$CECs compared to their $CD45^-$ siblings in an animal tumor model (23), we observed that $CD45^+$CECs consistently expressed higher levels of ROS in comparison to their $CD45^-$ counterparts. It is worth noting that splenic CECs had significantly higher levels of ROS compared to their mates in the bone marrow. This could be explained by a higher proportion of $CD45^+$CECs in the spleen versus the bone marrow. Higher numbers of $CD45^+$CECs indicates the abundance of progenitors versus precursors in the spleen of neonatal mice (34). Recently, we reported that CECs from the human cord blood/placenta, and likewise CECs in the PBMCs of HIV-infected patients and anemic individuals express ROS (31). Specifically, we found that CECs from the human cord blood had substantial levels of NOX2 mRNA, while the other NOX paralogous (NOX 1, 3, 4, 5, DUOX1, and 2) were undetectable (31). Although the endogenous ROS generation by RBCs has been documented (36), CECs had significantly higher ROS production capacity compared to their mature counterparts. More importantly, we showed CECs release mitochondrial ROS, which its function can be abrogated by ROS-scavenger Apocynin but not by N-acetyl cysteine (31).

Previously, we have reported that CECs in neonatal mice express arginase-II and this enzymatic activity was required for their immunosuppressive properties (13, 14). In particular, CECs *via* depletion of arginine in the microenvironment suppress immune cell activation (13) and impair phagocytosis of $CD11b^+$ cells *in vitro* (14). Similar observations have been made for other suppressor cells associated with tumor progression or persistent infection (37, 38). Nevertheless, measuring arginase-II activity in human CECs was technically impossible as they get lysed when exposed to the fixation/perming buffer. In contrast, mice CECs can be permed and stained intracellularly for arginase-II and any other intracellular molecules such as cytokines (e.g. TGF-β) (25). Despite this obstacle, we have been able to detect mRNA of arginase-II in human CECs from the cord blood (17, 31) but performing such studies on neonatal CECs was impossible because of the limited sample size.

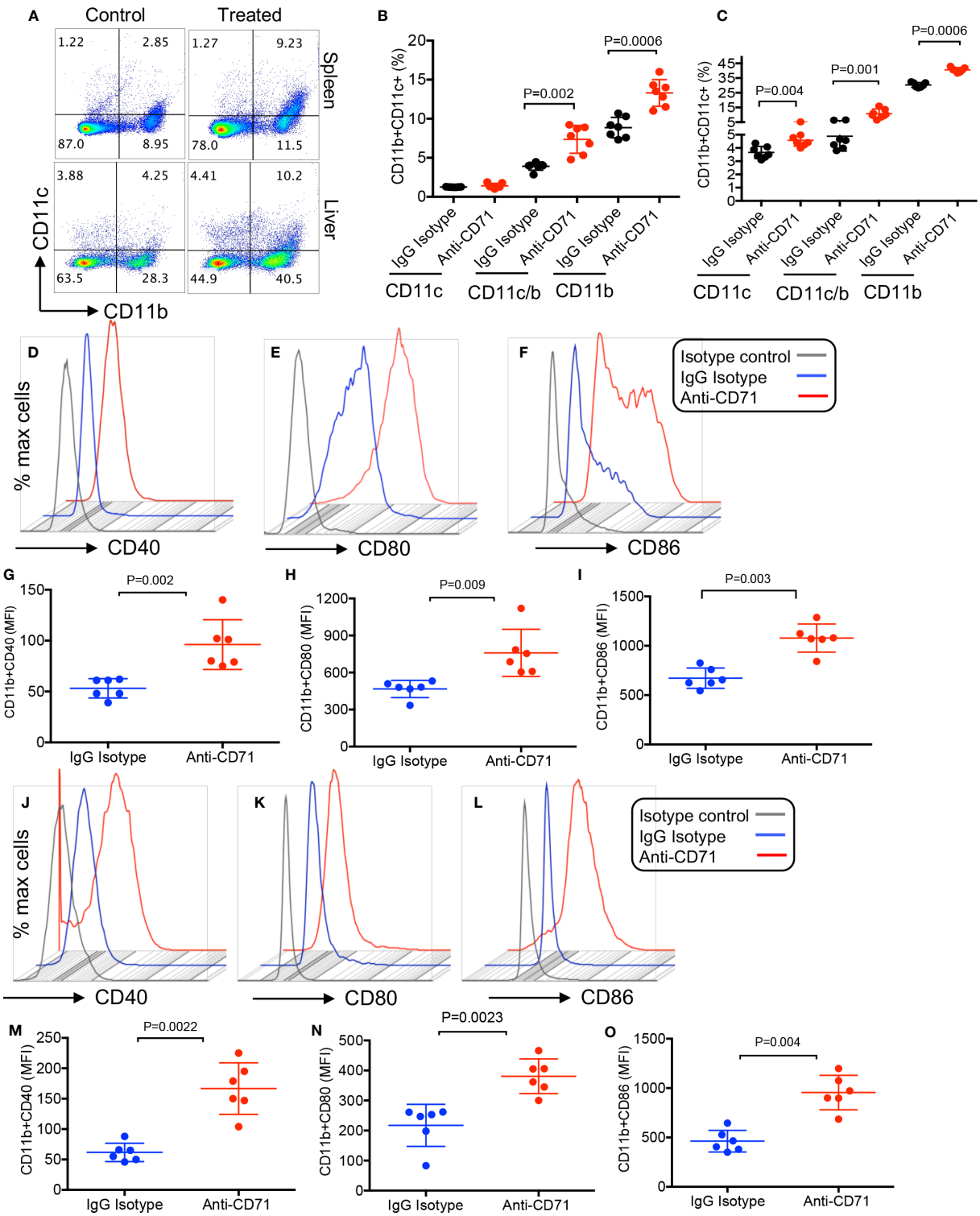

FIGURE 6 | Increase of effector immune cells 48 h post the depletion of CECs and 24 h after oral infection with *Listeria monocytogenes*. **(A)** Representative plots and **(B)** quantification of CD11b+, CD11c+, and CD11b+CD11c+ cells in the spleen and **(C)** the liver. **(D–F)** Representative plots, and **(G–I)** quantification of activation markers CD40, CD80, and CD86 in splenic CD11b+ by mean fluorescence intensity (MFI). **(J–L)** Representative plots, and **(M–O)** quantification of activation markers CD40, CD80, and CD86 in hepatic CD11b+ by mean fluorescence intensity (MFI). Differences in mean values analyzed by two-tailed Student's t test; p value is shown; N ≥ 6/group, from at least two experiments.

We have previously shown that CECs in neonatal mice exhibit immunosuppressive properties *in vitro*, and their depletion *in vivo* was associated with an early increased in the recruitment and influx of protective immune cells (e.g. Ly6G neutrophils, $CD11b^+$, $CD11c^+$, and NK cells) into the lungs in a model of pertussis (14). This early recruitment and/or activation of innate immune cells into the lungs of anti-CD71 treated mice more likely contributed to the clearance of bacteria and protection against *Bordetella pertussis* infection in neonatal mice (14). Likewise, we found that CECs populate the spleen and liver of neonatal mice and compromise their innate immune system against the pre-natal pathogen *L. monocytogenes*. Listeriosis is 18 times more common in pregnant than non-pregnant women and is responsible for 6% of all sepsis and 4% of meningitis presenting in the first 48 h of life (39, 40). We found that the deletion of CECs triggered a surge in the presence of immune effector cells in the spleen and liver of neonatal mice. This initial response was accompanied by a subsequent decline in the tissue bacterial load. The lower bacterial load in the periphery of treated mice with the anti-CD71 antibody may be the reason for a fewer L.m in the brain of animals. These observations support the notion of immunosuppressive properties of CECs which renders neonates more susceptible to infection. This was supported by significantly lower bacterial load in different tissues (e.g. the spleen, liver and brain) and prolonged survival of neonatal mice when CECs were partially depleted. The immunosuppressive properties of CECs goes beyond the innate immunity as they hinder systemic/mucosal cellular and humoral responses against infection (15). Despite the extreme limitations in the volume of blood specimens from human newborns/infants, we were able to perform some basic studies to evaluate the immunological properties of human neonatal CECs. Our observations proved that CECs from the PBMCs of human infants suppress the production of TNF-α by monocytes and IFN-γ by T cells *in vitro*. Interestingly, these immunosuppressive effects were partially reversed in the presence of apocynin, a ROS scavenger. Although these observations provide a novel mechanistic role for these physiologically abundant CECs in human infants, partial abrogation of their immunosuppressive functions suggest the presence of another factor(s). Therefore, we believe that CECs in human infants may utilize other immunomodulatory molecules such as arginase-II to mediate their immunosuppressive actions. VISTA could be another potential inhibitory molecule utilized by CECs either *via* interaction with its ligand or through the promotion of Tregs (25).

Taken together, we have demonstrated that CECs are abundant in human newborns. They have immunosuppressive capabilities and therefore may contribute to the compromised innate immune response to pathogens in newborns. In addition, it is possible to speculate that their abundance at the time of neonatal immunization programs may interfere with the adaptive immune responses to vaccination.

More importantly, our findings provide additional support to the notion of active immune suppression in the neonatal period. Thus, the impaired immune response seen in newborns could be explained, in part, by the abundance of CECs and other suppressor cells (e.g. myeloid-derived suppressor cells (MDSCs) (41) to dampen robust immune responses to external (pathogens) or internal antigens (e.g. microbiome). Our observations with recent discoveries in the field (42), support the concept that the neonatal immunity is not under-developed but instead tightly regulated, smart, highly dynamic, and complex. Therefore, such a highly regulated immune system due to the presence of immunosuppressive cells (e.g. CECs and MDSCs) in newborns/infants may explain one potential reason for the mainly asymptomatic COVID-19 infection in this age group (43–49). Compelling evidence indicated that innate immune hyperactivation in driving the acute disease in SARS-CoV-2–infected adults (50). It makes sense to suggest that the differential immune components in the young may prevent excessive and potentially damaging immune responses to COVID-19 infection. Similarly, trained innate immunity and the abundance of long-lived MDSCs in children (51) diminishes any excessive inflammatory response to pathogens such as SARS-CoV-2 (52). Thus, the biased immune tolerance than resistance strategy (53) in newborns/infants might be protective against COVID-19 infection (54). A deeper understanding of immune components and mechanistic pathways responsible for the immune regulation in newborn is required for an effective therapeutic approach to promote their health.

We can acknowledge several limitations in this study a) our sample size might appear to be small for some age groups but obtaining blood from human newborns was extremely challenging and most parents were not willing to participate in the study. b) the other limitation was the blood volume, performing functional studies on 0.5–1 ml blood was almost impossible. We have been fortunate to collect a few ml of blood from some infants for performing those functional studies. c) Due to the ethical issues, we were unable to obtain the sex/exact age of donors, thus, some variation might be related to these factors. Based on our observations we believe CECs are higher in day-1 compared to day-7 and putting 1- to 7-day-old newborns in one group might explain some of the observed variations in the frequency of CECs.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Human Review Ethics Boards at the University of Alberta approved human studies with the ethics # Pro0046080 and Pro00063463. All study participants gave written informed consent to participate in this study. Studies related to human newborns were mainly performed in Mexico. The Ethics

Committee of the Hospital de la Mujer (Women´s Hospital), the Mexican Ministry of Health approved the study (Reg. HM-INV/2018:02.09). In addition, some neonatal blood specimens were collected at the University of Alberta Hospital from infants who had elective operations. The appropriate Institutional Human Review Ethics Boards at the University of Alberta approved such studies (ethics # Pro00001408). Parents gave written informed consent form to participate in the neonatal related studies in Mexico and Canada. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. The animal study was reviewed and approved by the Animal ethics board of the University of Alberta in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Canadian Council for Animal Care with animal ethics # AUP00001021.

AUTHOR CONTRIBUTIONS

MV-L organized sample collection and processing, gave technical and logistic support and guidance, supervised data collection, analyses and reviewed the manuscript. VH-M and CR-E collected samples and performed the immunological studies. JM-R contributed in blood sample collection from children in Mexico. LW and BM assisted in recruitment of study subjects, sample collection in Edmonton and provided insight. OO performed the animal studies. SE conceived the research, supervised all of the study, assisted and performed some of the immunological assays, analyzed the data and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.597433/full#supplementary-material

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Immuno-Modulation of Hematopoietic Stem and Progenitor Cells in Inflammation

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Lifelong blood production is maintained by bone marrow (BM)-residing hematopoietic stem cells (HSCs) that are defined by two special properties: multipotency and self-renewal. Since dysregulation of either may lead to a differentiation block or extensive proliferation causing dysplasia or neoplasia, the genomic integrity and cellular function of HSCs must be tightly controlled and preserved by cell-intrinsic programs and cell-extrinsic environmental factors of the BM. The BM had been long regarded an immune-privileged organ shielded from immune insults and inflammation, and was thereby assumed to provide HSCs and immune cells with a protective environment to ensure blood and immune homeostasis. Recently, accumulating evidence suggests that hemato-immune challenges such as autoimmunity, inflammation or infection elicit a broad spectrum of immunological reactions in the BM, and in turn, influence the function of HSCs and BM environmental cells. Moreover, in analogy with the emerging concept of "trained immunity", certain infection-associated stimuli are able to train HSCs and progenitors to produce mature immune cells with enhanced responsiveness to subsequent challenges, and in some cases, form an inflammatory or infectious memory in HSCs themselves. In this review, we will introduce recent findings on HSC and hematopoietic regulation upon exposure to various hemato-immune stimuli and discuss how these challenges can elicit either beneficial or detrimental outcomes on HSCs and the hemato-immune system, as well as their relevance to aging and hematologic malignancies.

Keywords: hematopoietic stem cells, BM environment, inflammation, infection, immune-memory

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CELLULAR HETEROGENEITY IN EARLY HEMATOPOIESIS AND THE BM NICHE IN STEADY STATE

Lifelong replenishment of all mature blood and immune cells is sustained by a rare population of hematopoietic stem cells (HSCs) through a hierarchically organized lineage commitment program. In steady-state, the adult HSC pool is relatively quiescent but upon cell cycle entry, a stepwise specification of long-term reconstituting HSCs to progressively multi-, oligo- and uni-potent

hematopoietic progenitors (HPCs) restricted to the myeloid, lymphoid, and megakaryocyte-erythroid lineages supply the total blood cell pool (1). This program shows flexibility and durability to sudden hematopoietic perturbations such as blood loss or inflammation and reflects strict control over HSC self-renewal versus differentiation, as exhaustion or an imbalance in either will readily amount in hematopoietic failure and/or hematologic malignancies. The section will review current findings on HSC heterogeneity and its contribution toward steady-state hematopoiesis and briefly, cover essential concepts of the BM niche relevant later in the text for understanding the impact of a perturbed or stressed BM environment on HSCs.

The recent advancement in single-cell-based techniques and analysis (e.g., single-cell transplantation, RNA/ATAC-sequencing) has been revealing in terms of HSC biology in both native and stress hematopoiesis (2). The traditional roadmap of hematopoiesis, where HSCs were once assumed homogeneous with identical differentiation ability is currently being reassessed. The HSC population is in fact heterogeneous as clarified from single-cell transplantation and lineage-tracing experiments with certain HSC subsets being biased toward either myeloid or lymphoid lineages.

A large pool of multipotent progenitors (MPPs) rather than HSCs has been thought to drive steady-state hematopoiesis. Supportive of this are several publications that utilize lineage-tracing of genetically-labeled HSCs and barcoding *via* transposon tagging (3, 4). In contrast, Sawai et al., report of *Pdzk1ip1*-GFP-labeled HSCs as the ultimate source of continuous lymphopoiesis and myelopoiesis under steady-state conditions, which can be accelerated by a systemic interferon response (5). Chapple et al., utilize two independent HSC tracers, *Krt18* and *Fgd5* to likewise support this model, and additionally report a robust HSC contribution toward platelet and myeloid lineages (6). Although with no lineage bias as described in the previous study, Lu et al., similarly claim all donor-derived HSC clones regenerate the blood homogeneously under homeostasis, while in perturbed or conditioned states, a small fraction of engrafted HSC clones will dominantly expand and exhibit lineage bias (7). While this issue remains unresolved, the heterogeneity of HSCs nonetheless adds an additional layer of complexity in understanding HSC biology and requires consideration when interpreting functional readouts of steady-state and stress hematopoiesis, including infection and inflammation.

In addition to the well-established HSC immunophenotypes, lineage$^-$Sca-1$^+$c-Kit$^+$ (LSK), endothelial protein C (EPCR) (8), and the SLAM family proteins (9) used for the isolation of phenotypic hematopoietic stem and multipotent progenitors (HSPCs), others have been described to reflect HSC function by enriching for distinct lineage bias upon transplantation. Recently, Neogenin-1 (NEO1) was identified to distinguish NEO1$^+$ HSCs primed toward myelopoiesis at the cost of lymphopoiesis from NEO1$^-$ HSCs that show a balanced differentiation into both myelopoiesis and lymphopoiesis (10). NEO1$^+$*Hoxb5*$^+$ HSCs expand with age while NEO1$^-$*Hoxb5*$^+$ HSC counts remain unchanged as in young mice. Additionally, in vWF-GFP reporter mice, the megakaryocytic gene involved in platelet aggregation encoding von Willebrand factor (vWF) (11) was found to be expressed in ~60% of phenotypically defined HSCs (LSKCD150$^+$CD48$^-$CD34$^-$) with a higher expression of Mpl, encoding the thrombopoietin (TPO) receptor essential for megakaryocyte (Mk) and platelet production (12). This platelet-primed HSC subset with myeloid bias gives rise to lymphoid-biased HSCs, and thus are considered higher up in the hierarchical tree (11). The deficiency of Mitofusin 2, a regulator for mitochondrial fusion and tethering to the endoplasmic reticulum, results in reduced differentiation potential toward the lymphoid lineages (13). Several reports claim that platelet-biased HSCs residing at the apex of the HSC hierarchy are a major contributor of daily platelet production (14, 15). Indeed, a population of stem-like Mk-committed progenitors, primed but quiescent during steady-state and activated only during acute inflammation to replenish the depleted platelet pool has also been reported (16). Lineage skewing of HSCs toward platelets has been similarly observed in the BM of aged mice, where dysfunctional aged macrophages with an enhanced inflammatory signature fail to efficiently clear presiding apoptotic cells. The resulting increase in IL-1β is thought to induce the observed megakaryocytic HSC bias (17). Mks as a distinct lineage segregated from other hematopoietic lineages is so far implicated from several studies. HSC subtypes briefly noted here have been ably reviewed by Yamamoto et al., in which they discuss how emerging concepts of HSC heterogeneity presented *via* recent platelet and red blood cell lineage analyses may require a redefining of the "stemness" concept (18).

HSCs reside within the BM niche, a myriad of cellular, molecular and physical components of the BM microenvironment that maintain HSCs through the release of certain niche factors (19, 20). The perivascular niche has been well-described and is comprised of endothelial cells (ECs) and CXCL12 abundant reticular (CAR) cells, leptin receptor (LepR)$^+$ cells, and nestin$^+$ cells, with the latter three showing considerable overlap and high expression levels of stem cell factor (SCF) and CXCL12. Niche constituents are crucial in regulating HSC identity as demonstrated by several deletion studies. For example, SCF deletion in LepR$^+$ cells and ECs eliminates quiescent and transplantable HSCs from the BM (21). Depletion of Mks (22, 23) and periarteriolar NG2$^+$ stromal cells (24) results in HSC proliferation and are likewise thought to promote HSC quiescence. Interestingly, lineage-biased HSCs appear to occupy distinct BM microenvironments. Recently, myeloid-biased vWF$^+$ HSCs were found to be enriched in Mk niches, while lymphoid-biased vWF$^-$ HSCs were situated near quiescence-regulating arteriolar niches (25). Similar to HSCs, niche components are heterogeneous and form complex microenvironments with multiple inputs from cellular constituents. Together, a combination of cells coordinates the maintenance of the hematopoietic system both during steady-state and under perturbed situations.

INFLAMMATION-STRESSED EARLY HEMATOPOIESIS

Inflammation is the physiological reaction of the body to tissue injury or foreign insult and triggers a protective response involving blood and immune cells, vessels, and various molecular mediators. This is best illustrated in the case of infection; immune cells at local sites are activated through self or non–self-antigen recognition, and subsequent waves of innate and acquired immunity are coordinated to ensure host defense (26). In contrast to secondary lymphoid organs primarily tasked with immune activation, primary lymphoid organs including the BM had been long regarded immune-privileged with only minor exposure to the immune response. BM-residing HSCs and memory immune cells were thus assumed exempt from immune insults that can cause cell exhaustion or death, and reserved for prospective life-threatening invasions. HSCs were considered safely shielded in a dormant state through transcriptional and epigenetic regulators and their role in the initiation and resolution of inflammatory insults was presumed minimal.

Recent findings however highlight the dynamic response of HSCs toward inflammation. HSCs directly sense inflammation through their extracellular and intracellular receptors, rapidly lose quiescence and proliferate in response to an external milieu of inflammatory factors and infectious agents. Common inflammatory signals reported to impact primitive hematopoiesis include interferon (IFN)-α (27–29), IFN-γ (30, 31), tumor necrosis factor (TNF)-α (32), transforming growth factor (TGF)-β (33), interleukin (IL)-1 (34), IL-6 (35, 36), and macrophage colony-stimulating factor (M-CSF) (37); infectious agents include pathogen-associated molecular patterns (PAMPs) derived from microbes and danger-associated molecular patterns (DAMPs), both of which are recognized by pattern recognition receptors (PRRs). The activation of respective downstream signaling pathways in HSCs may result in their mobilization, proliferation, or differentiation to boost immune cell production (38, 39). Infection restricted to peripheral tissues/organs is primarily dealt with by immune cells at local sites that will get activated, consumed, and ultimately replenished by HSPCs (**Figure 1**). In the case of a systemic microbial spread due to severe infection or sepsis, HSPCs in the BM are activated to proliferate and drive myelopoiesis at the expense of lymphopoiesis. This is known as emergency myelopoiesis and involves the *de novo* generation and release of immature and mature neutrophils from the BM (38, 40).

Inflammatory cytokines are major regulators of stress hematopoiesis (39) (**Table 1**). Essers et al., reported that IFN-α produced by plasmacytoid dendritic cells (DCs) upon TLR9 activation (49) activated dormant HSCs and caused their entry into the cell cycle. During chemotherapy, the proliferative capacity of HSCs is likewise enhanced upon stimulation with IFN-α. The proliferative stress ensued with 5-fluorouracil (5-FU) treatment caused a profound reduction of WT HSCs, while IFNRα$^{-/-}$ HSCs were mostly unaffected (27). IFN-γ upregulation during *Mycobacterium avium* infection similarly activated HSCs and resulted in their increased cycling and proliferation (30). However, the effect of IFNs on HSCs can be diverse, as described in the case of the tick-borne pathogen *Ehrlichia* infection, where a robust production of IFNα/β impaired hematopoiesis through HSPC depletion and enforced HSPC quiescence (50). IL-1 produced by several cell types such as macrophages, ECs, and epithelial cells (51) directly stimulated HSCs and skewed their differentiation potential toward myeloid lineages through activation of PU.1, a transcription factor regulating the myeloid differentiation program (34). Likewise, upon LPS challenge, M-CSF secreted from ECs, macrophages, or fibroblasts was found to affect PU.1, and HSCs with higher PU.1 levels were primed toward myeloid differentiation (37). TNF-α mainly secreted by macrophages, T cells, and natural killer cells (52) promotes HSC survival and simultaneously, their myeloid differentiation *via* an NF-κB-PU.1-dependent mechanism (32). An alternative pathway in response to bacterial infection, driven by intermediate lineage-committed HPCs *via* osteoblast-derived IL-7, which is a crucial cytokine for lymphopoiesis has also been reported (48).

The effects of transient cytokine stimulation on HSC regulation is overall beneficial in fighting infection, but can also be detrimental when sustained by impairing HSC function as reported in the cases of chronic *Mycobacterium avium* infection (53), IFN-α (27) and LPS challenges (54), and IL-1 receptor stimulation (34). These detrimental effects may stem from the accumulation of DNA damage and double-strand breaks induced by various HSC activators (55). While the TGF-β presented by non-myelin Schwann cells is essential for steady-state HSC maintenance (33), continuous TGF-β stimulation *in vitro* appears to reduce HSC cell division and suppress their reconstitution ability (56). Interestingly, the proliferation of lymphoid-biased HSCs but not myeloid-biased HSCs, as defined by the Hoechst dye efflux efficiency or "side population" was suppressed (57). Upregulation of TGF-β was found upon *Trypanosoma cruzi* infection (58), which suggests chronic infection may also differentially impact the function of HSCs. Collectively, these findings illustrate how HSCs respond to various cytokine stimulations by adjusting their proliferative capacity as well as their differentiation program. The duration and/or magnitude of the stimulation are possible determinants for the cellular fate of HSCs (i.e., self-renewal, differentiation, or apoptosis).

Toll-like receptors (TLRs) are a family of transmembrane receptors that serve as the first-line innate immune sensor for a variety of infection-derived PAMPs and DAMPs (59). The TLRs primarily expressed on HSPCs are TLR2 and TLR4; both bind to bacterial ligands and induce their myeloid differentiation (60). Lymph-duct circulating HSPCs also express TLRs and differentiate into myeloid cells upon their ligation (61), indicating that TLR expression may serve as a means of immuno-surveillance, to sense infection at local sites and increase hematopoietic production upon need. Alternatively, the expression of bacteria-sensing receptors on HSPCs evolved to deal with life-threatening infections in the devastating case innate immune cells ever fail in combat and systemic bacterial infiltration follows. A proof-of-principle study involved an acute challenge with lipopolysaccharide (LPS), a gram-negative bacterial component recognized by TLR4, and subsequent activation of quiescent HSCs to proliferate and differentiate into

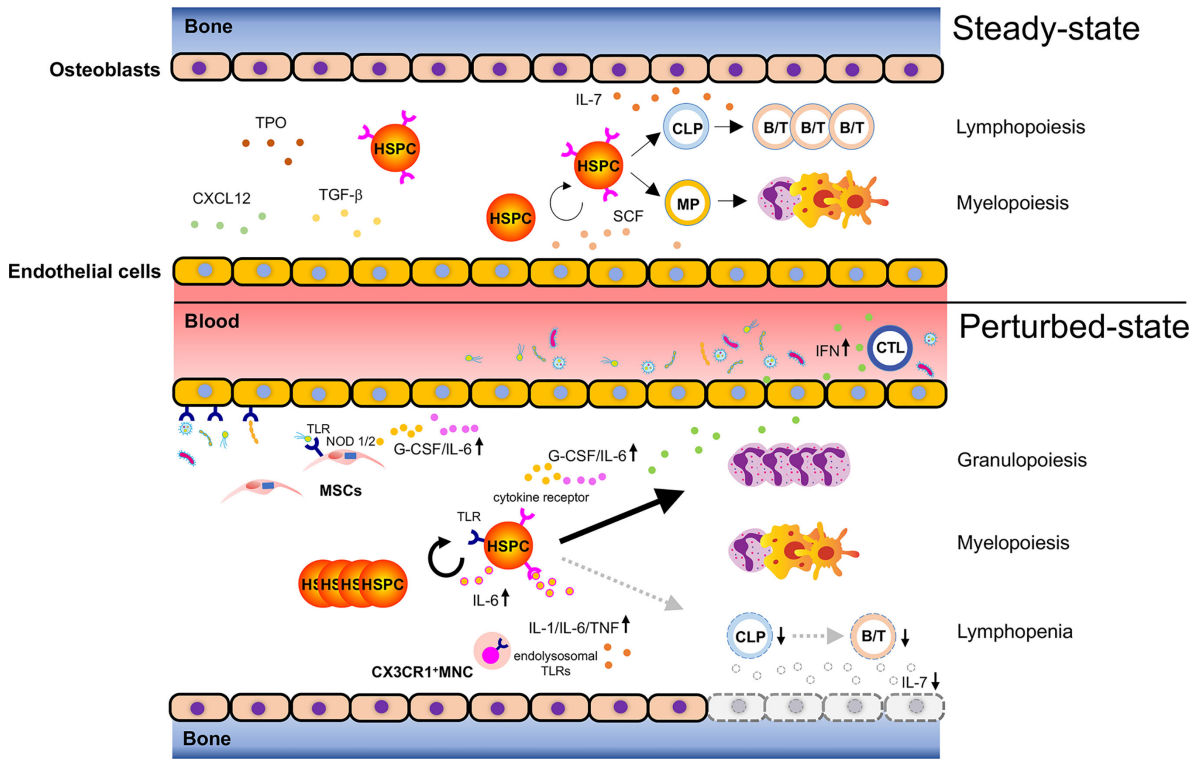

FIGURE 1 | Bacteria-induced activation of HSPCs. Steady-state hematopoiesis (upper): Hematopoietic stem and progenitor cells (HSPCs) self-renew and differentiate into myeloid progenitors (MPs) and common lymphoid progenitors (CLPs) to produce mature cells. The divisional manner of HSPCs toward either self-renewal or maturation (myelopoiesis/lymphopoiesis) is tightly controlled to sustain lifelong hematopoiesis. Hematopoiesis under infection (lower): Bacterial components reach the bone marrow (BM) *via* systemic blood circulation to activate pattern recognition receptors (PRRs) such as toll-like receptors (TLRs) expressed on HSPCs and promote their proliferation. Bacteria-associated molecules reach the BM and can alternatively activate TLRs and NOD1/2 on endothelial cells or mesenchymal stromal cells (MSCs), leading to the secretion of inflammatory cytokines such as G-CSF and IL-6. These secreted cytokines promote granulopoiesis by acting on HSPCs. Cytotoxic T lymphocytes (CTLs) respond to bacterial infection and produce inflammatory cytokines such as IFNs, which migrate to the BM and activate corresponding receptors expressed on HSPCs. This results in reduced HSPC self-renewal and enhanced myelopoiesis. Severe bacterial infection such as sepsis rapidly ablates osteoblasts and induces lymphopenia due to lack of osteoblast-derived IL-7. CX3CR1$^+$ mononuclear cells (MNCs) sense bacteria-derived molecules such as bacterial DNA *via* endolysosomal TLRs and secrete the inflammatory cytokines, IL-1, IL-6, and TNF, which control the expansion of hematopoietic progenitors, and shift the hematopoietic program toward myelopoiesis. Taken together, bacterial challenges induce HSPC activation and myelopoiesis directly and indirectly at the expense of lymphopoiesis.

myeloid cells (60–62), while upon repetitive stimulation or chronic LPS challenge, HSC numbers increase but their reconstitution capacity decrease, somewhat recapitulating HSC aging (54, 63, 64). The elicited downstream pathway appears bacterial species-dependent, as *Pseudomonas aeruginosa*, a gram-negative bacteria induced HSC expansion through TLR4 (65), whereas *Staphylococcus aureus*, a gram-positive bacteria showed similar HSC expansion but through a TLR-independent pathway (66). Live *Salmonella Typhimurium* induced proliferative stress in HSCs, albeit through TLR4-dependent and -independent mechanisms (54). Besides bacteria, the fungus *Candida albicans* also expanded HSC-containing LSK cells of the BM *via* TLR2 and prompted their differentiation into granulocytes, monocytes, macrophages and DCs (67). Of note, upon TLR2 and TLR4 activation, HSPCs were also capable of secreting IL-6, a particularly important regulator of myelopoiesis in an auto- and paracrine manner (36). Finally, sustained increase in Sca-1$^+$ HSPCs is a hallmark of bacterial and viral infections as described above, but also parasitic infections as well, as demonstrated in the malaria mouse model elicited by the *Plasmodium berghei* sporozoite, *via* direct HSC and progenitor proliferation (68).

Apart from invading pathogens, commensal bacteria or the microbiota can also regulate hematopoiesis. Microbiota depletion by antibiotics pretreatment induced atrophy of the thymus and spleen, and suppressed hematopoiesis in the BM by reducing HSC, MPP, and CLP numbers in a Stat1-dependent manner, and not *via* TLR signaling (69). Similarly, germ-free (GF) mice showed lower HSC, MPP, and CLP counts, and a selective functional defect in GMP and myelopoiesis (70). This phenotype was reversible and could be rescued with administration of the nucleotide-binding oligomerization domain (NOD) 1 ligand which activated mesenchymal stem cells (MSCs) to produce the inflammatory cytokines, IL-7, IL-6, TPO, SCF, and Flt-3. These results suggest peptidoglycan (PGN), the NOD1 ligand derived from the microbiota modulates daily hematopoiesis (71). Recently, CX3CR1$^+$ monocytes were found

TABLE 1 | The role of inflammatory cytokines or chemokines on steady-state and stress hematopoiesis.

Cytokines	Which cells produce	Effect on HSC function	Reference
SCF	Endothelial cell, MSC	HSC maintenance	Morrison Nature 2014 (20)
CXCL12	Endothelial cell, MSC, CAR cell		
Thrombopoietin (TPO)	Hepatocyte		Decker Science 2018 (41)
Transforming growth factor β (TGF-β)	Schwann cell		Yamazaki Cell 2011 (33)
Fms-like kinase 3 (Flt-3)	Ubiquitous	Myeloid differentiation	Gabbianelli Blood 1995 (42)
Interferon (IFN)-α	Plasmacytoid dendritic cell (DC)	Impaired HSC reconstitution capacity	Esser Nature 2009 (27)
		HSC exhaustion	Sato Nat Med 2009 (28)
			Pietras J Exp Med 2014 (29)
IFN-γ	T cell	Impaired HSC reconstitution capacity	Baldridge Nature 2010 (30)
		Impaired HSC maintenance	de Bruin Blood 2013 (31)
Granulocyte colony-stimulating factor (G-CSF)	MSC, endothelial cell	Myeloid differentiation	Boettcher J Immunol 2012 (43)
			Boettcher Blood 2014 (44)
Granulocyte-macrophage colony-stimulating factor (GM-CSF)	MSC, endothelial cell, macrophage, T cell		Weisbart Nature 1985 (45)
			Shi Cell Research 2006 (46)
Macrophage colony-stimulating factor (M-CSF)	Endothelial cell, macrophage, fibroblast		Mossadegh-Keller Nature 2013 (37)
Interleukin (IL-1)	Macrophage, Endothelial cell		Pietras Nat Cell Biol 2016 (34)
	Epithelial cell		
Interleukin (IL-3)	T cell		Suda J Cell Physiol 1985 (47)
IL-6	Ubiquitous		Zhao Cell Stem Cell 2014 (36)
	HSPC (LSK)		
IL-7	Osteoblast	Decrease of CLPs and induction of lymphopenia	Terashima Immunity 2016 (48)
TNF-α	Macrophage, T cell, natural killer cell	Myeloid differentiation	Yamashita Cell Stem Cell 2019 (32)

The table summarizes the role of chemokines and cytokines upon steady-state or stress hematopoiesis. SCF, stem cell factor; MSC, mesenchymal stem cell; CAR cell, CXCL12-abundant reticular cell; TNF, tumor necrosis factor.

to co-localize with HSPCs near blood vessels in the steady-state BM. These monocytes sensed commensal bacteria-derived molecules *via* their endolysosomal TLRs (TLR-3, -7, and -9) to produce tonic levels of the inflammatory cytokines, IL-1β, IL-6, and TNF-α and control proliferation and myeloid differentiation of HSPCs (70). Thus, microbiota-derived molecules circulate the blood in both physiological and pathological conditions (72), reach the BM, and are captured by specific hematopoietic and non-hematopoietic cells to fine-tune hematopoiesis.

INFLAMMATION-STRESSED BM MICROENVIRONMENT

Upon tissue insult, various BM cells have been reported to influence either HSCs or hematopoiesis, including adipocytes (73), endothelial vessels (74), osteocytes (75), neurons (76), macrophages (77) and Schwann cells (33) among others. The BM microenvironment has likewise been investigated at single-cell resolution during homeostasis and under stress hematopoiesis (78). The necessity of stromal cells for efficient HSC expansion and maintenance under perturbed conditions has become apparent from studies by several groups. Co-transplantation of $CD73^+CD105^-Sca1^+$ BM stromal cells with donor-derived HSCs after irradiation resulted in efficient repairing of the damaged niche and improved HSC expansion (79). The transplanted stromal cells were localized within clusters of HSCs, indicating the efficient expansion of HSCs following their transplantation relied on local interactions with stromal cell progenitors. Guo et al., reported the importance of Jagged-2 induction in vascular niches after myeloablation for HSPC expansion and reacquisition of HSPC quiescence (80). Recently, a subset of $apelin^+$ ECs was shown to be critical not only for the maintenance of steady-state hematopoiesis but also after myeloablative injury. $Apelin^+$ ECs expanded substantially and mediated the regeneration of the vascular niche and subsequent hematopoietic reconstitution after BM transplantation *via* pleiotrophin (81). Thus, reciprocal interactions between the niche and HSCs are vital in determining efficient hematopoietic reconstitution under stressed conditions.

Infection-induced HSPC activation is mediated by a combination of direct and indirect pathways involving PRRs such as TLRs and NODs expressed on hematopoietic and non-hematopoietic cells (**Tables 2** and **3**). In particular, granulocyte-colony stimulating factor (G-CSF) secreted from TLR4-expressing ECs is essential and sufficient to activate GMPs and drive emergency myelopoiesis (43). *Escherichia coli* infection rapidly mobilized HSCs to the spleen *via* two innate immune sensors, nucleotide-binding oligomerization domain (NOD)-like receptor 1/2 and TLR4, both of which are expressed on radio-resistant cells, presumably stromal cells. Their activation synergistically induced G-CSF secretion for efficient HSC mobilization and neutrophil differentiation (84). LPS challenge also drives vascular remodeling in the BM, proliferation of ECs and increase in their permeability, and accompanies HSPC proliferation and neutrophil mobilization from the BM (85). Del-1 is an extracellular matrix protein expressed by cellular components of the HSC niche, including ECs and CAR cells. Del-1 deficiency attenuated emergency myelopoiesis and HSPC expansion both in steady-state and in response to LPS and G-CSF injections (86). Intravital BM imaging revealed parasitic *Trichinella spiralis* infection dramatically increased HSC motility within the BM and their migration to other BM spaces (87). Parvovirus B19 caused

TABLE 2 | Pattern recognition receptors expressed by hematopoietic cells that regulate steady-state and stress hematopoiesis.

Receptors	Ligands	Cell type	Species	Signaling	Function	Reference
TLR2	*Candida albicans*	LSK ($Lin^-Sca\text{-}1^+c\text{-}kit^+$)	Mouse	TLR2-Myd88/ Dectin1	Differentiation into DCs	Yanez PLoS One 2011 (67)
	Pam3CSK4	Lin^- $CD34^+$	Mouse Human	TLR2-ROS	Differentiation into macrophages with lower levels of inflammatory cytokines	Yanez Eur J Immunol 2013 (82)
TLR2/4/9	Pam3CSK4 LPS CpG	Common dendritic cell progenitor (CDP)	Mouse	CXCR4 down-regulation and CCR7 up-regulation	DC expansion in inflamed lymph nodes and support of DC homeostasis	Schmid Blood 2011 (83)
TLR4	LPS of *Pseudomonas aeruginosa*	LSK ($Lin^-Sca\text{-}1^+c\text{-}kit^+$)	Mouse	TLR4	Dysfunctional HSC expansion	Rodriguez Blood 2009 (65)
	LPS	HSC (CD150+CD48-LSK)	Mouse	TLR4	Increased HSC number but decreased HSC reconstitution potential	Esplin J Immunol 2011 (64)
	LPS	HSC (CD150+CD48-LSK)	Mouse	TLR4-Id1	Increased HSC number but induced HSC dysfunction	Zhao PLoS One 2013 (63)
	LPS *Salmonella Typhimurium*	HSC ($CD150^+CD135^-CD34^-CD48^-$ LSK/ $CD150^+CD34^-CD48^-CD41^-$ LSK)	Mouse	TLR4-TRIF-ROS-p38	Proliferative stress-induced HSC dysfunction	Takizawa Cell Stem Cell 2017 (54)
TLR3/7/9	bacterial DNA	$CX3CR1^+$MNC	Mouse	TLR3/7/9	Inflammatory cytokine production by $CX3CR1^+$ MNCs induced MPP expansion and steady-state myelopoiesis	Lee Blood 2019 (70)

The table summarizes the role of pattern recognition receptors expressed in hematopoietic cells upon steady-state or stress hematopoiesis. TLR, Toll-like receptor; DC, dendritic cell; Pam3CSK4, Pam3Cys-Ser-(Lys)4; ROS, reactive oxygen species; LPS, lipopolysaccharide; CCR, CC chemokine receptor; CXCR, CXC chemokine receptor; MNC, mononuclear cell; CX3CR1, CX3C chemokine receptor 1; HSC, hematopoietic stem cell; MPP, multipotent progenitor.

TABLE 3 | Pattern recognition receptors expressed by non-hematopoietic cells that regulate steady-state and stress hematopoiesis.

Receptors	Ligands	Cell type	Signaling	Species	Function	Reference
TLR4	LPS	Non-hematopoietic cell	TLR4 not IL-1R	Mouse	G-CSF-mediated emergency myelopoiesis	Boettcher J Immunol 2012 (43)
		Endothelial cell	TLR4-Myd88			Boettcher Blood 2014 (44)
TLR4 NOD1/2	LPS PGN	Non-hematopoietic cell	G-CSF up-regulation and CXCL12 down-regulation	Mouse	G-CSF-induced HSC mobilization to spleen	Burberry Cell Host Microbe 2014 (84)
NOD1	PGN	MSC	NOD1	Mouse	Regulation of steady-state hematopoiesis *via* cytokine production by MSCs	Iwamura Blood 2017 (71)

The table summarizes the role of pattern recognition receptors expressed in non-hematopoietic cells upon steady-state or stress hematopoiesis. TLR, Tol- like receptor; LPS, lipopolysaccharide; G-CSF, granulocyte-colony stimulating factor; NOD, Nucleotide-binding oligomerization domain-containing protein; PGN, peptidoglycan; MSC, mesenchymal stem cell.

transient erythroid aplasia by infecting MSCs and upregulating their expression of IL-6 and TNF-α (88). Treatment with IFN-α or pI:C, a ligand for TLR3 and a mimetic of viral infection modulates hematopoiesis *via* hematopoietic- but also niche-expressed IFN-α receptor. Both challenges increased EC proliferation in the BM partly through vascular endothelial growth factor (VEGF) (89). Taken together, these findings indicate bacterial, viral, or parasitic infections can induce HSPC activation also through niche-dependent pathways.

CHEMOTHERAPY- AND IRRADIATION-INDUCED INFLAMMATION

Similar to naturally occurring infections, quiescent HSCs are recruited to actively divide and regenerate the hematopoietic system in response to artificial BM ablating agents, such as irradiation or chemotherapy. Here, HSC activation is likely caused by a transient surplus of systemic cytokines that occur after irradiation- or chemotherapy-induced BM suppression. Cytokine levels in the serum or BM, including SCF and TPO were elevated due to their reduced consumption by surrounding hematopoietic cells (90, 91). Specifically, the elevation of TPO, SCF, IL-3, FLT3, and CXCL12 after lethal irradiation protected HSPCs from apoptosis and improved the survival of irradiated mice (92). Other cytokines of note include TNF-α, IL-1β, and IL-6 detected in the serum (93) and additionally IL-1α, IFN- α/β, and GM-CSF in several cell types (94). Thus, not only local but also systemic cytokine levels determine the fate of HSCs post BM suppression.

Low mitochondrial membrane potential in steady-state HSCs is maintained by extracellular adenosine supplied by surrounding

myeloid progenitors, known to possess an anti-inflammatory effect (95). After 5-FU administration, the ablation of surrounding myeloid progenitors will result in low adenosine and consequently high mitochondrial activity and reactive oxygen species (ROS) production in HSCs. This is essential for initiating HSC cellular division and hematopoietic repopulation (95), but contradicts with a previous study claiming the negative regulation of ROS on HSC maintenance (96), and high mitochondrial membrane potential with reduced hematopoietic repopulating ability in steady-state and IFN-α-stimulated HSCs (97). Thus, the response of activated HSCs toward ROS is context-dependent and markedly differs from quiescent HSCs in steady-state. This is further evidenced in HSCs with enhanced mitochondrial activity and ROS levels possessing more potential for rapid regeneration of Mks and platelets after 5-FU administration (98). Here, TPO injection enhanced mitochondrial activity in HSCs and primed their differentiation toward the Mk-lineage. Not only are Mks a rich source of inflammatory cytokines and chemokines released upon acute injury and inflammation, but they can also cooperate with neutrophils to trap invading pathogens (99, 100). Among the cytokines, C-X-C motif ligand 4 (CXCL4) (22) has been reported to increase hematopoietic recovery of 5-FU-induced BM suppression (101). Fibroblast growth factor 1 (FGF1) supplied by Mks also contributed to the expansion of HSCs after chemotherapeutic stress by counteracting TGF-β inhibitory signaling (23). Furthermore, Mks help expand endosteal niche-residing osteoblasts after irradiation through the secretion of platelet-derived growth factor (PDGF)-BB, and thereby support hematopoietic recovery (102). Taken together, HSC-generated Mks and platelets serve as an essential source of hematopoietic recovery factors that regulate HSCs directly and indirectly through the BM niche.

Since BM injury induced by irradiation or chemotherapy affects not only hematopoietic cells but also non-hematopoietic cells within the BM, the reconstruction of the niche may well be key for a successful hematopoietic recovery. Angiopoietin-1 supplied from osteoblasts protects HSCs from BM suppression (103). Of note, angiopoietin-1 secreted by $LepR^+$ stromal cells can also negatively influence hematopoietic regeneration after irradiation (104). Adipocytes are an additional BM niche component found to proliferate extensively after irradiation or chemotherapy to promote hematopoietic regeneration by supplying SCF to HSCs, which under normal circumstances is provided by $LepR^+$ cells and ECs (73). Other factors besides SCF, including adiponectin (105) and leptin (indirectly *via* adipogenesis) can support HSC proliferation after irradiation (106). Moreover, a radio-resistant $CD105^-CD73^+NGFR^{hi}$ stromal subset expressing high levels of hematopoietic cytokines was found to support hematopoietic regeneration after irradiation (107). Co-transplantation of MSCs overexpressing PDGF-β improved the engraftment of transplanted HSCs *via* enhanced HSC self-renewal and expansion (108). Niche regeneration precedes HSC regeneration after irradiation, and by enlarging the niche pool, a better environment to facilitate HSC engraftment can be achieved. Thus, BM niches are also affected by BM injury, and these alterations contribute to the regeneration of the hematopoietic system.

Since irradiation or chemotherapy damages the DNA of hematopoietic cells, molecules relevant for the DNA damage and repair machinery play a key role in hematopoietic recovery. For instance, histone deacetylase 8 (HDAC8), which modulates p53 activation contributes to HSC survival by blocking apoptosis. HDAC8-deficiency showed hematopoietic failure and increased lethality after the administration of 5-FU (109). Similarly, deficiency of the growth arrest and DNA-damage-inducible protein (Gadd45a), a key tumor suppressor showed efficient recovery of the hematopoietic system through enhanced proliferation of HSPCs, although at the expense of their genomic integrity (110). These phenotypes are attributed to a decrease in HSPC apoptosis due to a greater resistance to DNA damage. Additionally, deficiency of Rap1, a member of the shelterin proteins decreased double-strand DNA break repair through the non-homologous end-joining pathway, and consequently HSC survival after irradiation or chemotherapy (111). Thus, the ability of HSCs to respond efficiently to DNA damage is one of several factors that determines HSC survival under stress conditions. However, since excess resistance to DNA damage will increase the risk for pathogenesis, particularly in the case of Fanconi anemia (55) and leukemogenesis (110), an appropriate balance is required for a healthy hematopoietic recovery.

After BM injury, HSCs and their niches respond to a damaged BM environment by calling for an alternate response compared to steady-state conditions, which may continuously adjust until a return to quiescence. These serial changes may well dictate the appropriate and balanced supply of stem cells and differentiated cells, and the efficient regeneration of the hemato-immune system and BM niche. Further studies are needed to clarify such possibilities.

IMMUNE-MEMORY IN HSPCs

Host immune responses can be divided into a rapidly reacting innate response that is relatively non-specific, and a slowly developing adaptive response that is highly specific to the antigens of invading pathogens. After clearance of an infection, the latter can form a type of immunological memory, ensuring a swift and robust response against future infections and lifelong immune protection. The concept of immunological memory was restricted to adaptive immunity but has since been extended to include innate immunity in the last decade. Indeed, various innate immune cells (i.e., monocytes, macrophages, natural killer (NK) cells) show a long-term adaptation of increased reactivity upon secondary stimulation, a state termed trained immunity (112). Epigenetic reprogramming such as histone modifications and chromatin reconfigurations established during a previous challenge is the basis for trained immunity. Upon stimulation with the TLR2 ligand β-glucan, epigenetic profiling of monocyte to macrophage differentiation has been especially revealing in terms of trained immunity signatures (113).

β-glucan pre-exposed macrophages produced more inflammatory cytokines such as TNF-α and IL-6 after a secondary challenge with tripalmitoyl glyceryl cysteine, a TLR2 ligand compared to naive macrophages. In contrast, LPS pre-exposure induced immune-tolerance in macrophages. Epigenetic marks in the promoter (ACp1) and distal elements (Ace1) of H3K27ac were altered by β-glucan, whereas LPS exposure induced changes in a small subset of the dynamic distal regulatory elements (Ace5) of H3K27ac. A shift in cellular metabolism is also a key driver of trained immunity, as is the case for β-glucan trained monocytes, from oxidative phosphorylation to aerobic glycolysis *via* the mTOR-HIF1α pathway (114). This metabolic switch may enable rapid cytokine and metabolite production to combat intruding pathogens and is not restricted to glycolysis but also glutaminolysis, accumulation of fumarate (115), the mevalonate pathway and cholesterol synthesis (116).

The paradox that short-lived myeloid lineages (monocytes and DCs) with a turnover of every 5 to 7 days retain trained immunity features lasting several months to years served as a motive for investigating long-lived HSPCs and their potential to be trained (117). Recent studies report trained immunity in HSPCs after acute/chronic stimulation by inflammatory cytokines and pathogen-derived agents, such as LPS, β-glucan, or BCG (**Figure 2A**). Relevant epigenetic, metabolic and key signaling pathways that activate or exhaust stem cell activity will be described here (**Figure 2B**).

β-glucan stimulation induced expansion of the myeloid-biased CD41^{+} HSC and MPP subset of the BM that persisted well after transplantation (117). A metabolic shift toward enhanced glycolysis, the mevalonate pathway and cholesterol synthesis in HSPCs was observed. Initial exposure to β-glucan enhanced the response of HSPCs to a secondary systemic LPS challenge by expansion of the LSK and MPP population and an elevated DNA damage response (lower γ-H2AX levels) (117). Kaufmann et al., similarly reported cell expansion and enhanced myelopoiesis of BCG-educated HSCs and MPPs in the BM *via* an IFN-γ-mediated pathway. These trained HSCs generated epigenetically-modified macrophages with better protection against *Mycobacterium tuberculosis* infection (118). Primary LPS stimulation likewise elicited trained immunity in HSCs, enabling a faster and more robust response against a secondary *Pseudomonas aeruginosa* infection (119). Here, a single LPS challenge induced HSC expansion 1 day post injection, which returned to homeostatic levels within 5 days. Transcriptome analysis revealed significant gene expression changes 1 day post injection but a return to normal expression patterns after 4 weeks. Analysis of chromatin opening/closing sites showed

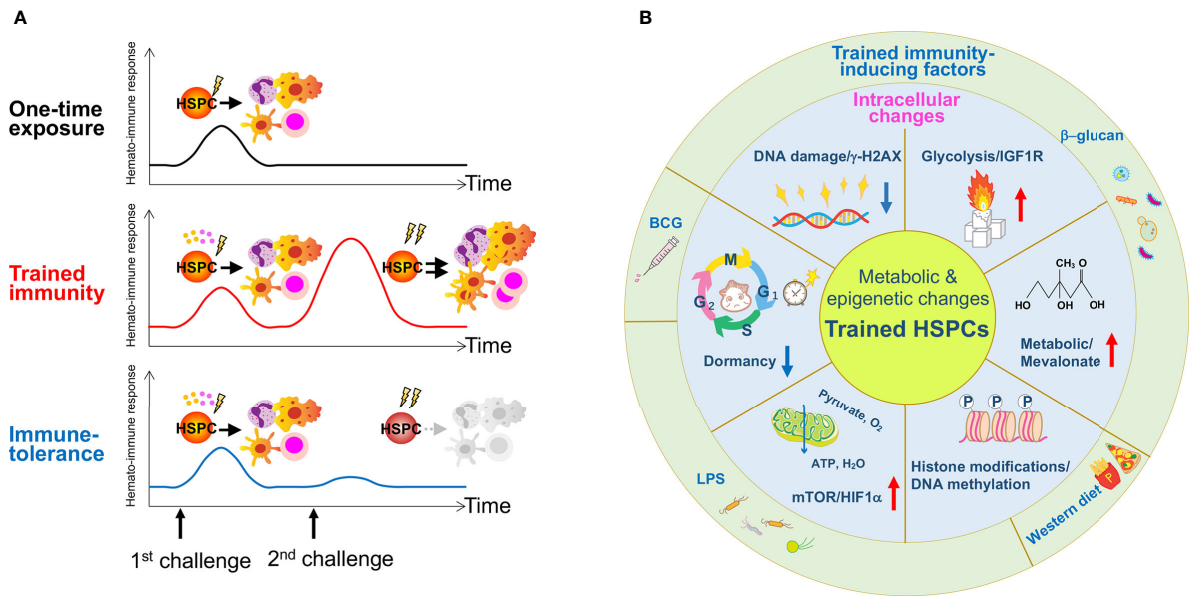

FIGURE 2 | The concept of trained immunity in HSPCs and reported stressors for its induction. **(A)** A one-time exposure to an immunological challenge drives proliferation and differentiation of HSPCs to enhance host immunity. A primary challenge by innate immune insults such as BCG, β-glucan or a Western-type diet induces epigenetic or metabolic changes at the cellular level in HSPCs, and activates them directly *via* cell intrinsic changes or indirectly *via* cytokine production such as IL-1β and GM-CSF. A secondary challenge such as LPS re-stimulation enhances overall immune response, cytokine production and myelopoiesis (trained immunity). Due to memory formation, HSPCs respond better to a secondary challenge and produce more reactive immune cells that can exert robust immune responses against the infection. A hypothetical scheme of immune-tolerance is shown. Immune-tolerance induces immune suppression upon a secondary challenge, which impairs HSPC function and their potency to differentiate into myeloid cells. As a result, immune responses decline and renders the host more susceptible to infection. **(B)** The schematic figure summarizes findings published in previous reports and highlights the role of inflammation on trained HSPCs. Several types of inflammation-causing components including β-glucan, a Western-type diet and BCG affect HSPCs at the intracellular level. These factors induce metabolic and epigenetic changes such as enhanced glycolysis and cholesterol biosynthesis, histone modifications, changes in cell cycle state and an increase in DNA damage.

newly created open chromatin regions in HSPCs (CD135$^-$LSKs) relevant for the immune response and myeloid differentiation, which were retained 4 weeks post injection. These open chromatin regions were significantly lost in HSCs deficient in TLR4 and C/EBPβ, a mediator of TLR signaling, and demonstrated LPS-TLR4-C/EBPβ-mediated epigenetic memory formation in HSCs (119). Thus, trained immunity confers a protective outcome on HSPCs and ultimately host defense by enhancing the reactivity of mature cells derived from "trained HSPCs" toward a secondary infectious challenge.

Constitutive stimulation by inflammation or infection can also result in immune-tolerance, a state of reduced reactivity (**Figure 2A**). LPS pre-exposure induced immune-tolerance in monocytes by inactivating epigenetic marks in lipid metabolism and phagocytosis-related genes; these were partially reverted upon β-glucan stimulation (120). Similarly, BCG vaccination induced H3K4me3 activation in human monocytes *via* the upregulation of IL-1β, a key mediator of trained immunity (121). Given that HSPCs chronically stimulated by IL-1β prior to transplantation showed compromised hematopoietic regeneration possibly due to immune-tolerance (34), the induction of trained immunity or immune-tolerance may depend on cell type and/or the duration of the signal, and possibly metabolic adaptability. Recently, a "sterile" Western-type diet was reported to trigger trained immunity in the *Ldlr*$^{-/-}$ atherosclerosis mouse model through proliferation and functional reprogramming of GMPs into activated and potentially harmful monocytes (122). Hypercholesterolemia reprogramming of HSCs has also been described (123, 124). Thus, the relevance of immune-tolerance is readily implicated in chronic inflammatory diseases and possibly for the treatment of certain autoimmune diseases such as type 2 diabetes.

EFFECT OF IMMUNE INSULTS ON HEMATOLOGIC MALIGNANCIES

Hematopoietic homeostasis is perturbed when the immune system is challenged, such as in cases of infection or inflammation. A systemic increase of inflammatory cytokines and chemokines will stimulate effector immune cells, stromal cells and HSPCs to rapidly replenish consumed innate immune cells at peripheral sites. Stress-induced cell cycle activation of quiescent HSCs will boost hematopoiesis to restore tissue homeostasis, but may come with dire consequences if chronically sustained (i.e., chronic inflammation and autoimmune diseases). Chronic immune stimulation induces cell stress, DNA damage and various hematopoietic dysfunctions, as observed in patients with sickle cell disease often developing myeloid malignancies, possibly from the associated cytokine storm that can cause somatic gene mutations and myeloid neoplasms (125). Persistent cytokine or PAMP stimulation *via* TNF, IFN, and IL-6 signaling and HSC dysfunction have been well-documented and may readily impact the initiation and progression of hematologic malignancies and BM failure (126).

The risk for developing hematologic malignancies increases exponentially with age (127–129). Although not a hematopoietic challenge per se, aging displays a chronic inflammatory phenotype often associated with expansion of the HSC compartment and lineage-bias toward myeloid and megakaryocytic cells (130). The increase in inflammatory factors, IL-6, IL-1, and C-reactive protein accompanies aging (131) and is basis for the emergence of inflammaging (132). As reported by Mann et al., aged and young HSCs display contrasting responses toward inflammatory stress (133). A myeloid-biased subset, which expands with age and are further marked by CD61 expression shows a poor response to prolonged infectious challenges, and possibly are prone to myeloid leukemia development.

Closely associated with aging is clonal hematopoiesis of indeterminate potential (CHIP), a precursor state where mutations in leukemia-associated driver genes are acquired in individuals with no prior history of hematologic diseases, and thus posing a neoplastic progression risk (134). Somatic genes with high potential to develop into hematopoietic malignancies upon mutation include epigenetic modifiers, splicing factors, proliferation signaling molecules and DNA-damage regulators such as DNMT3A, TET2, ASXL1, JAK2, SF3B1, PPM1D and TP53, all known to be mutated in prominent hematologic malignancies (135, 136). Clonal hematopoiesis is a predictor state with adequate potential toward the development of hematologic malignancies (137), while the indeterminate potential aspect of the name reflects the uncertainty behind why only a small population of individuals displaying clonal hematopoiesis develop into full-blown leukemia. Specific mutations may augment inflammation and drive HSC proliferation, while the inflamed environment may further foster genetic ablations in some HSCs and result in their selective expansion. Indeed, several patient studies report of cases where inflammatory conditions promote clonal hematopoiesis (138, 139), and illustrate how HSC impairment upon inflammatory stress may provoke their malignant transformation. This possibility is supported by epidemiological evidence where a history of infection/autoimmunity strongly correlates with hematologic malignancies (140, 141).

A prominent driver mutation in hematological neoplasms is TET2. A recent study reported the abnormal expansion of myeloid cells in *Tet2*-deficient mice (142). Cull et al., found LPS induced *Tet2* transcription in macrophages, while *Tet2* loss enhanced the secretion of the pro-inflammatory cytokines, IL-6, IL-1β, and TNF and the expression of LPS-induced genes associated with an inflammatory state (143, 144). This alteration toward an inflammatory environment may favor *Tet2*-mutant HSPC expansion (145). Interestingly, Meisel et al., reported a breach in the intestinal barrier and subsequent translocation of bacteria result in increased IL-6 production. The risk for development into a pre-leukemic myeloproliferation state was heightened in mice with *Tet2*-deficinent expression in hematopoietic cells, which was similarly recapitulated in *Tet2*-deficient germ-free mice upon colitis induction or in response to systemic bacterial stimuli such as treatment with a TLR2-agonist (146).

This study highlights the requirement for microbial-dependent inflammation in the development of pre-leukemic myeloproliferation.

TLR signaling is also essential for the inflammatory response by shaping HSC fate and blood cell output and if dysregulated, contributes to the loss of HSC potential and/or their malignant transformation (147). Recently, aberrant TLR signaling and its downstream effector molecule, Myd88 has been linked to myelodysplastic syndrome (MDS) and acute myeloid leukemia (148–150). As HSCs express several TLRs enabling their direct stimulation, a causal link between innate immune signaling, HSC dysfunction and hematologic malignancies can be readily imagined, as supported by the following studies. The expression of TLR2 and TLR4 genes was found higher in patients with myelomonocytic and monoblastic acute leukemia (151). Huang et al., reported enhanced innate immune response pathways in chronic myeloid leukemia mouse models (152). MDS patients were found to overexpress TLR1, TLR2, TLR4 and TLR6 in human $CD34^+$ cells (153, 154). Activation of the NF-κB pathway contributed to HSPC apoptosis in MDS, possibly *via* a family of Nod-like receptors (NLRs) and inflammatory-mediated cell death, or pyroptosis. Particularly, the NLR protein 3 (NLRP3) inflammasome overexpressed in MDS HSPCs increased secretion of IL-1β and IL-18, and caused pyroptotic cell death and eventual cytological dysplasia (147, 155, 156). An inflamed environment (i.e., chronic inflammation) was found to promote MDS progression by providing MDS HSPCs with a competitive advantage over normal HSPCs. The mechanistic basis for their clonal dominance occurred *via* a switch from canonical to noncanonical NF-κB signaling in TLR-TRAF6 primed HSPCs that ultimately sustained myeloid expansion (157). A novel perspective designating the BM niche as the driving force for the initiation and evolution of MDS pathogenesis has been elaborated upon in another review (158) Thus, inflammation is a key determinant for the competitive advantage of MDS HSPCs over normal HSPCs.

Abnormal activation of autoreactive T cells and a shortage in stem cells have been reported in both aplastic anemia (AA) patients and mouse models as the ruling cause of BM failure and appears central to the pathophysiology of acquired AA (159). Increased $CD4^+$ helper T cells and activated $CD8^+$ cytotoxic T cells can be found in the patient's BM and are suspected as culprits in HSPC and BM destruction (160). The $CD4^+$ T cells dominant in acquired AA secrete IFN-γ and TNF-α, and have been reported to inhibit $CD34^+$ colony formation. The adenylate-uridylate–rich element (ARE)-deleted mouse model that constitutively expresses IFN-γ, revealed IFN-γ alone could disrupt CMP generation, prevent hematopoietic differentiation and recapitulate AA pathology (161). Apart from AA, the most often inherited bone marrow failure syndrome, Fanconi anemia is associated with defective DNA repair and genomic instability, which are also primary hallmarks of aging. In addition to pI:C injections, Walter et al., demonstrated the ability of other physiological stimuli (i.e., IFN, G-CSF, TPO or serial bleeding) to cause DNA damage in LT-HSCs *in vivo* within similar ranges of pI:C, enforce HSC exit out of quiescence, and accelerate failure of the hematopoietic system as observed in Fanconi anemia patients (55). These findings collectively illustrate a causal link between HSC dysfunction induced by chronic immune stimulation and progression toward hematopoietic failure and hematologic malignancies.

DISCUSSION

Since the initial establishment of the hematopoietic differentiation tree, our understanding of the hematopoietic system, and of the HSC population situated at its apex undergoes continuous refinement. Formerly presumed unresponsive to tissue insult, HSCs in fact show high adaptability under various scenarios and actively cooperate with downstream hematopoietic progenitors, mature cells, and environmental stromal cells as frontline responders to preserve blood homeostasis. However, their ability to respond deftly through self-renewal and differentiation at times brings about detrimental consequences. In this review, we sought to address the latest understanding of HSC biology, in terms of heterogeneity, functionality, and adaptability in steady-state versus perturbed conditions with a particular emphasis on infectious and inflammatory challenges.

From ontogeny to aging, the functional readout of a single HSC in terms of repopulation ability and lineage output differs immensely, leading to the concept of clonality and heterogeneity. Only recently through the development of single-cell approaches can we now address the most basic questions: How many HSCs are born during definitive hematopoiesis formation (162)? Are all HSCs identical in terms of lineage fate (7, 15)? Do all HSCs equally contribute to daily hematopoiesis? Are HSC responses equal under perturbed conditions (133)? The list of questions is ever-expanding. However, transplantation-based studies to test for HSC functionality, where the recipient is subjected to irradiation is a non-physiological setting and should be interpreted with caution as the readout reflects lineage potential enforced on a single HSC rather than its native fate. For example, expanding HSCs after 5-FU treatment contain elevated ROS levels due to high mitochondrial activity but also high repopulating ability (95) and contrasts with steady-state HSCs, where high mitochondrial activity normally implies reduced repopulating ability (96). HSCs can perhaps change their cell fate, depending on the surrounding environment. However, whether this reflects genuine HSC heterogeneity or simply activation of an emergency program remains unresolved. The implementation of new technology to assess lineage output in unperturbed states, such as the inducible sleeping beauty transposon system enabling barcoding of single cells and lineage reconstitution by sequencing (4, 163) or the HUE mouse system (164) is beneficial here. Of note, the definition of HSCs (i.e., long-term, short-term and differences amongst the MPP subset) is still ambiguous and their exact contribution to steady-state hematopoiesis remains controversial. Different conclusions may thus be drawn depending on the experimental system at hand and must be examined carefully.

It is now clear that the BM is not immune-ignorant but a prominent lymphoid organ that receives a large spectrum of hemato-immunological insults. Likewise, BM-residing HSCs are not just quiescent sleeping cells but directly respond to insults not limited to infection, inflammation but also the regeneration of the BM after toxic agents or irradiation. Depending on the type of DAMPs, PAMPs, cytokines and growth factors involved and the strength/duration of the stimulation, HSCs will alter their fate toward myelopoiesis, granulopoiesis or even bypass progenitors altogether to directly orchestrate on-demand hematopoiesis. HSCs positioned at the interface of perturbed hematopoiesis will execute distinct emergency programs to integrate and fine-tune responses to maintain hematopoietic integrity. However, such beneficial effects of HSC activation can be counteracted by chronic inflammatory conditions. HSC dysfunction upon chronic inflammation or aging as the cause of clonal hematopoiesis and in certain cases leukemic transformation are all readily imaginable scenarios, although direct causality remains to be demonstrated. Emerging reports of trained immunity in HSPCs and mature cells derived from "trained" progenitors with an enhanced protective function provide a novel opportunity for interpretation. The well-established immune response against inflammatory or infectious stimuli may have well been under the influence of HSC trained memory and should be revisited. How HSC memory is formed and maintained, and to what degree trained immunity in HSCs dictate host immune defense are areas yet to be explored. Whether the metabolic shift in HSCs induced by memory formation alters the depth of HSC quiescence, population hierarchy and functional heterogeneity, and ultimately clonal hematopoiesis are primary but still unresolved questions. Despite the risk for potential collapse of the hematopoietic system, HSCs nonetheless persist at the frontline not only as an integrative hub for incoming inflammatory signals, but also execute tissue repair in organs beyond the blood system. Trained immune memory in HSCs offers one more additional perspective in elucidating the true nature of HSCs.

In line with this, counterpart immune/stromal niche components also regulate steady-state and stress hematopoiesis. The importance of stromal cells as a major source of HSC maintenance and activation factors for HSC homeostasis is clear. As observed by the apelin^{+} EC subset tasked to regenerate the BM after irradiation (81), ECs are highly heterogeneous in terms of their identity and function. Other BM constituents, including adipocytes that proliferate and secrete SCF post irradiation to promote BM regeneration (73), certain MSC progenitors that maintain both lymphoid progenitors and HSCs *via* CXCL12 (165), as well as a subset of regulatory T cells with high CD150 expression that localize in HSC niches and maintain HSC quiescence are additional examples of HSC interaction with immune/stromal heterogeneity (166). Thus, the heterogeneity of HSCs and their counterpart niche cells become vital when interpreting stressed conditions such as inflammation, infection and the onset of hematologic malignancies. Nonetheless, the bigger question would be, whether this so-called heterogeneity of HSCs (among others) is a distinct population or a continuum where cells retain the ability to transform back and forth. Single-cell RNA-sequencing, despite its immense power offers only a snapshot analysis and may not reflect the true nature of these cells.

Finally, the recently proposed concept of immune memory in HSPCs is a prime topic with clinical relevance. Only several studies till now have demonstrated trained immunity at the level of hematopoietic progenitors *via* β-glucan, LPS and BCG, and more can be expected. Besides transcriptional and epigenetic reprogramming and a metabolic shift as key characteristics of trained immunity, much remains to be revealed. For example, the similarities and differences in trained immunity between different stimuli, or whether the formation of a trained memory is mutually exclusive or synergistic. Regarding the duration of trained immunity as well as the cellular/molecular mechanisms associated with it, whether the stability of different signatures, e.g., chromatin modification, histone/DNA methylation, RNA splicing impact the half-life of the memory formed is a primary question among others. Furthermore, what determines whether a cell is able to form a memory? Is memory a privilege granted only to the hematopoietic compartment or do niche/stromal cells possess this ability as well and does this affect their interaction with HSCs? Although yet to be demonstrated, this possibility can readily be imagined as certain stromal cells also take part in the immune response by secreting inflammatory cytokines and chemokines and express PRRs. BCG vaccination induced trained immunity in human monocytes *via* IL-1β (121), whereas IL-1β re-stimulation damaged the repopulation ability of HSCs post transplantation (34). The latter situation may imply the induction of immune-tolerance, and a possible relevance with aging-associated HSC dysfunction due to IL-1 upregulation observed in the elderly (131). It is important to understand the determinants for dictating trained immunity versus immune-tolerance, and whether the type of stimuli or threshold of signal strength or duration determines the choice for one over the other. Lastly, trained immunity may possibly have detrimental outcomes, as in instances of autoimmune diseases (167), so can HSC memories be a predisposition for future hematopoietic malignancies, say in terms of CHIP progression to MDS? More studies are expected in the near future.

AUTHOR CONTRIBUTIONS

All authors studied the literature and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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MINI REVIEW
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The Role of the Bone Marrow Microenvironment in the Response to Infection

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Hematopoiesis in the bone marrow (BM) is the primary source of immune cells. Hematopoiesis is regulated by a diverse cellular microenvironment that supports stepwise differentiation of multipotent stem cells and progenitors into mature blood cells. Blood cell production is not static and the bone marrow has evolved to sense and respond to infection by rapidly generating immune cells that are quickly released into the circulation to replenish those that are consumed in the periphery. Unfortunately, infection also has deleterious effects injuring hematopoietic stem cells (HSC), inefficient hematopoiesis, and remodeling and destruction of the microenvironment. Despite its central role in immunity, the role of the microenvironment in the response to infection has not been systematically investigated. Here we summarize the key experimental evidence demonstrating a critical role of the bone marrow microenvironment in orchestrating the bone marrow response to infection and discuss areas of future research.

Keywords: bone marrow, niche, hematopoiesis, infection, microenvironment

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INTRODUCTION

The bone marrow microenvironment is the collection of cells and structures that together support blood cell production in the bone marrow. The architecture, composition, and function of the microenvironment has been recently reviewed in great detail (1, 2). The bone marrow architecture is defined by the enclosing bone tissue and the blood vessels that irrigate it. Small arterioles penetrate the BM through the bone and give rise to a dense network of sinusoids that drains through a central vein (3). The endothelial cells that form the BM vessels are major components of the microenvironment *via* their production of cytokines that support and regulate hematopoietic stem cells and other progenitors (4–6). Additional major sources of hematopoietic supportive cytokines are $Ng2^+$ cells that ensheath arterioles (7–9); a network of perivascular cells [defined as $LepR^+$, Cxcl12-abundant reticular cells, or Nestin-GFP^{dim} cells depending on the genetic reporter used to prospectively isolate them (3–6, 10–13)]; and non-myelinating Schwann cells (14). Stepwise hematopoiesis takes place in the space between these stromal cell types. Additionally, many hematopoietic and non-hematopoietic (stromal) cells cooperate to regulate blood cell production. Examples of these include: megakaryocytes that—in addition to platelet production—function by restricting hematopoietic stem cells (HSC) proliferation (15–17); macrophages which provide a niche for red blood cell production [reviewed in (18)] but also crosstalk with stromal (non-hematopoietic) cells (19–21) and neutrophils (22) to regulate HSC release into the circulation; and

dendritic cells which function as antigen-presenting cells, but also control HSC trafficking by targeting the endothelium (23). As the main source of immune cells, the bone marrow is a major player in -and target of- the response to infection [reviewed in (24–26)]. Here, we focus on the role of the microenvironment in orchestrating the response of the bone marrow to infection.

FUNCTIONAL CONSEQUENCES OF INFECTION IN THE BONE MARROW

Below we summarize the diverse effects of infection on hematopoiesis and discuss the experimental evidence demonstrating the role of the microenvironment in orchestrating these responses. Note that these are not independent phenomena and, in many cases, take place simultaneously and are mediated by the same mechanisms. However, the BM response to infection is also pathogen-specific (27–29) probably due to differences in the signaling pathways used to detect the infection and pathogen dosage (27). The main BM responses to infection are:

1. Emergency myelopoiesis: Neutrophils, monocytes, and dendritic cells (DC) are consumed in great quantities during infection. Emergency myelopoiesis is the main mechanism used by the bone marrow to produce large numbers of myeloid cells to replenish those consumed in the periphery. Emergency myelopoiesis has been traditionally divided into **emergency granulopoiesis**—the process of emergency neutrophil production—and into **emergency mono/DC-poiesis**—the process of emergency production of monocytes and dendritic cells. It is characterized by proliferation and preferential commitment of multipotent progenitors toward myeloid fates and of lineage-committed progenitors toward neutrophil (emergency granulopoiesis) or mono/DC fates (emergency monopoiesis). Both are also associated with rapid release of hematopoietic progenitors and myeloid cells into the circulation (see point two below). Several studies support a specific role of the microenvironment in orchestrating emergency myelopoiesis. Lipopolysaccharide (LPS) triggers emergency granulopoiesis *via* toll-like receptor 4 (TLR4) activation. Boettcher et al., used bone marrow transplantation to generate chimeric mice in which TLR4—or its downstream adaptor Myd88—was knocked out in the stroma or in hematopoietic cells. These experiments showed that TLR4 expression in the stroma was necessary and sufficient to trigger emergency granulopoiesis in response to LPS. *Tie2-cre:Myd88*$^{fl/fl}$ mice, which lack MyD88 in hematopoietic and endothelial cells, are unable to induce emergency granulopoiesis after LPS treatment or *Escherichia coli* infection indicating that TLR4 signaling in endothelial cells orchestrates emergency granulopoiesis. In this setting, emergency granulopoiesis is likely mediated by endothelial cell secretion of granulocyte colony-stimulating factor (G-CSF), the major cytokine that supports granulopoiesis (30, 31). Similarly, the Kelsoe group showed that in response to the adjuvant alum, the BM initiated IL1 mediated emergency granulopoiesis (32). *Il1r1*$^{-/-}$ chimeric mice studies indicated that IL1 acted on stromal cells to trigger emergency granulopoiesis and G-CSF release (the specific identity of these G-CSF producing stromal cells is not known). Alum-induced emergency granulopoiesis was abrogated in G-CSFR knockout mice or in WT mice injected with neutralizing antibodies against G-CSF (32, 33). Interestingly- and in the same manuscript- the Kelsoe group showed that loss of neutrophils in the absence of inflammation was sufficient to induce G-CSF-dependent and -independent progenitor proliferation, similar to that observed during emergency granulopoiesis (33). This suggests that any infection that results in neutrophil depletion in the BM will also lead to G-CSF secretion by stromal cells which in turn will trigger emergency granulopoiesis. A critical role for neutrophils in mediating emergency granulopoiesis is further supported by the work of Kwak et al., who showed that, during inflammation induced by intraperitoneal injection of heat inactivated *E. coli*-myeloid cells (likely neutrophils), produced reactive oxygen species that stimulated emergency granulopoiesis (34).

   Emergency myelopoiesis can also be triggered—in a G-CSF independent manner—by stromal-mediated cytokine release. Chou et al., found that *Toxoplasma gondii* infection induced emergency granulopoiesis by expanding granulocyte monocyte progenitors and inhibited erythropoiesis by reducing megakaryocyte erythrocyte progenitors. *T. gondii*-induced emergency granulopoiesis was blocked in IL6 knockout mice (28). Chimeric mice studies showed that IL6 expression in stromal cells was necessary for emergency granulopoiesis. VCAM^{+}PDGFRα^{+} mesenchymal cells—which are a subset of the LepR^{+} perivascular cells based on scRNAseq studies (35)—purified from infected mice expressed higher IL6 than those purified from control mice. These studies thus suggest that *T. gondii* induces IL6 release from perivascular cells which in turn re-programs hematopoietic progenitors toward neutrophil fates and emergency granulopoiesis (28). Schürch et al., found complex crosstalk between cytotoxic T- cells, mesenchymal stromal cells, and myeloid progenitors that drives emergency granulopoiesis in response to lymphocytic choriomeningitis virus (LCMV) infection (36). They found that transfer of LCMV-specific effector cytotoxic T cells caused expansion and proliferation of multipotent progenitors and myeloid progenitors and monocyte release into the circulation. Interferon gamma (*Ifng*) knockout T cells were unable to induce emergency myelopoiesis whereas *Ifngr*$^{-/-}$ mouse chimera studies showed that IFNGR expression in the stroma was required for emergency myelopoiesis. Only CD45^{-}CD31^{-}CD51^{+}Sca-1^{+} stromal cells expressed functional IFNGR and these cells released IL6 upon IFNγ stimulation. Mouse chimeras lacking IL6 in stromal cells did not induce emergency myelopoiesis after cytotoxic T cell transfer (36). Together these studies indicate complex crosstalk where LCMV infection leads to the generation of LCMV cytotoxic T cells. These in turn produce IFNγ which

targets bone marrow stromal cells to elicit IL6 production that then acts on hematopoietic progenitors to induce emergency myeloid production (36).

2. Mobilization: In addition to increased myeloid cell production, emergency myelopoiesis also encompasses the release of mature myeloid and hematopoietic stem cells and progenitors (HSPC) into the circulation. Mobilized progenitors can migrate to the spleen and other organs where they can differentiate *in situ*; this is advantageous in clearing local and systemic infections (27, 37). Many infections also cause stem and progenitor proliferation and loss of HSC (24–26). When HSC proliferate, they lose stem cell potential and can quickly become exhausted (38, 39). Infection-induced HSPC mobilization might also be used to replenish empty niches in distal bones thus maintaining the normal HSC pool (40). There is clear evidence indicating that hematopoietic cell mobilization in response to infection is regulated in a non-cell autonomous manner by the microenvironment.

   The chemokine CXCL12 and its receptor CXCR4 are the major signals regulating neutrophil (41, 42) and HSPC (43) retention in the bone marrow. CXCL12 is produced by perivascular stromal cells and endothelial cells (4, 6, 13, 43). G-CSF inhibits CXCL12 production in the BM and infections that cause increases in G-CSF, or reductions in CXCL12 (27, 30, 31, 44), will also elicit neutrophil and HSPC mobilization. Mechanistically, G-CSF functions by binding G-CSFR in a monocyte lineage hematopoietic cell. Through an unknown mechanism, these hematopoietic cells induce the downregulation of CXCL12 in BM stromal cells, triggering neutrophil and progenitor release to the circulation (21). A great example of this regulation is the work by Burberry et al. The bacterial wall contains LPS-sensed *via* the receptor TLR4 and the downstream mediators MyD88 and TRIF- and peptidoglycan-sensed *via* the receptors NOD1 and NOD2 and the adaptor RIPK2. Burberry et al., found that systemic infection elicited HSPC mobilization to the spleen. This mobilization was mostly abolished in $Trif^{-/-}$ and $Ripk2^{-/-}$ mice. Using bone marrow transplantation to generate chimeric mice with hematopoietic or stromal deletions of TLR4 and NOD1, they demonstrated that expression of these receptors in stromal cells was both necessary and sufficient for HSPC mobilization. Both receptors synergized to drive G-CSF expression by stromal cells (likely endothelial cells). Blocking G-CSF *via* antibody injection or by using G-$CSFR^{-/-}$ mice completely abolished HSPC mobilization after infection (27). In an elegant experiment, the same group formally demonstrated that the mobilized HSPCs generate immune cells that function in clearing the infection. They transferred splenocytes from control or mobilized mice into recipients that were then infected with *E. coli*. The mice transferred with the mobilized splenocytes had dramatically lower levels of *E. coli* colonies in the spleen and liver (27).

   Mobilization of inflammatory monocytes in response to *Listeria monocytogene*s infection is also controlled by the stroma in a G-CSF-independent manner. Using $Ccr2^{-/-}$ and $Ccl2^{-/-}$ mice (*Ccl2* encodes MCP1) Shi et al., found that low doses of LPS induced rapid monocyte mobilization in a CCR2/MCP1 dependent manner. Mouse chimeras showed that *Ccl2* deletion in non-hematopoietic cells completely abolished LPS-induced monocyte mobilization. Using an MCP1 reporter mouse, Shi et al., demonstrated that LPS quickly (2 hours) induces MCP1 expression in perivascular stromal cells that are tightly associated with sinusoids. Conditional *Ccl2* deletion in perivascular cells using *Nestin-cre* mice greatly reduced monocyte egress from the BM and reduced bacteria clearance in a model of *L. monocytogenes* infection (45).

   During homeostasis, several types of hematopoietic cells regulate HSPC release into the circulation. Bone marrow macrophages and monocyte-lineage cells specifically crosstalk with bone marrow perivascular cells promoting CXCL12 production. Loss of bone marrow macrophages leads to reductions in CXCL12 and HSPC release (19–21). In addition, trafficking and phagocytosis-by bone marrow and intestinal macrophages- also control HSPC release during homeostasis (22, 46). A recent study also showed that bone marrow dendritic cells regulate HSC release through the BM, likely by modulating permeability of sinusoids *via* CXCL1-CXCR2 signaling (23). Since infection causes massive changes in the numbers of these hematopoietic components of the microenvironment, it is highly likely that the same pathways will also participate in regulating HSPC mobilization during infection.

3. Hematopoietic injury: Infection frequently causes bone marrow aplasia and inefficient hematopoiesis as well as loss of functional HSC (24–26). While some of these are mediated by direct effects of cytokines like IFNα and IFNγ on hematopoietic cells (47, 48), several lines of experimental evidence support a role of the microenvironment in these processes.

   In the steady state, most hematopoietic stem cells are quiescent. Loss of quiescence and proliferation causes cumulative damage to HSC leading to their functional exhaustion (38, 39, 47, 48). Kobayashi et al., demonstrated that treatment with the bacterial second messenger c-di-GMP induced emergency myelopoiesis and loss of HSC quiescence. In agreement, c-di-GMP also caused a three-fold loss of bone marrow HSC without impairing surviving HSC function. Mouse chimera experiments indicated that loss of HSC required expression of STING—the c-di-GMP receptor—in both hematopoietic and non-hematopoietic cells (49).

   Many infections cause loss of B cell lymphopoiesis (50–53). B cell production in the bone marrow is maintained by CXCL12 and IL7-producing perivascular cells and CXCL12 producing osteoblasts (4, 6, 12, 54). Injection of adjuvants mimic the suppression of lymphopoiesis observed during infection (50, 55). Using this experimental paradigm, Ueda et al., showed that TNFα caused reduction of CXCL12- which is produced only by stromal cells in the bone marrow- leading to B cell egress from the BM (55). Similarly, the Link Laboratory showed that G-CSF targets monocyte-lineage

cells and this in turn induces downregulation of CXCL12, IL7, and other B cell supportive cytokines in perivascular stromal cells and osteoblasts (56, 57). Note that the same studies also observed that G-CSF treatment depleted perivascular cells and osteoblasts (56, 57). In agreement, Terashima et al., found that sepsis-induced G-CSF release caused loss of osteoblasts, reduced production of IL7 by the surviving osteoblasts, and depletion of common lymphoid progenitors leading to inefficient lymphopoiesis (52). Together these studies suggest that infections that cause increases in TNFα and/or G-CSF in the bone marrow will suppress lymphopoiesis by directly destroying the niche and inhibiting the ability of the surviving niche cells to support lymphopoiesis. The bone marrow is also the main reservoir for long-lived plasma cell and memory T cells (58–60). Both cell types are in contact with CXCL12- and IL7-producing perivascular cells (54) and require CXCL12 and other microenvironment-produced signals for maintenance in the BM (61). These suggest that the G-CSF mediated destruction/inhibition of the microenvironment described above will also impact the ability of the BM to recruit and maintain plasma cells and memory T cells.

4. Infection-induced remodeling and damage of the microenvironment: The bone marrow microenvironment is remarkably dynamic and can be extensively remodeled after myeloablation (62, 63), aging (64), and leukemia (35, 65). Infection is no exception and several pieces of data indicate extensive injury and remodeling of bone marrow stromal populations in response to infection or stimulation with bacterial cell wall components.

   It is becoming clear that endothelial cells and blood vessels in the bone marrow undergo rapid angiogenesis and remodeling in response to infection. Scumpia et al., observed dilated sinusoids as soon as 12h after cecal ligation in a mouse model of sepsis, suggesting that infection affects bone marrow vessel permeability (66). Many infections lead to increased levels of IFNα. Prendergast et al., found that pIpC treatment (an IFNα inducer) or IFNα injection caused a threefold increase in the number of BM endothelial cells, upregulation of adhesion molecules, vascular dilation, and permeability in wild-type but not *Ifnar*$^{-/-}$ mice. Surprisingly, mouse chimera experiments showed that IFNAR expression in stromal cells or hematopoietic cells is sufficient to activate the endothelium (67). In agreement, Vandoorne et al., found increased sinusoids, endothelial proliferation, and angiogenesis, and increased vascular permeability in response to LPS (68). Increased permeability correlates with neutrophil egress from the BM (68), suggesting that vascular remodeling facilitates mobilization. Non-endothelial stromal cells are also remodeled by infection. As discussed in point three, G-CSF-mediates the destruction of CXCL12-producing perivascular cells and osteoblasts (52, 56, 57). Additionally, c-di-GMP—which targets stromal cells *via* STING to cause loss of HSC (49)—also caused vascular dilation and loss of endothelial cells and perivascular stromal cells and that this required STING expression in stromal cells. The specific mechanisms for this stromal destruction are not known, but c-di-GMP upregulates G-CSF (49), strongly suggesting a role for this cytokine in remodeling the microenvironment in this experimental paradigm. In addition to these *in vivo* studies, there is evidence showing that many pathogens can directly infect endothelial and BM stromal cells and reduce their ability to support hematopoiesis *in vitro* (69–73). Together these studies demonstrate that infection massively remodels the microenvironment that supports hematopoiesis—likely *via* direct and indirect mechanisms—and suggest that destruction of the microenvironment might be a major driver for loss of hematopoietic function during infection.

DISCUSSION

The manuscripts discussed above provide overwhelming evidence for the role of the microenvironment in orchestrating the bone marrow response to infection. However, a common limitation is that most studies have focused on one aspect of the response to infection (emergency myeloid cell production, mobilization, hematopoietic and stromal injury). Thus it is still not possible to know if these are aspects of a single response controlled by common pathways (e.g., G-CSF) or individually controlled processes that allow fine-tuning of the response through the kinetics of the infection. The mechanisms through which infection damages the stromal compartment and how these structures regenerate are highly interesting; especially in light of recent scRNAseq studies demonstrating that the stromal compartment of the bone marrow is highly heterogeneous (35, 74, 75); and suggesting that specific components of the microenvironment provide unique niches supporting the differentiation of distinct lineages (1, 4, 6, 10, 12, 75). Whether some infections preferentially affect some niches but not others remains open. It is well established that aging negatively impacts HSC function, biases hematopoiesis toward myeloid cell production, and dramatically remodels the microenvironment (64, 76). The answers to these questions will provide critical insights into how the bone marrow functions during stress, and lead to the development of new therapies to preserve/improve bone marrow function during infectious challenges.

AUTHOR CONTRIBUTIONS

CBJ, JZ, and DL conceived and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Hematopoietic Stem Cell Niches and Signals Controlling Immune Cell Development and Maintenance of Immunological Memory

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Studies over the last couple of decades have shown that hematopoietic stem cells (HSCs) are critically dependent on cytokines such as Stem Cell Factor and other signals provided by bone marrow niches comprising of mesenchymal stem and progenitor cells (MSPCs) and endothelial cells (ECs). Because of their critical roles in HSC maintenance the niches formed by MSPCs and ECs are commonly referred to as HSC niches. For the most part, the signals required for HSC maintenance act in a short-range manner, which imposes the necessity for directional and positional cues in order for HSCs to localize and be retained properly in stem cell niches. The chemokine CXCL12 and its Gαi protein coupled receptor CXCR4, besides promoting HSC quiescence directly, also play instrumental roles in enabling HSCs to access bone marrow stem cell niches. Recent studies have revealed, however, that HSC niches also provide a constellation of hematopoietic cytokines that are critical for the production of most, if not all, blood cell types. Some hematopoietic cytokines, namely IL-7 and IL-15 produced by HSC niches, are not only required for lymphopoiesis but are also essential for memory T cell maintenance. Consequently, hematopoietic progenitors and differentiated immune cells, such as memory T cell subsets, also depend on the CXCL12/CXCR4 axis for migration into bone marrow and interactions with MSPCs and ECs. Similarly, subsets of antibody-secreting plasma cells also reside in close association with CXCL12-producing MSPCs in the bone marrow and require the CXCR4/CXCL12 axis for survival and long-term maintenance. Collectively, these studies demonstrate a broad range of key physiological roles, spanning blood cell production and maintenance of immunological memory, that are orchestrated by stem cell niches through a common and simple mechanism: CXCL12/CXCR4-mediated cell recruitment followed by receipt of a maintenance and/or instructive signal. A fundamental flaw of this type of cellular organization is revealed by myeloid and lymphoid leukemias, which target stem cell niches and induce profound transcriptomic changes that result in reduced hematopoietic activity and altered mesenchymal cell differentiation.

Keywords: hematopoietic stem cell niches, lymphopoiesis, myelopoiesis, CXCR4, WHIM syndrome, leukemia

INTRODUCTION

The hematopoietic system is composed of a multitude of cell types with different properties and functionalities. Hematopoietic cells develop from a rare population of hematopoietic-lineage restricted stem and progenitor cells that, in adult mammals, reside and differentiate in the bone marrow. Many studies over the last several decades have revealed a collection of extrinsic factors, such as cytokines and chemokines, that are required for hematopoietic stem and progenitor cell maintenance and activation. For the most part, these factors are locally produced in the bone marrow by a collection of cells that form the HSC niche. Several excellent reviews on the topic of stem cell niches have already been published (1, 2) and is not the focus of this review. Instead, we will focus on the bone marrow niches and signals involved in hematopoietic progenitor differentiation, as well as the bone marrow niches required for long term maintenance of adaptive immune cells. We will discuss a significant body of work showing that the niches controlling HSC maintenance overlap with the niches promoting lymphopoiesis and differentiation of at least some myeloid cell subsets. Furthermore, we will also discuss how these same niches play important roles in the maintenance of memory T cell subsets and long-lived plasma cells.

INSTRUCTIVE SIGNALS AND NICHES CONTROLLING HEMATOPOIETIC CELL DIFFERENTIATION

Hematopoietic stem and progenitor cells differentiate into lymphoid, myeloid, erythroid and megakaryocyte lineages in response to extracellular signals, predominantly cytokines, produced in the local microenvironment. Hematopoietic cytokines generally act to promote and/or maintain commitment to a specific cell lineage (e.g. lymphoid, myeloid, etc.), to induce progenitor cell proliferation, and/or to signal progenitor cell survival. Although some stochasticity is likely involved in HSC differentiation decisions, hematopoietic cell production is overtly reduced without access to lineage-specific hematopoietic cytokines. Therefore, the cellular sources of hematopoietic cytokines define the local microenvironments, or niches, where specific hematopoietic cell lineages are normally produced. We will focus on the cellular sources of key hematopoietic lineage-specific cytokines in bone marrow and on the guidance cues that allow hematopoietic progenitor cells to access these niches.

Lymphoid Cells and Their Niches

The lymphoid compartment is composed of B and T cell subsets (each greater than 10^8 cells in mice) and of innate lymphoid cells (ILCs). All lymphoid subsets differentiate from lineage-restricted Common Lymphoid Progenitors (CLPs). CLPs express the interleukin (IL)-7 receptor complex formed by IL-7Rα and the common γ chain (3), and the large majority of lymphocyte subsets depend on IL-7/IL-7R signaling for their development and survival. Consequently, in IL-7 or IL-7Rα deficient mice, B and T lymphocytes are reduced by 10-100 fold (4–6), and ILC subsets are also significantly reduced (7). Besides IL-7, FMS-like tyrosine kinase 3 ligand (FLT3L) has also been shown to contribute significantly to lymphopoiesis in that combined deficiency in IL-7 and FLT3L results in essentially undetectable B and T lymphocyte production (8–10). In terms of IL-7/IL-7R dependency, the single exception is Natural Killer (NK) cells, which are normally produced in IL-7-deficient mice but are overtly reduced in IL-15 or IL-15 receptor-deficient mice (11–13). Although several other extrinsic factors have been found to play measurable roles in lymphopoiesis *in vitro* and/or *in vivo* (14), these cannot compensate for the absence of IL-7 or IL-15 and therefore will not be discussed here.

Both IL-7 and IL-15 act as short-range signals, necessitating proximity between lymphoid progenitor "client" cells and the cells producing them. IL-15 requires binding to its IL-15Rα chain for trans-presentation to client cells expressing the IL-15 signaling receptor heterodimer formed by IL-2Rβ and the common γ chain. IL-7 acts as a soluble cytokine, but its expression is very low, and some evidence suggests that it can be tethered onto the cell surface through binding to glycosaminoglycans (15). For these reasons, a niche promoting IL-7-dependent lymphopoiesis exists in the vicinity of cells producing IL-7, and for NK cells the niche must be formed by the cells producing and/or presenting IL-15.

A study using *Il15* reporter mice identified the cellular sources of IL-15 in the bone marrow as being mostly composed of CXCL12, VCAM1, and platelet-derived growth factor receptor beta (PDGFRβ)-expressing cells (16), which stands in agreement with prior studies that showed IL-15 expression in CXCL12+ stromal cells (17). Using a dual *Il7* and *Il15* reporter mouse, considerable overlap between the IL-7+ and IL-15+ mesenchymal stromal cell populations was discovered in bone marrow (16). Although insightful, these studies did not demonstrate that lymphoid progenitors were indeed dependent on IL-7 or IL-15 produced by mesenchymal stromal cells *in vivo*, as other cells, such as dendritic cells, can also produce IL-15 under inflammatory conditions (18).

Mesenchymal stem/progenitor cells (MSPCs) identified by surface expression of the Leptin receptor (LEPR) and PDGFRα/β constitute about 90% of all *Il7*-expressing cells in the bone marrow, the remaining 10% being predominantly sinusoidal endothelial cells (19). Side-by-side comparison between *Il7*-GFP knock-in reporter mice, and *Il7*-cre recombinase mice crossed with Rosa26lox-stop-lox-YFP mice, a strategy that allows for cell lineage tracing of *Il7*-producing cells and *Il7*-past producer cells, also revealed that *Il7*-expressing MSPCs differentiate into osteolineage cells, such as osteoblasts and osteocytes, that do not express *Il7*. Conditional *Il7* deletion in MSPCs resulted in significant reduction of IL-7-dependent B-lineage-committed CLP numbers, B cell progenitors, and overall reduced B cell production, whereas conditional *Il7* deletion in endothelial cells caused a small but significant reduction in proB and preB cell numbers that did not impact the overall size of the B cell compartment. Importantly, MSPC differentiation into osteolineage cells coincides with halted *Il7* expression, and

thus, *Il7* deletion from mature osteoblasts and osteocytes has no quantitative impact on B cell production (19). It should be noted that these findings diverge from prior studies proposing that osteolineage cells form a major niche for lymphopoiesis. However, these prior studies relied on mouse models in which osteolineage cells were selectively modified or ablated by conditional gene targeting approaches that presumed to be selective in osteoblasts but that are now known to act in MSPCs with variable efficiency. In depth analyses of these studies have recently been reviewed (2, 14).

An interesting feature of IL-7-producing MSPCs is that these cells express the highest amounts of CXCL12 in bone marrow (19). CXCR4, the CXCL12 receptor, attracts not only HSCs but also lineage-restricted hematopoietic progenitor cells, such as multipotent progenitor cells (MPPs), CLPs, and early B-lineage progenitor cells to the vicinity of MSPCs (19–21). HSCs require CXCR4/CXCL12 for long-term maintenance not only because CXCR4 signaling promotes HSC quiescence directly (20, 22–24) but also because it may enable cells to encounter Stem Cell Factor (SCF, encoded by *Kitl*). Consistent with this possibility, CXCL12+ cells and SCF+ stromal cells overlap by more than 99%, and conditional *Kitl* or *Cxcl12* deletion from MSPCs result in similar phenotypes: significant reductions in HSC numbers and hematopoietic reconstitution capacity (25–27). While direct evidence of reduced ckit signaling in CXCR4-deficient HSCs is still lacking. there is evidence supporting this model in downstream hematopoietic progenitors. Specifically, *Cxcr4* conditional deletion in MPPs, or in CLPs, resulted in impaired lymphopoiesis due to a significant reduction in IL-7 receptor signaling, as measured by STAT5 phosphorylation (19). CXCR4 deletion at the B-lineage-committed proB cell stage also reduces developing B cell numbers in the bone marrow due to their premature mobilization into the periphery (28–30), possibly in combination with reduced IL-7R signaling at the IL-7-dependent proB and preB cell stages. Likewise, CXCR4/CXCL12 plays essential roles in NK cell development as it presumably guides NK progenitors toward IL-15 niches formed by MSPCs (17).

But, CXCR4 signaling plays other roles than acting as a chemoattractant receptor. For example, CXCR4 signaling promotes conformational changes in the integrin heterodimer α4β1 (VLA-4) that result in its transactivation and adhesion to fibronectin and VCAM-1 (31, 32). Hematopoietic stem and progenitor cells, and lymphoid progenitors, are exquisitely dependent on α4β1 signaling for movement and retention within the bone marrow. Defects in integrin-mediated adhesion reduce hematopoietic stem and progenitor differentiation in bone marrow, in part due to their premature release into the bloodstream (29, 30, 33, 34). Furthermore, *in vitro* studies suggested that CXCR4 may also act as a signaling receptor capable of influencing cell decisions. CXCR4 signaling drives HSC proliferation directly *via* transcriptional control of cyclin D1 and MAD1 (23), and in preB cells, CXCR4 signaling activates ERK to facilitate *Igk* germline transcription (35).

Recent single cell transcriptomic analyses of non-hematopoietic bone marrow cells have provided unprecedented resolution of MSPC clusters and differentiation trajectories during homeostasis and leukemia (36–38). Collectively, these studies not only reinforce previous findings with *Cxcl12*, *Kitl*, *Il7*, and *Il15* reporter mice described above, but also expand our knowledge of the repertoire of hematopoietic cytokines that are expressed by MSPCs and ECs in the bone marrow. For example, we now know that *Flt3l*, *Csf1*, and *Il34*, important myeloid cytokines, are primarily expressed by MSPC clusters that also express *Il7*, *Il15* and *Cxcl12* (37). Combined, these data support a model in which MSPCs not only control HSC maintenance and lymphopoiesis but may also control the development of essential myeloid cell subsets.

Myeloid Cells and Their Niches

Most myeloid cells develop from lineage-restricted common myeloid progenitors (CMPs), with the notable exception of megakaryocytes, which may have alternative developmental pathways (39), and mast cells, for which CMP and granulocyte-monocyte progenitors (GMP)-dependent and -independent pathways have been reported (40–44). In the classical model of hierarchical hematopoietic cell development, CMPs undergo differentiation into two major intermediate progenitors, GMPs and megakaryocyte-erythroid progenitors (MEPs), both of which retain cKit expression (the SCF receptor) at relatively high levels. Defects in *Kit* (encodes cKit) or in *Kitl* ultimately lead to reduced myelopoiesis *in vitro* and *in vivo* (45), but given the critical role this pathway plays in HSC maintenance, it is difficult to separate these effects from specific roles in myeloid progenitor maintenance and/or differentiation.

Phagocytes, such as neutrophils, basophils, eosinophils, and monocytes, differentiate from highly proliferative GMPs in response to cytokines such as macrophage colony-stimulating factor (M-CSF, encoded by *Csf1*), granulocyte-macrophage colony-stimulating factor (GM-CSF, encoded by *Csf2*), and granulocyte colony-stimulating factor (G-CSF, encoded by *Csf3*). *Csf1* and *Il34*, which can signal through the M-CSF receptor (46), are critical for the development of monocyte-lineage cells *in vivo*, including bone resorbing osteoclasts (47–49), and as mentioned earlier, are both primarily expressed by bone marrow MSPCs and some ECs. Like SCF, M-CSF can be produced in soluble and membrane-bound forms (50). Therefore, M-CSF-dependent cells likely require physical proximity to the cellular niches producing it. Consistent with this possibility, transgenic expression of the membrane-bound form of M-CSF in M-CSF-deficient op/op mice partially restores the development of several monocytic cell lineages including some tissue-resident macrophage populations and osteoclast progenitors (51, 52). Given the fact that CXCL12 is the most abundant chemoattractant produced by M-CSF-expressing cells, it is interesting to note that CXCR4 expression distinguishes early and proliferative M-CSF-dependent monocyte progenitor stages from the more mature inflammatory monocyte stage (53). Importantly, conditional CXCR4 deletion in MPPs resulted in significant reduction in inflammatory monocyte development (Miao et al. in preparation), which suggests that CXCR4/CXCL12 plays a role in localizing monocytic cells in the proximity of monopoietic cytokines. In contrast to monocytic differentiation, osteoclast differentiation likely requires movement away from M-CSF niches (MSPC/EC) toward sources of RANKL produced by osteolineage cells (54–56).

In adult mice, most dendritic cell (DC) subsets differentiate from a common monocyte/dendritic cell progenitor in response to two cytokines: M-CSF and FLT3L (49, 57). Although the bone marrow niches supporting DC development and DC lineage choices have not been functionally defined, the fact that both cytokines are predominantly expressed by MSPCs and some ECs in bone marrow suggests an overlap with HSC niches. Consistent with this model, plasmacytoid DCs depend on CXCR4/CXCL12 for development in bone marrow (58). Whether conventional or monocyte-derived DC subsets are also dependent on CXCR4 remains unclear.

Due to their very short life span (6-8 h in the resting state), neutrophils are the most abundantly produced granulocyte population with an estimated rate of 5-10 x 10^{10} cells/day (59). G-CSF is the major cytokine promoting neutrophil development during both homeostasis and emergency states (59, 60). G-CSF acts at multiple stages during neutrophil development, starting at the GMP stage. A recent study examining GMP localization in bone marrow tissue noted that while GMPs are seemingly scattered throughout the bone marrow parenchyma under homeostasis, upon myelosuppressive treatment and leukemia, GMPs undergo proliferative bursts in large clusters around perivascular niches surrounded by lineage+ cells (61). GMP expansion during regeneration and leukemia was fueled by G-CSF, presumably secreted by sinusoidal endothelial cells, and by IL-1. The mechanisms of GMP clustering remain unstudied, but it is likely that CXCR4/CXCL12 plays some role in this process. Under homeostatic conditions, conditional CXCR4 deletion in MPPs (bypassing HSCs) reduced CMP, GMP and granulocyte production (19), which suggests that myeloid progenitor localization is important for myelopoiesis *in vivo*. Paradoxically, during systemic inflammation and infection, CXCL12 production is sharply decreased at the mRNA and protein levels along with reductions in lymphopoietic cytokine production (62, 63). These changes in CXCL12 levels primarily reduce the retention and production of lymphoid lineage cells in the bone marrow, presumably to allow for the expansion of short-lived myeloid cells required for protective immunity and return to homeostasis. However, reduced CXCL12 production would be expected to also reduce neutrophil (and monocyte) production (19). Nevertheless, G-CSF production is dramatically increased in the early stages of the systemic inflammatory response (64–66), which likely compensates for the negative effects of lower CXCL12 levels.

As for other granulocytes, the niches (and rules) controlling their development are less well-defined. Elegant studies using mice with thymic rudiments devoid of hematopoietic function (due to a mutation in the transcription factor *Foxn1*) showed that adding back SCF is sufficient to support mast cell differentiation *in vivo* (67), in agreement with prior *in vitro* studies (68). Whether this is also the case in the bone marrow microenvironment remains unknown. In contrast, SCF alone is insufficient for basophil and eosinophil development, as these phagocytes require specific instructive cues for their development. Basophil development is still poorly understood, but well-controlled *in vitro* studies demonstrated that combinations of SCF and IL-3, the latter secreted by mast cells, promote their development (68). Therefore, it is reasonable to consider that cellular circuits formed by granulocyte progenitors, mast cells and SCF/CXCL12+ niches in bone marrow generate an appropriate environment for basophil development. Eosinophil development is characterized by an intermediate eosinophil progenitor stage marked by low cKit and high IL-5Rα expression, and is largely, but not entirely, dependent on IL-5 (69). In contrast to most hematopoietic cells, however, the majority of eosinophils do not differentiate in the bone marrow. Instead, they differentiate from eosinophil progenitors within peripheral tissues in response to IL-5 produced locally and primarily by ILC2 cells (70–72).

Niches for Megakaryocytes and Erythrocytes

While most hematopoietic cell lineages develop through well-defined stages, megakaryocyte development can take multiple pathways, including direct differentiation from HSC-like populations (39). Like all hematopoietic cell subsets, megakaryocyte development requires a combination of extrinsic cytokine signals for progenitor proliferation and differentiation into the final polyploid state. Early studies showed that thrombopoietin (encoded by *Thpo*) is the major cytokine that promotes megakaryopoiesis (73), and a recent elegant study showed that hepatocyte-derived, but not MSPC-derived thrombopoietin is essential for HSC maintenance and megakaryocyte development *in vivo* (74). However, the effects of thrombopoietin synergize with SCF in inducing megakaryocyte progenitor expansion and differentiation (75, 76). Whether such synergy can only occur in bone marrow niches or is also efficient in extramedullary sites is not entirely clear. Studies using CXCR4-deficient mice lend support to a model where megakaryocyte progenitor localization and differentiation takes place preferentially in bone marrow niches (77). Consistent with this model, CXCR4 expression is increased during megakaryocyte development (78), and these cells localize in perivascular niches in proximity to HSCs (79–81). In a similar manner, erythropoiesis relies on erythropoietin in synergy with SCF, which together coordinate the development of early uncommitted hematopoietic progenitors into erythroid lineage-committed developmental stages (82). Given the dependency on cKit/SCF it is not surprising that CXCR4/CXCL12 deficiency in the hematopoietic lineage also leads to erythropoietic defects (77, 83, 84).

Bone Marrow Niches Supporting Adaptive Immunity

Besides their critical function in HSC maintenance and hematopoietic progenitor differentiation, bone marrow niches formed by CXCL12+ cells are also instrumental for ensuring the longevity of adaptive immunity. Here, we will focus specifically on two major adaptive immune cell populations, plasma cells and memory T cell subsets.

Plasma Cell Niches

Plasma cells (PCs) are essential for humoral immunity against infections and can be divided into two sub-populations: short-lived and proliferative plasmablasts, and long-lived and quiescent

plasma cells (LLPCs). A large fraction of LLPCs reside in bone marrow and provide the body with long-term protection *via* constitutive antibody production (85–87). Besides the bone marrow, LLPCs also reside in secondary lymphoid organs, the gastrointestinal tract, and other mucosal-associated tissues (88). The microenvironments where LLPCs reside are thought to play important roles in LLPC survival because these cells lack the intrinsic ability to survive in the absence of extrinsic factors (89, 90). LLPC survival factors include ligands for the TNF superfamily member BCMA (B cell maturation antigen, gene symbol *Tnfrsf17*), namely B cell activation factor (BAFF, encoded by *Tnfsf13b*) and a proliferation-inducing ligand (APRIL, encoded by *Tnfsf13*), several cytokines of which IL-6 plays a prominent role, costimulatory B7 family members CD80 and CD86, CD44, CXCL12, and adhesion receptors (91).

PC survival factors can be divided into two groups: signals that directly control the expression of anti-apoptotic molecules (e.g. *Bcl2* family members) and factors that control PC localization in appropriate niches (e.g. chemokines, adhesion receptors). Of the signals that directly promote PC survival, BCMA is perhaps the most impactful as BCMA-deficient mice show the largest reduction in antigen-specific PC numbers in the bone marrow (92, 93). Consistent with such a prominent role, BCMA signaling induced by BAFF or APRIL promotes the expression of the anti-apoptotic *Bcl2* family member, *Mcl1* (93, 94). Interestingly, the dependence on BCMA for PC survival is tissue-specific, as BCMA does not control the expression of *Mcl1* or other *Bcl2*-family members in splenic PCs, and BCMA-deficiency does not reduce splenic PC numbers (93).

BAFF and APRIL bind to BCMA (95, 96) with APRIL binding with higher affinity (97, 98). The loss of both signals impairs PC survival in bone marrow (94, 96). BAFF is produced by myeloid precursors and neutrophils (99, 100), and APRIL is expressed by myeloid precursors, monocytes/macrophages, eosinophils and megakaryocytes in bone marrow (99–103). PCs have been found in close proximity with cells expressing BCMA ligands in bone marrow, and genetic models of megakaryocyte or eosinophil deficiency revealed small but significant reductions in bone marrow PC numbers (99–103). Besides BCMA ligands, myeloid cells may also provide other soluble and membrane-bound PC survival signals such as CD28 ligands and cytokines, which in the case of IL-6 can synergize with APRIL or other soluble factors secreted by stromal cells to extend PC survival (104). In turn, *in vitro* studies showed that interactions between PCs and stromal cells induced further IL-6 production (89). Similarly, secretion of IL-6 by DCs can also be induced by interactions with PCs through CD28-CD80/CD86 (105). These findings suggest that positive feedforward mechanisms driven by complex cellular interactions between PCs and niche cells may operate *in vivo* in a manner reminiscent of cell circuits between proB cells and IL-7-producing MSPCs (14, 21). However, it should be noted that very few MSPCs, osteolineage cells, and endothelial cells in bone marrow express IL-6 *in vivo* under homeostatic conditions (37), and that single deficiency in *Il6* does not cause measurable reductions of PC numbers *in vivo* (90).

Although some PCs may be able to differentiate in the bone marrow environment (106, 107), the large majority of PCs differentiate from activated B cells in secondary lymphoid organs and require a coordinated change in chemoattractant responsiveness to migrate into the bone marrow (108, 109). CXCR4 in particular is essential for PC homing into the bone marrow (110) and once inside this compartment, PCs localize adjacent to CXCL12-expressing cells (111) where they remain relatively static over short periods of time (112). The mechanisms underlying this non-motile behavior have not been elucidated but it is likely the result of high CXCR4 signaling in combination with integrin-mediated adhesion, namely α4β1- and αLβ2-mediated interactions with fibronectin and ICAM1 expressed on CXCL12-producing cells (89, 113). Signaling induced by αLβ2 and α4β1 activation may also prevent apoptosis *via* the phosphatidylinositol-3-kinase (PI3K)-protein kinase B (AKT) pathway (114, 115). Besides myeloid lineage cells, perivascular DCs also localize in CXCL12+ niches (116) and may contribute to PC survival *via* direct delivery of CD80 and CD86 signals (105). Combined, these studies demonstrate that a complex network of cells and signals promote PC survival and suggest that PC localization in bone marrow niches is a pre-requisite for receiving survival signals.

But, can PCs destined to reside in bone marrow niches survive elsewhere? The answer varies by the type of PC. Under conditions of acute immunization with model antigens, PCs specific for T-independent antigens depend on CXCR4 for homing into the bone marrow. Accordingly, defects in this process result in decreased PC numbers in bone marrow and reduced antigen-specific IgM and IgG production. PCs developing from T-dependent antigens from acute immunizations also require CXCR4 for bone marrow homing, but serum antigen-specific IgM, IgG and IgA concentrations remain normal (28). These data lend strong support to a model where LLPCs generated from acute immunizations destined for bone marrow niches can survive in peripheral sites and secrete antigen-specific antibodies for extended periods of time (117). Hence, PC localization in bone marrow niches may play other roles that are independent of antibody secretion (118, 119).

Bone Marrow Niches for T Cells

Most lymphoid organs contain large numbers of naïve T cells due to the activity of transcriptional programs controlled by Krüppel-like Factor 2 (KLF2), which coordinates the expression of a key set of genes responsible for T cell trafficking (120, 121). Naïve T cell re-circulation is essential for naïve T cell survival due to the fact that these cells depend on external signals, such as IL-7 and Sphingosine 1-phosphate (S1P), for homeostatic survival (122, 123). While both of these signals could theoretically be provided in bone marrow niches, naïve T cells seldom migrate into this compartment due to very low CXCR4 expression, a process that is actively regulated by Sin1-dependent mammalian target of rapamycin complex-2 (mTORC2) signaling and FOXO1 inactivation (124). The signals upstream of mTORC2-FOXO1 remain undefined. The bone marrow is, however, a reservoir for some T cell subsets. Long-lived CD4+ and CD8+ central memory (CM) T cells undergo cytokine-driven

homeostatic self-renewal to ensure long-term maintenance of the memory T cell pool. CM CD8+ T cells mainly depend on IL-15 for homeostatic self-renewal, whereas CM CD4+ T cells are more dependent on IL-7 than IL-15 (125, 126). Even though sources of IL-7 and IL-15 exist in multiple organs throughout the body (16, 127–129), as previously discussed, both cytokines are also produced by bone marrow MSPCs (37). Importantly, CXCR4-mediated homing to the bone marrow is essential for the survival of CM T cells (130–133).

Given the fact that CM lymphocytes reside in the bone marrow and share niches with HSCs and their descendants, mechanisms have evolved to keep antigen-experienced lymphocytes under control. Regulatory T cells (Tregs), a T cell population that is essential for immune tolerance, seem to play such roles in bone marrow niches. Under homeostatic conditions, Tregs account for 20% to 40% of CD4+ T cells in bone marrow. Bone marrow Tregs differ from peripheral Tregs in their increased ability to express IL-10 and the checkpoint receptor CTLA-4 (134). This is particularly relevant in bone marrow transplantation, where Tregs have been described to promote tolerance to allogeneic HSCs (135). In the scurfy mouse model, Treg deficiency results in profoundly abnormal hematopoiesis (136), although it is difficult to separate local effects in the bone marrow microenvironment from systemic inflammation and the ensuing emergency myelopoiesis (60, 62, 63). Besides their role in maintaining peripheral tolerance, some evidence suggests that Tregs can also directly control the homeostasis of several hematopoietic cell lineages. For example, bone marrow Tregs promote HSC quiescence *via* adenosine secretion (137), although it is unclear if this is through direct crosstalk with HSCs, or through indirect effects on CM and other T cell subsets. Tregs also promote LLPC survival and osteoclast differentiation through CTLA-4 (134). The fact that Tregs maintain a state of immune privilege in bone marrow for the homeostasis of HSCs and other hematopoietic cells provides another elegant example of the multifunctionality of HSC niches in bone marrow.

LESSONS FROM WHIM SYNDROME

A key feature of most cellular receptors is their ability to undergo ligand-induced receptor internalization and signal termination. This is particularly relevant for physiological control of chemoattractant receptor function as it allows cells to desensitize from one chemoattractant and respond to other cues emanating from adjacent locations (138, 139). More than 95% of hematopoietic cells in bone marrow express CXCR4 and migrate toward sources of CXCL12. Most, if not all, hematopoietic cell lineages utilize the CXCR4/CXCL12 pathway during development from HSCs and downstream multipotent progenitors. Like most chemoattractant receptors, CXCR4 is internalized upon binding to CXCL12, and CXCR4 desensitization is an important mechanism of controlled hematopoietic cell exit from the bone marrow (33). Defects in CXCR4 desensitization alone can cause numerous physiological defects in hematopoietic cell development and recirculation that result in immune deficiency, as evidenced by patients afflicted with WHIM syndrome.

WHIM syndrome is an extremely rare combined immunodeficiency disorder caused predominantly by heterozygous nonsense and missense mutations in the cytoplasmic tail of CXCR4, the most common being the replacement of Arginine 334 by a stop codon, which deletes the last 19 amino acids of the C-terminus domain (140, 141). The cytoplasmic domain controls CXCR4 desensitization by recruiting GPCR kinases (GRKs) followed by phosphorylation of serine/threonine residues and β-arrestin recruitment (142, 143). Therefore, mutations in the cytoplasmic tail of CXCR4 are typically gain-of-function, resulting in increased CXCR4 signaling in response to its ligand CXCL12 (144). The frequency of WHIM syndrome is estimated to be 0.23 per million births (145). The WHIM acronym is defined by disease symptoms: **W**arts, **H**ypogammaglobulinemia; **I**nfections, which are common in the respiratory and mucosal tracts, patients being particularly susceptible to Human Papilloma Virus infections; and **M**yelokathexis, the retention of neutrophils in bone marrow that is responsible for very low neutrophil counts in peripheral blood. Most patients also present with peripheral blood panleukopenia, particularly B lymphopenia and a paucity of plasmacytoid dendritic cells (Majumdar and Murphy, 2018).

Given the dominant effects of CXCR4/CXCL12 at multiple stages of hematopoietic cell development, gain-of-function mutations in CXCR4 are also expected to impact blood cell production. A mouse model of WHIM syndrome generated by Balabanian and colleagues reproduced several hematological defects seen in WHIM patients, including peripheral blood leukopenia, that could be reversed by CXCR4 antagonism (146). Likewise, WHIM patients treated with a low dose of a CXCR4 antagonist also restore leukocyte numbers in peripheral blood and have reduced susceptibility to infectious diseases (147). In the WHIM mouse model, peripheral blood leukopenia was mostly caused by reduced B cells and neutrophils. Besides mature B cells, CD4 and CD8 T cells were also significantly reduced in the spleen, suggesting defective B and T cell development in primary lymphoid organs. In contrast, neutrophil development was seemingly intact, with mature neutrophils being somewhat increased in the bone marrow perhaps as a consequence of reduced egress from this compartment (146).

A combination of serendipity and careful molecular and cellular studies of a single WHIM patient revealed surprising insights into the delicate balance between HSC quiescence, activation, and multilineage differentiation. Identified at the NIH as patient WHIM-09, this patient spontaneously cured leukopenia and susceptibility to infectious diseases, due to a chromothripsis event in chromosome 2 of presumably a single hematopoietic progenitor cell, which eliminated the $Cxcr4^{R334X}$ allele along with 163 other genes and switched a hyper-responsive for an haploinsufficient CXCR4 state (148). Studies in mice showed competitive advantage in hematopoietic reconstitution of HSCs haploinsufficient for CXCR4, thus providing a plausible explanation for why chromotryptic

"cure" of *Cxcr4*^{R334X} allele in a single hematopoietic progenitor resulted in the replacement of leukocytes expressing the R334X mutant CXCR4 by the single wild-type allele (148). Paradoxically, in the WHIM-09 patient, lymphoid cells only differentiated from R334X-expressing hematopoietic progenitors, while myeloid cells differentiated from hematopoietic progenitors expressing wild-type CXCR4, albeit at reduced amounts due to CXCR4 haploinsufficiency. The fact that short-lived myeloid cells such as neutrophils are only produced by CXCR4 wild-type expressing hematopoietic progenitors decades after chromothripsis occurred strongly suggests that deletion of the *Cxcr4*^{R334X} allele occurred in a single HSC, which expanded and self-renewed over time. However, this model is not easily compatible with the fact that lymphoid lineage cells develop from hematopoietic progenitors carrying *Cxcr4*^{R334X} alleles. One possibility is that one or several of the 163 genes deleted are directly or indirectly critical for lymphopoiesis. Alternatively, expression of hyper-responsive R334X CXCR4 in a few hematopoietic progenitors diluted in a sea of hematopoietic stem and progenitor cells expressing 50% of wild-type CXCR4 confers a very strong competitive advantage for contacts with niche cells providing lymphopoietic factors (19, 21). It should be noted that lymphoid progenitors are particularly sensitive to efficient CXCR4 desensitization for their proper development from hematopoietic progenitors (149), and thus studies are needed to fully understand how changes in CXCR4 signaling intensity translates into alterations in hematopoietic cell lineage decisions.

LEUKEMIA AND ITS IMPACT ON BONE MARROW NICHES, HEMATOPOIESIS, AND IMMUNE CELLS

The tight regulation over cell proliferation, differentiation, and quiescence carried out by the HSC niche is severely disrupted in the context of malignancy (150). Studies over the last few years have revealed that several types of blood cancers interact with MSPCs and ECs and alter their ability to produce homeostatic cytokines and chemokines. Mouse models of chronic myelogenous leukemia (CML) showed significant reductions in the expression of HSC niche factors, including *Cxcl12*, *Lepr*, *Kitl* and *Angpt1*, and concomitant expansion of osteolineage cells (151, 152). The expansion of osteolineage cells contributed to bone marrow fibrosis, a phenomenon also observed in CML patients (153). These observations have been reinforced by single-cell RNA-sequencing of stromal and endothelial cells of mice transplanted with acute myeloid leukemia (AML), which revealed a block in adipogenic and osteogenic differentiation programs in MSPCs and osteolineage cells, as well as a reduction of *Cxcl12* and *Kitl* in MSPCs and arteriolar ECs (36). In addition, *Angpt1* and *Il7* expression in MSPCs was also decreased. Furthermore, in myeloproliferative neoplasms (MPNs), hyperactivated hedgehog (HH) signaling, predominantly driven by the overproduction of HH ligands by malignant cells, results in decreased numbers of bone marrow MSPCs and osteoblasts and the downregulation of niche-derived HSC-maintenance factors (154). Specifically, *Kitl* and *Cxcl12* are downregulated especially in endothelial cells and CXCL12-abundant reticular (CAR) cells, and *Jagged1* is downregulated in endothelial cells (154).

Besides myeloid malignancies, lymphoid malignancies have also been shown to alter the bone marrow microenvironment. An early study using adoptive transfer of the Nalm6-GFP preB Acute Lymphoblastic Leukemia (ALL) cell line found reduced *Cxcl12* expression in poorly-defined bone marrow niches, resulting in displacement of normal hematopoietic stem and progenitor cells (155). More recently, our group demonstrated that pre-malignant preB cells with unrepaired double-stranded DNA breaks induce the downregulation of *Il7* transcription in bone marrow MSPCs, while BCR-ABL preB ALL cells downregulate both *Il7* and *Cxcl12* in MSPCs *via* undefined mechanisms (21).

In addition to the reduced expression of key niche factors, the overproduction of pro-inflammatory cytokines by niche cells has also been observed in the early stages of multiple hematological malignancies. IL-1β is one of the first pro-inflammatory cytokines abnormally increased in the development of MPN and CML in mice (156), and clinically, such elevated levels of IL-1β in CML patients have been associated with poor prognosis (157). Other cytokines and growth factors such as IL-6, thrombopoietin, and CCL3 have also been suggested to render the bone marrow microenvironment pro-inflammatory in AML, ALL, MPN, and CML (152, 156, 158, 159).

This pro-inflammatory milieu disrupts normal HSC niche function primarily in two ways. First, it damages sympathetic nerve fibers that innervate arterioles, which are essential for maintaining MSPC quiescence (156, 160). In AML, sympathetic neuropathy has been correlated with skewed Nestin (gene symbol *Nes*)-producing niche cell fate determination, wherein MSPCs primed for the osteoblastic lineage significantly expand at the expense of HSC-maintaining arteriole-associated Neuron-glial antigen 2 (NG2)-expressing niche cells (161). Second, the inflammatory signals, possibly in combination with HH signaling, reduce CXCL12 expression in niche cells (151, 156, 161).

Together, these niche changes impair normal hematopoiesis while favoring the growth of leukemic malignancies (21, 161–163). In AML patients, total hematopoietic progenitors (CMPs, GMPs and MEPs) are reduced (164), and likewise, bone marrow samples from pediatric cases of ALL have lower levels of myeloid progenitor cells and erythroid progenitor cells than control samples (158). One possible explanation for the selective growth of leukemic cells is that other pathways could complement the CXCR4/CXCL12 axis in malignant cells to enable their migration. For instance, some studies have shown that Bruton's Tyrosine Kinase (BTK) signaling is important for mediating leukemic cell migration toward CXCL12 in chronic lymphocytic leukemia and multiple myeloma (165, 166). As such, malignant cells may have an advantage in the competition for niche occupancy when CXCL12 concentration becomes limiting. Furthermore, other studies have shown that as leukemia progresses, leukemic cells

become less dependent on certain niche factors than normal hematopoietic cells. For example, while pre-leukemic stem cells (LSCs) in AML are highly dependent on niche-derived Wingless-type (Wnt) signals in a manner similar to long-term HSCs, established LSCs and AML are unresponsive to Wnt inhibitors due to cell-intrinsic activation of Wnt signaling (167). Hence, AML cells downregulate niche factors to which normal hematopoietic cells are more sensitive, possibly as a method to gain a competitive advantage.

On the other hand, defects in the bone marrow microenvironment can themselves initiate the development of hematopoietic malignancies (150). Activating mutations of the protein tyrosine phosphatase SHP2 in MSPCs marked by *Nes*-cre, *Prx1*-cre, *Lepr*-cre or *Osx*-cre all result in MPNs, in part due to excessive production of CCL3 by MSPCs (168). In addition, deletion of Dicer1 in *Osx*-cre-expressing MSPCs led to myelodysplasia and even AML in a small percentage of mice (169). In this case, Dicer1 deficiency resulted in reduced expression of Sbds, the gene that is mutated in Schwachman-Bodian-Diamond syndrome, a human bone marrow failure and leukemia pre-disposition condition. Importantly, deletion of Sbds using *Osx*-cre also led to myelodysplasia. In other studies, Sipa1 and retinoic acid receptor gamma (RARγ) deficiency in radio-resistant cells led to the development of myeloproliferative syndromes (170, 171). Interestingly, an activating mutation of beta-catenin in mouse osteoblasts led to the development of AML, where wild type hematopoietic stem and progenitor cells acquired chromosomal aberrations and the ability to propagate disease autonomously even after transplantation into a wild type environment (172). This demonstrates that dysregulation of the bone marrow niche can even enable the transformation of mutant hematopoietic cells.

In summary, leukemic cells can cause profound transcriptional changes in critical bone marrow niche cells, though the molecular mechanisms underlying these alterations remain poorly defined. It is possible that the molecular crosstalk between malignant cells and the bone marrow niche gradually remodels normal niche behavior to foster leukemic growth and attenuate normal hematopoiesis. Also poorly understood is whether leukemias affect the long-term maintenance of adaptive immune cells that require access to critical bone marrow survival niches, though recent studies suggest that may be the case (173, 174).

CONCLUDING REMARKS AND UNANSWERED QUESTIONS

The bone marrow niches formed by MSPCs and endothelial cells provide an array of soluble and membrane-bound cytokines and chemoattractants that not only control HSC maintenance but also support hematopoietic progenitor commitment into multiple hematopoietic cell lineages. Besides orchestrating unperturbed hematopoiesis, these niches also contribute to long-term maintenance of immunological memory through the production of key homeostatic cytokines such as IL-7 and IL-15. These observations raise a number of physiologically relevant questions: What factors and mechanisms control the size of the HSC compartment under homeostasis? Is there direct competition between HSCs, hematopoietic multipotent progenitors, and adaptive immune cells for common factors, and if so, how is homeostasis of each cellular compartment achieved? HSCs, hematopoietic progenitors, plasma cells and memory T cells are critically dependent on CXCR4 for bone marrow homing, and therefore it is likely that these cells compete for proximity or even direct contact with niche cells. Consistent with this model, CXCL12 production is highest in MSPCs producing critical HSC maintenance factors and cytokines. However, there is evidence indicating that HSC niches are not saturated and can sustain up to 2- to 3-fold increased numbers of HSCs for extended periods of time (175). It is possible that heterogeneous production of additional chemoattractants may control the differential localization of HSCs, hematopoietic progenitors, and adaptive immune cells in the proximity of distinct niche cells. Future studies are needed to further examine the intricate relationship between HSCs, hematopoietic progenitors, and terminally differentiated immune effector cells. The recent advances in HSC visualization *in vivo* should allow these questions to be addressed (34, 176).

An increasing body of work strongly indicates that the production of hematopoietic cytokines and chemoattractants by HSC niche cells is regulated by soluble inflammatory cytokines (e.g. TNFα, IL-1β), but may also be regulated by other cues provided by leukemic cells. The fact that inflammation induces a switch in blood cell production from homeostatic and balanced lymphoid and myeloid production to an emergency state of increased myelopoiesis suggests that these distinct cell lineages compete for limiting factors in the local microenvironment. Leukemic cells and non-leukemic hematopoietic cells are also likely to compete for certain types of factors (e.g. anabolic nutrients) such that leukemic cells may exploit hard-wired mechanisms of cytokine/chemokine production to gain competitive advantage by reducing the fitness of non-leukemic hematopoietic progenitors. Furthermore, it is still unclear if leukemia or inflammation-induced changes in cytokine/chemokine production by individual niche cells are reversible, or if altered cytokine/chemokine production is coordinated with niche cell differentiation and/or survival. Future studies addressing these questions are likely to uncover novel mechanisms and pathways that may be applied therapeutically to reduce leukemic cell competitive advantages, to improve HSC transplantation, and to enhance the lifespan of immunological memory against pathogens.

AUTHOR CONTRIBUTIONS

RM, VL, and NK made extensive review of the literature listed and drafted different sections of the review. YM and JF drafted a section of the review focused on the WHIM syndrome with help

from SZ. RS drafted a section of the review focused on blood cell malignancies with help from NK. JP wrote the manuscript with input from all authors. All authors contributed to the article and approved the submitted version.

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Mobilized Multipotent Hematopoietic Progenitors Stabilize and Expand Regulatory T Cells to Protect Against Autoimmune Encephalomyelitis

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Achieving immunoregulation *via in vivo* expansion of Foxp3$^+$ regulatory CD4$^+$ T cells (Treg) remains challenging. We have shown that mobilization confers to multipotent hematopoietic progenitors (MPPs) the capacity to enhance Treg proliferation. Transcriptomic analysis of Tregs co-cultured with MPPs revealed enhanced expression of genes stabilizing the suppressive function of Tregs as well as the activation of IL-1β–driven pathways. Adoptive transfer of only 25,000 MPPs effectively reduced the development of experimental autoimmune encephalomyelitis (EAE), a pre-clinical model for multiple sclerosis (MS). Production of the pathogenic cytokines IL-17 and GM-CSF by spinal cord-derived CD4$^+$ T-cells in MPP-protected recipients was reduced while Treg expansion was enhanced. Treg depletion once protection by MPPs was established, triggered disease relapse to the same level as in EAE mice without MPP injection. The key role of IL-1β was further confirmed *in vivo* by the lack of protection against EAE in recipients of IL-1β–deficient MPPs. Mobilized MPPs may thus be worth considering for cell therapy of MS either per se or for enrichment of HSC grafts in autologous bone marrow transplantation already implemented in patients with severe refractory multiple sclerosis.

Keywords: transcriptome, multiple sclerosis, IL-1β, expansion, stability, Tregs, mobilization, multipotent hematopoietic progenitors

INTRODUCTION

Autologous transplantation of hematopoietic progenitors (HSCs) is being performed in patients with severe, refractory, autoimmune diseases and singularly, multiple sclerosis (1–4). Although beneficial effects of the procedure have been ascribed to the resetting of a naïve, non-activated immune system, as well as a rebound of diverse regulatory cell subsets (5), the hypothesis that selective HSC subsets might exert an active immunoregulatory role should not be neglected.

Hematopoietic stem cells and progenitors are present in the bone marrow (BM) at various stages of differentiation. Long-term HSC (LT-HSC) and short-term HSC (ST-HSC) are endowed with self-renewing potential and upon transplantation replenish on the long-term or on the short-term, respectively, the entire blood system. They give rise to a series of multipotent hematopoietic progenitors (MPP) with decreasing self-renewal capacity, that differentiate toward committed progenitors and more mature cells of the various hematopoietic lineages. Four MPP subsets have been identified (MPP1-4) that, in addition to their c-kit and Sca-1 expression shared with LT- and ST-HSCs, can be distinguished immunophenotypically by the gain of expression of CD34, CD48, and CD135 (Flt3), the Flt3-L receptor and loss of CD150 expression (6).

Taking into consideration that HSCs are increasingly isolated from the peripheral blood after mobilization, the possibility that mobilized HSCs exhibit immunoregulatory properties was explored. Using a cocktail of hematopoietic growth factors composed of G-CSF and Flt3-ligand (Flt3-L) for a synergistic HSC mobilization from the BM to the periphery, we could show previously that MPP were enriched by mobilization and acquired the capacity to enhance the proliferation of TCR-activated $Foxp3^+$ Tregs (7, 8). The promotion of Treg expansion also took place *in vivo*, and accounted for the protection against spontaneous type 1 diabetes (TID) in Non Obese Diabetic (NOD) mice induced by adoptive transfer of as few as 10,000 mobilized MPP per mouse. Both contact- and soluble factor-dependent mechanisms (7, 8) were involved in this acquired property of mobilized MPP.

Tregs play an important role in the control of tolerance in multiple sclerosis as well as in its animal model of experimental autoimmune encephalomyelitis (EAE). Considerable efforts are being produced aiming at promoting expansion of Tregs *in vitro* with IL-2, rapamycin, activation with anti-CD3/CD28 mAb-coated beads (9, 10) or with minute foreign antigen doses (11), for subsequent administration to patients with autoimmune diseases. Notably, Treg cell therapy may require billions of cells (12). Promoting the expansion of Tregs directly *in vivo* thus represents a therapeutic strategy worth of interest. Interleukin-2 (IL-2) at low dose has been demonstrated to expand preferentially Treg and numerous trials in a host of clinical settings are underway (13). However, it remains interesting to develop alternative strategies susceptible to confer highly selective expansion of Treg with no expansion of effector T-cells. We therefore investigated whether adoptive transfer of mobilized MPP could be used to protect against EAE by selectively promoting *in vivo* Treg expansion.

We herein report that MPP promote Treg proliferation and survival both *in vitro* and *in vivo*. Transcriptomic analysis demonstrated that Treg co-cultured with MPP display enhanced expression of several genes contributing to the stabilization of their regulatory function, reduced apoptosis and enhanced plasticity enabling them to effectively control neuro-inflammation. Adoptively transferred mobilized MPP effectively reduce the EAE score by an IL-1β–driven mechanism. Furthermore, the key role of Treg in the protection against EAE conferred by MPPs was demonstrated by disease relapse occurring upon depletion of Treg undertaken once protection was established. Therefore, *in vivo* expansion of functional Treg can be efficiently induced by MPP, and MPP-based cell therapy could represent a therapeutic strategy against autoimmune diseases either per se or as a an enrichment of autologous HSCT, already implemented in patients with severe multiple sclerosis (1–5).

MATERIALS AND METHODS

Mice

Wild type C57BL/6J, C57BL/6 Foxp3-GFP-KI, and C57BL/6 IL-$1\beta^{-/-}$ (obtained from CDTA, Orléans, France) mice were bred in our animal facility under specific pathogen-free conditions. Live animal experiments were conducted according to the EU Directive 2010/63/EU for animal experiments under an animal study proposal approved by the Paris Descartes University Ethical Committee for Animal Experimentation and the French Ministry of Research and Higher Education, number 3846-2015070622031545v4.

EAE Induction

Active EAE was induced in 10- to 12-week-old female mice by s.c. immunization at two sites, upper back and lower back, with 200 μg myelin oligodendrocyte glycoprotein $(MOG)_{35–55}$ peptide (MEVGWYRSPFSRVVHLYRNGK) emulsified in CFA containing 400 μg heat-killed Mycobacterium tuberculosis H37Ra (Hooke Laboratories, Lawrence, MA, USA), on day 0. Additionally, mice received 300 ng pertussis toxin (Hooke Laboratories, Lawrence, MA, USA) i.p. in 0.1 ml/mouse on days 0 and 1. Clinical signs of EAE were assessed daily with a 0- to 5-point scoring system, as follows: 0, no obvious changes in motor function compared to non-immunized mice; 0.5, tip of tail is limp; 1, limp tail; 1.5, limp tail and hind leg inhibition; 2, limp tail and weakness of hind legs; 2.5, limp tail and dragging of hind legs; 3, limp tail and complete paralysis of hind legs or paralysis of one front and one hind leg; 3.5, limp tail and complete paralysis of hind legs, in addition to: mouse is moving around the cage, but when placed to its side, is unable to right itself; 4, limp tail complete hind leg and partial front leg paralysis; 4.5, complete hind leg and partial front leg paralysis, no movement around the cage, mouse is not alert; 5, mouse does not move any more in the cage. Mice with score ≥4 for two consecutive days and mice with score 5 were euthanized. Disease scores over the course of the 35-d experiments were totalized for each animal, and the mean for the experimental group was expressed as a cumulative EAE score.

In Vivo Mobilization Treatments and Isolation of Mobilized Multipotent Progenitors

Mobilized progenitor cells (MPP) were prepared as follows: Wild type or IL-$1\beta^{-/-}$ C57BL/6J mice (8- to 12-week-old) were

injected s.c. for four consecutive days with human recombinant G-CSF (200 μg/kg/day) (Zarzio 48 MU/0.5 ml, Sandoz) and recombinant murine Flt3L (20 μg/kg/day) (Immunotools, Friesoythe, Germany). Total splenocytes were magnetically sorted for c-kit$^+$ cells with an automated magnetic cell sorter (Robosep, StemCell Technologies, Vancouver, BC, Canada), further stained with the mAbs directed against CD34 (BD Biosciences, Le Pont de Claix, France), Sca-1 (anti-Ly6A/E) and CD11b (eBioscience, ThermoFisher Scientific, Illkirch, France), and electronically sorted into c-kit^{high}Sca-1^{high}CD34$^+$CD11b$^{-/low}$ cells with the FACS Aria II cell sorter (BD Biosciences). Each mouse received intravenously 25,000 cells of the above subset at the same day of immunization with MOG_{35-55}/CFA. Intravenous treatment with anti-CD25 mAb (PC61) (200 μg/ml) or the control isotype antibody was performed at day 17 of the disease, once protection by MPP was established.

Assessment of Differentiation Potential of Sorted Mobilized MPP

Electronically sorted MPP were cultured on plates at 20,000 cells/ml, over OP9 or OP9Δ4 stromal cells at a 1:5 ratio, in OPTIMEM medium (Gibco) supplemented with 10% FCS, 1% antibiotics, 0.1% β-mercaptoethanol, SCF (1 ng/ml), Flt3L (10 ng/ml) (Immunotools), and IL-7 (8 ng/ml) (Peprotech, Neuilly-sur-Seine, France). After 7 days of incubation, cells were harvested, stained with appropriately labeled mAbs against CD4 (clone RM4-5), CD8 (clone 53-6.7), CD3 (clone 145-2C11), B220 (clone RA3-6B2), Gr1 (clone RB6-8C5), CD11c (clone HL3) all from BD Biosciences, NK1.1 (clone PK136, Sony) and CD11b (clone M1-70), ckit (CD117, clone 2B-8), Sca1(anti-Ly6A/E, clone D7) and PDCA-1 (clone eBio927) from eBioscience, and analyzed by flow cytometry for lineage determination.

Isolation of Immune Cells from the Spinal Cord

Spinal cord isolated from control and MPP-recipient mice were incubated for 30 min in digestion buffer of DNAse and Liberase (27 WU/ml) in PBS 1× at 37°C, mixing every 5 min. EDTA (500 μl, 100 mM) was added for 1 min to end the digestion. Cells were passed through a 100 μm cell strainer, using a syringe plunger (back side) to smash the tissue. A Percoll separation was performed resuspending the cells first in 3 to 5 ml of 40% Percoll and underlayed with the same volume of 70% Percoll (in PBS) followed by centrifugation for 35 min at 1300*g* (2800 rpm) without brake. Cells at the interface were collected with a Pasteur pipette and diluted 10 times with complete medium RPMI 10% fetal cell serum (FCS), centrifuged and resuspended in 2 to 3 ml of complete medium.

Staining of Cells for Flow Cytometry Analysis

To block nonspecific Fc receptor binding, cells were preincubated for 10 min at room temperature with FcR blocker 2.4G2 mAb. Cells were then stained with appropriately labeled mAbs against CD4 (clone RM4.5), c-kit (CD117) (clone 2B-8, eBioscience), CD11b (clone M1-70, eBioscience), Sca-1 (anti-Ly6A/E) (clone D7, eBioscience), CD34 (clone RAM34, BD Biosciences) as well as CD150 (Clone TC15-12F12.2, Sony Biotechnology, Weybridge, Surrey, UK), anti-CD48 (clone HM48-1, Biolegend) and Flt3 (clone A2F10, eBioscience). Nuclear Foxp3 and Ki67 expression was measured by FACS analysis as per manufacturer's instructions (eBioscience). Cytokines were measured at day 4 after a cell culture of lymph node cells isolated from control or MPP-recipient mice, re-stimulated with the MOG_{35-55} peptide and in spinal cord cells the same day. Intracytoplasmic expression of cytokines was assessed after a 5-h stimulation with PMA (10 ng/ml) plus ionomycin (500 ng/ml) in the presence of Brefeldin A (2 mg/ml) for lymph nodes, and 3 h for spinal cord, followed by fixation/permeabilization with PFA/saponin and subsequent staining with specific antibodies including APC-labeled anti-IFN-γ (clone XMG1.2), PE-labeled anti–GM-CSF (clone MP1-22E9), APC-labeled anti-IL-17 (clone eBio17B7) (all from eBioscience) or isotype controls. Membrane and intracellular antigen expression were analyzed in a FACS Canto II cytometer (BD Biosciences) using FlowJo software (Treestar).

Proliferation Assays

Proliferation Assays. CD4$^+$CD25^{high} (all Foxp3$^+$) cells isolated from the secondary lymphoid organs were magnetically sorted from the spleen of Foxp3-GFP-KI C57BL/6J mice. They were loaded with 5 μM carboxyfluorescein succinimidyl ester (CFSE) (Life Technologies) and cultured (5×10^4 cells per well) in RPMI medium 1640 supplemented with 5% (vol/vol) FCS (Life Technologies), 1% antibiotics, and 5×10^{-5} M β-mercaptoethanol. Cells were plated in 96-well round-bottomed culture plates, either alone or with sorted MPP at 1:1 T:MPP cell ratios, and stimulated with 2.5 μg/ml of anti-CD3 mAb (clone 145–2C11) and 5 μg/ml of anti-CD28 mAb (clone 37.51, eBioscience) for 4 days. Inhibitors were added at 5 to 20 μg/ml: anti-CD137L (clone TKS-1, Biolegend), anti-CD80 (clone 16-10A1, Biolegend), anti-GITRL (clone 5F1, Biolegend), anti-CD86 (clone GL-1, BD Biosciences), anti-OX40 (polyclonal goat IgG, R&D Systems), anti-TGFβ (clone 2G7, grown in our laboratory), anti-IL10 (clone JES052A5, R&D).

Microarray Experiment

To analyze the possible mechanisms involved in mouse CD4$^+$CD25^{high} regulatory T-cells (Treg cells) expansion promoted by mobilized MPP (MPP), we evaluated the transcriptomes of activated Treg extracted from simple culture (Treg control group) or from co-culture with MPPs (Treg + MPP). Treg and MPP were sorted as previously described. Treg were cultivated for 3 days with anti-CD3 and anti-CD28 alone or together with freshly purified MPP. After 3 days, CD4$^+$ cells (Treg) were sorted for extraction of RNA using RNeasy Micro kit (QIAGEN). Its quality was verified in an Agilent Bioanalyzer. Total RNA was amplified and converted to biotinylated cRNA according to the manufacturer's protocol (Illumina TotalPrep RNA Amplification Kit; Ambion). Paired biological replicates (3 for each group) were hybridized to the Sentrix BeadChips Array

mouse WG-6 v2 (Illumina) and gene expression analysis was performed using GeneChip Mouse Genome 430 2.0 arrays (Affymetrix), as recommended by the manufacturer.

Transcriptomics

Fluorescence values corresponding to raw expression data for each 6 samples were extracted from CEL files using the R oligo package (https://bioconductor.org/) with the corresponding platform definitions (pd.mouse430.2). Probe annotations were added using affycoretools (Bioconductor) with the mouse4302.db database. Internal positive and negative controls, and ambiguous or unknown probes were removed, which left 39.444 probes, corresponding to 21,108 unique and well-annotated genes. Briefly, quality control steps, data normalization, unsupervised explorations and functional annotations were conducted as described previously [https://doi.org/10.1007/s12035-018-1128-3]. Statistical analyses were achieved using linear modeling with empirical Bayes, *p values* were computed by applying a moderated two-way t-test and adjusted for false discovery rate (FDR) following the Benjamini–Hochberg procedure. Hierarchical clustering heat maps were obtained on gene-median-centered data with uncentered correlation as similarity metric. Volcano plot were rendered using EnhancedVolcano (Bioconductor). Additional pathway analyses were performed with the Reactome platform (https://reactome.org) and with ReactomePA (Bioconductor) for over-representation tests in mouse. Target enrichment were obtained with EnrichR [https://doi.org/10.1093/nar/gkw377]. For all experiments, an FDR or q-value < 0.05 indicated statistical significance.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism (GraphPad Software, La Jolla, CA). Disease curves were analyzed using two-way ANOVA test, with Bonferroni post-test. Cell proportions were analyzed using one way ANOVA with Bonferroni or Tukey's post-test. Data are shown as mean ± s.e.m. $P < 0.05$ was considered statistically significant.

RESULTS

Mobilized MPP Characterization

C-kit$^+$ spleen cells derived from mobilized C57Bl/6 mice were sorted for c-kit^{hi}Sca-1^{hi} CD34$^+$ CD11b$^-$ cells (**Figure 1A**). Using SLAM markers (14), FACS analysis revealed that 80% of the mobilized sorted cells displayed a CD48$^+$CD150$^-$CD135$^-$(Flt3$^-$) phenotype (**Figure 1B**), and thereby corresponded to the described MPP3 subset, reported to be prone to myeloid differentiation (15). Reflecting the continuum of the MPP differentiation process, approximately 20% CD150$^+$ cells were detectable among the mobilized c-kit$^+$Sca-1$^+$CD34$^+$CD11b$^-$ cells, most probably corresponding to progenitors at the MPP2 (CD48$^+$CD150$^+$CD135$^-$) stage. To assess the capacity of the sorted mobilized progenitors to differentiate into multiple hematopoietic lineages, in keeping with their MPP phenotype, we cultured them on OP-9 and OP-9Δ4 stromal cells, the latter permitting differentiation into the T-cell lineage that requires Notch pathway stimulation conferred by the expression of the Notch ligand Delta-4. A myeloid differentiation bias was also

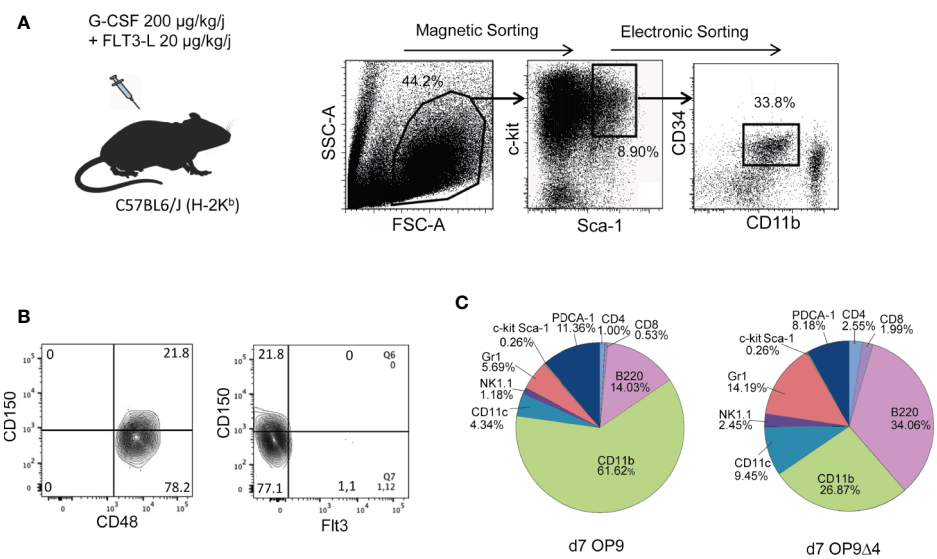

FIGURE 1 | Preparation and characterization of mobilized MPP. **(A)** Upon mobilization with G-CSF and Flt3L, spleen c-kit$^+$ cells were magnetically sorted, further stained with c-kit, Sca-1, CD11b and CD34 and cell-sorted as c-kit$^+$Sca-1$^+$ CD34$^+$ CD11b$^{-/low}$ cells. **(B)** SLAM markers including CD150 and CD48 and Flt3 were used for characterization of mobilized cell sorted c-kit$^+$Sca-1$^+$ CD34$^+$ CD11b$^{-/low}$ progenitors as 80% MPP3 (CD150$^-$) and 20% MPP2 (CD150$^+$). **(C)** The differentiation properties of mobilized MPP were assessed after 7 days of co-culture upon OP9 and OP9Δ4 stromal cells in the presence of SCF (1 ng/ml), IL-7 (8 ng/ml) and Flt3L (10 ng/ml). Cells were recovered and stained for FACS analysis with different lineage markers. Percentages of the different subsets resulting from MPP differentiation are indicated.

noted upon *in vitro* differentiation of the mobilized MPP. Yet, their differentiation potential into both myeloid and lymphoid hematopoietic lineages remained detectable (**Figure 1C**).

Mobilized MPP Enhance Treg Proliferation: Transcriptomic Analysis

Treg sorted from Foxp3-GFP-KI mice and co-cultured *in vitro* at 1:1 ratio with MPP showed enhanced proliferation, assessed by CFSE dilution over 4 days in response to stimulation by anti-CD3/anti-CD28, compared to Treg cultured alone (**Figure 2A**). Furthermore, MPP did not increase the proliferation of activated $CD4^+CD25^-$ cells (**Figure 2B**). Thus, MPP specifically increased the *in vitro* expansion of TCR-activated Treg cells. Neutralization assays demonstrated that the molecular pathways (Jagged-Notch3 and GM-CSF-CD116) previously shown to be implicated in the NOD mouse (7, 8) were not implicated in the C57BL/6 strain, nor were TGF-β, IL-10, CD80, CD86, CD40, OX40, ICOS, or GITR on co-cultures, as neutralizing antibodies to these molecules had no significant effect on Treg survival and proliferation. These data raised the question about the mechanism used by MPPs to promote Treg expansion. We therefore performed microarray experiments on sorted Treg after incubation with anti-CD3/anti-CD28 either alone (Treg; N=3) or in presence of MPP during 4 days (Treg+MPP; N=3). Resulting transcriptomes finally constituted a curated dataset including 39,444 probes, corresponding to 21,108 unique genes. Unsupervised K-means clustering clearly delineated two groups of samples, with Treg on one hand and Treg+MPP on the other (**Figure 3A**). These presented with a strongly correlated gene expression profile, divided in two clusters of 4,035 and 4,247 unique genes, down- (cluster c8) and upregulated (cluster c9) in Treg+MPP, respectively.

The two dysregulated clusters represented a wide proportion (39.2%) of the whole transcriptome, with a significant differential expression between the two groups (**Figure 3B**), thus driving the sample classification. A compilation of the most relevant functional annotations, pathways and transcription factor targets (with GO, Reactome and EnrichR, respectively) were dissected for these two clusters (**Figure 3C**). Under-expressed genes in Treg+MPP (cluster c8) were mainly involved in the positive regulation of apoptosis, the Wnt calcium signaling pathway (non-canonical), or in TCR binding and signaling. The cell cycle, especially the replication during the S phase, was also found decreased, as well as the establishment of the immunological synapse through focal adhesion and adherens junction (all FDR < 0.05; **Supplementary Table S1**). This cluster presented with a large number of genes that are transcription targets of FOXO1 (n=530; q-value = 6.1e-22), an important regulator of cell death acting downstream of CDK1, PKB/AKT1, and STK4/MST1, and was also enriched in targets of FOXP3 (n = 55; q-value = 2.1e-4). Conversely, main over-expressed functions and pathways in Treg+MPP (from cluster c9) included the MHC complex, acute inflammatory response and cytokine-mediated signaling, TNFα on cytokine activity and cytokine-cytokine receptor interaction. Positive T cell differentiation, T cell activation and T cell mediated cytotoxicity were also increased

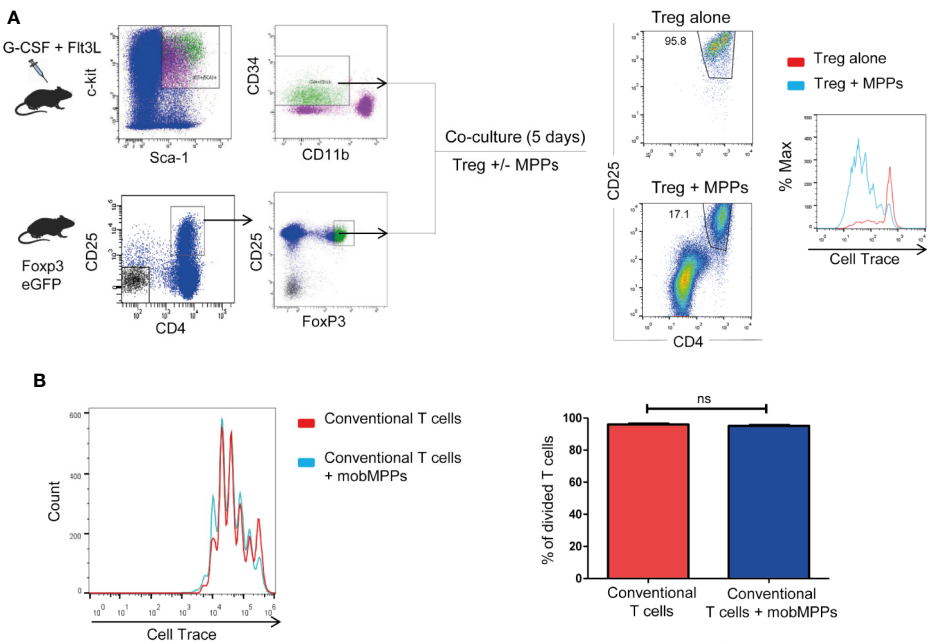

FIGURE 2 | MPP promote Treg proliferation *in vitro*. Role of IL-1β. **(A)** MPP were isolated from mobilized C57BL/6 donors as in **Figure 1A** and Tregs were isolated from Foxp3 eGFP mice as $CD4^+CD25^+$ Foxp3 $(GFP)^+$ cells and loaded with Cell-Trace. Tregs were stimulated for 4 days with anti-CD3/anti-CD28 in the absence or presence of MPP at a 1:1 ratio. Cell Trace incorporation was measured in the $CD4^+CD25^+$ gate. **(B)** Lack of effect of MPP co-cultured with CFSE-incorporated $CD4^+CD25^-$conventional T cells (ratio 1:1). ns: Not statistically significant by Student's *t*-test.

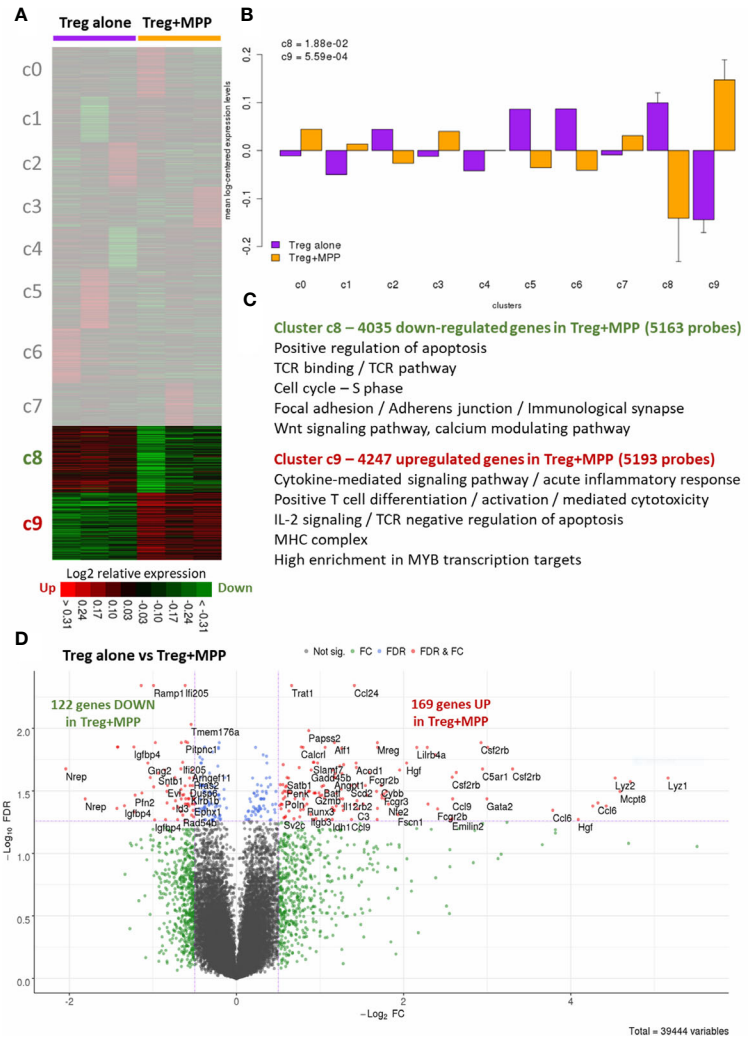

FIGURE 3 | Overview of the changes induced by MPPs on the whole transcriptome (39,444 probes) of Tregs. Tregs were recovered after culture for 4 days either alone (Treg alone) or with MPPs (Treg+MPP) at a 1/1 ratio. **(A)** Unsupervised K-means clustering. The heat map delineates two large clusters of co-expressed genes (green: down-regulated genes; red: upregulated genes; black: median genes; 10 clusters labeled c0 to c9). **(B)** Grouped mean-log2-centered gene expressions in Treg alone vs. Treg+MPP for each of the 10 clusters, with standard deviation. *P* values are indicated for the significant clusters c8 and c9 (two-sided t-test). **(C)** Main enriched functional annotations for the down-regulated (c8) and upregulated (c9) K-means clusters in Treg+MPP, respectively. All FDR and q-values < 0.05. **(D)** Supervised analyses with Bayesian linear models. Volcano plot derived from the resulting statistics of Treg alone vs. Treg+MPP. FDR, False discovery rate; FC, Fold-change; FDR cutoff: 0.05; FC cutoff: –1.5 and +1.5-fold.

in Tregs cultured with MPPs, as well as TCR negative regulation of apoptosis *via* IL-2 signaling. Glycolysis was also upregulated (**Supplementary Table S2**). Transcription targets of MYB, which prevents the differentiation of Tregs into effector T cells (16), and whose deletion leads to fatal immune pathology, were highly enriched in this cluster (n = 284; q-value = 8.7e-10).

For higher stringency and identification of core differential genes, we then performed statistical analyses with Bayesian linear models on the entire dataset and identified 291 differential genes between Treg and Treg+MPP (FDR < 0.05), 122 being down-regulated and 169 upregulated in the presence of progenitor cells, respectively (**Figure 3D**; **Supplementary Table S3**). These results completely overlapped (100%) those obtained with unsupervised methods, with wider ranges of over-expression (up to more than 30-fold) than under-expression (4-fold). Among the strongly increased genes were Lyz1 and Lyz2 (35.8-fold and 23.1-fold, respectively), coding for lysozymes related to the C-MYB transcription factor network and the innate immune system pathway. Other genes, such as Ccl6 (20-fold), coding for a small CC chemokine involved in myeloid cell recruitment, Mcpt8 (24.1-fold), predicted to be involved in granzyme-mediated apoptotic signaling pathway, and Csf2rb (9.8-fold), encoding a common subunit for the type I cytokine receptors GM-CSF, IL-3, and IL-5, were also highly increased in Tregs cultured with MPPs. On the

other side, greatest decreases were observed for genes like Nrep (-4.1-fold), playing a role in the regulation of transforming growth factor beta receptor signaling pathway, Evl (-1.8-fold), a highly expressed gene in white blood cells, as well as its corresponding protein, a member of the Ena/VASP family, Igfbp4 (-2.5-fold), known to act as an apoptotic factor by reducing the growth of several cancers (17), or Bcl2l11 (-2.3-fold), coding for the BIM apoptotic activator, whose deficiency results in Treg enhanced survival and accumulation (18).

This highly specific gene signature (**Figure 3**) was explored further to study the molecular profile conferred to Treg by MPP in terms of expansion, plasticity and stabilization of their regulatory function, particularly in inflammatory settings. High expression levels were observed for Cd4, Il2ra (CD25), Il17ra (CD127), Il2rg, Ptprc (CD45), Tcrb-J, Foxp3, Foxo1, Foxo3, Lef1, Ctla4, Stat3, Cdkn1a (p21), Tgfb1, Tnfrsf1b, Tnfrsf4 (Ox40/CD134), Tnfrsf18 (Gitr), Ikzf2 (Helios), Ikzf1 (Ikaros), Nr4a3, Hif1a, and Cd28. Among these, genes with even enhanced expression after exposure to MPPs (up to 1.7-fold) were Ctla4, Tnfrsf4 (Ox40/CD134), Tnfrsf18 (Gitr), Ikzf2 (Helios), Tgfb1, Cdkn1a (p21), Stat3, Nr4a3, and Hif1a. Genes reaching high expression level status after increase included Gzmb (Granzyme B; +2-fold), Tbx21 (+2.5-fold), and Gata3 (+1.8-fold). Other increases concerned genes like Runx2, Runx3, Satb1, Cd8a (+3.9-fold), Il2, Il12rb2, Il4ra, Il10, Ifrg, Irf4, Stat5a, Prdm1, and Itgae (CD103). Cd4 expression was found to be slightly diminished in Treg+MPP but maintaining a high level, as well as Ikzf1 (Ikaros), Tcrb-J and Lef1 (down to -1.2-fold). Other highly expressed and decreasing genes included Il17ra (CD127), Il2rg, Ptprc (CD45), Foxo1, Foxo3 (-1.6-fold), Tnfrsf1b, and Cd28. Finally, Nrp1 (Neuropilin-1; -1.9-fold), Rarg, Dnmt3a, Ilr1, Wnt7a, Wnt10a, and Gata1 were also moderately down-regulated in Treg+MPP (**Supplementary Figures S1**, **S2**; **Supplementary Table S3**).

In brief, Treg exposed to MPP harbored a "specialized" molecular signature, with a stabilized phenotype $Cd4^{high}$, $Il2ra^{high}$, $Foxp3^{high}$, Il7ra–, Ctla4+, Tnfrsf18+, Ikzf2+, Tnfrsf4+, Itgae+, under the transcriptional regulation of increased Satb1, Runx2, Runx3, and Gata3 gene products, switching from FOXO control, with decreased gene expression in Treg+MPP. Moreover, they exhibit enhanced expression of Prdm1 (+1.3-fold; p-value=6e-3), recently reported to prevent methylation of Foxp3 within Treg in central nervous system inflammation (19), and Nr4a (+1.45-fold; p-value = 9e-5), that stabilizes Treg against their differentiation into effector T-cells (20), which is further confirmed by the unchanged Foxp3 expression observed here after contact with MPP. Treg survival is also improved with a finely controlled and reduced apoptosis by means of TCR signaling in response to increased IL-2, the inhibition of NF-κB pathway *via* the increase of TNFα activity, the FOXO-mediated transcription, and through p21 (Cdkn1a) cell replication inhibiting activity, which has been shown to ultimately promote Treg proliferation through enhanced anti-apoptotic control (21).

MPP exposed Treg also displayed molecular pathways activated by several cytokines (IL1, IL4, IL13, IL10, IL12) and several chemokines acting *via* STAT proteins (**Figure 3**; **Supplementary Tables S1**–**S3**; **Supplementary Figures S1**, **S2**). For a deeper understanding on IL-1β/TNFα–mediated inflammatory responses, we thus compiled a list of 51 genes involved and interacting with these pathways (from SigDB curated gene sets C2; https://www.gsea-msigdb.org/gsea/msigdb) along with top correlated genes extracted from gene dendrograms obtained from hierarchical clustering of each down-regulated and upregulated K-means clusters c8 and c9, respectively. Further hierarchical clustering of these 51 genes unravels two clusters of strongly correlated expression patterns, one down-regulated, and one strongly upregulated (up to 20-fold), representing an inflammatory-specific signature in Treg +MPP, completely in line with Il1b expression, but not Tnfrsf1a (**Figure 4A**). Among the genes dependent of the IL-1β pathways with enhanced expression in Treg co-cultured with MPP were Ifng (IFN-γ), Tbx21 and Ccl5 genes. In addition, pro-inflammatory Myd88 was upregulated and highly correlated with the inflammation signature.

Inflammation also affects hematopoietic progenitors. Stress hematopoiesis, triggered upon mobilization with G-CSF, prompts copious inflammatory cytokine production by MPP, including IL-1β (22), IL-1β expression is enhanced approximately 20 fold in peripheral blood $CD34^+$ cells compared to BM $CD34^+$ cells (23). The role of MPP-derived IL-1β in the promotion of Treg proliferation was confirmed by the reduction of the expansion effect provided by MPP onto Treg in the presence of a neutralizing antibody to IL-1β (**Figure 4B**). Moreover, efficiency of MPP isolated from IL-1β KO mice (prepared as in **Supplementary Figure S3**) in enhancing Treg proliferation *in vitro* was reduced relative to WT MPP (**Figure 4C**). Nevertheless, additional factors besides IL-1β are presumably implicated as the enhanced proliferation effect of MPP was not totally abolished in the absence or the neutralization of this cytokine.

Adoptive Transfer of Mobilized MPP Protects Against EAE and Reduces Pathogenic Cytokine Autoimmune Production

Role of IL-1β

To investigate whether MPP can expand Treg *in vivo* and thereby confer protection against immune inflammatory diseases, we chose a Th1/Th17 cell-driven animal model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE). We performed the adoptive transfer of as few as 25,000 mobilized MPP per mouse, cell-sorted as shown in **Figure 1A**, at day 0 of immunization with the myelin oligodendrocyte glycoprotein peptide (MOG_{35-55}) according to the administration protocol outlined in **Figure 5A**. MPP isolated from WT donors, but not from IL-1β–deficient donors (prepared as in **Supplementary Figure S3**), significantly reduced the clinical scores of EAE, relative to mice injected only with PBS (**Figure 5B**). Accordingly, both in the periphery (lymph nodes) and in the CNS (spinal cord) of MPP recipients, the $CD4^+$ T-cell production of the major pathogenic cytokines, IL-17, IFN-γ and GM-CSF measured at the peak of the disease (day 21) was

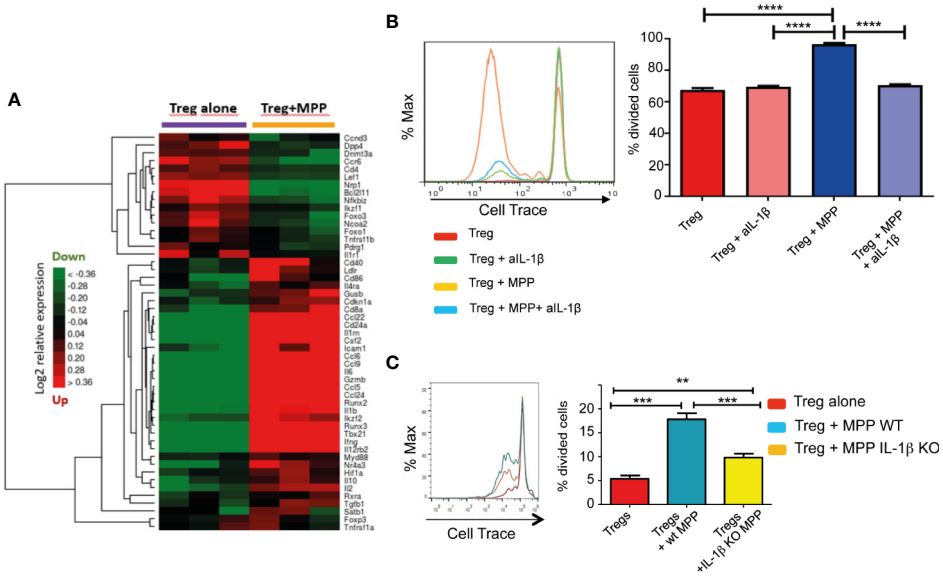

FIGURE 4 | Role of the IL-1β pathway in the modulation exerted by MPPs onto Tregs. **(A)** Hierarchical clustering heat map of targeted genes in the IL-1β pathway and the inflammatory response modulated in Tregs by MPPs. The signature was obtained by compiling a list of genes included in the Il-1β/TNFα pathway from the curated gene sets on MSigDB (https://www.gsea-msigdb.org/gsea/msigdb/) and adding top correlated genes of interest to the list. Green: down-regulated genes; red: upregulated genes; black: median genes. **(B)** Role of IL-1β in enhancing proliferation of Tregs: Tregs were cultured for 4 days with anti-CD3/anti-CD28 either alone or in the presence of MPPs and a neutralizing antibody to IL-1β. Representative FACS analysis (left) and mean ± s.e.m. of triplicates (right). ****$p < 0.0001$ by one-way ANOVA with Bonferroni post-test. **(C)** Tregs loaded with Cell Trace were co-cultured with MPPs isolated from mobilized WT or IL-1β KO mice. Cell Trace dilution was analyzed by FACS (left) and percentages of proliferated cells compared (right: mean ± s.e.m. of triplicates from 5 cumulated experiments), **$p = 0.0091$, ***$p < 0.001$, by one-way ANOVA with Tukey's multiple comparison test.

reduced (**Figures 5C, D**), again only in recipients of WT but not of IL-1 β–deficient MPP (**Figure 5B**). These data confirm the key role of this cytokine as well in the *in vivo* protection by MPP against the disease.

Peripheral and CNS Treg Proliferate at Higher Rates in MPP Recipients

Role of IL-1β

Percentages of Foxp3$^+$ Treg were enhanced in gated CD4$^+$ cells in lymph nodes and spinal cord of MPP recipient mice compared to control mice (**Figures 5E, F**). Treg proliferation, measured by nuclear staining of Ki67 within gated Foxp3$^+$ CD4$^+$ cells at the peak of the disease tended to be enhanced in the periphery as well as in the spinal cord of MPP recipients relative to control mice with EAE, however without reaching statistical significance (**Figures 5G, H**). This *in vivo* effect on Treg was also IL-1β dependent as, contrary to recipients of WT MPP, recipients of IL-1β KO MPP showed no increase in the percentages nor in the Ki67 expression of Treg in their spinal cord, compared to non-injected EAE controls ((**Figures 5E–H**).

The Protection Against EAE Conferred by Adoptively Transferred MPP, Once Established, Can Be Abrogated by Treg Depletion

To assess whether Treg that accumulated in MPP recipients played a non-redundant role in the observed protection against disease, MPP recipient mice with established protection were divided into two separate groups (n = 5 mice per group) that received one single injection at day 17 either of the anti-CD25 PC61 mAb (250 μg/mouse, i.p.), which depletes CD25^{hi} Treg cells or of a control isotype mAb (**Figure 6A**). While the same treatments applied to control EAE undergoing mice did not change significantly the disease scores, three days after the mAb injection, disease relapse was observed in the PC61-injected group of MPP recipients which by d25 reached the clinical score of the control EAE group (**Figure 6B**). Conversely, the MPP-recipient group injected with the same amount of an isotype mAb did not significantly change its mean clinical score, and remained protected against EAE till the end of the experiment (d30). Therefore, Treg play a key role in the protection conferred by adoptively transferred MPP since their removal, after protection is established, triggers disease relapse.

DISCUSSION

Mobilized hematopoietic progenitor subsets at the developmental stage of MPP3 as defined by SLAM markers, that remain multipotent although with a clear myeloid developmental bias, displayed the remarkable property to promote the expansion of TCR-activated Treg, without affecting the proliferation of CD4$^+$CD25$^-$ cells. The functional outcomes of this property were highly significant, since as few as 25,000 mobilized MPP

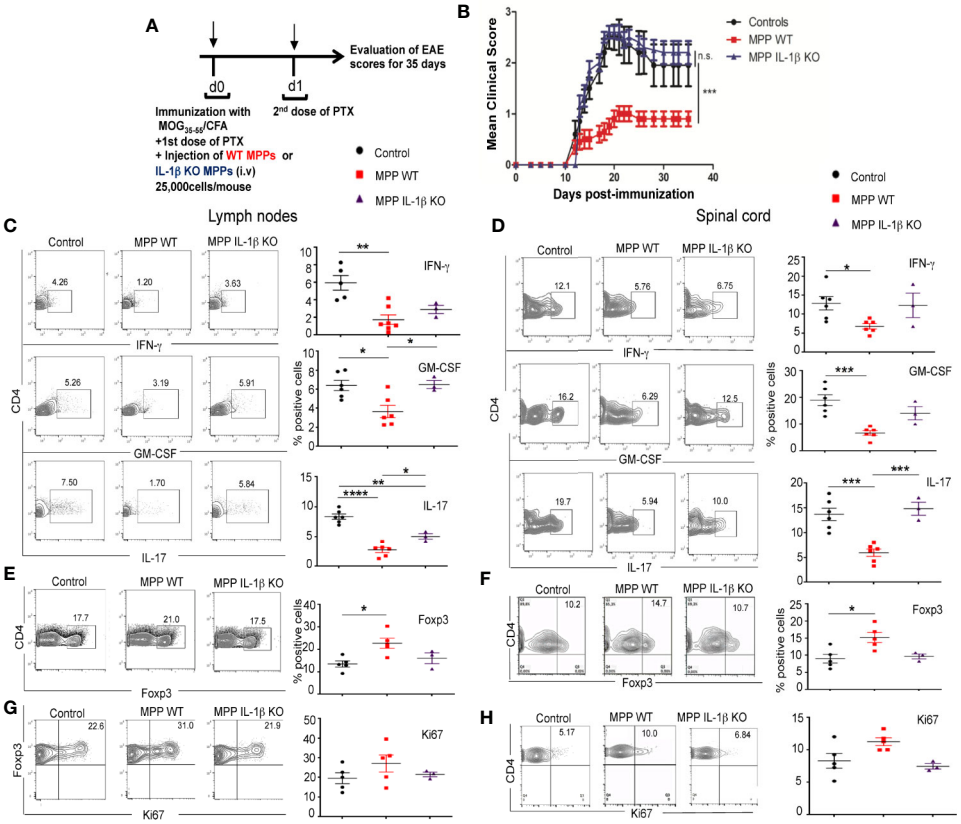

FIGURE 5 | Protective role of MPPs against EAE. Role of IL-1β. **(A)** EAE was induced by s.c. injection at d0 with an emulsion of MOG_{35-55} in CFA and MPPs, isolated either from WT or IL-1β –deficient C57BL/6 mobilized donors, were adoptively transferred by retro-orbital i.v. injection (25,000 cells per recipient). A first dose of PTX was injected i.p. on d0 and a second dose on d1. **(B)** EAE clinical score was assessed daily until d35 in control mice immunized for EAE, and in mice adoptively transferred with either WT or IL-1β–deficient MPPs (n = 10 mice per group). Mean ± s.e.m., ***, p= 0.0015 by two-way ANOVA, with Bonferroni post-test. **(C-F)** FACS analysis of gated $CD4^+$ cells isolated from cervical lymph nodes (left) and spinal cord (right) of mice from the 3 groups, at d18 after immunization. **(C, D)** Intracytoplasmic neuroinflammatory cytokine (IFN-γ, GM-CSF, IL-17) production. **(E, F)** Percentages of Foxp3 expressing $CD4^+$ cells. **(G, H)** Intranuclear Ki67 expression of $Foxp3^+$ $CD4^+$ cells, **(C, H)** left: representative contour plots, right: summary of data. ****$p < 0.0001$, ***$p < 0.0002$, **$p < 0.002$, *$p < 0.05$, by one-way ANOVA with Bonferroni post-test.

cells adoptively transferred per recipient conferred protection against EAE, and this protection was highly dependent on Treg as demonstrated by disease relapse upon Treg depletion by the PC61 mAb treatment in MPP-recipient mice.

The molecular mechanism by which C57Bl/6 mice derived MPPs afforded protection against autoimmune disease differs from what we had previously described in spontaneous diabetes in NOD mice in which mobilized MPP triggered both Notch- and GM-CSF-dependent signaling (7, 8). Genetic variations between strains may account for this inconsistency as it has been shown that GM-CSF effectively enhanced Treg proliferation in both the NOD and BALB/c strains but neither in the CBA nor the C57Bl/6 strains (24). Notably, both GM-CSF overproduction and persistent STAT5 phosphorylation have been reported to be amplified in autoimmune NOD bone marrow cells (25). The latter phenotype can be reproduced in non-autoimmune C57BL/6 bone marrow cultures by blocking M-CSF while stimulating with NOD level GM-CSF. Thus, the observed discrepancies between the NOD and the C57BL/6 strains in the molecular mechanisms of MPP on Treg expansion may at least in part originate in this dysregulation of GM-CSF affecting NOD myeloid cell differentiation. We herein show that in C57Bl/6 mice immunized with MOG_{35-55}, IL-1β, one of the cytokines reportedly released by stress-activated MPP (22), is a key molecular mediator promoting the expansion of TCR-activated Treg both *in vitro* and *in vivo*. IL-1β likewise appears involved in the Treg expansion properties of G-CSF mobilized human MPPs isolated from healthy donors characterized in D'Aveni et al. (submitted). Yet, whether autoimmune patients will exhibit a distinct molecular mechanism for MPP expansion of Treg will have to be explored.

This may appear paradoxical since IL-1β is mostly associated with inflammation in MS and EAE. An absolute requirement of IL-1R was demonstrated for the development of EAE (26–29). IL-1β is present in MS lesions and IL-1RA moderates the induction of EAE (30, 31). Both IL-1β and IL-

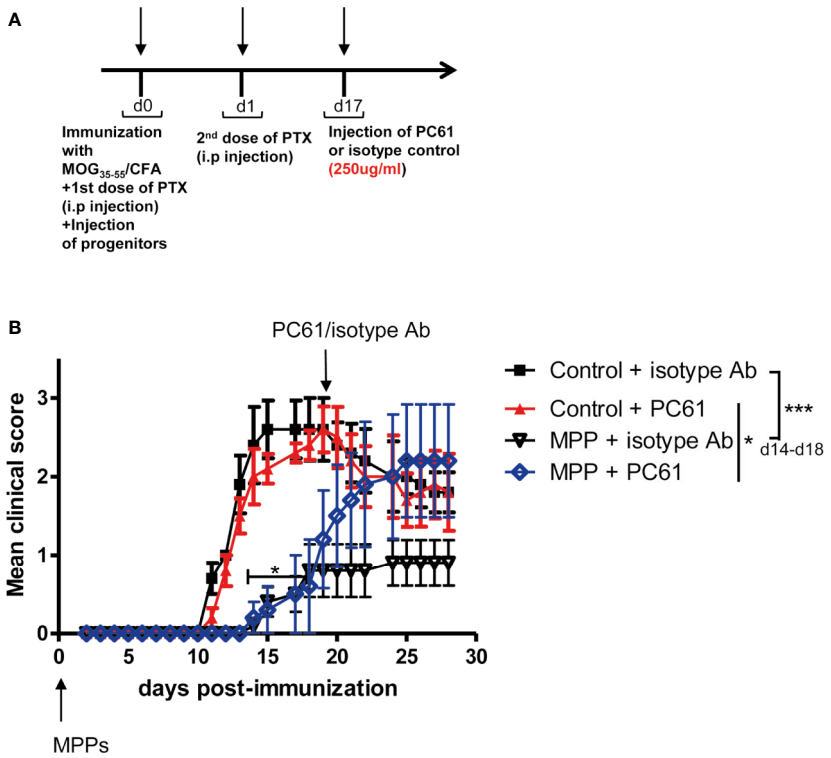

FIGURE 6 | Loss of protection against EAE by MPPs upon elimination of Treg. **(A)** Mice were immunized for EAE and injected at d0 with or without MPP. At d17, control and MPP recipient mice were separated into 2 groups of 5 mice, receiving either the anti-CD25 PC61 mAb or an isotype antibody (250 µg/mouse, i.p.). **(B)** EAE was scored daily. Clinical scores are indicated as mean ± s.e.m., $^{*}p = 0.0037$, $^{***}p < 0.0001$, using two-way ANOVA with Bonferroni post-test, when comparing controls with MPP recipients injected with isotype Ab. N.S. for comparisons of all other curves.

1RA appear in white and grey matter lesions in a model of relapsing EAE at sites of active demyelination (30). IL-1β contributes to the generation of GM-CSF^{+} Th17 cells in EAE (28, 32). An elevated IL-1β over IL-1RA corresponds to higher risks of developing MS (33). In addition, IL-1β plays a role in glutamate-mediated synaptic excitability and neurotoxicity taking place in EAE (34).

Conversely, IL-1β priming reduced EAE severity in rats (28, 29, 35). Additionally, the capacity of IL-1β to enhance proliferation of TCR-activated Treg, but not of resting Treg, was recently reported in several *in vitro* studies. IL-1β has been shown to promote TGF-β and IL-2–dependent Foxp3 expression in Treg and to display co-stimulatory effects on their expansion and differentiation (36). It has been proposed that Foxp3^{-} T cells may be the primary target of IL-1β and actually support Foxp3^{+} Treg expansion (37). Here, IL-1β produced by mobilized MPP was able to enhance the proliferation and survival of Foxp3^{+} Tregs isolated from Foxp3-GFP-KI mice, thus devoid of non Treg.

Foxp3^{+} Treg are endowed with plasticity that enables them to specialize in selectively regulating effector T cell responses (38). Several of the genes dependent of the IL-1β pathways that showed enhanced expression in Treg after interaction with MPP, including Ifng (IFN-γ), Tbx21 (39), and Ccl5 (20), have been reported to confer to Treg the capacity to suppress Th1 and Th17 inflammatory cells, most appropriate to control EAE disease. Moreover, they exhibit enhanced expression of Prdm1, recently reported to prevent methylation of Foxp3 within Treg in central nervous system inflammation (19). Indeed, CD4^{+} T cells of MPP recipients displayed reduced IFN-γ, IL-17 as well as GM-CSF cytokine production. This suggests that MPP may confer to Treg the capacity to better suppress effector Tcells with the same gene set expression, i.e. Th1 and Th17 cells (38). Therefore, Treg expanded in contact with MPP have acquired the capacity to provide robust cell therapy against immune inflammatory diseases.

Moreover, they exhibit enhanced expression of Nr4a, that stabilizes Treg against their differentiation into effector Tcells (20). This stabilization is further confirmed by the high and unaffected expression level of Foxp3. Their survival is improved with a reduced apoptosis by means of TCR signaling in response to increased IL-2, the inhibition of NF-κB pathway *via* the increase of TNFα activity, the FOXO-mediated transcription, and through p21 (Cdkn1a) inhibiting activity. Their cell cycle is also slowed at the end of G1 stage through DNA synthesis inhibition in the S phase, stemming from increased p21 expression (Cdkn1a), which is in turn regulated by RUNX3 and p53. Coupled with a stringent control of apoptotic decrease,

a reduced cell cycle by overexpression of Cdkn1a, and independent from Foxp3, ultimately promotes Treg generation, as was reported after ionizing radiation of Langerhans cell (21). Altogether, MPP confer to Treg stability, enhanced survival and a highly functional state along with the phenotype Cd4^{high}, Il2ra^{high}, Foxp3^{high}, Il7ra−, Ctla4+, Tnfrsf18+, Ikzf2+, Tnfrsf4+, Itgae+.

Interaction with innate signals was reported to confer to HSC and particularly MPP the capacity to release numerous and copious amounts of cytokines and chemokines (22). Both murine and human HSC stimulated by innate signals contribute to long-term increased anti-infectious response termed trained immunity (40, 41). In systemic lupus erythematosus, an autoimmune disease with a humoral component, TLR-stimulated HSC produce IL-17 and IL-21 and expand Th17 and Tc17 cells (42), contributing to disease severity. On the other hand, hematopoietic progenitors further engaged into differentiation, in the myeloid (43), B (44–46) and pDC (Letscher et al., submitted) lineage pathways have instead been conferred immunoregulatory properties upon innate activation. Such innate activated progenitors provide protection against inflammation, autoimmune diseases, but may as well limit anti-cancer defense. They target and suppress effector T cells or favor the accumulation of diverse regulatory cell subsets. Our present data demonstrate that in organ-specific autoimmune diseases such as Type 1 diabetes and EAE, G-CSF mobilized MPP exert unique regulatory properties indirectly *via* a major mature regulatory cell type, Foxp3^{+} Treg, that they not only expand *in vivo* but also confer stabilization and fitness for controlling inflammatory settings.

The data herein presented advocate for the use of mobilized MPP as a promising tool for cell therapy of autoimmune diseases, either per se or as a complement of autologous HSC transplantation already performed in MS patients with severe, non-responsive disease. Although G-CSF was reported to provide protection in two different models of EAE in mice (47, 48), in MS patients, mobilization with G-CSF administrated alone caused disease flare (49). However, this deleterious effect could be controlled if G-CSF was associated with steroids (50). The CXCR4 antagonist plerixafor that induces rapid mobilization (51) might be worth evaluating for its potential morbidity in the MS setting and for the capacity of MPP mobilized with this alternative agent to expand Treg to the same extent as those obtained after G-CSF mobilization. In addition, the effectiveness of MPP at different disease stages remains to be evaluated. Indeed, the data presented herein demonstrate the capacity of mobilized MPP to prevent EAE induction. For a putative application to MS, their ability to reduce EAE remains to be evaluated with an adoptive transfer once clinical signs are detectable.

DATA AVAILABILITY STATEMENTS

The datasets generated and analyzed for this study can be found in the GEO repository https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE155148. All other data are available upon reasonable request.

ETHICS STATEMENT

The animal study was reviewed and approved by Paris Descartes University Ethical Committee for Animal Experimentation and the French Ministry of Research and Higher Education, number 3846-2015070622031545v4.

AUTHOR CONTRIBUTIONS

Conception: FZ. Experimental design and execution: SK, MD'A, HL, ET, PG, VA, CG, GF, and FZ. Data acquisition: SK, MD'A, HL, ET, PG, VA, CG, GF, JR, and FZ. Data analysis and interpretation: SK, MD'A, PG, ET, SH, J-CC, NC, and FZ. Preparation/revision of the manuscript: SK, MD'A, SH, MT-R, OH, FZ. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.607175/full#supplementary-material

SUPPLEMENTARY FIGURE 1 | Expression levels of plasticity- and stability-effector genes and their relative fold-change in Tregs cultured with MPPs when compared with Tregs alone. Mean expression levels are indicated with the standard deviation for each group (top-right corner). All FDR < 0.05; FC: Fold change; Blue: Treg alone; Orange: Treg+MPP.

SUPPLEMENTARY FIGURE 2 | Expression levels of additional genes of interest in Tregs cultured with MPPs when compared with Tregs alone. Mean expression levels are indicated with the standard deviation for each group. Down-regulated and upregulated genes are all members of K-means clusters c8 and c9, respectively. All p-values < 0.05; Blue: Treg alone; Orange: Treg+MPP.

SUPPLEMENTARY FIGURE 3 | Isolation of MPPs derived from IL-1b deficient C57BL/6 donor mice. Splenocytes recovered from G-CSF + Flt3L mobilized mice were magnetically sorted as c-kit+ cells, stained with CD34, Sca-1 and CD11b and sorted as c-kit+ Sca-1+ CD34+ CD11b-/low cells by flow cytometry.

SUPPLEMENTARY TABLE 1 | Functional annotations of the genes in K-means cluster c8, under-expressed in Treg cultured 4 days in presence of MPP *versus* Treg alone. FDR (false discovery rate obtained by bootstrapping 50 times) cutoff is at 0.05. Enrichments in GO (Gene Ontology) terms as well as gene symbols are provided for each significant GO category. Table is sorted by decreasing enrichment.

SUPPLEMENTARY TABLE 2 | Functional annotations of the genes in K-means cluster c8, over-expressed in Treg cultured 4 days in presence of MPP *versus* Treg alone. FDR (false discovery rate obtained by bootstrapping 50 times) cutoff is at 0.05. Enrichments in GO (Gene Ontology) terms as well as gene symbols are provided for each significant GO category. Table is sorted by decreasing enrichment.

SUPPLEMENTARY TABLE 3 | Statistics obtained after supervised differential gene expression analyses. Moderated t-test on linear modeling with empirical Bayes was applied on Treg alone vs. Treg+MPP. FDR: False discovery rate by Benjamini-Hochberg. Fold change is indicated in plain and centered on zero.

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Mobilized Multipotent Hematopoietic Progenitors Promote Expansion and Survival of Allogeneic Tregs and Protect Against Graft Versus Host Disease

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Allogeneic Hematopoietic Stem Cell Transplantation (Allo-HSCT) is routinely performed with peripheral blood stem cells (PBSCs) mobilized by injection of G-CSF, a growth factor which not only modulates normal hematopoiesis but also induces diverse immature regulatory cells. Based on our previous evidence that G-CSF-mobilized multipotent hematopoietic progenitors (MPP) can increase survival and proliferation of natural regulatory T cells (Tregs) in autoimmune disorders, we addressed the question how these cells come into play in mice and humans in an alloimmune setting. Using a C57BL/6 mouse model, we demonstrate that mobilized MPP enhance the immunosuppressant effect exerted by Tregs, against alloreactive T lymphocytes, both *in vitro* and *in vivo.* They do so by migrating to sites of allopriming, interacting with donor Tregs and increasing their numbers, thus reducing the lethality of graft-versus-host disease (GVHD). Protection correlates likewise with increased allospecific Treg counts. Furthermore, we provide evidence for a phenotypically similar MPP population in humans, where it shares the capacity to promote selective Treg expansion *in vitro.* We postulate that G-CSF-mobilized MPPs might become a valuable cellular therapy to expand donor Tregs *in vivo* and prevent GVHD, thereby making allo-HSCT safer for the treatment of leukemia patients.

Keywords: allogeneic HSCT, graft versus host disease, mobilization, multipotent progenitors, regulatory T cells, expansion, alloreactivity, mixed lymphocyte reaction

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INTRODUCTION

Allogeneic Hematopoietic Stem Cell Transplantation (allo-HSCT) is the only therapy that can cure some hematological malignancies resistant to standard anti-cancer treatment. Its success relies on the capacity of donor T cells to eliminate residual tumor cells, called the Graft-versus-Tumor or GVT effect. The drawback is that donor T cells also target alloantigens expressed on the cells of the recipient's organs, which leads to life-threatening complications, resulting in graft-versus-host disease (GVHD). Administration of Granulocyte Colony Stimulating Factor (G-CSF) is routinely used for allo-HSCT as a procedure to mobilize and harvest peripheral blood stem cells (PBSC) from healthy donors. Even though PBSC comprise a higher proportion of T cells than bone marrow (BM) allografts, they do not increase the incidence of acute graft-versus-host disease (GVHD) (1). To explain this paradox, we and other investigators have demonstrated that mobilization with G-CSF induces myeloid-derived suppressor cells (MDSCs) capable of reducing alloreactive T lymphocyte proliferation (2). Among these, some subsets have been associated with a lower incidence of acute GVHD. Unfortunately, in the context of alloimmunity, suppressing conventional T cells could enhance infection and relapse incidence (3). This adverse effect led to the implementation of an alternative strategy to enhance the contribution of regulatory T cells (Tregs). It has indeed been reported that freshly isolated donor Tregs (4, 5) or *ex vivo* expanded Tregs (6) added to donor $CD3^+$ T cells early after allo-HSCT, reduced GVHD, while maintaining the GVT effect. We knew also that G-CSF could mobilize murine $CD117^+Sca\text{-}1^+CD34^+CD11b^{-/low}$ multipotent progenitor precursor cells (mobMPP), which promoted, in turn, the expansion of regulatory T cells. The latter prevented spontaneous autoimmune type 1 diabetes in the NOD mouse model (7, 8) and modulated experimental autoimmune encephalomyelitis (EAE) in the C57BL6/J mouse model (9). These findings prompted us to investigate whether mobMPP could likewise contribute to Treg expansion during allo-HSCT. Having confirmed that *in vitro* these progenitors did effectively enhance Treg survival and proliferation in both mice and humans, we examined whether they could protect recipient mice from acute GVHD and improve their survival through expansion of natural allo-antigen-specific donor Tregs early after allo-HSCT. Furthermore, we verified whether human G-CSF-mobilized MPP shared similar properties.

MATERIALS AND METHODS

Mice

BALB/cJ ($H2^d$), C3H/HeJ ($H2^d$) and C57BL6/J ($H2^b$) mice were purchased from Janvier (Le Genest Saint Isle, France). Congenic (CD45.1) C57BL6/J ($H2^b$), $Foxp3^{GFP}$-C57BL6/J and CD45.1-$Foxp3^{GFP}$-C57BL6/J mice were kindly provided by Lucienne Chatenoud (Institut Necker Enfants Malades, INSERM U1151, Paris, France). All mice were bred and housed in a specific pathogen-free facility in microisolator cages and used at 8–12 weeks of age in protocols approved by the local Ethical Committee (CEEA34.0AP.018.11).

Recovery of mobMPP cells

C57BL6/J mice (B6) were injected subcutaneously for 5 consecutive days with recombinant human G-CSF (200 µg/kg/day) (Neupogen, Amgen, France) and recombinant murine Flt3-L (20 µg/kg/day) (Immunotools, Friesoythe, Germany). At day 6, spleen cells labeled with mAbs directed against mature lineage markers (CD4, CD8, Gr-1, CD45R, Ter119, CD11b) were depleted with anti-rat Ig-coated magnetic beads (Invitrogen Dynal, ThermoFisher Scientific, Cergy Pontoise, France). The lineage negative (lin^-) fraction was electronically sorted, as previously described (7). Briefly, mobMPP were stained with appropriately labeled mAbs: CD34, CD117, Sca-1, CD11b (eBioscience), after incubation with purified anti-CD16/32-mAbs to block non-specific FcR binding and electronically sorted as $CD117^+Sca\text{-}1^+CD34^+CD11b^{-/low}$ cells.

Human peripheral blood samples (10 ml EDTA-tube) from rhG-CSF-treated (filgastrim 10 µg/kg/day for 5 consecutive days) healthy allogeneic donors (randomly selected at Necker Hospital and Saint Antoine Hospital in Paris) were collected after informed consent. Ficoll-purified peripheral PBMC were stained with the following antibodies: CD34, CD38, CD45RA, CD90 (eBioscience, Life Technologies, Villebon-sur-Yvette, France) and sorted (FACS Aria II, BD Bioscience, Le Pont de Claix, France) as $CD34^+CD38^+CD90^-CD45RA^-$ mobilized MPP cells, and mobilized hematopoietic stem cells (mobHSC) identified by their $CD34^+CD38^-$ $CD90^+CD45RA^-$ phenotype.

Proliferation Assays

In mice, conventional T cells from B6 mice were purified by negative selection using a CD4 T cell depletion kit (Miltenyi Biotec, Bergisch-Gladbach, Germany). Purity was routinely above 98%. Natural Tregs were stained with appropriately labeled mAbs against CD4 and CD25 and electronically sorted as $CD4^+CD25^{high}$.

T cell activation (including conventional $CD4^+$ T cells, conventional $CD4^+CD25^-$ T cells and natural $CD4^+CD25^{high}$ Tregs) was performed with allogeneic dendritic cells or, alternatively by non-specific TCR activation, with anti-CD3 and CD28 antibodies. Mature dendritic cells were harvested from the spleen of BALB/cJ (or C3H/HeJ) mice with a pan-DC selection kit (Miltenyi Biotec). B6 T cells were stained with 5 µM carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen, Life Technologies, Villebon-sur-Yvette, France). In Mixed Lymphocyte Reaction (MLR), B6 T cells were mixed with DC stimulators at a ratio of five T cells for one DC, and plated in a 96-well round bottom culture plate (5.10^4 T cells/well). In some experiments, mobMPP were added at 5.10^4/well (1:1 T cell ratio), 10.10^4/well (2:1 T cell ratio) or 15.10^4/well (3:1 T cell ratio). In some experiments, Tregs were added at 5.10^4/well (1:1 T cell ratio) alone or with mobMPP at a 1:1:1 T cell ratio. Non-specific Treg activation was performed in a 96 round bottom plate coated with 10 µg/ml anti-CD3 mAb (clone 2C11) and 10 µg/ml anti-CD28 mAb (Biolegend) for 2 h before the addition of T cells (50,000/well). In some experiments, monoclonal IL-2 blocking antibody was used at 10 µg/ml (R&D Systems). Cells were incubated in custom RPMI 1640 medium supplemented with 10% fetal calf serum, 50 µM 2-mercaptoethanol and 100 U/ml penicillin-streptomycin (Life Technologies, Villebon-sur-Yvette, France).

Conventional human T cells were purified by negative selection using a pan T cell depletion kit (Miltenyi Biotec). Purity was routinely above 98%. Human naïve Tregs were sorted (FACS Aria II, BD Bioscience, le Pont de Claix, France) as $CD3^+CD4^+CD25^+CD45RA^+$ cells and conventional T cells were sorted ad $CD3^+CD4^+CD25^-$ T. They were plated at 50,000 cells/well in round-bottom 96-well microculture plates with or without mobMPP at a 1:1 T cell ratio in a final volume of 200 µl. Non-specific T cell activation was induced with 10 µg/ml anti-CD3 mAb (clone UCHT1, R&D Systems) and 10 µg/ml anti-CD28 mAb (clone 37407, R&D systems) for 2 h before adding T cells (50,000/well). In some experiments, monoclonal IL-1β blocking antibody was used (mouse anti-human clone 2805, R&D Systems).

Flow Cytometry

In vivo, for some experiments, allo-HSCT was performed with T cells from congenic CD45.1-$Foxp3^{GFP}$ B6 mice. Thereafter, donor natural Tregs were analyzed at days 8 and 30 post-transplantation, in lymph nodes and spleen using mAbs against CD45.1, CD3, CD4, CD25 (eBioscience).

In vitro, cultured Tregs were characterized with anti-CD4 and anti-CD25 (eBioscience). Foxp3 was measured following surface staining with fixation/permeabilization working solution according to the manufacturer's guidelines (eBioscience). Intracellular IL-2 cytokine (eBioscience) staining was performed after a 3-h incubation of cultured Tregs +/− mobMPP with PMA (10 ng/ml) plus ionomycin (500 ng/ml) and Brefeldin A (2 mg/ml) at 37°C and 5% CO_2.

In human healthy donors, mobMPP were stained with appropriately labeled mAbs against CD34, CD38, CD45RA, CD90, CD13 and CD33 (eBioscience). Tregs were labeled with anti- CD3, -CD4, -CD25 and -CD45RA, (eBioscience). To confirm that naïve Tregs were all $Foxp3^+$, sorted naïve Tregs $CD3^+CD4^+CD25^+CD45RA^+$ were fixed/permeabilized (Fix/Perm solution, eBioscience) and stained according to the manufacturer's instructions (eBioscience). For flow cytometric characterization of freshly sorted mobMPP, intra-cellular cytokine (IL-1β) staining was performed after 3-h incubation with brefeldin A (2 mg/ml). Flow cytometric characterization of Tregs +/−mobMPP after 4 days of culture, intracellular cytokine (IL-2, IFN-γ and TNF-α, eBioscience) staining was performed after 3-h incubation with PMA (10 ng/ml) and ionomycin (500 ng/ml) and brefeldin A (2 mg/ml) at 37°C and 5% CO_2. Samples were analyzed on a FACS Canto II cytometer (BD Biosciences) using FlowJo software (Treestar, Ashland, OR).

Acute GVHD

BALB/cJ recipients were lethally irradiated using 600 cGy total body X-ray irradiation on day -1 followed by intravenous caudal vein infusion of 10^7 T cell-depleted B6 donor bone marrow (BM) cells, 2×10^6 purified B6 T cells, with or without 0.35×10^6 B6 mobMPP on day 0. Control groups were transplanted with BM cells alone (syngenic group) or BM + T cells (control T cell group). For some experiments, 2×10^6 purified CD45.1-$Foxp3^{GFP}$-B6 T cells were used for allo-HSCT to analyze donor Tregs on day-8 and day-30 post-HSCT by flow cytometry. In other experiments, allo-HSCT was carried out with 0.35×10^6 CD45.1B6 mobMPP and 2×10^6 purified CD45.2-$Foxp3^{GFP}$-B6 T cells to evaluate donor mobMPP in lymph nodes and spleen at day-15 post-HSCT by confocal microscopy. AlloHSCT was performed with donor cells lacking Tregs, using BALB/cJ recipients having received a lethal 600 cGy total X-ray body irradiation on day- -1 followed by intravenous injection into the caudal vein of 10^7 T cell-depleted B6 donor bone marrow (BM) cells, 2×10^6 purified B6 $CD25^-$ T cells (control $CD25^-$ T cells group), and +/− 0.35×10^6 B6 mobMPP on day-0. All recipient mice were monitored daily for survival, and at least twice a week for weight and acute GVHD score. The clinical scoring system was based on six parameters as previously described (2): weight loss, posture, activity, fur texture, skin integrity, and diarrhea. A severity scale of 0–2 was used for each parameter, with a maximum global score of 12.

Confocal Microscopy Analysis of Recipient Lymph Nodes and Spleens

Spleens and lymph nodes from allografted BALB/c j mice were harvested on day 15 and immediately embedded in OCT (Sakura), snap frozen in liquid nitrogen, and stored at -80°C. Cryosections (7–9 µm) were cut, fixed in cold (-20°C) acetone for 10 min, rehydrated in washing buffer (TBS, pH 7.6), and exposed to blocking buffer (0.5% BSA and 10% goat serum in PBS) for 30 min at room temperature. Sections were then treated for 60 min at room temperature with rat anti-mouse CD3 and mouse biotin anti-CD45.1 antibody (eBioscience). Controls were set up without primary antibodies. Sections were washed 3–4 times, incubated with the secondary antibody followed by conjugated streptavidin-CY5 (20 min at room temperature). Sections were washed and mounted in Fluoromont-G (Southern Biotech). Images were acquired by confocal microscopy with a LSM 700 (Zeiss). Fluorescence of single channels was measured, and control reference was systematically done. Pictures were taken at 40× magnification. Images were analyzed and processed with ImageJ version 1.46.

Statistical Analysis

The results were averaged in each group. Statistical analyses were performed using GraphPad Prism software version 8, and data are presented as mean ± SEM. Before analysis, the normality assumption was examined. Comparisons were performed by one-way ANOVA with Tukey's multiple comparison when comparing more than two groups with normal distribution of values, by unpaired *t*-test for comparing two groups with normal distribution of values. Survival curves were compared by log-rank test. A p-value < 0.05 was considered statistically significant.

RESULTS

MobMPP Inhibit Alloreactive T Cell Proliferation by Expanding Alloreactive Tregs in Mice

We evaluated the effect of splenic mobMPP on alloreactive T cell proliferation induced by allogeneic dendritic cells during mixed lymphocyte reaction (MLR). We failed to observe a significant immunosuppression when either $CD4^+CD25^-$ T cells (**Figure 1A**) or total $CD4^+$ (including $CD25^+$ and $CD25^-$) T cells (**Figure 1B**)

were used, even if mobMPP were added at higher ratios (**Figure 1C**). By contrast, mobMPP became strongly immunosuppressant on alloreactive T cell proliferation when cultured with Tregs at a 1:1:1 ratio (**Figure 1D**). In fact, we observed that Tregs activated by allogeneic dendritic cells or alternatively by anti-CD3 and -CD28 antibodies, were expanded in terms of cell counts and proportion of divided cells, assessed by CFSE dilution, from day-4 of co-cultures (**Figure 1E**). To ensure that expanding Treg cells maintain their phenotype, we studied activated Tregs (+/− mobMPP) after 4 days of culture. We observed that Tregs maintained their $CD4^+CD25^{high}Foxp3^+$ phenotype (**Supplementary Data, Figure S1A**) and did not produce IL-2 in contrast with activated conventional T cells (**Supplementary Data, Figure S1B**). Notably, we observed that mobMPP did not produce IL-2 either (**Figure S1B**). In addition, Treg expansion by mobMPP occurred even in the presence of neutralizing anti-IL-2 antibody, demonstrating that mobMPP promoted Treg expansion by an IL-2 independent mechanism (**Supplementary Data, Figure S1C**).

Adoptively Transferred mobMPP Prevent Acute GVHD and Improve Donor Treg Expansion

We further investigated how mobMPP affected allogeneic HSCT in a murine model. Donor cells were electronically sorted as lin^-, $CD117^+$, $Sca\text{-}1^+$ $CD34^+$, $CD11b^-$ cells from the spleen of B6 mice having received G-CSF and Flt3-L injections (mobMPP). BALB/c mice allografted with 2.10^6 T cells together with mobMPP showed a significantly improved survival, as compared to allogeneic controls without mobMPP (**Figure 2A**). This group of recipients was protected against GVHD and weight loss (**Figure 2B**). Mice allografted with 2.10^6 $CD25^-$ T cells (devoid of Tregs) together with mobMPP did not survive significantly

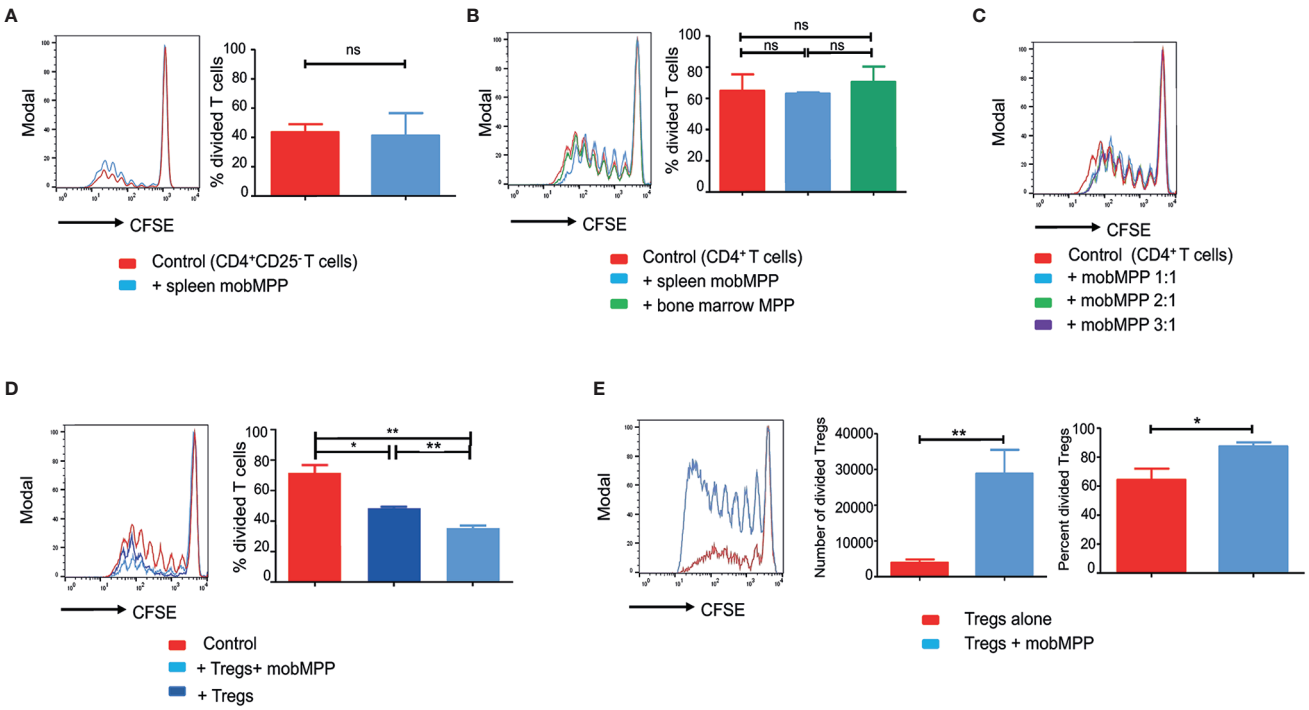

FIGURE 1 | G-CSF-mobilized MPP can suppress MLR by enhancing $Foxp3^+$ Treg proliferation. **(A)** CFSE dilution was assessed in B6 $CD4^+CD25^-$ T cell (50,000/well) activated by BALB/c DC (10,000/well) cultured alone (red) or with splenic mobMPP (blue) at a 1:1 ratio (50,000 $CD4^+CD25^-$ T:50,000 mobMPP/well). Percentage of divided T cells were 43.5± 3.17% in control, versus 41.23± 8.9% in coculture with mobMPP ($p = 0.80$). Data from 3 independent experiments (n = 3) were compared using Student's unpaired *t*-test (ns, not significant). **(B)** Total B6 $CD4^+$ T cells (containing both $CD25^-$ and $CD25^+$ $CD4^+$ T-cells) activated by BALB/c dendritic cells were cultured alone (red), with splenic mobilized MPP at a 1:1 ratio (light blue), or with non-mobilized bone marrow MPP at a 1:1 ratio (green). Percentages of divided T cells were 64.88± 10.48% in control, versus 63 ± 1.30% and 70.43 ± 17.19% in coculture with mobMPP ($p = 0.87$) or bone marrow MPP ($p = 0.73$), respectively. Data were compared using one-way ANOVA with Tukey's multiple comparison test (ns, not significant). **(C)** Total B6 $CD4^+$ T cells (containing both $CD25^-$ and $CD25^+$ $CD4^+$ T-cells) CFSE dilution is similar when B6 $CD4^+$ T cells activated by BALB/c DC were cultured alone or with mobMPP at 1:1 ratio (50,000 $CD4^+$ T cells:50,000 mobMPP/well). A higher number of MobMPP in the well (1:2 ratio (50,000 $CD4^+$ T cells: 100,000 mobMPP/well), or 1:3 ratio (50,000 $CD4^+$ T cells: 150,000 mobMPP/well) does not impact total B6 $CD4^+$T cell proliferation (one experiment, n = 1). **(D)** B6 $CD4^+CD25^-CD45.1$ T cells activated by BALB/c DC were cultured alone (red) or with CD45.2 Tregs at a 1:1 ratio (dark blue), or with CD45.2 Tregs and CD45.2 mob-MPP at a 1:1:1 ratio (light blue) (n = 3). Percentages of CD45.1 divided T cells were respectively 71.54 ± 5.27% in control, 48.52 ± 0.86% in the presence of Tregs, ($p = 0.0125$, *) and 35.49 ± 1.66% with both Tregs and mobMPP, (**$p = 0.0028$). The percentage of divided T cells was higher when both Tregs and mobMPP were present, rather than Tregs alone (**$p = 0.0023$). Data were compared using Student's unpaired *t*-test (ns, not significant). **(E)** B6 Treg cells activated with allogeneic BALB/c DC were cultured alone (red) or with mobMPP (blue) (n = 11). The number of Tregs in proliferation (≥ division 1) is lower when Tregs are cultured alone (3918 ± 905 vs. 28792 ± 6659, **$p = 0.0014$). Percentages of Tregs in proliferation (≥ division 1) are also lower when Tregs are cultured alone (64.28 ± 25.66% vs. 87.40 ± 9.26%, *$p = 0.0108$, using Student's unpaired *t*-test).

longer than allogeneic controls without mobMPP (**Figure 2C**), underlining the strict requirement for Tregs for the protective effect of mobMPP. Eight days after allo-HSCT, we found that mesenteric and inguinal lymph nodes contained an increased percentage of Tregs among the donor CD45.1 $CD4^+$ population, by contrast with spleen. This percentage could reach 42% one month after allo-HSCT in surviving recipients (**Figure 2D**). This accumulation of Tregs in recipient inguinal lymph nodes was confirmed by confocal microscopy, which revealed also that mobilized MPP and expanded Tregs were in close contact with each other (**Figure 2E**).

Adoptive Transfer of mobMPP Modulates Alloreactive T Cell Proliferation In Vivo

We further assessed whether *in vivo* expanded Tregs exhibited specific alloreactive immunosuppressive functions. For this purpose, we performed MLR with 50,000 $CD25^-$ B6 T cells/well. At day-8 post-transplantation, Tregs expanded from B6 CD45.1 donor cells in BALB/c recipients in the presence or absence of mobMPP were electronically sorted and added during MLR, at a very low ratio (10 T cell: 1 Treg). We observed that *in vivo* expanded Tregs could totally suppress MLR when B6 T cells were activated not only by BALB/c but also by C3H DC, demonstrating that *in vivo* expanded Tregs were highly suppressive and capable of hampering alloreactive responses non-specifically (**Figure 3A**). We further tested $CD45.1^+CD4^+CD25^{low}$ allografted T cells, on day 8 after transplantation. During MLR, only $CD4^+CD25^{low}$ T cells sorted from BALB/c mice transplanted with mobMPP exhibited a significantly reduced proliferation after stimulation by BALB/c DC, compared with proliferation of $CD4^+CD25^{low}$ T cells from allografted mice that had not received mobMPP. However, this suppression did not occur in the presence

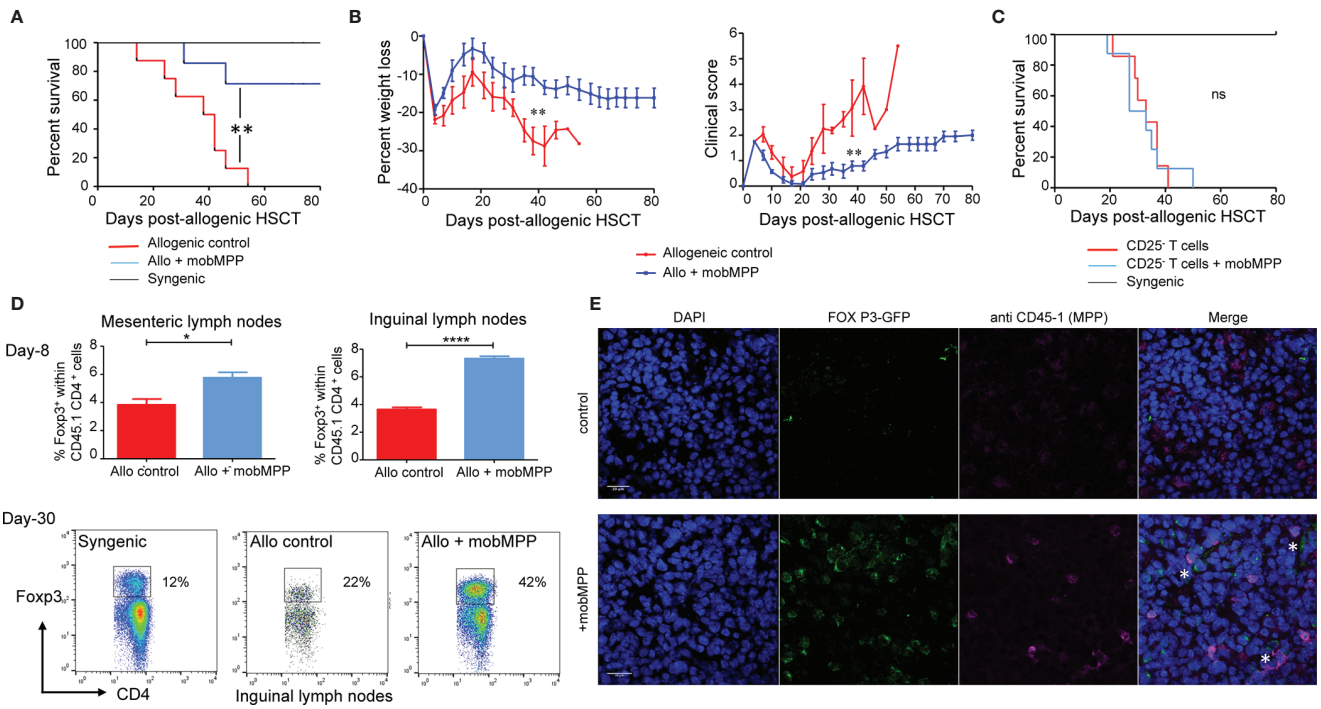

FIGURE 2 | G-CSF mobilized MPP prevent GVHD by enhancing $Foxp3^+$ Tregs *in vivo*. **(A)** Lethally irradiated (6 Gy) BALB/cJ recipients received 1×10^7 B6 T cell-depleted BM cells alone (black, n = 4) or together with 2×10^6 purified B6 T cells (allogeneic control, red, n = 7). In a third group 0.35×10^6 B6-derived mobMPP were co-transferred with the two other populations (blue, n = 7) on day 0. Less than 40% of mice having received co-transferred mobMPP died over 80 days whereas median survival was 40 days without mobMPP (**p = 0.004). Data shown represent pooled results from 2 independent experiments. Results were compared with Kaplan-Meier survival curves. **(B)** Mean weight loss of BALB/cJ recipients at day 40 post-HSCT (median survival group) was: -10.57 ± 2.28% in mice co-injected with mobMPP (n = 6) vs. -27.44% ± 3.60% in allogeneic controls (n = 4). Mean clinical GVHD scores in recipients at day 40 post-HSCT was: 0.8 ± 0.19 in mice co-injected with mobMPP (n = 6) vs. 3.06 ± 1.1 in allogeneic controls (n = 4). Data were compared using Student's unpaired *t*-test (**$p \leq 0.01$). **(C)** Lethally irradiated (6 Gy) BALB/cJ recipients received 1×10^7 B6 T-cell depleted BM cells alone (black, n = 4), with 2×10^6 purified $CD25^-$ B6 T cells (allogeneic control, red, n = 7) or with additional 2×10^6 purified $CD25^-$ B6 T cells and 0.35×10^6 B6-derived mobMPP (blue, n = 7). Median survival in mice having received allogeneic HSCT plus $CD25^-$ T cells was 30 days vs. 33 days in mice with co-transferred mobMPP (p = 0.52). Results were compared with Kaplan-Meier survival curves (ns, not significant). **(D)** Percentage of $Foxp3^+$ Tregs in allogeneic $CD45.1^+CD4^+$ B6 T cells were studied in control BALB/c mice (red) or in BALB/c mice transplanted with mobMPP (blue) (n = 3). The percentage of Tregs in the $CD45.1^+CD4^+$ T cells in mesenteric lymph nodes were 3.84 ± 0.71% vs. 5.76 ± 0.67%, (*p=0.0274), respectively. The percentage of Tregs in the $CD45.1^+CD4^+$ T cells in inguinal lymph nodes were 3.64 ± 0.28 vs. 7.31 ± 0.29%, (****$p < 0.0001$, using Student's unpaired t-test). **(E)** Representative confocal fluorescence micrograph of inguinal lymph nodes. BALB/c mice were sacrificed at day 15 post-allogeneic HSC and inguinal lymph nodes were harvested for confocal microscopy. Top: inguinal lymph node from control allografted BALB/c mice. Below: inguinal lymph node from allografted BALB/c mice with CD45.1 mobMPP (DAPI, blue; Foxp3, green; CD45.1 red; merged images show Foxp3/CD45.1 colocalization, indicated by *). Bar: 0.2 µm.

of C3H DC, suggesting that natural Tregs already suppressed alloreactive CD25^{low} T cells from day-8 post-transplantation onwards (**Figure 3B**). Therefore, mobilized MPP have contributed to expand highly suppressive donor natural Tregs that immediately suppressed alloreactive T cells in BALB/c recipients. It can therefore be concluded that *in vivo* Treg expansion by mobMPP confers allo-antigen specific T cell suppression to control GVHD in allo-HSCT.

Characterization of a Human mobMPP Counterpart Mobilized by G-CSF

We analyzed the CD34$^+$ fraction of PBSC from 30 healthy donors. It has been previously reported that human HSC are enriched in the Lin$^-$CD34$^+$CD38$^-$CD90$^+$ cell compartment of non-mobilized cord blood and bone marrow cells. By contrast, in mobilized PBSCs, the majority of the CD34$^+$ cells are CD38$^+$CD90$^-$ CD45RA$^-$ and homogeneously expressed low levels of CD13 and CD33 (**Figure 4A**). We distinguished mobMPP as CD34$^+$CD38$^-$CD90$^-$CD45RA$^-$ and mobilized hematopoietic stem cells (mobHSC) as CD34$^+$CD38$^-$CD90$^+$CD45RA$^-$. We tested these two main subsets in co-culture with naïve Tregs (described in **Figure 4A**). Only the CD34$^+$CD38$^+$CD90$^-$ CD45RA$^-$ subset (mobMPP) could increase naïve Treg proliferation (**Figure 4B**). MobMPP did not affect conventional (CD4$^+$CD25$^-$) T cell proliferation (**Figure 4C**). After four days of culture, naïve Treg cells were still Foxp3$^+$ (**Figure 4D**). To further investigate how mobMPP could enhance naïve Treg proliferation, we first prevented cell-to-cell contact in transwell cultures. We observed that naive Treg proliferation, induced by mobMPP, was not totally abolished in transwell cultures (**Supplementary Data, Figure S2A**). However, neither naïve Treg cells nor mobMPP were able to produce IL-2, TNF-α, and IFN-γ, the main cytokines involved in T cell proliferation (**Supplementary Data, Figure S2B**). Taking into account a previously suggested mechanism in mice (9), and higher IL-1β production reported in CD34$^+$ cells from PBSC relative to steady state bone marrow cells (10), we observed that 5% of freshly sorted mobMPP produce IL-1 β (**Supplementary Data, Figure S2C**). When neutralizing IL-1β by an IL-1β blocking antibody, naïve Treg survival and proliferation were reduced, although not fully abolished (**Supplementary Data, Figure S2D**).

DISCUSSION

Acute and chronic graft-versus-host diseases (GVHD) are major causes of morbidity and mortality after allo-HSCT. In mice, reduced frequency of Tregs contributes to both of these disorders (11). In humans, the incidence of natural Treg in peripheral blood (PB) is very low, amounting only to 1-2% (12), which is probably an overestimation because of contaminating CD25$^+$ T-effector cells (13). This percentage is inversely correlated with the

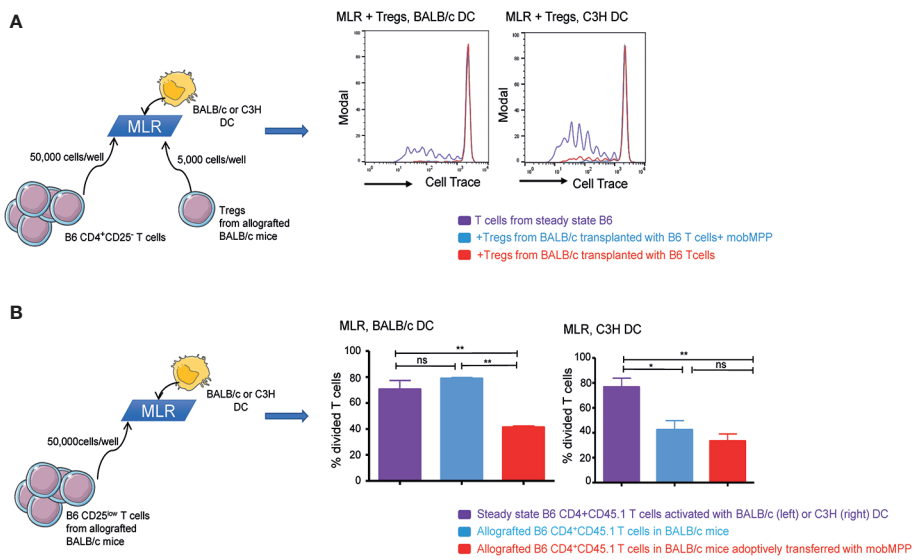

FIGURE 3 | G-CSF mobilized MPP induce tolerance. **(A)** Steady state B6 CD4$^+$CD45.1$^+$ T cells activated by BALB/c (left) or C3H (right) DC were cultured alone (purple) or with Tregs in 10:1 ratio (50,000 T cells: 5,000 Tregs). Tregs were sorted from the spleen of allografted control BALB/c mice (red), or from allografted BALB/c mice with mobMPP (light blue) (n = 3). CFSE dilution within CD45.1$^+$ T cells were compared. (One representative experiment out of 3). **(B)** Left: Steady state B6 CD4$^+$CD25^{low}CD45.1$^+$ T cells activated by BALB/c DC were analyzed for CFSE dilution (purple) and compared to CFSE dilution of allografted B6 CD4$^+$CD25^{low}CD45.1$^+$ T cells in BALB/c mice with (light blue) or without (red) adoptive transfer of mobMPP. Percentage of steady state B6 CD4$^+$CD25^{low}CD45.1$^+$ T cells in division is 70.57 ± 6.81% (purple) vs. 41.37 ± 1.03% (red), vs. 70.57 ± 6.81% (light blue), respectively $^{**}p \leq 0.005$ by one-way ANOVA with Tukey's multiple comparisons test. Right: Steady state B6 CD4$^+$CD25^{low}CD45.1$^+$ T cells activated by C3H dendritic cells were analyzed for CFSE dilution (purple) and compared to CFSE dilution of allografted B6 CD4$^+$CD25^{low}CD45.1$^+$ T cells in C3H mice with (light blue) or without (red) adoptive transfer of mobMPP. Percentage of steady state B6 CD4$^+$CD45.1 T cells in division is 76.60 ± 7.18% (purple) vs. 42.40 ± 7.35% (red), vs. 33.37. ± 5.69% (light blue), respectively, n = 3, ($^*p = 0.0275$, $^{**}p = 0.0097$; ns, not significant, by one-way ANOVA with Tukey's multiple comparisons test).

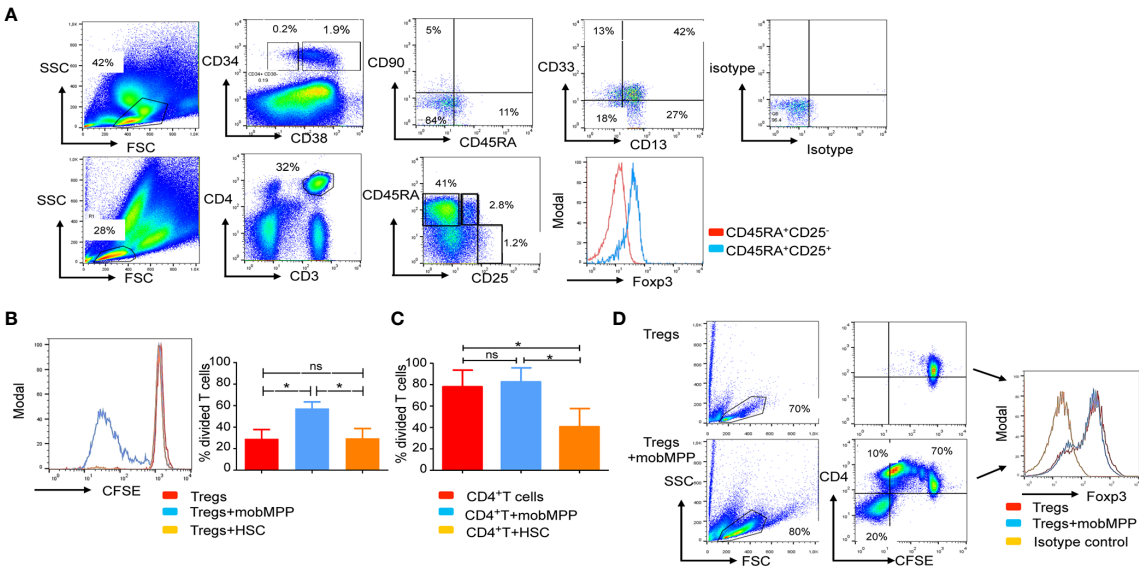

FIGURE 4 | Human mobilized MPP share features of immature myeloid cells endowed with naïve Treg proliferation properties. **(A)** Human G-CSF mobilized peripheral blood stem cells (PBSC) were analyzed by flow cytometry. We first distinguished the $CD34^+$ fraction from the $CD34^-$ fraction of cells. In the gated $CD38^+$ $CD34^+$ fraction, a population of cells that lacked CD90 and CD45RA, homogeneously expressed CD13 and CD33 and was identified and termed mobMPP. Mobilized hematopoietic stem cells (mobHSC) were distinguished as $CD34^+CD38^-CD45RA^-CD90^-$. Below, in the same PBSC, naïve Tregs defined as $CD3^+CD4^+CD45RA^+CD25^+$ were analyzed by flow cytometry. All gates were based on isotype control. **(B)** Anti-CD3 and -CD28 activated naïve Treg cell proliferation after 4 days of culture alone (red) with mobMPP (light blue) or with mobHSC (orange) at a 1:1 ratio (n = 3). Percentages of divided naïve Tregs were in control = 29.17 ± 8.58% vs. 60.77 ± 2.847% with mobMPP vs. 29.67 ± 9.06% with mobHSC, respectively. Data were compared using one-way ANOVA with Tukey's multiple comparison test (ns, not significant, $*p = 0.0371$). **(C)** Anti-CD3 and -CD28 activated $CD4^+CD25^-$ T cell proliferation after 4 days of culture alone (red) with mobMPP (light blue) or with mobHSC (orange) at a 1:1 ratio (n = 3). Percentages of divided T cells were in control = 78.03 ± 8.945 vs. 82.57 ± 7.583% with mobMPP, vs. 40.70 ± 9.815% with mobHSC respectively. Data were compared using one-way ANOVA with Tukey's multiple comparison test (ns, not significant, $*p = 0.0289$). **(D)** Naïve Treg cells activated by anti-CD3 and anti-CD28 were cultured alone or with mobMPP. After 4 days of culture, intra-nuclear Foxp3 was measured.

severity of acute (14) and chronic (14) GVHD. Among the strategies proposed to modulate and prevent GVHD, adoptive transfer of natural Tregs can reproducibly and efficiently prevent GVHD in murine models, but requires a high Treg dose (infusion of one Treg for one conventional donor T-cell) (15). Thus, in murine models, Treg expansion for adoptive transfer has been explored in a variety of approaches (16). In humans, co-cultures with diverse antigen-presenting cells have been investigated, such as donor-derived stimulated B cells, that expand the pool of alloreactive Tregs present in the blood (17, 18), as well as DCs (19) and artificial APCs (20). However, depending on the state of activation of Tregs, their proliferative capacity remains highly variable (21, 22) and the procedure is time-consuming. Furthermore, *ex vivo* expansion of natural Tregs (nTregs) must be anticipated far ahead of allo-HSCT. The safety and efficacy of *ex-vivo* expanded nTreg infusions have been attested (23, 24) and confirmed with a trend toward a lower incidence of acute grade II-IV GVHD. Even though, the technical obstacles associated with direct Treg adoptive transfer would make an alternative strategy leading to an enhancement of donor Tregs *in vivo* following allo-HSCT preferable. Mobilized MPP are interesting candidates for such an approach. They are readily available during HSCT, since the hematopoietic stem cells used are increasingly recovered from peripheral blood after G-CSF mobilization. Our evidence for the capacity of G-CSF-mobilized MPP to modulate autoimmunity (type 1 diabetes and EAE) through $Foxp3^+$ Treg expansion and stabilization provides a further argument for their possible benefit during allo-HSCT. Starting from these findings, we explored whether their immunosuppressive functions applied also to allo-HSCT.

Our results show that mobMPP can selectively enhance survival and proliferation of activated Tregs *in vitro*, both in mice and humans. Their failure to promote proliferation of $CD25^-$ cells may be considered an advantage over less selective options, such as IL-2 treatment (25), for which it is difficult to determine efficacious low doses, and foresee off-target effects, their short *in vivo* half-life being also problematic. Therefore, cellular therapy with mobilized MPP may provide a new method to expand *in vivo* alloantigen specific Tregs conferring protection against GVHD. Indeed, we observed that donor nTregs in mobMPP co-transferred recipients were rapidly expanded at allopriming sites and already exerted allospecific T-cell suppression at day-8 post-transplantation.

We observed that only mice given at least $0.35.10^6$ mobMPP had significantly improved survival. In fact, cohorts receiving $0.1.10^6$ mobMPP at the time of transplantation, did not significantly improve their survival (data not shown). The impact

of higher mobMPP doses (>0.35.10^6/mouse) on recipient outcomes was not studied. First, to avoid using excessive number of donor mice since one mobilized B6 mice was necessary to obtain 1.10^5 mobMPP. Moreover, we assume that the dose of 3.5.10^5 mobMPP represents a relevant ratio for coinjection with 2.10^6 $CD3^+$ T cells containing approximately 1.4 to 1.8.10^5 natural Tregs in mice. Notably, the number of mobMPP required for protection in the allogeneic setting of GVHD is 10 to 30-fold higher than in autoimmune settings where as few as 10,000 and 25,000 mobMPP were sufficient to provide protection against spontaneous type 1 diabetes (7, 8) and Experimental Autoimmune Encephalomyelitis (9), respectively. This difference may reflect the multiple target tissues in GVHD where mobMPP may have to migrate to and control the allogeneic response, in contrast with the tissue specificity characterizing the autoimmune experimental settings.

Tregs from GVHD patients exhibit multiple defects, including instability of Foxp3 expression, impaired suppressive functions, decreased migratory capacity and increased apoptosis (26). Since we demonstrated the stability of MobMPP-expanded Tregs at the molecular level (9), we assume that *in vivo* expanded nTregs are less prone to differentiate into effector T cells during the cytokine storm post-HSCT (27, 28), a differentiation reported in inflammatory responses (29). In the same manuscript, we demonstrated that mobilized MPPs increase the transcription factor t-Bet in Tregs which enables them to suppress pathogenic Th1 cells and induces the expression of chemokine receptors, particularly CXCR3, which condition the migration of cells toward Th1 inflamed GVHD target organs (30). Finally, we demonstrated that Treg expansion by mobilized MPPs was partly mediated through IL-1β in mice. In the present study we identified a human mobMPP counterpart sharing most likely a similar mechanism to promote Treg expansion. IL-1β has been previously reported as a mediator involved in the induction of $Foxp3^+$ Tregs in response to CD8α DCs exposed to GM-CSF (31). Moreover, these Tregs had a significantly higher ability to maintain Foxp3 expression when activated in the presence of IL-1β, which enhanced their capacity to suppress the effector T cell response *in vitro* and ongoing experimental autoimmune thyroiditis *in vivo* (32).

In humans the expression of inflammatory cytokine receptors IL-1R1 and TNFR2 has been described for being higher on resting mature Tregs than on naïve or memory T cells. While both receptors were upregulated upon activation through the T cell receptor (TCR), on all T cell subsets, IL-1R1 expression was maintained at significantly higher levels on activated Tregs than among other T cell subsets (33). Collectively, these results suggest that IL-1β-IL-1R signaling between mobMPP and Tregs could be a potent and safe mechanism to enhance natural Tregs *in vivo* in mice and should be explored in humans. It is noteworthy that the effect of IL-1β on GVHD depends critically on the timing of its intervention during allo-HSCT (34). Notably, in humans, IL-1β blockade during the period of initial T-cell activation had no impact on the cumulative incidence of acute GVHD (35).

Altogether, our results support the notion that mobilized MPPs could become useful as a novel cellular therapy to expand functional alloantigen specific Tregs *in vivo* that may lead to a considerable improvement of allo-HSCT safety by preventing GVHD.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by REG Allo NCTC02194868, CPP 24/04/2014, CCTIRS 18/06/2014, CNIL 17/04/2015. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Paris Descartes University Ethical Committee and Ministry of Education and Research Ethical Committee (CEEA34.0AP.018.11).

AUTHOR CONTRIBUTIONS

MD'A and FZ designed the experiments, interpreted the data, and wrote the manuscript. MD'A, A-BN, VAA, AB, PG, MDC, and GF performed the experiments. MG-T provided technical support, and analyzed and interpreted the confocal microscopy. M-TR and OH provided the human HSC grafts. FZ, M-TR, and OH provided a critical review of the data and manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.607180/full#supplementary-material

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REVIEW
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Hematopoietic Stem Cells in Type 1 Diabetes

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Despite the increasing knowledge of pathophysiological mechanisms underlying the onset of type 1 diabetes (T1D), the quest for therapeutic options capable of delaying/reverting the diseases is still ongoing. Among all strategies currently tested in T1D, the use of hematopoietic stem cell (HSC)-based approaches and of teplizumab, showed the most encouraging results. Few clinical trials have already demonstrated the beneficial effects of HSCs in T1D, while the durability of the effect is yet to be established. Investigators are also trying to understand whether the use of selected and better-characterized HSCs subsets may provide more benefits with less risks. Interestingly, *ex vivo* manipulated HSCs showed promising results in murine models and the recent introduction of the humanized mouse models accelerated the translational potentials of such studies and their final road to clinic. Indeed, immunomodulatory as well as trafficking abilities can be enhanced in genetically modulated HSCs and genetically engineered HSCs may be viewed as a novel "biologic" therapy, to be further tested and explored in T1D and in other autoimmune/immune-related disorders.

Keywords: type 1 diabetes, hematopoietic stem cells, autoimmune response, NOD mouse model, genetic modulation

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INTRODUCTION

Hematopoietic stem cells (HSCs) have been extensively used as an effective therapeutic approach in hematological malignancies and have demonstrated to be safe in human subjects (1). Over the last 10 years, several studies documented the extraordinary immunoregulatory properties of HSCs, which render them a potential useful tool in the fight for immune-mediated diseases (2). Despite being in limited number in the circulating blood of healthy individuals, HSCs are extremely potent and able to suppress the immune system response, as several *in vitro* and *in vivo* studies have shown (2). Based on these premises, the use of HSCs has been tested in numerous autoimmune diseases such as type 1 diabetes (T1D), multiple sclerosis (MS), systemic sclerosis, systemic lupus erythematosus and Chron's disease, with relevant benefits (3–6). Indeed, HSCs may reset the immune response, thus reshaping the chronic derangement of the immune system to a more self-tolerant state (7, 8). Interestingly, it has been also demonstrated that the bone marrow-derived and blood HSCs are altered in some autoimmune conditions such as T1D and MS, with HSCs being

scanty in the circulation and often unable to exploit their immunoregulatory function (9–11). Here we are presenting major advances in the preclinical and clinical studies of HSCs in T1D. We report recent insights coming from novel T1D *in vivo* research and provide an update on the most relevant clinical studies that have been performed by using HSCs in human subjects with T1D. In this perspective, we envision to consider HCSs as a novel "biologic", which can be personalized and modeled, as a novel relevant therapeutic option in T1D.

HSCs IN TYPE 1 DIABETES: THE MURINE SCENARIO

The rationale behind the use of HSCs in autoimmune disease such as T1D has been extensively studied in the last decade by taking advantage of the NOD mouse model. This mouse spontaneously develops autoimmune diabetes at the age of 12–15 weeks, with severe hyperglycemia (12, 13). However, signs of activation of the immune system against pancreatic islets are already visible at 8–10 weeks of age when the NOD mouse shows insulitis with an abundant T cell infiltrate (12). Over the last two decades, two major HSCs-based strategies have been pursued to prevent the onset of experimental autoimmune diabetes in murine models: (i) HSCs have been infused to induce mixed chimerism and to re-establish the peripheral deletion of autoreactive T cells, (ii) HSCs have been genetically engineered to reshape the immune reservoir and facilitate tolerance towards auto-antigens. The use of HSCs infusion was extremely successful in preventing diabetes onset in NOD mice through the induction of a mixed chimerism. Indeed, a deletion of autoreactive T cells generated at the thymus level (14) as well as the re-establishment of immune tolerance in the periphery were obtained. Furthermore, in the presence of a tolerogenic network between donor Regulatory T cells (Tregs) and host-donor dendritic cells (DCs), costimulatory pathways, particularly PDL-1, play a major role (15). However, the HSC-mediated chimerism, despite effective in reshaping the autoimmune response, requires the use of myeloablative agents/approaches, which may further limit translational applications (16, 17). Given that common polymorphisms exist in MHC class II in T1D patients and in NOD mice, which confer a higher risk of developing T1D, genetically engineering of single HSCs to express the proper and protective MHC class II, held great promises in the new therapies in T1D (18). Indeed, the introduction of new protective MHC class II through lentiviral delivery in HSCs of NOD mice was able to prevent the onset of T1D, mainly through the deletion of autoreactive T cells which did not engage in the MHC class II-mediated response (19, 20). While this approach was again limited by the need of immune ablation for the HSCs infusion, which is feasible in NOD mice but at high risk in humans, it paved the way for exploring genetic engineering of HSCs to better exploit their multiple properties in autoimmunity. *Ex vivo* genetic manipulation of NOD HSCs, to encode proinsulin and transgenically target MHC class II, successfully prevented T1D onset (21, 22). Also, HSCs can be engineered for tolerogenic purposes such as those aimed at inducing tolerance to autoantigens or at replacing genetic alleles associated with increased disease susceptibility (23). In view of this, some studies explored whether HSCs in diabetic NOD mice are altered and might be fixed through genetic engineering or pharmacological modulation. Elevated levels of CXCL12 (SDF-1) in bone marrow-HSCs of NOD mice have been suggested to alter trafficking of HSCs and Tregs in the periphery, thus favoring the onset of T1D (24). The use of ADAM3100, which antagonizes the CXCL12 receptor SDF-1, was associated with increased mobilization of HSCs and T cells, and delayed onset of experimental autoimmune diabetes in NOD mice (24). Recently, a defect in PDL-1 expression has been demonstrated in HSCs of NOD mice, which was associated with a reduced immunomodulatory function (9, 25). Genetic and pharmacological modulation of PDL-1 on HSCs restored the HSCs immunomodulatory properties, reset the immune balance and prevented the onset of T1D. In summary, all the aforementioned studies support the use of *ex vivo* manipulation of HSCs in the NOD mouse model as a successful tool to delay the onset of autoimmune diabetes. Genetic engineering of HSCs has been recently employed in a humanized mouse model in which *ex vivo* manipulated human HSCs successfully restored the development of functional Tregs and rescued the autoimmune IPEX syndrome (26). Recently, the introduction of the NOD-Rag1null IL2rγnull Ins2Akita (NRG-Akita) mouse, a humanized mouse model available in diabetes research which develops spontaneous hyperglycemia, fostered studies in the field (27, 28). This model, in which human immune cells can be infused without being rejected, may be extremely useful in testing the potency of newly genetically engineered human HSCs in the diabetes prevention.

HSCs IN TYPE 1 DIABETES: THE HUMAN LANDSCAPE

In the last 20 years, autologous hematopoietic stem cells transplantation (AHSCT) has been used in several clinical trials to treat refractory autoimmune disease such as multiple sclerosis (MS), systemic sclerosis (SSc), systemic lupus erythematosus (SLE), Crohn's disease (CD), type 1 diabetes (T1D) and a range of other immune-mediated disorders (29). With regard to patients with T1D, the use of AHSCT obtained significant insulin independence and a well-preserved glycometabolic control in the short and mid-term follow-up (**Figure 1A** and **Table 1**) (17, 31, 34, 35). Also, an increase in C-peptide levels and C-peptide area under the curve (AUC) measurement were detectable in AHSCT-treated T1D patients as compared to baseline, and only minor adverse events were registered in the mid-term (25, 36). A reduction of the T-helper-1 and T-helper-17 subsets was also observed in the short-term (37). Interestingly, a cost-effectiveness analysis conducted in patients with T1D undergoing AHSCT as compared to patients with T1D remaining on insulin therapy demonstrated that AHSCT provides some benefits over time depending on the

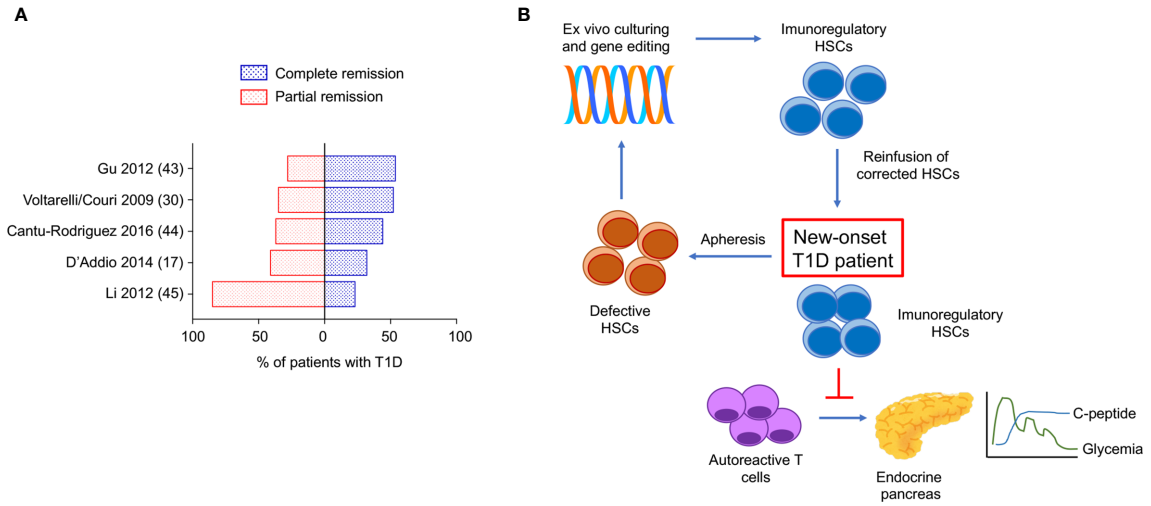

FIGURE 1 | Complete/partial remission of type 1 diabetes obtained with AHSCT in the long-term. Proposed genetic engineered HSC-based approach to target type 1 diabetes. **(A)** Proportion of patients with T1D undergoing AHSCT who achieved complete remission (insulin independence) and partial remission (low dose exogenous insulin requirement) at the latest timepoint analyzed within each clinical study registered in ClinicalTrials.gov available as publication. **(B)** Use of genetically engineered HSCs to target T1D: proposed approach. T1D, type 1 diabetes; AHSCT, autologous hematopoietic stem cell transplantation.

TABLE 1 | Summary of main characteristics, clinical outcomes and results obtained in the clinical studies conducted in T1D and registered in ClinicalTrials.gov.

Clinical Study	N of pts Follow-up Type of study	Clinical outcomes	Main results
Autologous Hematopoietic Stem Cell Transplantation for Early Onset Type 1 Diabetes (NCT00807651)	28 pts T1D 3 years Monocentric prospective	EIR, HbA1c, C-peptide and anti-GAD level	Insulin independence: 53.6% Increased C-peptide level (30)
Safety and Efficacy Study of Autologous Stem Cell Transplantation for Early Onset Type I Diabetes Mellitus (NCT00315133)	23 pts T1D 5 years Monocentric prospective	C-peptide level Morbidity/ mortality EIR changes HbA1c level	Insulin independent: 52% Low EIR: 35%; C-peptide AUC increase, HbA1c <7% (31)
Hematopoietic Stem Cell Transplantation in Type 1 Diabetes Mellitus (NCT01121029)	15 pts T1D 3 years Monocentric prospective	EIR C-peptide and HbA1C	Insulin independent: 44% HbA1c decrease: 2.3% Mortality: 0% (32)
Efficacy and Safety Study of Autologous Hematopoietic Stem Cell Transplantation to Treat New Onset Type 1 Diabetes (NCT01341899)	13 pts T1D 4 years Monocentric prospective	C-peptide and HbA1C Islet autoantibodies Immune profile Survival	3/13 pts: no insulin 11/13 pts low EIR, reduced HbA1C low autoantibodies increased C-peptide (33)
Stem Cell Mobilization (Plerixafor) and Immunologic Reset in Type 1 Diabetes (T1DM) (NCT03182426)	60 pts T1D 2 years Monocentric prospective	C-peptide AUC, EIR HbA1C <7% Hypoglycemia Autoantibodies titer	Not available

pts, patients; T1D, type 1 diabetes; AUC, area under the curve.

duration of preserved glycated hemoglobin levels achieved with AHSCT, but overall being cost-effective for treatment of T1D if the AHSCT lasts from 3 to 8 years (38). Long-term follow-up analyses for AHSCT-treated T1D patients were only reported for a few studies, due to the high number of patients lost at follow-up and the worldwide spreading of the studies, which also accounted for a small sample size, missed randomization process, lack in standardized procedures and enrolment of a heterogenous patients' population (39). Despite all these limitations, a minor percentage of relapse in the autoimmune disease was evident between 4 and 6 years of follow-up after the AHSCT, which varied among centers and lead few patients to resume insulin treatment (40, 41). Moreover, a subgroup of AHSCT-treated patients exhibited a prolonged remission and remained insulin independent for more than 4 years, thus leading to hypothesize that the response to the AHSCT treatment may differ in patients with T1D (17, 35, 42). Indeed, Malmegrim and Colleagues demonstrated that a different immune profile exists between patients experiencing short and prolonged remission, with the latter exhibiting lower frequencies of effector-memory CD4 T cells and islet-specific autoreactive CD8 T cells, paralleled by a detectable expansion of immunoregulatory T cells (35). Moreover, the favorable outcome of AHSCT in T1D was also associated with a less islet-specific autoreactive immune profile at baseline, thus delineating a subgroup of patients with T1D who may benefit the most from an AHSCT-based strategy (43, 44). This also emphasized the importance of the conditioning regimen, which

may need to be employed in association with AHSCT in patients with a high level of autoimmune response. In summary, results of the use of HSC-based approach, primarily the AHSCT, in patients with T1D (**Table 1**), suggest two major observations: (i) AHSCT treatment has to be limited to a subgroup of T1D patients and it requires high-level immunosuppression to obtain long-term effect, and (ii) the immune profile of T1D patients plays a central role in the achievement of long-term insulin-independence when using HSC-based strategies. Therefore, the infusion of a subset of HSCs, rather than the whole HSCs pool, such as in AHSCT, endowed with immunoregulatory properties may provide additional benefits in terms of balancing autoimmunity and achieving the proper clinical and metabolic outcomes.

CONCLUSIONS AND FUTURE DIRECTIONS

The use of HSCs has hold great promises in the treatment of autoimmune diabetes, however, in the last decade. The results obtained in clinical trials with the use of AHSCT in T1D suggest a potential novel approach to treat autoimmune diseases, despite all the aforementioned limitations. The use of a selected subset of HSCs endowed with immunoregulatory properties, without the need of additional immunosuppressive agents remains unexplored so far and deserves more investigation and testing from the scientific community. Patients with T1D who may benefit the most from this therapeutic approach need to be carefully identified, probably based on disease stages, degree of cellular and humoral autoimmune response, presence or not of diabetic ketoacidosis (17). The recent findings on the use of teplizumab in patients at risk for T1D (45) confirmed that immune ablation aimed at preventing T1D onset is a hot topic. HSCs, endowed with immunomodulatory properties, may offer a potent immunoregulatory effect without inducing T lymphocytes depletion, which is commonly observed with teplizumab. Indeed, several studies demonstrated that in absence of "healthy" HSCs central tolerance may be difficulty obtained. Autoimmune disorders, particularly type 1 diabetes, are associated with altered HSCs, which fail in exerting their immunomodulatory properties. Strategies aimed at targeting this defect successfully delayed diabetes onset in murine models. Feasibility and effectiveness in of the *ex vivo* manipulation and genetic engineering of HSCs are well-established in mouse models, while studies on safety for translational purposes are still required. In view of this, the use of humanized mouse model may accelerate the translation from murine experiments to human studies. The outstanding results collected in the past and ongoing clinical trials are encouraging in pursuing the research around the use of genetic engineered-HSCS in type 1 diabetes. Therefore, in our opinion, genetic modulation to reset HSCs physiological function, may find an interesting field of application not only in type 1 diabetes (**Figure 1B**) but in other autoimmune conditions too. Finally, in the era of the development of biologic therapy to treat immune-mediated diseases, we envision genetically engineered HSCs as a novel "biologic" agent and a "natural immunosuppressant" to be considered in the portfolio of alternative therapeutic options in type 1 diabetes and autoimmune diseases.

AUTHOR CONTRIBUTIONS

IP and EA wrote the paper. AB, MB, AM, CL, VU, AS, and AA, collected clinical and preclinical data. GZ edited the paper. FD'A and PF conceived the idea, wrote and edited the paper. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: PF and MB hold a patent of modulated HSCs and founded Altheia Science.

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Sex Matters: Physiological Abundance of Immuno-Regulatory CD71+ Erythroid Cells Impair Immunity in Females

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Mature erythrocytes are the major metabolic regulators by transporting oxygen throughout the body. However, their precursors and progenitors defined as CD71+ Erythroid Cells (CECs) exhibit a wide range of immunomodulatory properties. Here, we uncover pronounced sexual dimorphism in CECs. We found female but not male mice, both BALB/c and C57BL/6, and human females were enriched with CECs. CECs, mainly their progenitors defined as CD45+CECs expressed higher levels of reactive oxygen species (ROS), PDL-1, VISTA, Arginase II and Arginase I compared to their CD45– counterparts. Consequently, CECs by the depletion of L-arginine suppress T cell activation and proliferation. Expansion of CECs in anemic mice and also post-menstrual cycle in women can result in L-arginine depletion in different microenvironments *in vivo* (e.g. spleen) resulting in T cell suppression. As proof of concept, we found that anemic female mice and mice adoptively transferred with CECs from anemic mice became more susceptible to *Bordetella pertussis* infection. These observations highlight the role of sex and anemia-mediated immune suppression in females. Notably, enriched CD45+CECs may explain their higher immunosuppressive properties in female BALB/c mice. Finally, we observed significantly more splenic central macrophages in female mice, which can explain greater extramedullary erythropoiesis and subsequently abundance of CECs in the periphery. Thus, sex-specific differences frequency in the frequency of CECs might be imprinted by differential erythropoiesis niches and hormone-dependent manner.

Keywords: CD71+ erythroid cells, females, immune system and sex, anemia and immunity, anemia and infection

INTRODUCTION

The role of sex as an important biological variable has received significant attention in recent years. In 1993, the National Institutes of Health (NIH) decided to fill this gap by including women in clinical trials/studies to ensure that research outcomes apply to the whole population (NIH Revitalization Act of 1993 Public Law 103-43). With the emergence of personalized medicine and rejection of the one-size-fits-all therapeutic approaches, the significance of sex as a variable, and its impact on preventive, diagnostic, and therapeutic strategies have become more prominent (1). Males and females show explicit differences in their immunological profiles under physiological and pathological conditions. Generally, females mount a more robust cellular and humoral immune response, which results in efficient pathogen elimination and greater vaccine efficacy (2, 3). However, a strong immune response increases the risk of autoimmune diseases which is four times higher in females (3). In the context of innate immunity, there are several differences between males and females in terms of antigen recognition by Toll-like receptors (TLRs), frequency, and functionality of innate immune cells such as natural killer (NK) and dendritic cells (DCs) (4–6). Similarly, the impact of sex on different aspects of adaptive immunity has been widely documented. For example, it has been reported that human females have higher CD4+ T cells whereas CD8+ T cells are more abundant in males (3, 7). Although the Th1–Th2 dichotomy in males and females has been inconsistent (8), naïve CD4+ T cells in human females tend to produce more IFN-γ whereas their counterparts in males produce more IL-17 (9). Mouse studies on regulatory T cells (Tregs) have provided contradictory results but a human study has reported higher Tregs in males versus females (10). It is well acknowledged that genes associated with sex chromosomes, reproductive organs, and sex hormones are the main mechanisms behind such differences (2, 3, 6). Other environmental factors such as nutrition, microbiome, and lifestyle can affect immune responses in both sexes but these are usually considered as gender-associated factors (3).

Despite the extensive work on the influence of sex on different immune cell lineages, the effect of sex on erythrocytes has not been fully understood. Early studies have shown that men and women differ in their erythrocyte parameters including the size, count, and hemoglobin content (11). For example, reticulocytes were shown to be more enriched in the blood of women than men and suggested to be a compensatory response to the blood loss during menstruation (12). Moreover, higher levels of bone marrow-derived circulating progenitor cells with differential potentials for multiple lineages such as hematopoietic and endothelial cells in females are reported before the menopause age (13, 14). However, the impact of sex on the frequency of erythroid precursors and progenitors has not been well investigated.

In recent years, the physiological and pathological abundance of immunomodulatory erythroid precursors/progenitors defined as CD71+ Erythroid Cells (CECs) has received significant attention (15–17). CECs co-express CD71, the transferrin receptor, and TER119, the erythroid lineage marker, in mice but CD71 and CD235a in humans (18, 19). CECs mediate their immunosuppressive functions *via* cell–cell interaction and/or soluble mediators such as Arginase I (Arg I), Arginase II (Arg II) and reactive oxygen species (ROS) (15, 17, 19–22). CECs are highly abundant in neonatal mice up to 4-weeks of age regardless of sex and similarly up to 6 months in human newborns (18, 19, 23). Because of the extramedullary erythropoiesis (EE) (21), CECs expand in the peripheral blood during pregnancy in humans and mice (24, 25). EE induction requires hematopoietic stem cell (HSCs) activation and mobilization, which depends on the estrogen receptor-α (ERα) in HSCs (26). This suggests that sex hormones can influence EE that takes place mainly in the spleen and liver (21). Although adult mice and humans have a significantly lower frequency of CECs compared to neonates (18), there is no evidence about the potential impact of sex on their proportion. Therefore, we decided to investigate the frequency of CECs and their immunological properties in the spleen of female and male mice using two commonly used mouse strains, BALB/c and C57BL/6, and also in the peripheral blood of men and women.

MATERIAL AND METHODS

Animals

Male and female BALB/c and C57BL/6 mice were purchased from the Charles River Institute. All animals were maintained and bred under pathogen-free conditions within the animal care facility at the University of Alberta. Throughout our studies, we used age-matched male and female mice (8–10 weeks). For anemia induction, BALB/c mice were injected intraperitoneally (i.p.) with 60 μg of the anti-TER119 antibody (Bio XCell).

Ethics Statement

This study was conducted under the recommendations in the Guide for the Care and Use of Laboratory Animals of the Canadian Council for Animal Care. The protocol was approved by the Animal Ethics Board of the University of Alberta (Protocol # AUP00001021). Similarly, the appropriate Institutional Review Boards at the University of Alberta approved the human studies (Protocol # Pro00046080). All study participants gave written informed consent to participate in the study.

Human Sample Collection and Processing

Blood samples were obtained from both male and female age-matched healthy controls. The study subjects were between the age of 20 and 50 without any underlying conditions and no clinical evidence of anemia. For some studies, blood samples were collected from females a week before their menstrual and 3–5 days' post-menstrual cycle. Thereafter, peripheral blood mononuclear cells (PBMCs) were isolated over Ficoll–Hypaque gradients. For CEC isolation, blood samples were stained using anti-CD71 or isotype control biotin-conjugated antibody and fractioned using streptavidin-linked magnetic beads (Miltenyi Biotec) (19).

Antibodies and Flow Cytometry

Fluorophore or biotin-conjugated antibodies with specificity to mouse cell surface antigens and cytokines were purchased from the BD Biosciences or Thermo Fisher Scientific. Specifically, the following antibodies were used for mice: anti-CD71 (R17217 and C2F2), anti-Ter119 (TER-119), anti-CD45 (30-F11), anti-VISTA (MIH64), anti-PDL-1 (MIH5), anti-CD11b (M1/70), anti-CD11c (N418), anti-CD3 (145-2C11), anti-CD4 (GK1.5), anti-CD8a (53-6.7), anti-IFN-γ (XMG1.2), anti-F4/80 (6F12), anti-CD169 (Siglec-1, 3D6.112), anti-VCAM-1 (51-10C9), anti-Arg I (IC5868A R&D) and anti-Arg II (ab81505, Abcam). For human studies, the following fluorophore or biotin-conjugated antibodies with specificity to surface markers or cytokines were used: anti-CD3 (HIT3a), anti-CD4 (RPA-T4), anti-CD8 (RPA-T8), anti-CD45 (H-130 or 2D1), anti-IFN-γ (4SB3), anti-CD71 (MA712), Ki67 (20Raj1), anti-CD69 (FN50), anti-Arg II (ab81505, Abcam), and anti-CD235A (HIR2). ROS staining (Sigma) was performed by flow cytometry per the manufacturer's protocols and our previous reports (25, 27). Live/dead fixable dead cell staining (ThermoFisher) was used to exclude dead cells in flow cytometry. For proliferation studies, CFSE labeling was performed per our previous protocols (28, 29). Paraformaldehyde fixed cells were acquired by flow cytometry using the LSRFORTESSA flow cytometer (BD) and analyzed with FlowJo software (version 10.7.1).

Co-Culture and Stimulation

For *in vitro* intracellular cytokine staining, human PBMCs or mice splenocytes were cultured and stimulated with anti-CD3/CD28 (3 and 1 μg/ml, respectively) in RPMI media supplemented with 10% FBS for 6 h in the presence or absence of CECs according to our previous reports (30, 31). For co-culture, a fixed number (1×10^5) of isolated T cells were seeded onto 96-well round bottom plates individually or with isolated CECs at different ratios with Brefeldin A (10 μg/ml). For some experiments, L-arginine (2 mM) was added at the time of stimulation to abrogate the effects of Arg II *in vitro* (19). For mice studies, splenocytes were harvested, and single-cell suspension was made by grinding between sterile frosted glass slides in RBC lysis buffer and filtering through nylon mesh as we have reported elsewhere (19). Splenocytes were washed by centrifugation and used for subsequent *in vitro* studies. In some experiments, T cells were isolated using the T cells isolation kit (Stem Cell Technologies) and CECs using a biotin-conjugated antibody and fractioned using streptavidin-linked magnetic beads (Miltenyi Biotec). To distinguish donor cells in co-culture experiments, we used mice with different congenic markers (e.g. CD45.2 and CD45.1 in the case of C57BL/6) of labeled donor cells with the CFSE dye (BALB/c mice).

Infection Model

Bordetella pertussis strain Tomaha I was cultured on Bordet–Gengou agar supplemented with 15% sheep blood as we described elsewhere (32, 33). Adult female or male (8 weeks) anemic or non-anemic mice were anesthetized by inhalation of isoflurane and administered intranasally with 50 μl of 5×10^6 colony-forming units (CFUs) of *B. pertussis* (34).

Adoptive Cell Transfer and Purification

Splenocytes from anemic mice were processed into single cell suspensions by grinding between sterile frosted glass slides in RBC lysis buffer and filtering through nylon mesh as we have reported elsewhere (19). As described above, CECs were purified by negative selection using biotin-conjugated antibodies and streptavidin linked magnetic beads (Miltenyi Biotec) according to our previous reports (19). Isolated CECs from anemic mice (1×10^7) first stained with the CFSE-dye then injected intravenously into the tail vein of recipient mice 24 h prior infection. Control mice were administered through the tail vein with 1×10^7 mature red blood cells. The single cell suspension of splenocytes was processed in the absence of RBC lysis buffer then mature RBCs and CECs were isolated by positive selection using biotin-conjugated anti-TER119 antibody (Thermo Fisher Scientific). In the next step, we used the anti-CD71 antibody on PE followed by anti-PE beads (Miltenyi Biotec) to exclude CECs.

Statistical Analysis

Statistical analyses were carried out using the GraphPad Prism (version 8) software using the appropriate statistical tests for various data sets. All statistically significant values were identified as having a p-value rate of <0.05.

RESULTS

Female Mice Have a Higher Proportion of CECs in Their Spleens

We have previously reported that CECs expand during pregnancy and are important in feto-maternal tolerance in mice (24). Therefore, we decided to quantify the frequency of CECs in the spleen of adult female and male mice by examining two commonly used mice strains; BALB/c and C57BL/6. We found significantly higher percentages and absolute numbers of CECs in the spleen of adult females compared to male BALB/c mice (**Figures 1A–C**, and **Supplemental Figure S1A**). Although BALB/c males had significantly higher body weight (**Supplemental Figure S1B**), their spleen weights were similar to females (**Supplemental Figure S1C**). Similarly, we observed significantly higher percentages and absolute numbers of CECs in the spleen of adult C57BL/6 female mice (**Figures 1D–F**). We also noted that C57BL/6 male mice had significantly higher body weight but similar spleen weight compared to female mice (**Supplemental Figures S1D, E**). Interestingly, the frequency of CECs was significantly higher in the spleen of BALB/c mice, both sexes, compared to their C57BL/6 counterparts (**Figures 1G, H**). Furthermore, to prevent potential confounding factors such as variation between litters, we decided to quantify the frequency of CECs in littermates when reaching adulthood (8 weeks). Once again, we found a significant difference in the frequency of CECs between male and female mice regardless of the mouse strain (**Figure 1I**). Overall, these observations confirmed a higher frequency of splenic CECs in females compared to males in both strains of mice.

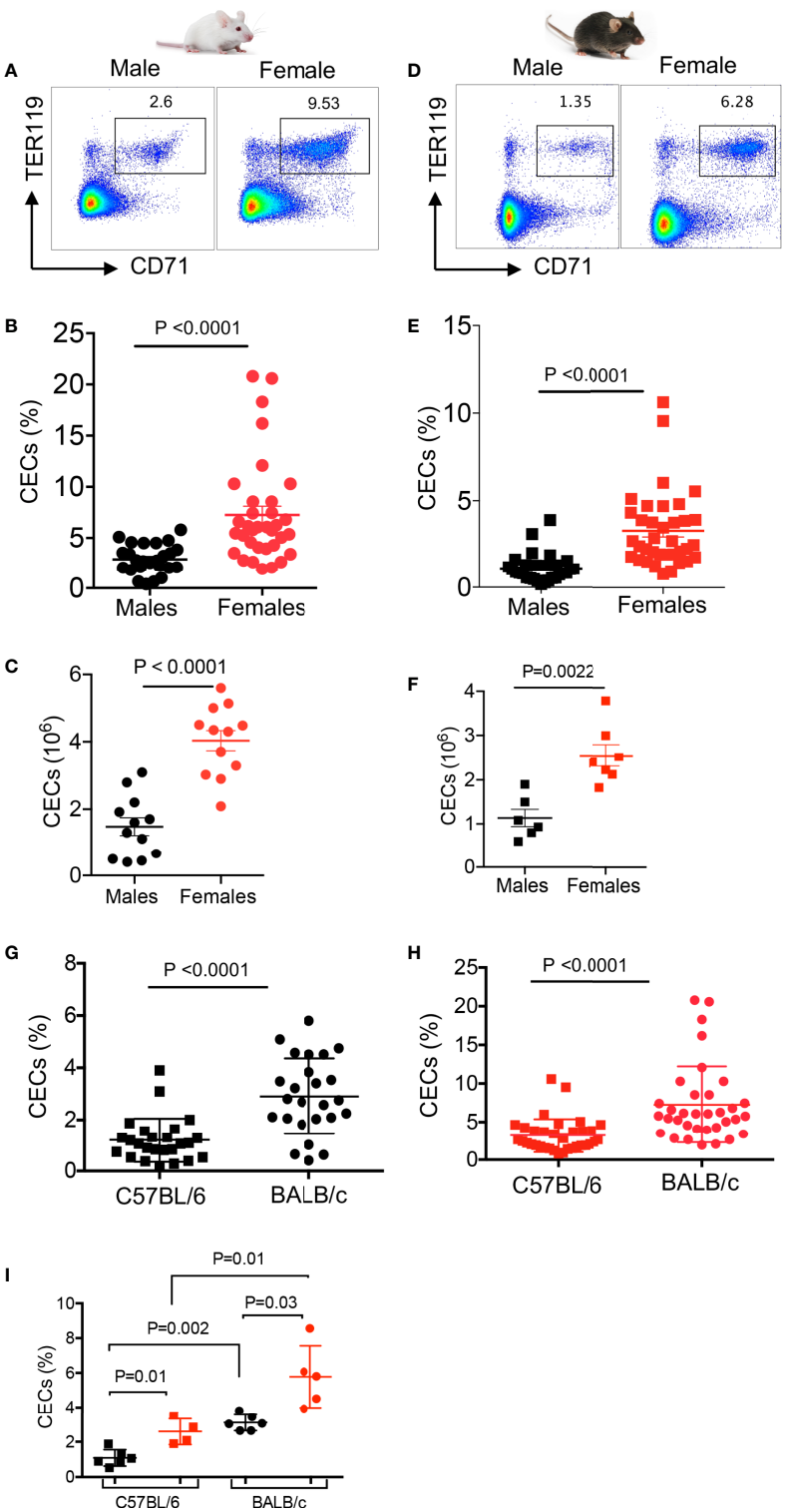

FIGURE 1 | Higher frequency of CECs in female versus male mice. **(A)** Representative flow cytometry plots, **(B)** cumulative data of percentages, and **(C)** absolute numbers of CECs in spleens of BALB/c male and female mice. **(D)** Representative flow cytometry plots, **(E)** cumulative data of percentages, and **(F)** absolute numbers of CECs in spleens of C57BL/6 male and female mice. **(G)** Cumulative data comparing percentages of CECs in spleens of C57BL/6 and BALB/c male mice. **(H)** Cumulative data comparing percentages of CECs in spleens of C57BL/6 and BALB/c female mice. **(I)** Cumulative data comparing percentages of CECs in spleens of C57BL/6 male and female, and BALB/c male and female littermates at 8 weeks old. Each point represents data from an individual mouse, representative of at least three independent experiments. Bar, mean ± standard error.

Splenic CECs in Adult Mice Express ROS, Arg II, and Arg I

The immunosuppressive properties of CECs *via* Arg II expression has been widely documented in neonatal mice (18, 19, 24). Therefore, we decided to further characterize the functionality of CECs in adult mice. We found a significantly higher percentages and absolute numbers of splenic CD45+CECs in female BALB/c mice compared to their male counterparts (**Figures 2A–C**). While in terms of percentages of CD45+CECs we did not find any significant difference between male and female C57BL/6 mice (**Figures 2D, E**), females had significantly higher numbers of CECs in their spleens when total cell count

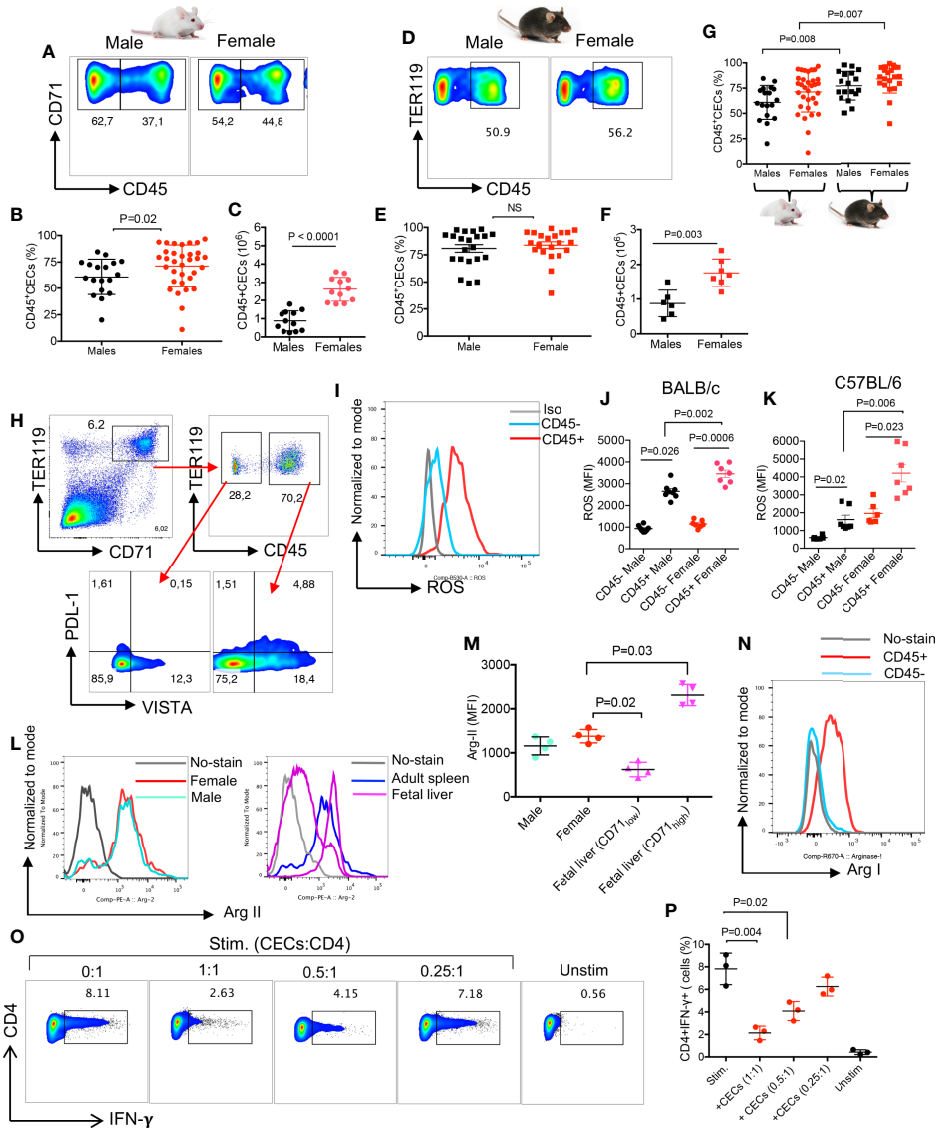

FIGURE 2 | CECs in adult mice express Arg II and ROS and suppress IFN-γ production by T cell. **(A)** Representative flow cytometry plots, **(B)** cumulative data of percentages, and **(C)** absolute numbers of CD45+/CD45-CECs in the spleen of BALB/c male and female mice. **(D)** Representative flow cytometry plots, **(E)** cumulative data of percentages, and **(F)** absolute numbers of CD45+/CD45−CECs in the spleen of C57BL/6 male and female mice. **(G)** Cumulative data comparing percentages of CD45+CECs in spleens of BALB/c and C57BL/6 male and female mice. **(H)** Representative flow cytometry plot of PDL-1 and VISTA expression in CD45− and CD45+ CECs from a female BALB/c mouse. **(I)** Histogram plots, and **(J)** cumulative data of the mean fluorescence intensity (MFI) for ROS expression in CD45− and CD45+CECs in male and female BALB/c mice. **(K)** Cumulative data of MFI for ROS expression in CD45+ BALB/c male and female C57BL/6 mice. **(L)** Histogram plots, and **(M)** cumulative data of Arg II expression (MFI) in total CECs in male versus female mice compared to fetal liver CD71^{low} and CD71^{high} CECs. **(N)** Representative histogram plot of Arg I expression in splenic CD45+ and CD45−CECs of a female BALB/c mouse. **(O)** Representative flow plots, and **(P)** cumulative data of IFN-γ production by CD4+ T cells in the absence or presence of CECs at different ratios (CECs : CD4) after stimulation with anti-CD3/CD28 antibodies for 6 h. Unstimulated (Unstim), stimulated with anti-CD3/CD28 (stim). Each point represents data from an individual mouse, representative of at least three independent experiments. Bar, mean ± standard error.

was evaluated (**Figure 2F**). However, we noticed that the frequency of CD45+CECs was significantly higher in both C57BL/6 male and female mice compared to their BALB/c counterparts (**Figure 2G**). CECs are a heterogeneous population of erythroid progenitors and precursors (21, 23). Erythroid progenitors downregulate CD45 as they mature; therefore, CD45+CECs reflect erythroid progenitors (35). These observations suggest that C57BL/6 mice possess more erythroid progenitors in their spleens. CECs are more abundant in the spleen of neonatal mice (19), however, the proportion of CD45+CECs appears to be much higher in adults compared to neonatal mice (**Supplemental Figure S1F**, and **Figure 2G**).

CECs express different co-inhibitory molecules such as PDL-1 and the V-domain Ig suppressor of T cell activation (VISTA) as we have reported elsewhere (24, 36). Therefore, we decided to measure the surface expression of PDL-1 and VISTA in CD45+ versus CD45−CECs. We found that CD45+CECs were the dominant cells expressing PDL-1 and VISTA compared to their CD45−CEC siblings (**Figure 2H**). However, there was no significant difference in the percentages of VISTA+ and PDL-1+ CECs between males and females in both mice strains (**Supplemental Figures S1G–J**). It is worth to mentioning that CECs from male C57BL/6 but not female mice appeared to have significantly a greater proportion of PDL-1+CECs compared to their counterparts in BALB/c mice (**Supplemental Figure S1K**). However, there was only a significant difference in the frequency of VISTA+CECs in female C57BL/6 compared to female BALB/c mice (**Supplemental Figure S1L**). The surface expression of PDL-1 and VISTA on adult CECs suggests that these cells similar to their counterparts in neonatal and pregnant mice may exert their biological functions *via* cell–cell interactions (e.g. PD-1: PDL-1) (15, 24). The higher expression of PDL-1 and VISTA in CECs of C57BL/6 versus BALB/c mice could be explained by the increased proportion of CD45+CECs in these mice compared to BALB/c mice (**Figure 2G**). Next, we measured the expression of ROS in CD45+ and CD45−CECs, which showed significantly higher ROS expression in CD45+CECs compared to their negative counterparts regardless of the mouse strain (**Figures 2I–K**), as we have reported in newborns (23). Also, we noted significantly higher ROS expression in CD45+CECs in both BALB/c and C57BL/6 female mice compared to their male counterparts (**Figures 2I–K** and **Supplementary Figure S2A**). However, we did not find any significant difference in the expression of ROS by CD45+CECs between C57BL/6 versus BALB/c female mice (**Supplemental Figure S2B**). Next, we decided to quantify the expression of Arg I/II as the most potent regulators of T cell proliferation and function (37, 38). In agreement with neonatal CECs (18, 19), we found a similar expression level of Arg II in splenic CECs of both female and male mice regardless of the mouse strain (**Figures 2L, M**). Since CECs in the fetal liver of mice express high levels of Arg II (24), we compared Arg II expression in splenic CECs of adult mice versus fetal liver. We found that although the $CD71^{low}$ subpopulation of fetal liver CECs expressed significantly lower levels of Arg II, the $CD71^{high}$ subpopulation expressed significantly higher levels of Arg II compared to their counterparts in the spleen of adult mice (**Figures 2L, M** and **Supplemental Figure S2C**). This is related to the abundance of CD45 expressing cells in the $CD71^{high}$ subpopulation (23). We also measured the expression of Arg I in CECs and found significantly higher expression of Arg I in CD45+CECs versus their negative counterparts in both sexes/mice strains (**Figure 2N** and **Supplementary Figures S2D, E**). Moreover, we observed that CD45+CECs in BALB/c female mice had significantly higher expression of Arg I compared to their counterparts in male mice (**Supplementary Figure S2D**). However, this was not the case for CD45+CECs in C57BL/6 mice (**Supplementary Figure S2E**). Finally, we noted significantly higher Arg I expression in CD45+CECs in female BALB/c compared to female C57BL/6 mice (**Supplementary Figure S2F**). These results suggest that CECs in adult mice mediate-immunosuppression possibly *via* cell–cell interactions and/or soluble factors such as ROS and Arg I/II.

Splenic CECs in Adult Female Mice Suppress T Cell Cytokine Production and Proliferation

As proof of concept, we isolated T cells and CECs, then performed intracellular cytokine staining for IFN-γ following stimulation with anti-CD3/CD28 antibodies in the presence or absence of CECs for 6 h. We found that CECs from BALB/c reduced IFN-γ expression by CD4+ T cells in a dose-dependent manner (**Figures 2O, P**). Next, we labeled T cells with CFSE, stimulated them with anti-CD3/CD28 antibodies in the absence or presence of CECs with purity >95% (**Supplemental Figure S2G**) for 3 days. We found that CECs suppressed both CD4+ and CD8+ T cell proliferation *in vitro* in a dose-dependent manner (**Figures 3A–D**). Since CD45+CECs were significantly higher in female BALB/c mice compared to their male counterparts (**Figures 2A, B**), and CD45+CECs had higher expression of PDL-1, VISTA, Arg I and ROS (**Figures 2H–K, N**), we reasoned to compare their immunosuppressive properties in female versus male BALB/c mice. These observations indicated a greater immunosuppression of T cell proliferation by CECs from female compared to their male counterparts (**Supplemental Figures S3A, B**). To better understand the mechanism underlying T cell suppression by adult CECs, we conducted similar proliferation assays in the presence and absence of CECs supplemented with or without L-arginine (2 mM). We observed that L-arginine supplementation partially but significantly reversed the immunosuppressive properties of CECs *in vitro* (**Figures 3E, F**). Although our data do not exclude the possibility that CECs *via* ROS or cell–cell interactions may also exhibit immunosuppressive properties, we believe Arg I/II expression is one mechanism of CECs-mediated T cell suppression in adult mice.

Anemia Promotes the Expansion of CECs in Female and Male Mice

As anemia is more common in women than men and we have observed a reverse correlation between the hemoglobin levels with percentages of CECs in COVID-19 patients, we sought to investigate whether anemia-induced CECs exhibit

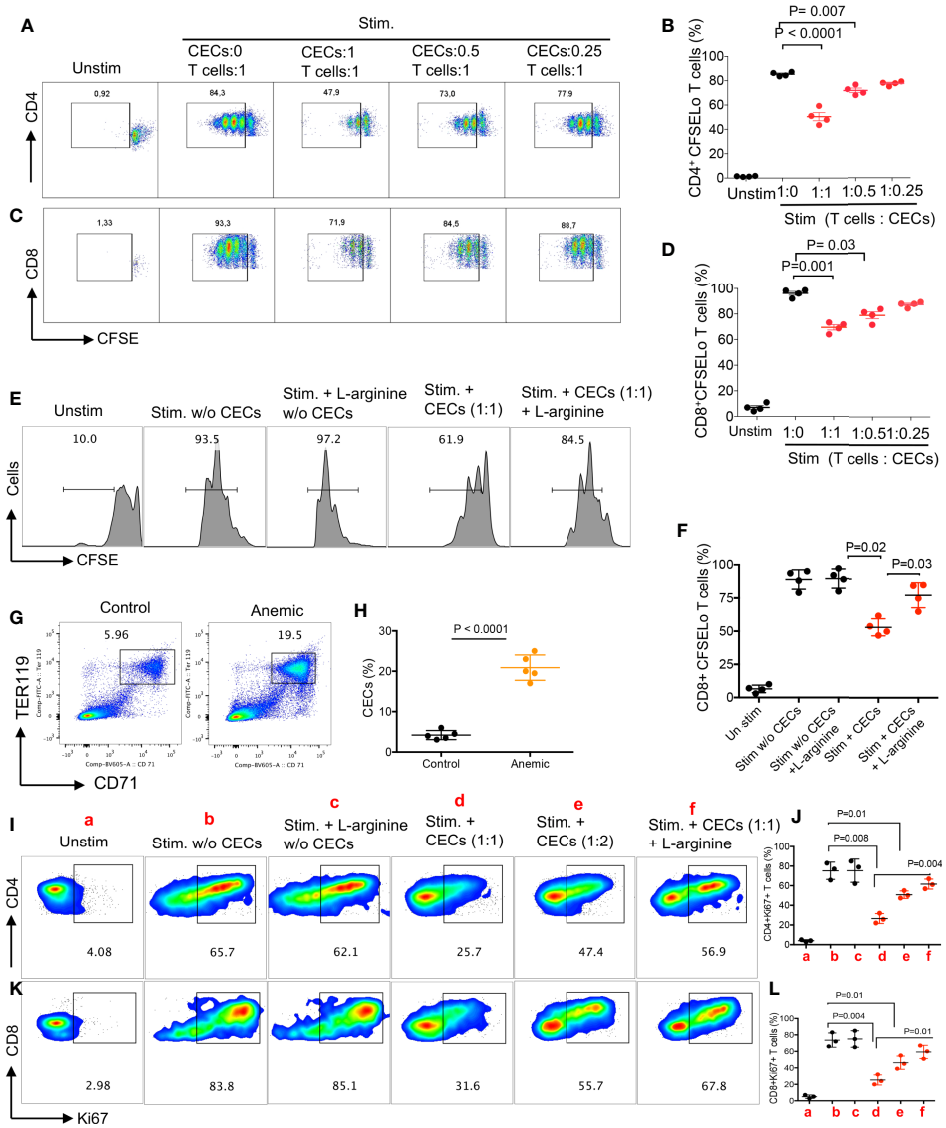

FIGURE 3 | CECs from female mice suppress T cell activation and proliferation. **(A)** Representative flow cytometry plots, and **(B)** cumulative data of CD4+ T cell proliferation as measured by CFSE dilution in the absence or presence of CECs at indicated ratios following stimulation with anti-CD3/CD28 for 3 days. **(C)** Representative flow cytometry plots, and **(D)** cumulative data of CD8+ T cell proliferation in the absence or presence of CECs at indicated ratios following stimulation with anti-CD3/CD28 for 3 days. **(E)** Representative flow cytometry plots, and **(F)** cumulative data of CD8+ T cell proliferation in the absence or presence of CECs at indicated ratios following stimulation with anti-CD3/CD28 for 3 days with or without (w/o) L-arginine supplementation (2 mM). **(G)** Representative flow cytometry plots, and **(H)** cumulative data of CECs in untreated (control) versus treated female mice with the anti-TER-119 antibody. **(I)** Representative flow cytometry plots, and **(J)** cumulative data for Ki67 expression in CD4+ T cells following stimulation for 24 h with anti-CD3/CD28, in the absence or presence of CECs (different indicated ratios) and with or without (w/o) supplementation with L-arginine (2 mM). **(K)** Representative flow cytometry plots, and **(L)** cumulative data for Ki67 expression in CD8+ T cells following stimulation for 24 h with anti-CD3/CD28, in the absence or presence of CECs (different indicated ratios) and with or without (w/o) supplementation with L-arginine (2 mM). Each point represents data from an individual mouse, representative of at least three independent experiments. Bar, mean ± standard error.

immunosuppressive properties. Adult female and male mice were injected i.p. with the anti-TER119 antibody and 5 days later evaluated for the frequency of splenic CECs. As shown in **Figures 3G, H**, the anti-TER119 antibody administration resulted in a significant expansion of splenic CECs in female and male mice (**Supplemental Figures S3C, D**). Similar to our previous report (19), we found that anemia-induced CECs did not suppress TNF-α production by CD11b+ cells *in vitro*. However, these isolated CECs from anemic mice suppressed the proliferation of both CD4+ and CD8+ T cells as measured by Ki67 (39) when stimulated with anti-CD3/CD28 antibodies *in vitro* (**Figures 3I–L**). Notably, the inhibitory property of CECs from

anemic mice on T cell proliferation was mainly Arg-dependent (**Figures 3I–L**). These observations show the differential capacity of anemia-induced CECs compared to the neonatal CECs (19) as neonatal CECs have lower proportion of CD45+CECs (**Supplemental Figure S1F**). Subsequently, we observed higher Arg II expression in anemia-induced CECs compared to their mature red blood cell counterparts (**Supplemental Figure S3E**). Although we did not find any significant difference in the percentages of CD4+ and CD8+ T cells in the spleen of male versus female mice (**Supplemental Figure S3F**), higher frequency/absolute number of CECs in females may predispose their T cells to a more pronounced CECs-mediated immunosuppression. Overall, these observations provide a novel insight into the impact of anemia-induced CECs on T cell function.

Anemia and Anemia-Induced CECs Enhance Susceptibility to *Bordetella pertussis* Infection

First, we evaluated the influence of anemia on infection susceptibility in female and male mice. As shown in **Figure 4A**, BALB/c female mice were injected (i.p.) with the anti-TER119 antibody (60 μg/injection) at 5 and 2 days before the infection, and control mice received the isotype control antibody (IgG2b). Mice were intranasally challenged with *B. pertussis* ($1–5 \times 10^6$ CFUs) (40). Two and four days later the bacterial load was quantified by the serial culture dilution of lung homogenates on the Bordet–Gengou (BG) agar media (41, 42). We found a significant increase in the bacterial load in the lungs of anemic female mice compared to the control group (**Figure 4B**). We performed similar studies on anemic male mice but the outcome was different. While anemic male mice exhibited significantly higher susceptibility to *B. pertussis* infection at day 2 post-infection, the bacterial load in the lungs of anemia male mice was not significantly different compared to controls at day 4 post-challenge (**Supplemental Figure S3G**). To avoid other potential effects of anemia in treated animals and to demonstrate a direct connection between the anemia-induced CECs and infection susceptibility, we decided to perform adoptive transfer of CECs. Splenic CECs from the anemic BALB/c female mice (8 weeks) were enriched and transferred (1×10^7 cells) *via* the tail vein injection into another group of female BALB/c mice (**Figure 4C**). In general, we use two to four spleens from healthy female mice for isolating 1×10^7 CECs. However, because of the expansion of CECs in anemic mice, we require one to two mice to obtain 1×10^7 CECs. Control mice received mature red blood cells. The presence of CFSE-labeled adoptively transferred CECs was confirmed in the spleen of recipient mice the next day by flow cytometry (**Figure 4D**) before the intranasal challenge with *B. pertussis* (5×10^6 CFUs). Two days later the bacterial load was quantified by the serial culture dilution of lung homogenates on the BG agar media, and we found enhanced susceptibility of CEC-recipient mice to *B. pertussis* infection (**Figure 4E**). This increased bacterial load remained significantly higher even during 4-day post-infection (**Figure 4F**). Thus, the enhanced susceptibility of anemic mice to *B. pertussis* infection supports the immunosuppressive property of CECs *in vivo*.

Higher Abundance of Central Macrophages in the Spleen of Female Mice

To better understand the mechanism underlying significantly greater percentages of CECs in the spleen of female versus male mice, we reasoned to determine EE in female versus male mice. To test this hypothesis, we studied the presence of erythroblastic islands (EBIs) (43) in the spleen of female and male adult mice. These islands are niches where central macrophages interact closely with RBCs in their different stages of proliferation/maturation and engulf free nuclei as they are extruded from the reticulocytes (44). We analyzed the presence of central macrophages defined as $CD11b^{-}CD169^{+}F4/80^{+}VCAM1^{+}$ according to a recent study (45). It is worth mentioning that under rapid stress erythropoiesis after treatment with phenylhydrazin (PHZ) the phenotype of splenic central macrophages appears to be different. During the recovery process from anemia early EBI niches are enriched with phenotypically more monocyte-like cells expressing high CD11b and Ly6C but low F4/80, CD169and Vcam-1 levels (46). As shown in **Figures 4G, H** and **Supplemental Figures S3H, I**, we observed significantly higher proportion and absolute number of central macrophages in spleens of female compared to male mice either BALB/c or C57BL/6 strain. Therefore, enriched central macrophages in the spleen of female mice might in part explain the abundance of EBIs in females.

Expanded CECs in the Peripheral Blood of Human Females Suppress T Cell Proliferation

We have previously reported a physiologically enriched proportion of CECs in the late stage of pregnancy in pregnant women (25). In this study, we compared the frequency of CECs in the peripheral blood mononuclear cells (PBMCs) of adult non-pregnant women compared to their age-matched males. We found females had significantly higher levels of CECs in their PBMCs compared to males (**Figures 5A–B**, and **Supplementary Figure S3J**). More interestingly, we noted a significant expansion of CECs in PBMCs of women post-menstrual cycle (**Figure 5C**).

Similar to mouse CECs, we observed that CD45+CECs were the most dominant source of ROS expression. However, CD45+CECs constituted a small proportion of CECs in human females (**Figures 5D, E**). Next, we decided to evaluate their immunosuppressive properties by performing the CFSE proliferation assay. Similar to our observations in mice, CECs from human females exhibited immunosuppressive properties in a dose-dependent manner when co-cultured with isolated T cells and stimulated with anti-CD3/CD28 antibodies for 3 days (**Figures 5F–I**). To better understand the mechanism associated with their immunosuppressive properties, we were unable to measure the expression of Arg II since human CECs get lysed once exposed to the permeabilization buffer during Arg II intracellular staining as reported elsewhere (25). However, we

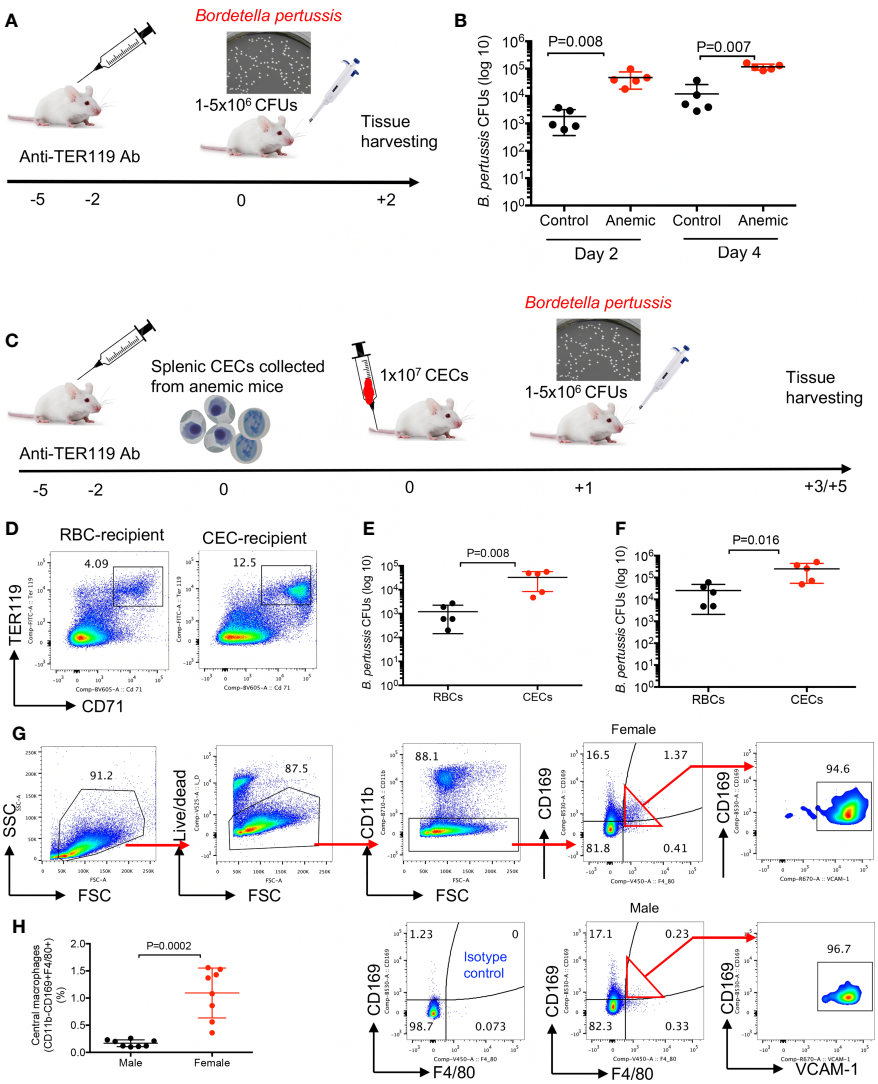

FIGURE 4 | Anemia and anemic-induced CECs enhance susceptibility to infection in female mice. **(A)** A schematic representation of the anti-TER119 antibody treatment and intranasal infection of mice with *B. pertussis* bacteria. **(B)** Cumulative data of number of isolated *B. pertussis* shown as colony forming units (CFUs) from the lung homogenates of control (IgG2b-treated) and anemic (anti-TER119-treated) mice 2 and 4 days' post-infection. **(C)** A schematic representation of the anti-TER119 antibody treatment, isolation of splenic CECs, tail vein injection of CECs and intranasal infection of mice with *B. pertussis*. **(D)** Representative flow cytometry plots of percentages of CECs in the spleen of control versus CEC-treated female mouse. **(E)** Cumulative data of number of isolated *B. pertussis* from the lung homogenates of control (RBC-injected) and CEC-injected mice 2, and **(F)** 4 days' post-infection. **(G)** Representative flow cytometry plots, and **(H)** cumulative data of central macrophages in female versus male mice. Each point represents data from an individual mouse, representative of at least two independent experiments. Bar, mean ± standard error.

have previously detected the Arg II gene in human CECs in pregnant women by qPCR (25). Notably, we were able to detect Arg II in COVID-19 patients since SARS-CoV-2 infection makes CECs resistant to the permeabilization buffer (22). Therefore, we decided to investigate whether CECs in human females *via* Arg II mediate immunosuppression *in vitro*. Isolated T cells were co-cultured with the isolated CECs with purity >95% (**Supplemental Figure S3K**) at a 1:1 ratio in the absence or presence of L-arginine (2 mM) following stimulation with anti-CD3/CD28 antibodies overnight. We observed that CECs suppressed both CD4+ and CD8+ T cell activation measured by CD69; however, L-arginine supplementation partially reversed this inhibitory effect (**Figures 5J, K**). Finally, we measured IFN-γ secretion by T cells following stimulation with anti-CD3/CD28 antibodies in the presence or absence of CECs. We found that CECs in a dose-dependent manner suppressed IFN-γ production by CD4+ T cells as measured by the intracellular cytokine staining (**Figures 5L, M**) and similarly in by CD8+ T cells (data not shown). These observations suggest that the abundance of CECs in human females can suppress T cell activation/proliferation.

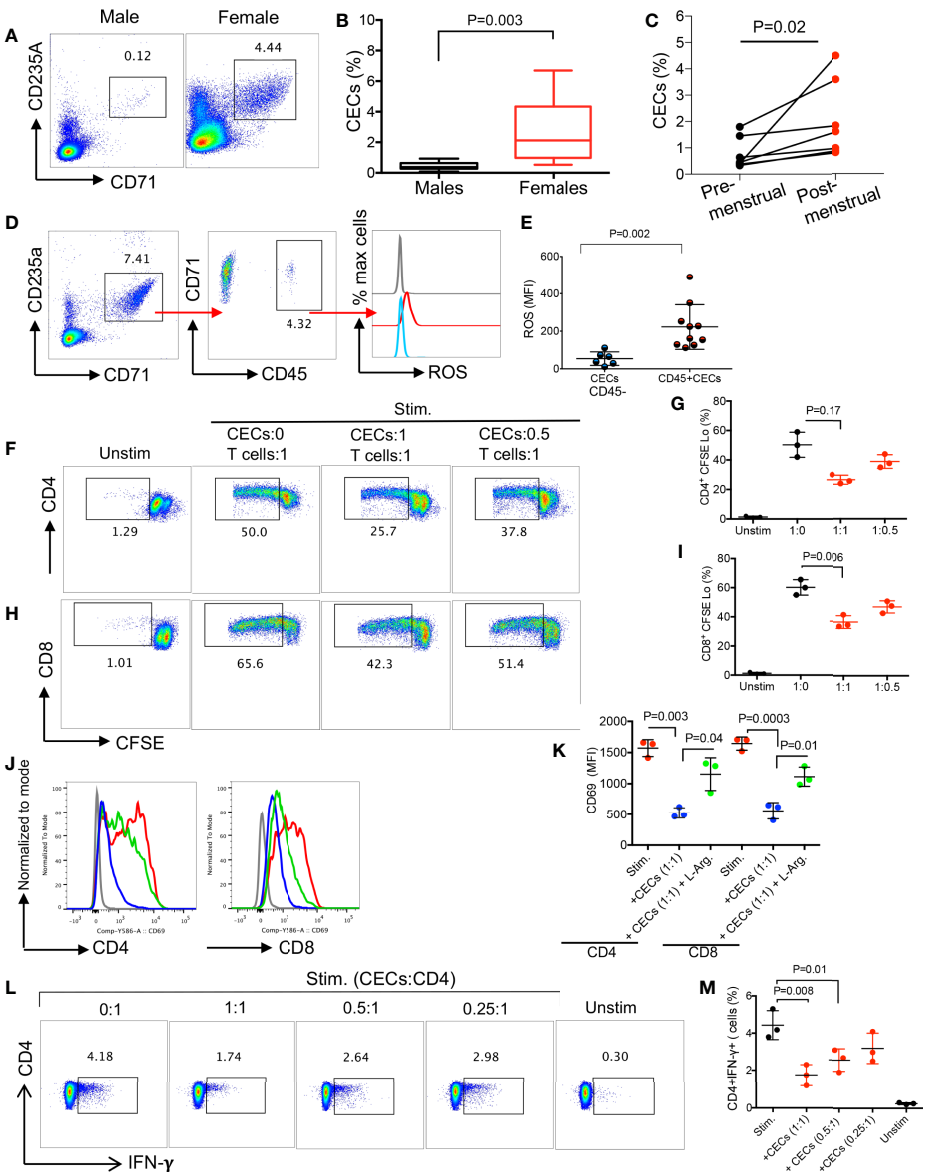

FIGURE 5 | CECs are more abundant in human females and suppress T cell activation and IFN-γ production. **(A)** Representative flow cytometry plots, and **(B)** cumulative data of percentages of CECs in males versus females (>15 subjects/group). **(C)** Cumulative data of % CECs in females pre-and post-menstrual cycle. **(D)** Representative flow cytometry plots of percent CD45+ subpopulation and ROS expression in CECs. **(E)** Cumulative data of MFI for ROS expression in CD45– and CD45+ CECs in women. **(F)** Representative flow cytometry plots, and **(G)** cumulative data of CD4+ T cell proliferation as measured by CFSE dilution in the absence or presence of CECs at indicated ratios following stimulation with anti-CD3/CD28 for 3 days. **(H)** Representative flow cytometry plots, and **(I)** cumulative data of CD8+ T cell proliferation in the absence or presence of CECs at indicated ratios following stimulation with anti-CD3/CD28 for 3 days. **(J)** Histogram plots, and **(K)** cumulative data of CD69 expression on CD4+ and CD8+ T cells in the absence or presence (1:1 ratio) of CECs following stimulation with anti-CD3/CD28 for 24 h with or without supplementation with L-arginine (2 mM) in females. **(L)** Representative flow plots, and **(M)** cumulative data of IFN-γ production by CD4+ T cells in the absence or presence of CECs at different ratios (CECs : CD4) after stimulation with anti-CD3/CD28 for 6 h. Unstimulated (Unstim), stimulated with anti-CD3/CD28 (stim). Each point represents data from an individual woman, data are from three different study subjects. Bar, mean ± standard error.

DISCUSSION

Sex as a biological variable affects immune responses to self and non-self-antigens, and it influences multiple aspects of innate and adaptive immunity (3, 47). However, the effect of sex on CECs has not been well appreciated in the past. In this study, we have provided a novel insight into the influence of sex on erythropoiesis as demonstrated by the differential frequency of CECs in males versus females. We show that suppression of T cells is a common property of both murine and human CECs that are enriched in the female. However, this was more pronounced in female than male BALB/c mice possibly because of a higher

proportion of CD45+CECs (erythroid progenitors) in females. CECs, particularly, CD45+CECs suppress T cell proliferation and activation *via* Arg I/II and ROS. Recent advancement in the field has provided more depth into the role of CECs in different physiological and pathological conditions. Immunosuppressive properties of physiologically enriched CECs have been widely described in the neonatal period (19, 20). Neonatal CECs *via* the expression of Arg II can suppress activation of myeloid-derived cells and impair humoral and cellular immune responses against infection in mice (18, 19, 30). In addition to Arg II, CD45+CECs in both neonatal mice and human newborns can suppress cytokine production by myeloid cells *via* ROS production and Arg I (23). Unlike Tregs that require days to exert their immunosuppressive functions (48), CECs exhibit their regulatory function (e.g. inhibition of cytokine production) in a matter of hours. Moreover, CECs expand during pregnancy in both humans and mice and play an important role in feto-maternal tolerance (24, 25, 49) as their depletion results in fetal resorption in an allogenic mouse model (24). In this study, we observed CECs were more abundant in females and they expand during the post-menstrual cycle. The increased frequency of CECs post-menstrual cycle and in anemic mice suggest that iron deficiency or blood loss in females might be a contributing factor for the expansion of CECs. It is well-documented that men and women have similar erythropoietin levels but women have lower hemoglobin levels (50). Although we were unable to measure hemoglobin levels in our study subjects, we have observed a negative correlation between the hemoglobin level and the frequency of CECs in COVID-19 patients (22). This observation may support the concept of anemia as a driving factor in the expansion of CECs in the periphery. However, further studies in larger cohorts are required to examine this hypothesis. In general, anemia disproportionally affects women (51) and the expansion of CECs might serve as a compensatory mechanism for anemia. Previous studies suggested that acute anemia causes tissue hypoxia, which enhances the production of erythropoietin (Epo). Subsequently, Epo mobilizes cells with erythroid lineage progenitors from the bone marrow to the spleen where they expand and mature (52). However, this idea has been challenged by the discovery of resident erythroid progenitors in the spleen of mice which upon stress erythropoiesis activate and expand (53). This may resemble resident stem cells in mammalian intestinal epithelium involved in homeostasis and the epithelium regeneration (54). The generation of erythrocytes outside of medullary spaces of the bone marrow defined as EE occurs at a very small rate in the spleen of adult mice (55). Under normal circumstances, erythrocytes following maturation enter the blood circulation (15, 21) while under pathological conditions EE is considered as the main cause for the abundance of erythroid precursors in the periphery (21). This may occur as a result of passive incontinence of hematopoietic cells release from the site of EE (56). Also, sex hormones may contribute to the higher CECs in females. For example, ERα is highly expressed on HSCs, and 17β-estradiol, an estrogen agonist of ERα, enhances splenic HSC proliferation and subsequently promotes EE in females in particular during pregnancy (57). In agreement, we found significantly more frequency of splenic central macrophages in female than in male mice. This suggests that female mice might have more splenic EBIs, however, further studies are required to prove this hypothesis. Although performing such comparison in human subjects was not feasible, there is a possibility to speculate a similar pattern in the spleen of human females compared to males. Nevertheless, this concept merits further investigations.

The EE has been reported in chronic conditions such as HIV and cancer (17, 27), which results in the expansion of CECs in the periphery. Even though the exact role of expanded CECs in females and in the context of anemia has not been well studied, we believe CECs may impair innate and adaptive immune responses against infections and cancer (17, 19, 30). However, this can be controversial since females represent 80% of all of the autoimmune diseases (58). Moreover, in the experimental autoimmune encephalomyelitis (EAE) model and PBMCs from multiple sclerosis patients, females exhibit greater Th1 responses, and IFN-γproduction compared to males (9). However, based on our observations the expansion of CECs might be beneficial in certain circumstances by preventing hyper-immune activation (25). Notably, anemia and iron-deficiency have been associated with the expansion of Tregs with a protective role in the EAE model (59). Although the authors in this study did not investigate the presence of CECs, it's likely that anemia-induced CECs may contribute to the induction of Tregs (36). It is worth mentioning that CECs-mediated impaired production of IFN-γas a potent inhibitor of erythropoiesis (60) may support the maintenance of erythropoiesis.

Moreover, the abundance of CECs in anemic individuals may in part explain the underlying mechanism of impaired immunity in anemia (61, 62). This concept was supported by the enhanced susceptibility of anemic mice to *B. pertussis* infection. Notably, the increased bacterial load in the lungs of CEC recipient mice demonstrate a direct connection between the anemia-induced CECs and susceptibility to infection. Although anemia-induced CECs did not suppress TNF-α production by myeloid cells *in vitro*, they may suppress innate immune response against *B. pertussis in vivo*. This hypothesis is supported by our previous observations that the depletion of CECs enhanced the recruitment of NK cells and antigen presenting cells (APCs) into the lungs of mice (18). Subsequently, this resulted in the elevation of protective cytokines (e.g. IFN-γ, TNF-α and IL-12) and lower *B. pertussis* in the lungs of mice (18). However, it is possible to suggest that this effect might be more pronounced and persistent in the context of chronic anemia and continuous expansion of CECs as we have shown in anemic mice. Of note, the consistency of the excessive *B. pertussis* infection rate in females is well documented (63). In agreement, we found female anemic more exhibited higher susceptibility to *B. pertussis* than their male counterparts. This explains the influence of sex in this respiratory infection, however, the role of CECs in this context merits further investigation. Taken together, our novel findings highlight the differential influence of sex on CECs and reveal that CECs should be considered as a sex-associated variable in both humans and mice. Considering the immunomodulatory properties of CECs, our observations could have implications in animal experimental design and data interpretation. However, we are aware of multiple study limitations such as the small

number of human females for pre-post-menstrual cycle studies that due to the pandemic we were restricted in recruiting more study subjects. Also, we were unable to correlate the frequency of CECs with the hemoglobin levels in healthy individuals although we have conducted such studies in COVID-19 patients (22). Another limitation of our study was related to using total CD4+ T cells instead of excluding Tregs when we performed co-culture studies with CECs. Further studies are required to determine whether exclusion of Tregs results in a different outcome.

Our data indicate that CECs in both male and female mice exhibit immunosuppressive properties; however, it was more pronounced in female mice. Because of the extremely low frequency of CECs in human males it was impossible to isolate enough CECs for performing functional studies.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Ethics Board at the University of Alberta. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by The Ethics Board at the University of Alberta.

AUTHOR CONTRIBUTIONS

SM performed most of animal studies and pre-post menstrual studies, analyzed the data, and wrote part of the introduction. PK performed a wide range of animal studies, including functional assays, comparing the frequency of CECs in male and female mice, and analyzed the data. MH performed some of animal studies for the frequency of CECs and central macrophages. IO performed some of the human related studies. SS assisted in blood collection and processing from human subjects. SE conceived the original idea, designed, and supervised all the research, secured resources, performed some of the functional assays, assisted in data analysis, and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.705197/full#supplementary-material

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Identification of a Hematopoietic Cell Population Emerging From Mouse Bone Marrow With Proliferative Potential *In Vitro* and Immunomodulatory Capacity

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There is continuing interest in therapeutic applications of bone marrow-derived mesenchymal stromal cells (MSC). Unlike human counterparts, mouse MSC are difficult to propagate *in vitro* due to their contamination with adherent hematopoietic cells that overgrow the cultures. Here we investigated the properties of these contaminating cells, referred to as bone marrow-derived proliferating hematopoietic cells (BM-PHC). The results showed that both BM-PHC and MSC had strong immunomodulatory properties on T cells *in vitro*, with PGE2 and NO involved in this mechanism. However, BM-PHC were stronger immunomodulators than MSC, with CCL-6 identified as putative molecule responsible for superior effects. *In vivo* studies showed that, in contrast to BM-PHC, MSC endorsed a more rapid xenograft tumor rejection, thus indicating a particular context in which only MSC therapy would produce positive outcomes. In conclusion, bone marrow contains two cell populations with immunomodulatory properties, which are valuable sources for therapeutic studies in specific disease-relevant contexts.

Keywords: mesenchymal stromal cells, Ly-6C, bone marrow-derived proliferating hematopoietic cells, CCL-6, immunomodulation

INTRODUCTION

There has been a continuing interest in the potential therapeutic applications of adult stem-like cells, referred to as mesenchymal stromal cells (MSC). These cells, residing in almost all postnatal organs and tissues, are heterogenous populations of fibroblast-like cells and have initially drawn attention due to their capacity to support hematopoiesis and differentiate into specific cell types (1–4). Within the bone marrow, MSC are known to reside in a complex microenvironment and together with hematopoietic stem cells (HSC) form a unique bone marrow niche (5, 6). HSC produce all blood cell lineages during homeostasis and stress in a highly dynamic program being tightly regulated by an interdependent network with MSC (5).

Abbreviations: MSC, mesenchymal stromal cells; BM-PHC, bone marrow-derived proliferating hematopoietic cells.

Among the various types of stem cells proposed for cell therapy (7), MSC were shown to have distinct advantages, which include convenient isolation (MSC can be rapidly obtained from bone marrow and adipose tissue by non-invasive methods), reduced immunogenicity, lack of ethical controversy, and trophic activity (8, 9). Although larger than other stem cells used in cell therapy, MSC can trigger the outcomes with no need of homing to the site of injury, as recent studies pointed towards a remote blood-borne-mediated pathway activated by transplanted MSC (10–13).

In preclinical settings, MSC demonstrated consistent ability to promote tissue healing, modulate inflammation and improve the outcomes in various animal models (14). All these positive *in vivo* effects are primarily due to a broad array of secreted bioactive factors, collectively referred to as MSC secretome, as it is now generally accepted that transplanted MSC do not survive for long *in vivo* (15, 16). The recognition that MSC create a microenvironment suitable for tissue repair has increased the interest in MSC therapy and this interest has been even fueled over the past years by multiple studies showing strong immunomodulatory properties (17, 18) with the principal effector being potent inhibition of T cell function (19, 20). Today, MSC are recognized as promising agents for the treatment of inflammatory disorders due to their immunomodulatory functions in contexts linked to auto/allo-immunity (21, 22).

Several mechanisms of immunomodulation have been proposed for bone marrow-derived MSC isolated from multiple species. Among these, MSC are capable of educating B cells and inducing regulatory B cell production (23). MSC can also polarize the responses of macrophages from a pro-inflammatory to an anti-inflammatory/reparative phenotype (24). They can also function to prevent the maturation of dendritic cells and the lytic ability of natural killer cells (1). Concisely, MSC could have extensive interactions with every major component of the innate and adaptive immune system, through a combination of wide-ranging molecular mechanisms involving paracrine activity, extracellular matrix remodeling, direct contact-based signaling, or extracellular vesicles (25). Identifying the particular molecules contributing to the positive effects in each clinical scenario is important for accelerating the transition into clinical practice, which is still considerably difficult.

Their heterogeneity and the absence of a specific MSC-defining antibody make these cells difficult to characterize. Therefore, mouse MSC are currently defined by using a panel of acceptable surface markers (including Sca-1, CD44, CD105), plastic adherent fibroblast-like growth and functional properties (26, 27). Besides, owing to the limited amount of these cells in the adult mouse, most of our knowledge of the biological properties of MSC has been obtained from the study of *in vitro* expanded MSC, rather than of endogenous (or primary) cells (22). Even so, while human and rat MSC are relatively easily obtained *in vitro*, the expansion of their mouse counterparts is far more difficult. Numerous reports documented that mouse bone marrow-derived MSC are frequently contaminated by hematopoietic progenitors that overgrow the culture during the initial passages. As a result, several strategies to deplete the contaminating cells and purify MSC cultures have been developed by various laboratories (28–32).

In this paper, we comparatively analyzed cells within MSC culture at different passages: an initial passage, at which the hematopoietic cells were prevailing, and two advanced passages, at which MSC culture was free of hematopoietic cells. We showed that both MSC and hematopoietic cells had high immunomodulatory effects on splenic T cells *in vitro*. Analysis of the secretome produced by these cells and inhibitory studies revealed both common and particular molecules involved in their effects. Our data showed that while both cell types had strong immunomodulatory effects on T cells *in vitro*, they were differing in other modulatory properties, such as anti-tumor effect *in vivo*.

MATERIALS AND METHODS

MSC Isolation

MSC were isolated as previously reported (28). Briefly, bone marrow aspirate was obtained from 6-week-old C57Bl/6 mice by flushing the medullar channels of the tibiae and femurs with 5 ml culture medium (DMEM containing 10% MSC-qualified FBS) using a syringe with a 26-gauge needle. A single cell suspension was thereafter obtained by passing the aspirate through increasing needle gauges (from 21 to 25), which was subsequently seeded on 10-cm cell-culture treated Petri dish and incubated at 37°C under 5% CO_2 atmosphere. The first two passages were performed at around 7-day intervals, by using 0.25% trypsin and gentle scraping with the rubber policeman. Recovered cells were plated at 5000 cells/cm^2. Starting from the third passage, the cells were trypsinized when reached around 80% confluency, without using the rubber policeman, and replated on 0.1% gelatin-coated plates at 5000 cells/cm^2.

Flow-Cytometry

Cells were trypsinized to obtain a single cell suspension and the density was adjusted to 10^6 cells/ml. One hundred-µl cell suspension was incubated with fluorescent-labeled antibody specific for CD45, Sca-1, CD44, CD29, CD90, CD73, CD105, CD11b, F4/80, CD206, Ly-6C, alone or in combination. All antibodies were purchased from BioLegend. After 30 minutes of incubation at 4°C, the cells were washed by centrifugation and resuspended in FACS buffer (PBS containing 2% fetal bovine serum) for flow cytometry analysis. Propidium iodide (0.2 ug/ml final concentration) was added before analysis to identify the live cells and at least 30,000 events were considered for each sample. Acquired data was analyzed using CytExpert software (Beckman Coulter). For multiple staining, the compensation matrix was obtained using compensation beads (Thermo Fisher Scientific) combined with fluorescent antibodies for every single-color sample.

Suppression of T-Cell Proliferation by MSC

To assess the ability of MSC to suppress T-cell proliferation, splenic T cells were isolated by nonadherence to nylon (33). T cells were CFSE-labeled and then co-cultured for three days with

anti-CD3/CD28 activating microbeads (in a cell: bead ratio of 1:1) in 96-well tissue culture plates at 10^5 cells per well in the presence or absence of irradiated MSC. Various numbers of MSC (ranging from 625 to 10,000 cells/well) were used to assess their immunosuppression capacity. MSC irradiation was performed 24 hours prior to the interaction with T cells, as previously described (34). The co-culture was maintained for three days, after which the proliferation of the fluorescent cells was analyzed using CFSE dye dilution assay and ModFit software. In experiments assessing various molecules as potential inhibitors for MSC immunosuppressive effect, these molecules were added simultaneously with the lymphocyte suspension at the time of co-culture initiation.

Cytokine Array

The profiles of the relative levels of cytokines in the conditioned medium produced by MSC culture at low and high passages were analyzed using Proteome Profiler Mouse XL Cytokine Array (R&D Systems). Briefly, the conditioned medium was incubated overnight with the array, followed by a wash step and incubation with a cocktail of biotinylated detection antibodies. Streptavidin-HRP and chemiluminescent detection reagents were then applied, and the signal produced at each spot (corresponding to the amount of protein bound) was detected with FUJIFILM Luminescent Image Analyzer LAS-3000. The pixel densities were analyzed with TotalLab Quant software.

LEGENDplex Assay

To assess soluble analytes secreted by MSC cultures at different passages, two LegendPLEX mouse panels (Th1/Th2 T Helper Cytokine Panel Version 2 and Mouse HSC Myeloid Panel) were used (BioLegend), according to the manufacturer's instructions. Briefly, the analytes were measured using bead-based sandwich immunoassays, which captured each soluble analyte between two antibodies. The analytes were bound by specific capture bead populations within a mixture of bead populations, which are differing in size and level of APC fluorescence, and each had specific antibody for a particular analyte on the surface. The concentration of each particular analyte was determined based on a known standard curve using the LEGENDplex™ data analysis software. The following panel of soluble analytes were measured in the supernatant of cells at various passages: IL-5, IL-34, GM-CSF, M-CSF, CXCL12, TGF-β1, SCF, IFNγ, IL-2, IL-4, IL-6, IL-10, IL-13, TNFα.

ELISA

Concentrations of IL-1ra, CCL-6, HGF, Fractalkine, and Tissue Factor were determined from conditioned medium, using mouse ELISA duo set kits (R&D Systems), following the manufacturer's protocols. Ang-2 and PGE2 were determined with a Mouse/Rat Angiopoietin-2 Quantikine ELISA Kit and Prostaglandin E2 Parameter Assay Kit, respectively (both from R&D Systems), following the manufacturer's protocols.

NO Determination

The ability of the cells to produce NO was assessed by measuring the concentration of nitrite in the culture medium using Griess reagent, according to the manufacturer's instructions. Briefly, 100 µl conditioned medium was incubated with 50 µl 1% sulfanilamide and 50 µl 0.3% N-1-naphthylethylenediamine dihydrochloride (in 2.5% H_2SO_4) for 30 minutes in the dark, to produce a colored azo product. The azo dye product was then spectrophotometrically quantitated based on its absorbance at 548 nm, using a freshly prepared sodium nitrite standard curve.

xCELLigence Analysis of Macrophage Activation

The effect of MSC on macrophage activation was evaluated with xCELLigence system (Roche Applied Science), using murine macrophage cell line Raw 264.7 and LPS (10 ng/ml) for cell activation. xCELLigence system monitors cellular events in real time by measuring electrical impedance in E-plates, as previously described (9). Cell activation is displayed by increasing cell index in cells treated with LPS. Briefly, 4 x 10^4 cells were seeded onto each E-plate well in 200 µl DMEM in the presence of LPS and 10% MSC-conditioned medium (CM) which was 10 times concentrated prior to analysis. Concentrated MSC-CM was obtained by ultrafiltration using centrifugal filter units with 3-kDa cut-off (Millipore) and stored in aliquots at -20°C until use. Controls of cells incubated with growth medium (negative control) and LPS-containing medium (positive controls) were also included.

Xenotransplantation of Tumor Cells

Mice were used in accordance to national and EU regulations for animal experimentation (Directive 2010/63/EU of the European Parliament) and all the procedures were approved by the Ethical Committee of ICBP. Mice were subcutaneously injected into the interscapular region with 50 µl of cell suspension composed of $2x10^6$ U87MG-luc2 cells, alone or mixed with 10^6 MSC. Tumor development was monitored by *in vivo* imaging system, as described (35). Briefly, mice were intraperitoneally injected with luciferin (150 mg/Kg body weight) and 15 minutes later, they were imaged in dorsal position with IVIS Spectrum system (Perkin Elmer). The following settings were used: field of view 6.6; binning factor 4; F-stop 2; exposure 15 seconds. Surface images were then analyzed using Living Image 4.3.1 software (PerkinElmer, Norway) and quantification of bioluminescence was performed by manually defining regions of interest and reported as photons/second/square centimeter/steradian. Six mice were sacrificed at 5 days after cell injection and cellular pellet was harvested for RNA isolation and Real-time RT-PCR analysis.

Real-Time RT-PCR Analysis

Total RNA (1 ug) was revers-transcribed into cDNA using High-Capacity RNA-to-cDNA Kit (Applied Biosystems). The qRT-PCR was carried out using SYBR™ Select Master Mix (Applied Biosystems) with 400 nM primer mix at a final reaction volume of 10 µL, on ViiA™ 7 Real-Time PCR System. The cycling conditions were: 50°C for 2 min (UNG activation step), followed by 95°C for 2 min (enzyme activation step), and 40 cycles of amplification (95°C for 1 sec and 60°C for 30 sec). Relative expression was calculated using the comparative CT method and S18 recognizing both human and mouse transcripts were used for normalization.

Statistical Analysis

Statistical analysis was performed with GraphPad Prism 7 software. Results were expressed as mean ± SD (*in vitro* studies) and mean ± SEM (*in vivo* studies). Statistical comparisons of the secretome at different passages were performed *via* one-way ANOVA with Bonferroni corrections test applied for multiple comparisons. *In vivo* studies and inhibition studies were analyzed by two-way ANOVA with Tukey corrections for multiple groups. $p<0.05$ was considered significant.

RESULTS

Characterization of Contaminating Hematopoietic Cells in Bone Marrow-Derived MSC Culture

Our strategy to purify mouse bone marrow-derived MSC in culture was based on serial passages through gentle trypsinization, by which MSC were detached and further propagated, while part of the hematopoietic cells remained attached to the substrate, being more resistant to trypsin. **Figure 1A** shows the decrease in the percentage of $CD45^{pos}$ cells in bone marrow-derived cell culture with each passage, until the culture became negative to CD45, after passage 6. Within these first passages, a proliferation of hematopoietic cells was observed, as the percentage of $CD45^{pos}$ cells increased from day 3 to day 5 after seeding (**Supplemental Figure 1**). We therefore named these cells bone marrow-derived proliferating hematopoietic cells (BM-PHC), a term that captures the origin and proliferative status of the contaminating $CD45^{pos}$ cells in the mouse MSC culture at low passages. It is worth mentioning that a large variability in the time course of the culture purification was noted, with certain batches of serum producing hematopoietic-free cultures early than others (data not shown).

Comparative characterization of BM-PHC and MSC was done at passage #3 (at which BM-PHC represented more than 80% of total viable cells) and passage #8 (at which culture MSC was free of hematopoietic cells). The results showed that BM-PHC were smaller-sized, however they expressed the whole panel of markers that are usually used to characterize MSC, except the endoglin (CD105). Thus, both cell populations were positive for Sca-1, CD44, CD29, and CD73 and were $CD90^{low}$ (**Figure 1B**). Comparative analysis of multipotency showed that BM-PHC could not generate adipocytes and chondrocytes *in vitro* (data not shown), as MSC did when cultured under appropriate conditions (28).

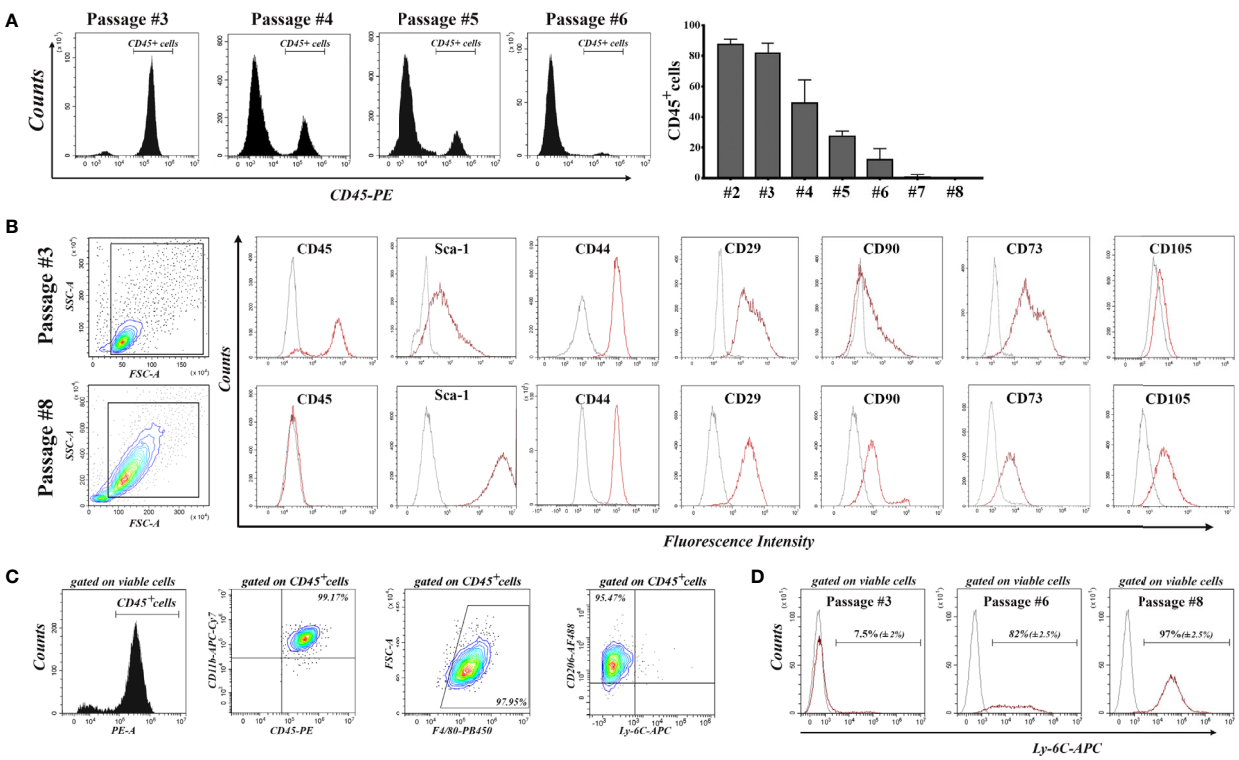

FIGURE 1 | Flow-cytometry characterization of cells emerged from mouse bone marrow aspirate during serial passages until total depletion of $CD45^{pos}$ cells. **(A)** The decrease in the percentage of $CD45^{pos}$ cells with each passage. Note that the culture becomes completely depleted of $CD45^{pos}$ cells after passage 6. Data are mean +/- S.D of at least 6 experiments. **(B)** Comparative analysis of the expression of cell markers in culture at passages 3 and 8. Note the presence of MSC markers (except CD105) on the cells at passage 3, when most of them are $CD45^{pos}$ cells. At passage 8, the culture contained no $CD45^{pos}$ cells, and cells are $CD105^{pos}$. **(C)** Characterization of hematopoietic cells at passage 3. Note that all $CD45^{pos}$ cells (around 80% of total viable cells in culture) are $CD11b^{pos}$/$F4\text{-}80^{pos}$/$CD206^{pos}$/$Ly\text{-}6C^{neg}$, being therefore asserted as anti-inflammatory cells. At least 3 different batches were analyzed and the results were similar. **(D)** Expression of Ly-6C in cell culture at increasing passages. Note that all MSC express Ly-6C in culture. Data are mean +/- S.D of at least 3 experiments.

Further characterization of BM-PHC showed a population of $CD11b^{pos}/F4/80^{pos}/CD206^{pos}/Ly\text{-}6C^{neg}$ cells, which pointed towards an anti-inflammatory macrophage phenotype (**Figure 1C**). Importantly, Ly-6C and Ly-6G were not expressed on BM-PHC however, Ly-6C was noticed on MSC. As the percentage of $CD45^{pos}$ cells decreased in culture, the percentage of $Ly\text{-}6C^{pos}$ cells increased and all cells at passage #8 were positive to Ly-6C (**Figure 1D**). Therefore, Ly-6C is being proposed as a genuine marker for C57Bl/6 -derived MSC.

Comparative Analysis of the Immunomodulatory Properties of BM-PHC and MSC

These small BM-PHC are the most abundant cells at passage #3, yet the function of these cells is unknown. Consequently, we comparatively evaluated the immunomodulatory properties of BM-PHC (as whole population at passage #3) and MSC at passages #6 (in which the percentage of contaminating cells was very low), and #10 (in which MSC had underwent several doublings in culture after total hematopoietic depletion), by co-culturing them with syngeneic splenic T cells in activating conditions. In corroboration to previous reports (36), our results showed a dose-dependent inhibitory effect of MSC on T cell proliferation (**Figure 2A** and **Supplemental Figure 2**), with no difference between MSC at passages #6 and #10. However, passage #3 was significantly more effective in suppressing T cell proliferation in comparison to passages #6 and #10, thus demonstrating a strong immunosuppressive effect of BM-PHC (**Figure 2A**).

We next evaluated the effects of BM-PHC and MSC on resting T cells in culture. The results showed a dose-dependent protective effect of MSC (**Figure 2B** and **Supplemental Figure 3**) at both passages #6 and #10, and again, a much more protective effect of BM-PHC (**Figure 2B**).

We then attempted to establish whether secreted soluble factors were involved in the immunosuppression properties of these cells. First, the impact of MSC on T cell proliferation was assessed at passage #6 in a transwell co-culture, without allowing direct cell contact. The results showed that, even in the absence of cell-to-cell contact, the immunosuppressive effect of MSC still remained significant (**Figure 2C**), thus emphasizing that the secretome produced by MSC was partially responsible for inhibition of T cell cycling. In addition, the secretome of either MSC or BM-PHC also had pro-survival effects on resting T cells in culture, albeit at lower extents than the cells (**Figure 2D**, in comparison to **Figure 2B**).

Together, these data demonstrated strong immunomodulatory properties of cells contaminating the MSC culture, which even surpassed the properties of MSC themselves, by promoting the viability of resting T cells and suppressing splenic T lymphocyte proliferation.

Comparative Analysis of the Secretomes of MSC and BM-PHC

The above data showed that the immunomodulatory effects of MSC and BM-PHC could be reproduced to a certain extent by the soluble factors secreted by these cells. To search for candidate molecules involved in the immunomodulatory properties of these cells, their CM was assessed by cytokine array. Around 22 proteins were identified at high levels (**Supplemental Figure 4**), with 6 of them having considerable differences in the secretion level between the two cell types. Specifically, Angiopoietin-2 (Ang-2), Hepatocyte Growth Factor (HGF), Fractalkine, Tissue Factor and Interleukin-1 receptor antagonist (IL-1ra) were secreted at higher levels by MSC than BM-PHC. On contrary, CCL6 (a mouse C-C motif chemokine), with chemoattractant properties for macrophages, B and T lymphocytes and eosinophils (37), was secreted at higher level by BM-PHC (**Figure 3A**).

The different secretion level of these molecules was further assessed by ELISA, using four different batches at different passages. The results confirmed the gradual decrease of CCL6 level with increasing passage (**Figure 3B**), thus suggesting that BM-PHC was the source of CCL6. Quantification of Ang-2 level in various batches at different passages showed batch-dependent secretion patterns, with some batches secreting high levels of Ang-2 at high passages (**Supplemental Figure 4**), and other batches secreting very low levels at all passages (**Supplemental Figure 5**). Quantification of IL-1ra revealed a high, yet wide-ranging secretion level between passages, with no validated increased level in MSC as compared to BM-PHC (**Supplemental Table 1**). On contrary, the increase in the secreted levels of Fractalkine and HGF with increasing passage was validated by ELISA, and very low levels of molecules were detected in BM-PHC (**Figure 3C** and **Supplemental Figure 5**). Similarly, Tissue Factor increased in MSC with passages (**Supplemental Figure 5**); however, it is worth mentioning that the levels of Tissue Factor were very low, ranging from 5 - 50 pg/ml. This data is important for intravascular therapeutic delivery of MSC, as Tissue Factor is the major determinant of cell product hemocompatibility (38). On the other hand, both cell types secreted high levels of Prostaglandin E2 (PGE2) at all passages, which were 3 orders of magnitude above the Tissue Factor level, with a median of around 4 ng/ml (data not shown). PGE2 was reported to be markedly increased in the inflammatory settings and has roles in inhibition of cytotoxic T cell development, division and function (39).

To get deeper insights into the composition of the secretome, two bead-based multiplex assay panels were used to quantify several mouse cytokines specifically associated to T helper or myeloid stem cells. The results showed no or minimal secretion levels of IL-2, -4, -5, -6, -10, -13, -34, as well as of Interferon γ (IFN-γ), Tumor Necrosis Factor α (TNF-α), Tissue Growth Factor β1 (TGF-β1), Granulocyte/Macrophage Colony Stimulating Factor (GM-CSF) and Stem Cell Factor (SCF), which thus confirmed the data obtained by cytokine array (**Supplemental Table 1**). Instead, very high levels of Macrophage Colony Stimulating Factor (M-CSF) were found in the CM of both cell types, with significantly higher levels secreted by MSC as compared to BM-PHC (**Figure 3D**). Likewise, CXCL12/SDF-1, a chemokine involved in stem cell homing and T cell chemoattraction had been found in higher levels in MSC than

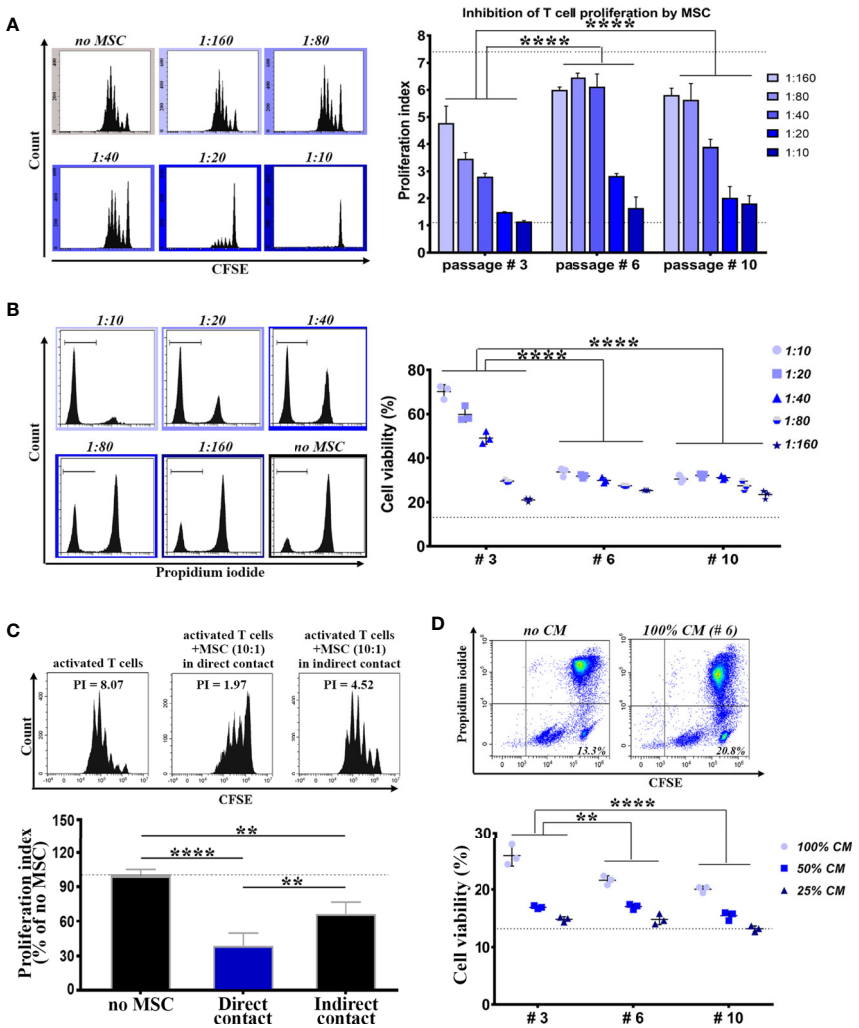

FIGURE 2 | Comparative analysis of the immunomodulatory properties of cells within culture at low, intermediate and high passages. **(A)** The immunosuppressive effect of cells on splenic T cell proliferation in vitro. T cells were cultured in the presence of activating beads (1:1 ratio) and increasing numbers of irradiated cells, so that to span the interval of MSC: T cell ratio between 1:160 and 1:10 (constant number of T cells). Shown data represent mean +/- SD of one representative experiment performed in triplicates. At least three experiments were performed with similar results. **(B)** The pro-survival effect of cells on the viability of resting T cells *in vitro*. T cells were cultured in resting conditions in the presence of increasing numbers of irradiated cells. Shown data represent mean +/- SD of one representative experiment performed in triplicates. At least three experiments were performed with similar conclusions. **(C)** The suppressive effect of MSC (passage #6) on T cell proliferation *in vitro*, in the absence of cell-cell contact between MSC and T cells. **(D)** The pro-survival effect of the conditioned medium (CM) produced by cells on resting T cells *in vitro*. Shown data represent mean +/- SD of one representative experiment performed in triplicates. At least six experiments were performed with same conclusion. $^{**}p < 0.01$, $^{****}p < 0.001$.

in BM-PHC. Both M-CSF and CXCL12 have been previously reported as being secreted by MSC and involved in the control of survival and differentiation of bone marrow progenitor cells (40). A summary of all molecules identified in MSC secretome is illustrated in **Supplemental Table 1**.

Candidate Molecules for the Immunosuppressive Effects of MSC and BM-PHC

The above data showed that BM-PHC secreted high levels of CCL6, whereas MSC secreted high levels of HGF, Fractalkine, M-CSF and CXCL12. However, both cells types secreted high levels of PGE2, previously reported to modulate the immunity (41) and the immunosuppressive properties of MSC (42). We therefore hypothesized that the immunosuppressive effect of both cell types is primarily mediated by PGE2, and to lesser extents by HGF and CCL6, which were differentially secreted by the two cell types. To test this hypothesis, proliferation studies of activated T cells in co-culture with MSC or BM-PHC in the presence of specific inhibitors of PGE2, HGF or CCL6 were done. BM-PHC were used for studying the effect of CCL6 and MSC for studying the effects of PGE2 and HGF. Our data showed that

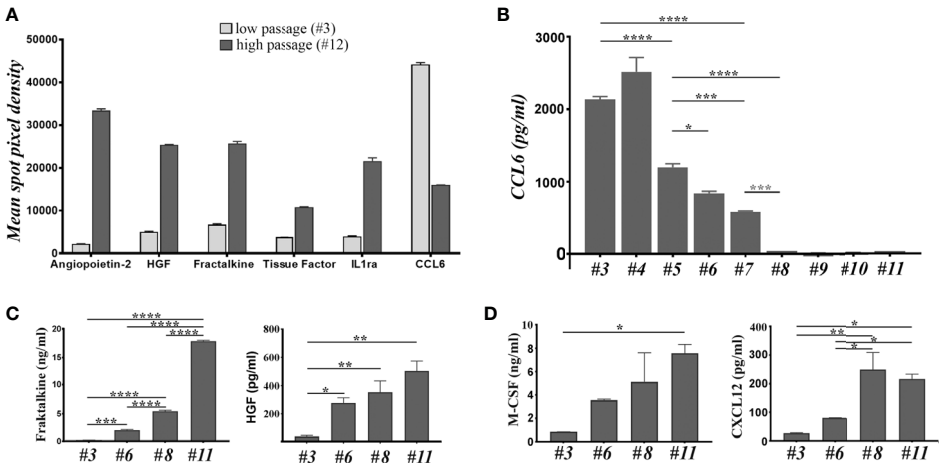

FIGURE 3 | The analysis of soluble factors secreted by cells at low and high passages. **(A)** The relative expression level of 6 cytokines identified by cytokine array in the secretome of cells at 3 versus # 12. The depicted cytokines were selected from the 111 soluble proteins detected by cytokine array based on two considerations: a high level of expression and high differences between the two passages. **(B)** ELISA quantification of CCL-6 in the secretome of cells at increasing passages. Note the high level of CCL-6 at low passages and its loss after passage 7. The data represent the results of one representative experiment from 5 experiments with different batches and similar conclusions. **(C)** ELISA quantification of Fractalkine and HGF in the secretome of cells at different passages. The data represent the mean+/- S.D. @ of at least 3 experiments performed in duplicates. Note that Fractalkine is secreted by cell culture at all passages, while HGF is completely absent from the culture at low passage. **(D)** The LegendPlex quantification of M-CSF and CXCL12 in the secretome of cell culture at different passages. Data illustrates one experiment performed in duplicates. Two independent experiments with different batches were analyzed and the results were similar. *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, ****$p < 0.001$.

NS398, a specific COX-2 inhibitor, partially reversed the suppressive effect of MSC and increased the proliferation index of T cells at doses ranging from 1 to 10 μM (**Figure 4A**). This effect was apparent only at 1:160, and not 1:10, cell ratio (MSC: T cell), which thus suggested a partial contribution of PGE2 on the inhibitory effects of MSC on T cell proliferation *in vitro*.

Interestingly, inhibition of CCL6 by using a specific neutralizing antibody significantly attenuated the inhibitory effect of BM-PHC at both 1:160 and 1:10 cell ratios (**Figure 4B**). This data showed a major suppressive role of CCL6 on T cell proliferation *in vitro*, which has not been previously reported and might explain the enhanced immunomodulatory properties of BM-PHC over MSC. However, the specific blocking of CCR1 (reported as the putative receptor of CCL6) (37), using BX471 small molecule, totally suppressed T cell proliferation (**Supplemental Figure 6A**), which probably reflected the involvement of other CC chemokines, such as CCL3, or CCL5 (which are synthetized by T cells and also signalize through CCR1 (43, 44), in cell survival and proliferation.

Similarly, inhibition of HGF signaling using SGX523 (a specific c-Met inhibitor) negatively impacted the T cell proliferation *in vitro* (**Supplemental Figure 6B**), which pointed towards the important role of HGF in cell proliferation and survival. However, addition of recombinant HGF protein (50-100 ng/ml) on activated T cells did not produce inhibitory effects (data not shown), suggesting that HGF was not involved in the suppressive effect of MSC on T cell proliferation in culture.

Collectively, these data indicated that CCL6 secreted by BM-PHC, yet not by MSC, might explain the superior effects of BM-PHC in inhibiting activated T cell proliferation *in vitro*, as compared to MSC. PGE2, which was secreted at high levels by both cell types, was found to be partially involved in the inhibitory effects on T cell proliferation, yet the degree of inhibition did not point towards PGE2 as the major inhibitory molecule. We therefore assumed that T-cell suppression might be also mediated by factors induced in the presence of activated T cells, by the cross-talk between the two cell populations.

As nitric oxide (NO) was previously reported to inhibit T-cell proliferation *in vitro* (45, 46), we investigated the effects of L-NAME, a specific inhibitor of NO synthase. As shown in **Figure 4C**, 1 mM L-NAME partially reversed the immunosuppressive effects of MSC and its effect was more pronounced in the presence of low numbers of MSC (at MSC: T cell ratio of 1:40 and above). This data suggested that NO was also an important suppressive factor of T cells *in vitro*. However, complete recovery was not achieved, implying that a synergistic immunosuppressive mechanism of MSC on T cell proliferation did exist.

Putative Mechanisms by Which MSC Induce Suppression of T Cell Proliferation

Since NO is known as a highly unstable molecule, we next investigated the context in which NO secretion occurred in cultured MSC. To this aim, supernatants from naïve MSC culture, as well as from the 3-day co-culture of MSC with T cells in activating or quiescent conditions, were used for nitrite determination by Griess reaction. The results showed that MSC

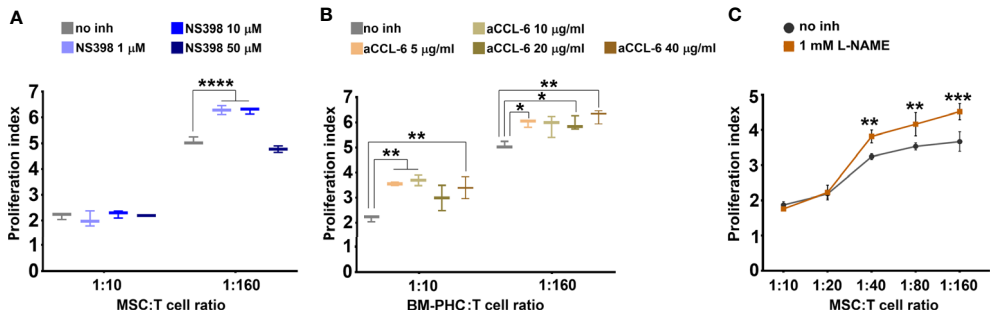

FIGURE 4 | The effects of cells on T cell proliferation in the presence of inhibitors. **(A)** Effect of different doses of NS398, a specific COX-2 inhibitor, on reversing the inhibitory effect of MSC. Note the capacity of NS398 at 1 and 10 μM to partially reverse the MSC effect on T cell proliferation at the lowest MSC: T cell ratio. **(B)** Effect of different doses of CCL-6 neutralizing antibody on reversing the inhibitory effect of BM-PHC. Note the reversing effects of anti-CCL-6 at both BM-PHC: T cell ratios. The graphics in a-b illustrate a representative experiment from at least four experiments performed with different batches, with the same conclusions. **(C)** Effect of L-NAME, a specific NO synthase inhibitor, on reversing the inhibitory effect of MSC. The graphic illustrates a representative experiment from three experiments preformed with different L-NAME doses: 100 nM, 500 nM, and 1mM. No dose-dependent effect of L-NAME was observed, yet the three doses produced the same reversing effects. *p < 0.05, **p < 0.01, ***p < 0.005, ****p< 0.001.

produced high levels of nitrite in the presence of activated T cells (**Figure 5A**). On contrary, neither naïve MSC, nor MSC in the presence of resting T cells did produce nitrite.

As MSC were previously reported to produce NO when activated by TNFα and IFNγ (46) and both these molecules were identified in the secretome of activated T cells (47), we measured the nitrite level in the culture medium of naïve MSC in the presence of various doses of TNFα and IFNγ, alone or in combination. The results showed small levels of NO secreted by MSC in the presence of TNFα, in a dose-dependent manner, and no NO secreted in the presence of IFNγ alone. However, the concomitant presence of the two cytokines in MSC culture resulted in a massive NO secretion (**Figure 5B**). No significant difference was found in the NO levels produced by MSC and BM-PHC (**Figure 5C**). It is therefore likely that, similar to PGE2, NO production was a common mechanism by which the two cell types induced the suppression of T- cell proliferation *in vitro*.

Given the anti-inflammatory behavior of MSC in the presence of inflammatory cytokines, we investigated whether the expression of pro-inflammatory protein Ly-6C was changed in MSC in our experimental setting. Flow-cytometry analysis revealed that TNFα produced a dose-dependent decrease in the expression of Ly-6C in MSC culture (**Figure 5D**). On contrary, IFNγ increased the Ly-6C expression in MSC culture, thus suggesting that the balance between the two cytokines dictates the overall expression of Ly-6C on MSC. We further determined Ly-6C expression in MSC in co-culture with activated T cells and found it increased, as compared to naïve MSC (**Figure 5E**). This data might be explained through increased level of INFγ being secreted over TNFα in this experimental setting.

By summarizing, the factors by which MSC exerted the immunosuppressive effects on T cell proliferation *in vitro* appear to involve the constitutive secretion of PGE2 and the induced secretion of NO. Besides these two molecules, BM-PHC appear to exert the immunosuppressive effects on T cell proliferation *in vitro* also by CCL6.

Capacity of BM-PHC and MSC to Inhibit Xenogeneic Tumor Formation in Immunocompetent Mice

Having the strong immunosuppressive effects of BM-PHC and MSC on T cell proliferation *in vitro*, we attempted to evaluate whether these two cell types would induce tolerance to tumor development in a model of xenotransplantation of tumor cells in adult mice with intact immune system. To this aim, 2 x 10^6 U-87 MG-luc2 cells (human glioblastoma - derived cells that constitutively expresses Luciferase) were subcutaneously injected in C57Bl/6J mice, either alone, or mixed with 1 x 10^6 BM-PHC or MSC. *In vivo* bioluminescence imaging demonstrated that, in the absence of cell therapy, the tumor rejection occurred between days 3 and 9 in all groups, with complete rejection occurring by day 11 (**Figures 6A, B**). Similar results with U-87 MG-luc2 cells injected into immunocompetent mice were previously reported (48). The group receiving tumor cells mixed with BM-PHC showed a transient increase in the luminescent signal within the first three days after transplant, followed by tumor rejection by day 9, a pattern similar to control group. This data suggests no significant effects of BM-PHC on tumor development.

In contrast, co-injection of U-87 with MSC resulted in a significantly more rapid rejection of the xenograft, indicating a direct anti-tumoral effect of MSC (**Figure 6A**). As tumor growth was visibly affected from the first day after implantation (**Figure 6B**), a direct impact of MSC on the innate immune cells invading the tumor was assumed. In vitro investigation of the effect of MSC secretome on the activation of macrophages showed a significant anti-inflammatory effect of MSC, by slowing down the cell index, indicative of macrophage activation in the presence of LPS (**Figure 6C**). A similar anti-inflammatory effect of MSC was noted *in vivo*, in the model of tumor xenotransplantation described above, where quantitative RNA analysis of tumors removed at five days after injection revealed a tendency of decrease in the CD45 mRNA level in U87+ MSC

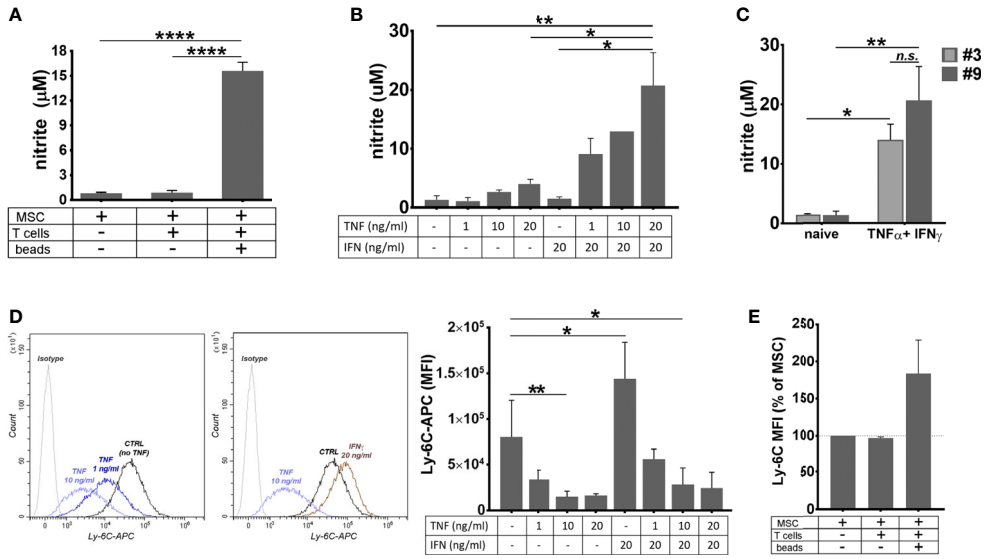

FIGURE 5 | The behavior of MSC in pro-inflammatory conditions. **(A)** The level of nitrite secreted by MSC in basal conditions, and in the co-culture with resting or proliferating T cells. The values represent mean+/- S.D. @ of two independent experiments performed in triplicates. **(B)** The levels of nitrite secreted by MSC after 48 hours of culture in the presence of TNFα and IFNγ. Note the synergic effect of the two cytokines on the secreted NO level. The values represent mean +/S.D of four experiments performed in duplicates with different MSC batches at passages 7- 10. **(C)** The levels of nitrite secreted by cell culture at low and high passages. The values represent mean +/S.D of at least two experiments performed in duplicates. **(D)** The attenuation of Ly-6C expression on MSC after stimulation with TNFα in the presence or absence of IFNγ. Note that, while TNFα produced a dose-dependent decrease in the expression of Ly-6C in MSC culture, IFNγ had an inverse effect, however in the presence of both cytokines, MSC decrease Ly-6C expression. The values represent the mean +/- S.D. from the three independent experiments. Representative histograms showing Ly-6C expression in MSC with and without cytokines are also given. **(E)** Ly-6C expression on MSC in basal conditions, and in the co-culture with resting or proliferating T cells (n= 2 experiments). *p < 0.05, **p < 0.01, ****p < 0.001, n.s., not-significant.

group, as compared to U87 group (**Figure 6D**). The level of CD45 gene expression was positively correlated with the immunohistochemically-quantified cell marker in solid tumors, as previously documented (49). Still, RT-qPCR analysis identified increased transcription of several pro-inflammatory genes e.g., IL-1β, IFNγ, COX-2, IL-12A (**Figure 6D**). A possible explanation for these results is that although MSC retained the anti-inflammatory properties *in vivo*, by reducing the number of hematopoietic cells infiltrated the transplant area, they underwent activation in the presence of tumor cells and consequently become polarized towards the inhibitory functionality for tumor development. However, due to the low number of animals used in this study, the statistical significance of the data was not reached. Therefore, additional studies are warranted to confirm this mechanism by which MSC act to inhibit tumor initiation *in vivo*.

Together, these data show that mouse bone marrow aspirate generates in culture two populations of proliferating cells with immunomodulatory properties, MSC and BM-PHC, which are valuable for therapeutic purposes. While both cell types inhibit the proliferation of activated T cells and promote the survival of resting T cells *in vitro*, the *in vivo* effects are divergent: MSC exert an anti-tumor effect, whereas BM-PHC may induce transplantation tolerance. Therefore, these two cell populations should be considered for cell therapy depending on the context.

DISCUSSION

The major findings of this study are summarized as follows: (i) bone marrow-derived MSC express high levels of Ly-6C; (ii) BM-PHC, the hematopoietic cells contaminating the MSC culture at initial passages, have strong immunomodulatory properties on T cells *in vitro*, which were partially mediated by CCL6; (iii) PGE_2 and NO, secreted by both MSC and BM-PHC, are common mediators of the suppression of T cell proliferation *in vitro*; (iv) MSC, yet not BM-PHC, exert anti-tumoral effects *in vivo*.

Increasing data on the immunomodulatory effects of MSC have shown that the mechanisms of action were largely paracrine-mediated (50). However, substantial batch-to-batch variation, as well as differences based on donor, tissue of origin, culture conditions and passage were observed (51). We showed here that MSC secrete constitutively high levels of IL-1ra, Fractalkine, PGE2, HGF. Among them, PGE2 was validated as being involved in the immunosuppressive effects of MSC on T cells *in vitro*. As IL-1ra and fractalkine were acknowledged as molecules with opposite roles in lymphocyte recruitment (52, 53), their concomitant production by MSC may not produce a major impact on the immunosuppressive function. On the other hand, HGF was proven not to affect T cell proliferation *in vitro*. Neither addition of recombinant HGF, nor inhibition of HGF receptor in our co-culture system did reverse the immunosuppressive effects of MSC. This may not be surprising,

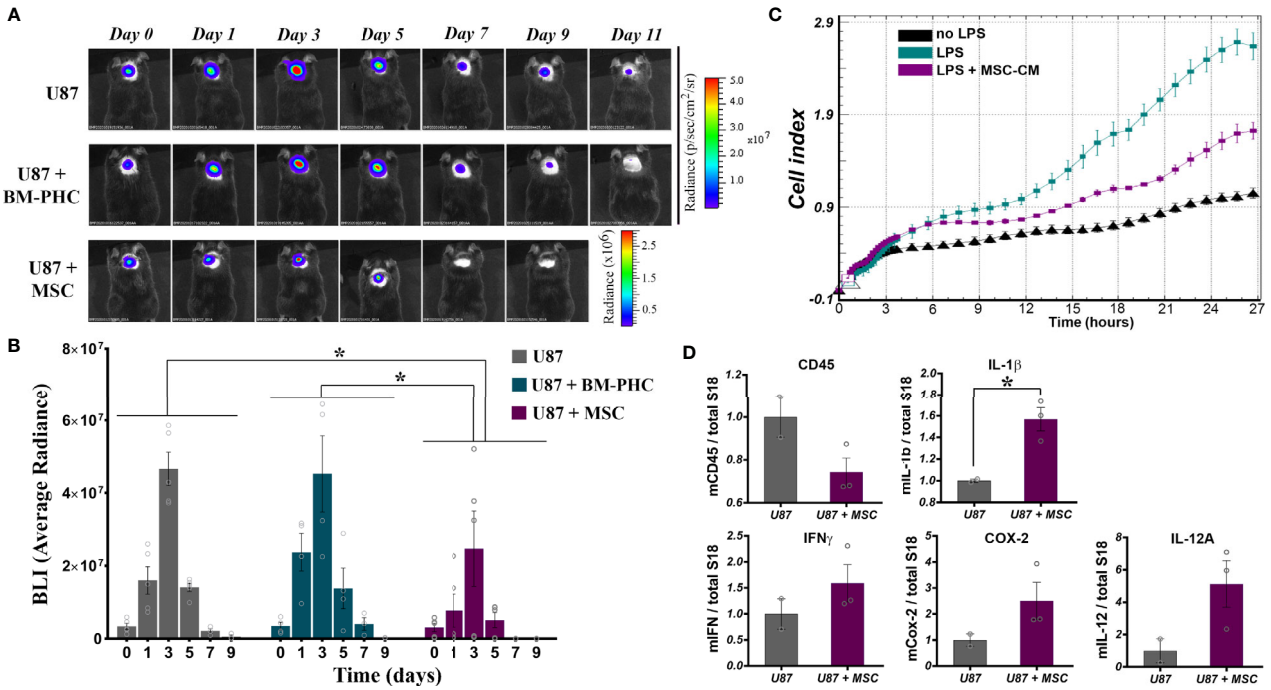

FIGURE 6 | The effects of BM-PHC and MSC on tumor development in immunocompetent mice. **(A)** A representative bioluminescent image of C57Bl/6 mice subcutaneously transplanted with U87 cells alone (U87) or in the presence of BM-PHC or MSC is given above for each time point analyzed. **(B)** The diagram illustrates the bioluminescence signal of the tumor cells in all three groups, determined as average radiance. Values represent means+/-SEM of n=4-5 animals/group. **(C)** Dynamic assessment (original recording) of the effect of MSC secretome on macrophage activation in the presence of LPS. The recording represents mean values obtained from triplicates of one representative experiment from 2 experiments. **(D)** Quantification by real time RT-PCR (n = 2-3 per group) of the relative expression of genes associated with inflammation in cellular aggregates of tumor cells extracted after 5 days from implantation. *p < 0.05.

as HGF mostly exerts its role on dendritic cells (54–56). Still, HGF has many other reported functions, such as induction of angiogenesis, promotion of cell proliferation and migration, and inhibition of apoptosis (57), which are accomplished through its receptor, c-Met. T cells were showed to express c-Met, which was reportedly involved in immune system activation against cancer cells overexpressing HGF (54). Our inhibitory studies showed that inhibition of c-Met by SGX523 negatively affected T lymphocytes, thus emphasizing that HGF-c-Met signaling was crucial for normal cellular processes both in MSC and T cells.

Another mechanism of MSC-mediated immunosuppression involves NO secretion (45, 46, 58). Our study showed that co-culture of MSC with activated T cells in the presence of L-NAME partially reversed the MSC inhibitory effect. It is important to emphasize that this effect was elicited by IFNγ and TNFα, which were actively secreted by activated T cells (59, 60), and in their absence MSC did not produced NO (40, 46). Indeed, our *in vitro* studies confirmed that only MSC stimulated with both IFNγ and TNFα produced high levels of nitrite in the culture medium.

In addition to the contribution of PGE2 and NO in MSC-mediated immunosuppression of T cell proliferation, we showed here a decline in Ly-6C expression on MSC in the presence of TNFα and an increase in the presence of IFNγ, which suggested that the anti- or pro-inflammatory behavior of MSC was decided by the balance between these two cytokines in various settings (19). The presence of Ly-6C on MSC has not been acknowledged before. It would be interesting to find out if this expression is a particularity of these cells (C57Bl/6-derived MSC) or is a more generalized characteristic of mouse MSC.

We also report here that BM-PHC reveal similarities with anti-inflammatory macrophages and share many characteristics with MSC, in terms of surface markers and immunomodulatory properties *in vitro*. These $CD45^{pos}$ cells with positive expression of F4/80 and negative expression of Ly-6C and Ly-6G are different from the cell subset termed myeloid-derived suppressor cells (MDSC), which are basically inflammatory cells, and have been defined based on the high expression levels of Ly-6C (monocyte-derived MDSC) and/or Ly-6G (polymorphonuclear-derived MDSC) and lack of F4/80 molecules (61–64). However, these cells share several biological properties with MDSC, such as myeloid origin, *in vitro* proliferation, and suppressive potential for T cell proliferation. Other similarities of BM-PHC with MDSC refer to the mechanisms they use to suppress immune functions, as previous reports indicated that MDSC used inducible NO synthase and arginase for suppressing immune functions (65). Besides, up-regulation of COX-2 and PGE2 by MDSC had also been mentioned among the mechanisms of immunosuppression (39).

BM-PHC secreted high levels of CCL6, which was partially involved in the suppressive effect of these cells on T cell proliferation. As CCL6 was not secreted by MSC, this molecule

might explain the superior immunosuppressive effects of BM-PHC over MSC. In harmony with our results, a previous study suggested a role of CCL6 in the antileukemic immune response and CCL6 down-regulation as a mechanism adopted by leukemic cells to evade the immune system (66). Furthermore, another study reported an apoptotic effect of CCL6 on several cell lines (67).

In conclusion, multiple cell populations with immunomodulatory properties can be obtained from bone marrow aspirate. They all may be valuable for therapeutic purposes, however the individual effect of each of them should be established in disease-relevant contexts.

A possible debating conclusion based on results reported in this paper is the antitumor effect of MSC *in vivo*. Extensive studies have been previously conducted and conflicting results have been reported with regards to the role of MSC in cancer therapy (68). On the one hand, there are studies boosting the conceptualization of MSC-based experimental cancer therapy by showing that MSC prevented tumor progression and metastasis though inhibiting angiogenesis or suppressing immune responses (69–73). On the other hand, several other studies reported the pro-tumorigenic properties of MSC (74–77). Similarly, although in a different context, MSC were demonstrated to delay the allograft rejection and generate a local immune privileged site (78).

Another debate that may also hinder the therapeutic potential of MSC is the significant safety concerns regarding the possible long-term tumor growth after MSC infusion, as previously reported in mice (79). Such *in vivo* spontaneous malignant transformation of mouse MSC have been previously documented particularly after long-term *in vitro* culture (80, 81), which sustained the hypothesis that cell characteristics are dynamics and change depending on intracellular and extracellular stimuli. In our experimental setting, MSC co-administrated with tumor cells generated a more rapid xenograft rejection in immunocompetent mice. While MSC apparently decreased the murine CD45 expression inside the tumor, the tumor microenvironment induced MSC polarization towards the inhibitory functionality, which resulted in rapid tumor annihilation. However, in this paper we have only focused on the fate of tumor cells, yet not followed the long-term effects of MSC transplant, therefore we cannot deliberate on malignant transformation of MSC *in vivo*. However, MSC remain unquestionably a promising therapy option for a variety of diseases, yet despite numerous *in vitro* and *in vivo* studies, there is much more that is still unknown and as such, more research and observations will be necessary to investigate the long-term effects of MSC therapies.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethical Committee of ICBP.

AUTHOR CONTRIBUTIONS

C-IM, MP, CN, ER, SP, and AB performed experiments. C-IM, MP, and AB analyzed and interpreted the data. AB designed the work and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.698070/full#supplementary-material.

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