RESEARCH ARTICLE

PRR signaling during *in vitro* macrophage differentiation from progenitors modulates their subsequent response to inflammatory stimuli

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ABSTRACT. Toll-like receptor (TLR) agonists drive hematopoietic stem and progenitor cells (HSPCs) to differentiate along the myeloid lineage *in vitro* and also *in vivo* following infection. In this study, we used an *in vitro* model of HSPC differentiation to investigate the functional consequences (cytokine production) that exposing HSPCs to various pathogen-associated molecular patterns (PAMPs) and *Candida albicans* cells have on the subsequently derived macrophages. Mouse HSPCs (Lin^- cells) were cultured with GM-CSF to induce macrophage differentiation in the presence or absence of the following pattern recognition receptor (PRR) agonists: Pam_3CSK_4 (TLR2 ligand), LPS (TLR4 ligand), depleted zymosan (which only activates Dectin-1), or inactivated *C. albicans* yeasts (which activate several PRRs, mainly TLR2 and Dectin-1). Our data show that only pure TLR2 ligand exposure (transient and continuous) impacts the inflammatory function of GM-CSF-derived macrophages, because Pam_3CSK_4-exposed HSPCs generate macrophages with a diminished ability to produce inflammatory cytokines. Interestingly, the Pam_3CSK_4-induced tolerance of macrophages (by transient exposure of HSPCs) is reinforced by subsequent exposure to *C. albicans* cells in GM-CSF-derived macrophages; however, the induced tolerance is partially reversed in M-CSF-derived macrophages. Therefore, the ability of macrophages to produce inflammatory cytokines is extremely dependent on how the HSPCs from which they are derived receive and integrate multiple microenvironmental signals (PRR ligands and/or CSFs).

Key words: hematopoietic stem and progenitor cells, macrophages, M-CSF, GM-CSF, TLRs, *Candida albicans*

Myeloid phagocytes play key roles in inflammation process and antipathogen defence. During infection, myeloid cells detect microorganisms and microbial components using pattern recognition receptors (PRRs), including toll-like receptors (TLRs) and are responsible for microbial killing, antigen processing and presentation to initiate adaptive immune response and for releasing proinflammatory cytokines to recruit and activate other leukocytes [1].

Because innate immune cells have a limited life span, their replenishment is essential during homeostasis and especially important during infection so that cells killed by microorganisms or consumed during the immune response can be replaced and immune surveillance increased. In most acute infections, myelopoiesis becomes the predominant form of cellular production (emergency myelopoiesis) while the development of other lineages (lymphoid and erythroid) is inhibited [2, 3]. It is well established that enhanced myelopoiesis during infection is coordinated by cytokines produced by differentiated cells in response to pathogens, but several recent reports have demonstrated a new mechanism of direct interaction of pathogens or their components with hematopoietic stem and progenitor cells (HSPCs) [4]. Murine and human HSPCs express functional TLRs and TLR signals provoke cell cycle entry and myeloid differentiation, indicating that TLR signaling in HSPCs can contribute to HSPC expansion and myeloid differentiation during infection [5-7].

Our group had previously demonstrated that *Candida albicans*, the most frequent cause of opportunistic fungal infections, induces proliferation of HSPCs and their differentiation toward the myeloid lineage both *in vitro* and *in vivo*. This response requires signaling through TLR2 and Dectin-1 and gives rise to functional macrophages able to internalize yeasts and secrete proinflammatory cytokines [8-11]. Although the concept of microbial components directly stimulating HSPCs to trigger the rapid generation of myeloid cells to fight the infection is certainly

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attractive, full exploration of the functional consequences is critical. In this context, we have previously shown that detection of pathogen-associated molecular patterns (PAMPs) by HSPCs impacts the antimicrobial function of the macrophages they produce [12]. Pure soluble TLR2 and TLR4 ligands generate macrophages with a diminished ability to produce inflammatory cytokines, whereas HSPC activation in response to *C. albicans* leads to the generation of macrophages that produce higher levels of cytokines than control M-CSF-derived macrophages (homeostatic–like macrophages) [13].

In this study, we have extended research on our previous studies to GM-CSF-derived macrophages (produced during the inflammatory state) to show that the functional phenotype of macrophages is extremely dependent on how HSPCs receive and integrate multiple microenvironmental signals (PRR ligands and/or CSFs).

MATERIALS AND METHODS

Mice

Lineage negative (Lin^-) progenitor cells were isolated from 8–12-week-old wild-type C57BL/6 female mice (Harlan Ibérica, Barcelona, Spain). The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Valencia, Generalitat Valenciana (Permit Number: 2014/072 type 2).

Purification of Lin^- cells and differentiation with CSFs and PRR agonists

Lin^- cells were purified as previously described [9, 10]. Briefly, murine bone marrow was obtained by flushing the femurs and tibias and the depletion of lineage-positive cells was performed by immunomagnetic cell sorting (negative selection) using MicroBeads and a cocktail of antibodies against a panel of lineage antigens [CD5, CD45R (B220), CD11b, Gr-1 (Ly-6G/C), 7-4 and Ter-119] according to the manufacturer's instructions (Miltenyi Biotec, Madrid, Spain). Purity of the sorted cells was assessed by labeling with anti-Lin cocktail and by flow cytometry analysis; as expected, no Lin-positive cells were detected.

Purified cells were immediately cultured in complete cell culture medium: RPMI 1640 medium supplemented with 2 mM L-glutamine, 5% heat-inactivated fetal bovine serum and 1% penicillin–streptomycin stock solution (Gibco, Barcelona, Spain). The complete culture medium was supplemented with two cytokines: 20 ng/mL stem cell factor (SCF, Peprotech, Rocky Hill, NJ) to support the survival of HSPCs and 50 ng/mL of granulocyte-macrophage colony-stimulating factor (GM-CSF, Peprotech, Rocky Hill, NJ) or 50 ng/mL macrophage colony-stimulating factor (M-CSF, Miltenyi Biotec, Madrid, Spain) to induce their differentiation to macrophages. Cultures also contained PRR agonists or inactivated *C. albicans* yeasts as stimuli, either for the first 24 hours or throughout the seven days of culture or during the last six days, as indicated in *figures 1A, 3A* and *4A*.

PAMPs and preparation of fungal cells

The stimuli used were Pam_3CSK_4 (1 μg/mL), Ultrapure *Escherichia coli* LPS (100 ng/mL), depleted zymosan (10 μg/mL) (Invivogen, San Diego, CA), or inactivated *C. albicans* ATCC 26555 yeasts (at a 1:7.5 ratio, murine cell:yeast) obtained as previously reported [14, 15]. Briefly, starved yeast cells were inoculated (200 μg dry weight of cells per mL) in a minimal synthetic medium and incubated for 3 hours at 28°C to obtain yeasts. For inactivation, fungal cells were resuspended (20×10^6 cells/mL) in 4% paraformaldehyde (fixation buffer, eBioscience, San Diego, CA) and incubated for 1 hour at room temperature. After treatment, fungal cells were extensively washed in PBS and brought to the desired cell density in cell culture medium. All procedures were performed under conditions designed to minimize endotoxin contamination as described elsewhere [15, 16].

Antibodies and flow cytometry analyses

The following antibodies were used in this study: APC-labeled anti-CD11b (clone M1/70.16.11.5 from Miltenyi Biotec), FITC-labeled anti-MHC class II (clone M5/114.15.2 from eBioscience) and PE-labeled anti-Ly6C (clone AL-21 from BD Pharmigen). Flow cytometry analyses were performed on an LSR Fortessa cytometer (BD Biosciences) and the data were analyzed with FACSDiva and FlowJo 10 software.

Measurement of cytokine production

Macrophages were plated in flat-bottomed 96-well plates at a density of 50,000 cells in 200-μL complete cell culture medium. Cells were challenged with the indicated stimuli for 24 hours and cell-free supernatants were then harvested and tested for TNF-α and IL-6 release using commercial ELISA kits (eBioscience, San Diego, CA). Unstimulated macrophages served as negative controls. Triplicate samples were analyzed in each assay.

Statistical analyses

Statistical differences were determined using one-way ANOVA followed by Dunnett's test for multiple comparisons and two-tailed Student's t-test for dual comparisons. Data were expressed as mean ± SD. Significance was accepted at $*P<0.05$, $**P<0.01$ and $***P<0.001$ levels.

RESULTS

Exposure of HSPCs to PRR agonists during differentiation alters macrophage expression of Ly6C and MHC class II

We investigated the consequences of *in vitro* exposure of HSPCs to PAMPs during differentiation by comparing the phenotype of the macrophages they produce. Lin^- cells were cultured with GM-CSF to induce macrophage differentiation in the presence or absence (control) of different PRR agonists: Pam_3CSK_4 (which only activates TLR2), LPS (which only activates TLR4), depleted zymosan (a Dectin-1-activating *Saccharomyces cerevisiae* cell wall preparation that has been treated with hot alkali to remove its TLR-stimulating properties), or *C. albicans* yeasts (which activate several PRRs, mainly TLR2 and Dectin-1). In these conditions, PRR agonists are present throughout differentiation (*figure 1A*, continuous expo-

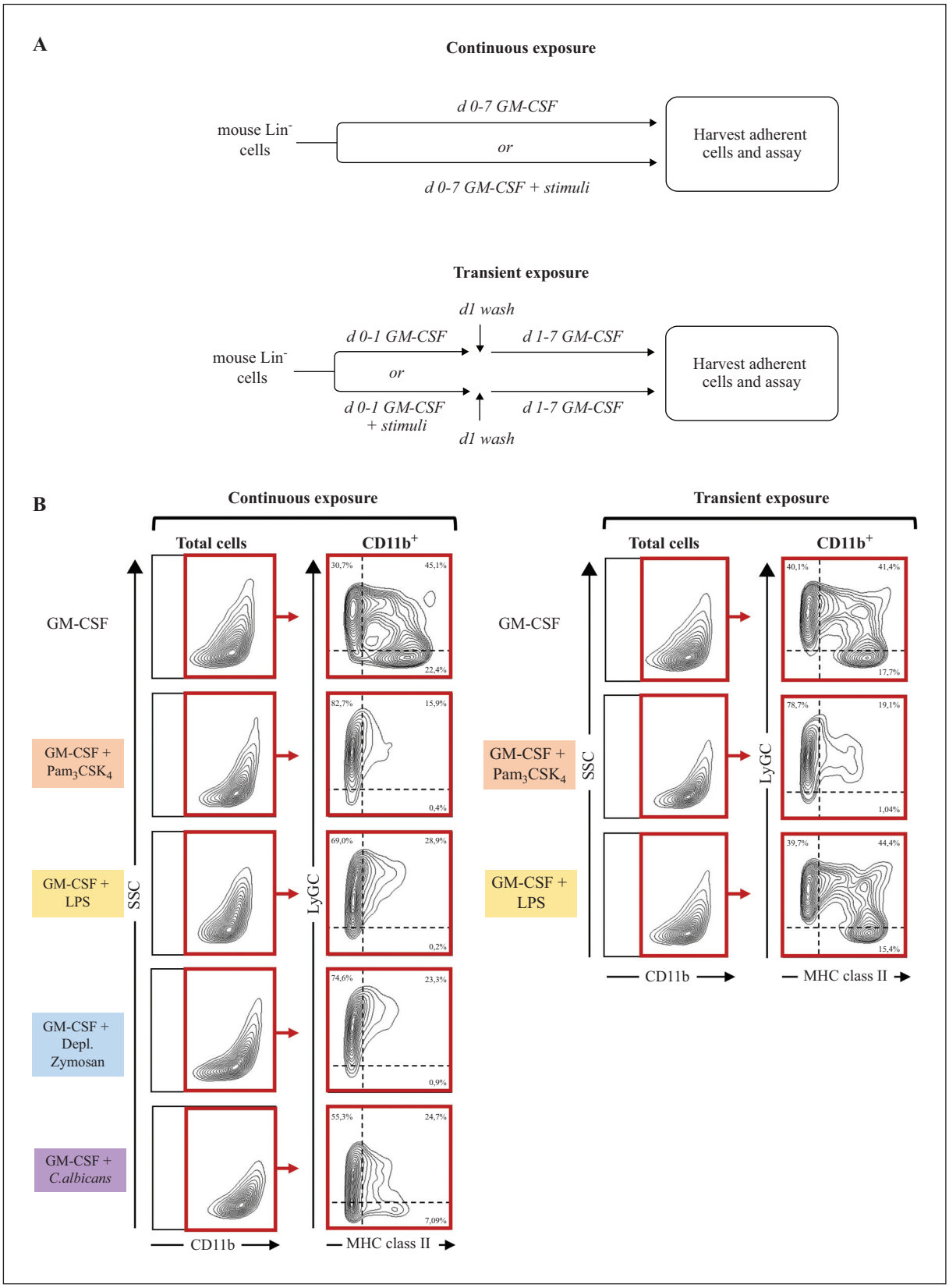

Figure 1

Effect of continuous or transient exposure to different stimuli (PAMPs) during *in vitro* differentiation of HSPCs into macrophages. (A) Schematic protocol (as described in Materials and methods). Lin⁻ HSPCs were plated at a density of 200,000 cells in 4 mL of complete medium with SCF and GM-CSF and incubated for seven days to induce macrophage differentiation in the absence or presence of different microbial stimuli for the seven days (continuous exposure) or the first 24 hours only (transient exposure). The stimuli used were Pam_3CSK_4 (1 μg/mL), LPS (100 ng/mL), depleted zymosan (10 μg/mL), or inactivated yeasts of *C. albicans* (1:7.5 murine cell:yeast ratio). (B) After seven days of culture, adherent cells were harvested, labeled with antibodies and analyzed by flow cytometry. Macrophages were gated as $CD11b^+$ and subsequently analyzed in an MHC class II *versus* Ly6C plot. The indicated percentages refer to cells analyzed in each contour-plot. Results shown are representative of three independent experiments.

sure), most closely reproducing the *in vivo* situation during an ongoing infection, when HSPCs in the bone marrow or infected tissues may interact with the microorganisms or their products. Moreover, we also investigated the consequences of exposing HSPCs to soluble TLR2 or TLR4 agonists prior to their differentiation (by adding these agonists during the first 24 hours of culture and then removing them by thoroughly washing the cells). This transient exposure model (*figure 1A*, transient exposure) enables us to define the phenotype of macrophages generated by previously exposed HSPCs and, thus, determines whether TLR signaling in HSPCs impacts the phenotype of macrophages produced after clearance of an infection.

In all cases, after seven days of culture, adherent cells were harvested and the phenotypic surface markers of the GM-CSF-derived adherent cells were analyzed by multicolor flow cytometry (*figure 1B*). It has been described that the output from culturing mouse bone marrow cells with GM-CSF is heterogeneous and comprises different CD11b-positive mature myeloid cells including granulocytes, macrophages and dendritic cells; macrophages are thought to be enriched in the adherent fraction [17]. In our culture conditions of Lin^- cells, the mature CD11b-positive adherent population obtained (GM-CSF-derived macrophages) was heterogeneous in terms of expression of MHCII and Ly6C, as roughly 30% cells were $Ly6C^+MHCII^-$, 22% cells were $Ly6C^-MHCII^+$ and 45% cells were $Ly6C^+MHCII^+$.

When Lin^- progenitors were cultured in the same conditions but in the presence of TLR ligands, the surface marker profiles were different. The $CD11b^+$ adherent macrophages generated in the presence of Pam_3CSK_4 and LPS exhibited increased Ly6C expression (from 30% to 83% and 69% single positive cells, respectively) and a significant decrease in MHC class II expression (from 22% to 0.4% and 0.2% single positive cells, respectively). The percentage of double positive cells $Ly6C^+MHCII^+$ decreased (from 45% to 16% and 29%, respectively). Depleted zymosan and *C. albicans* yeast treatment changed Ly6C and MHC class II expression by GM-CSF-derived macrophages in a similar way (*figure 1B*, continuous exposure), although in a lower extent than Pam_3CSK_4 and LPS. This was noted particularly in the case of yeasts as the percentage of $MHCII^+$ single positive cells remained at 7%. Interestingly, although LPS and Pam_3CSK_4 induced similar phenotypic changes upon continuous exposure, transient exposure to LPS did not change Ly6C or MHC class II expression, whereas transient exposure to Pam_3CSK_4 induced a similar phenotypic change to continuous exposure (*figure 1B*, transient exposure).

In summary, transient exposure of HSPCs to a TLR2 agonist (Pam_3CSK_4) was sufficient to induce a different phenotype with increased Ly6C expression and lower MHCII expression. The TLR4 agonist (LPS) induced the same changes but only following continuous exposure; similarly, the surface markers of GM-CSF-derived macrophages were changed in the same way by both *C. albicans* yeasts and the Dectin-1 agonist (depleted zymosan) continuous exposure.

Differential cytokine production by macrophages derived from HSPCs differentiated in the presence of PRRs agonists

Next, we wondered whether the exposure of HSPCs to PAMPs, including specific fungal ligands and *C. albicans* yeasts, impacts the ability of the generated macrophages to secrete proinflammatory cytokines (*figure 2*).

Lin^- cells were cultured with GM-CSF to induce macrophage differentiation, in the presence (continuous or transient) or absence of different PRR agonists, as indicated in *figure 1A*. After seven days, the adherent harvested cells were counted and equal numbers of macrophages were stimulated with TLR agonists (for 24 hours) to assess their ability to produce cytokines. Unstimulated macrophages served as negative controls. The production of TNF-α and IL-6 in response to Pam_3CSK_4 or LPS was significantly diminished in macrophages generated from HSPCs exposed (transiently or continuously) to the TLR2 ligand (Pam_3CSK_4) compared to GM-CSF-derived control macrophages (*figure 2*).

Interestingly, macrophages generated from LPS-exposed HSPCs produced similar proinflammatory cytokine levels to control macrophages. The secretion of TNF-α and IL-6 in response to Pam_3CSK_4 was slightly increased in macrophages generated from HSPCs in the presence of depleted zymosan compared to control macrophages, whereas *C. albicans* yeasts did not change the production of cytokines in any condition. These results indicate that exposure of HSPCs to a TLR2 agonist during differentiation (continuous or transient) causes a reduction in the ability of the macrophages derived from them in the presence of GM-CSF to produce proinflammatory cytokines. Neither differentiation in the presence of the soluble TLR4 ligand nor the Dectin-1 agonist (depleted zymosan particles) or yeasts provokes a consistent altered cytokine production of GM-CSF-derived macrophages.

C. albicans yeasts modulate the Pam_3CSK_4-induced tolerance phenotype

Tolerance in macrophages is a well-known process whereby cells that previously responded to LPS or Pam_3CSK_4 display a reduced ability to produce inflammatory cytokines upon subsequent stimulation [18]. Our data indicate that Pam_3CSK_4 tolerance also occurs in HSPCs, because transient exposure of Lin^- cells to Pam_3CSK_4 results in the generation of GM-CSF-derived macrophages with a reduced ability to produce inflammatory cytokines. Taking into account that it has been recently reported that β-glucan (the ligand of Dectin-1) reverses the LPS-induced tolerance of macrophages [19], we wondered whether specific fungal ligands and *C. albicans* yeasts are able to reverse the Pam_3CSK_4-induced tolerance that occurs in HSPCs.

Lin^- cells were cultured with GM-CSF in the presence or absence (control) of Pam_3CSK_4 on day 0, washed thoroughly to remove the Pam_3CSK_4 on day 1 and then cultured with GM-CSF to induce macrophage differentiation for further six days in the presence or absence of depleted zymosan or *C. albicans* yeasts, as indicated in *figure 3A*. The adherent harvested cells were then counted and equal numbers of macrophages were plated for stimulation with TLR agonists (Pam_3CSK_4 or LPS) to assess

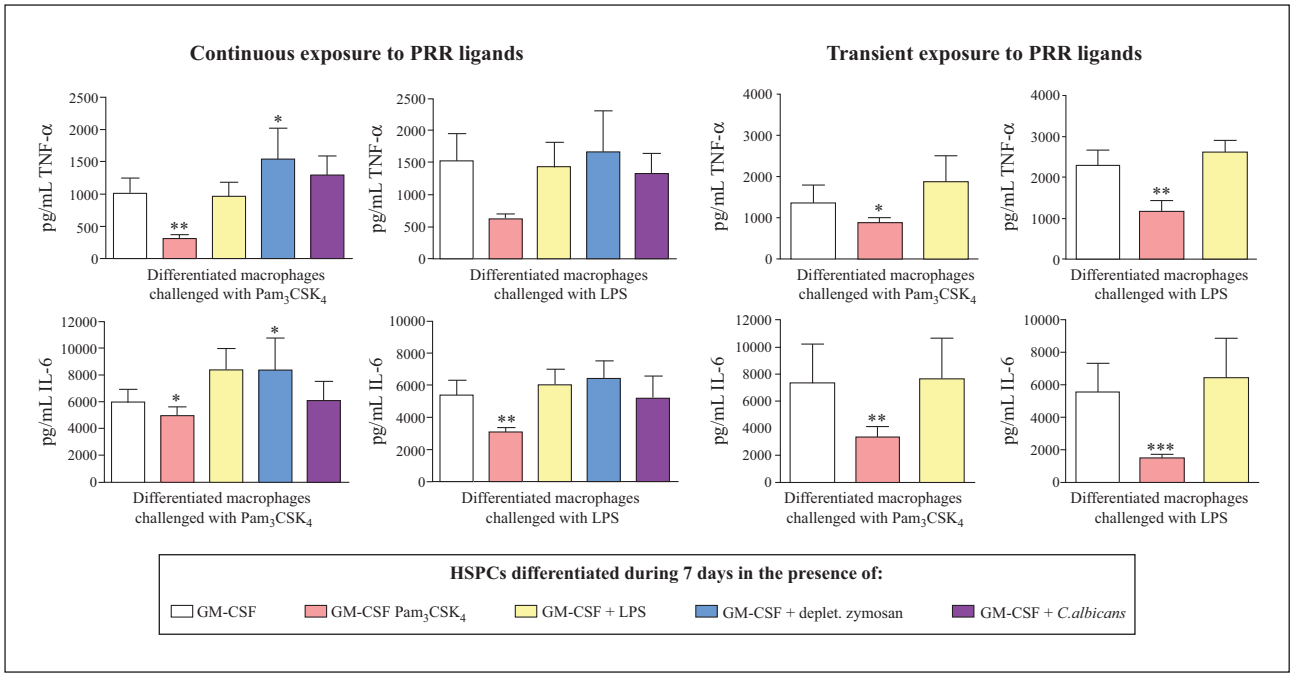

Figure 2

Cytokine production by adherent cells differentiated from HSPCs following transient or continuous exposure to PRR ligands. Macrophages obtained from HSPCs following continuous or transient exposure to different stimuli (as indicated in *figure 1A*) were plated at a density of 50,000 cells in 200-μL complete cell culture medium and challenged with Pam_3CSK_4 (100 ng/mL) or LPS (100 ng/mL) for 24 hours. TNF-α and IL-6 levels in cell-free culture supernatants were measured by ELISA. Results are expressed as means ± SD of pooled data from three experiments. *$P<0.05$, ** $P<0.01$ and *** $P<0.001$ with respect to cytokine production by control cells (macrophages derived from HSPCs differentiated with GM-CSF only).

their ability to produce cytokines. As expected, the production of TNF-α and IL-6 in response to both Pam_3CSK_4 and LPS was significantly diminished in macrophages generated from HSPCs exposed (transiently) to the TLR2 ligand (Pam_3CSK_4) compared to GM-CSF-derived control macrophages (*figure 3B*). However, neither depleted zymosan nor *C. albicans* yeasts (during the last six days of differentiation) reversed the lower production of cytokines by Pam3CSK4-tolerized GM-CSF-derived macrophages. Rather, they even decreased further the production of TNF-α and IL-6 in response to Pam_3CSK_4, while they did not modify the cytokine production in response to LPS.

These findings prompted us to investigate whether this reduced inflammatory responsiveness of GM-CSF-derived macrophages induced by sequential exposure to Pam_3CSK_4 and yeasts (during their differentiation process) may also occur in M-CSF-derived macrophages. In this context, we have previously shown that HSPC activation in response to *C. albicans* leads to the generation of M-CSF-derived macrophages that produce higher levels of inflammatory cytokines [13]. Lin^- cells were cultured with M-CSF to induce macrophage differentiation in the presence (transient) or absence of Pam_3CSK_4 and then cultured with M-CSF to induce macrophage differentiation for further six days in the presence or absence of depleted zymosan or *C. albicans* yeasts, as indicated in *figure 4A*. As previously reported [13], the production of TNF-α and IL-6 in response to Pam_3CSK_4 or LPS was significantly diminished in macrophages generated from HSPCs exposed (transiently) to Pam_3CSK_4 during their differentiation, compared to M-CSF-derived control macrophages (*figure 4B*). Our results showed that differentiation of Lin^- cells with M-CSF in the presence of fungal stimuli following a transient Pam_3CSK_4 challenge partially reversed the tolerized phenotype: the generated macrophages produced more cytokines than Pam_3CSK_4-tolerized macrophages but still significantly minor amounts than M-CSF control macrophages. This effect (partial reversion of tolerization) was stronger in response to *C. albicans* than to depleted zymosan. The reduced production of IL-6 by macrophages (following transient exposure of HSPCs to Pam_3CSK_4) was not affected by subsequent exposure during differentiation to depleted zymosan in M-CSF-derived macrophages (*figure 4B*).

Therefore, *C. albicans* yeasts reinforce the tolerized phenotype induced by the TLR2 ligand (Pam_3CSK_4) in GM-CSF-derived macrophages, while partially reversing it in M-CSF-derived macrophages.

DISCUSSION

Additional perspectives on hematopoiesis during infection have come from the discovery that HSPCs express functional TLRs and that TLR signals provoke myeloid differentiation [5, 20]. Various mechanisms govern HSPC responses to infection, including cytokine signaling, niche function and direct sensing of pathogen-derived molecules by HSPCs themselves [3, 4, 21, 22]. The mechanism by which HSPCs sense pathogens indicates a new role for TLRs because they are involved in instructing immune cell development following direct detection of microbes by HSPCs and may lead to generation of mature innate immune cells to fight the infection. However, a study of the functional properties of the generated myeloid cells is essential to discern whether pathogens may modulate HSPC responses to evade the immune system or, alternatively, this mechanism may be protective, allowing a rapid

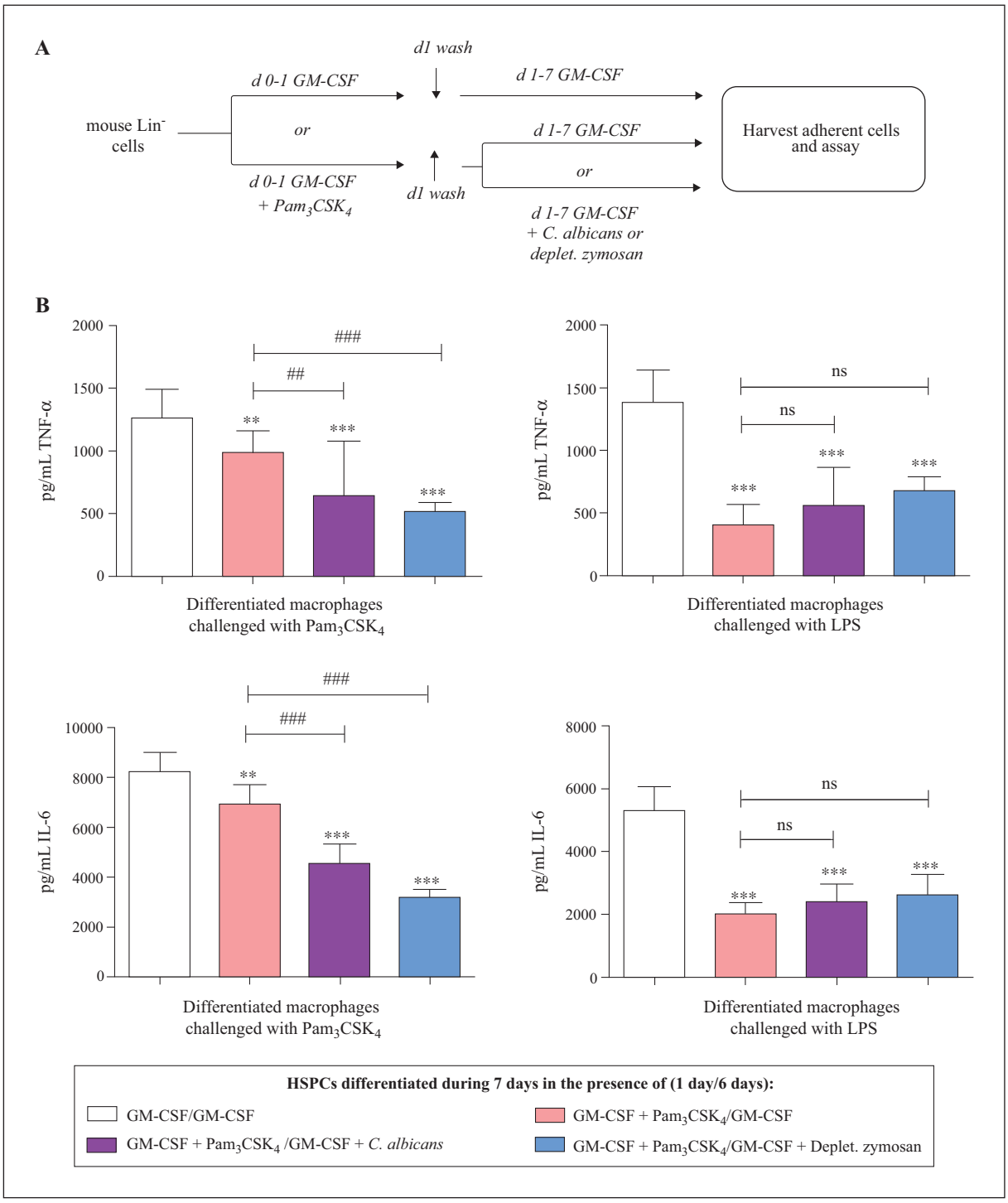

Figure 3

Cytokine production by GM-CSF-derived adherent cells obtained from HSPCs under the conditions indicated in the schematic protocol (A): Lin⁻ HSPCs were cultured (as described in Materials and methods) on day 0 with GM-CSF for 24 hours in the presence or absence of Pam_3CSK_4 (1 μg/mL). Cells were washed thoroughly on day 1 and then cultured with GM-CSF for further six days in the presence or absence of depleted zymosan (10 μg/mL) or inactivated yeasts of *C. albicans* (1:7.5 murine cell:yeast ratio). (B) Adherent cells were harvested and plated at a density of 50,000 cells in 200 μL of complete cell culture medium and challenged with Pam_3CSK_4 (100 ng/mL) or LPS (100 ng/mL) for 24 hours. TNF-α and IL-6 levels in cell-free culture supernatants were measured by ELISA. Results are expressed as means ± SD of pooled data from two experiments. ns: non significant, ** $P<0.01$ and *** $P<0.001$ with respect to cytokine production by control cells (macrophages derived from HSPCs differentiated with GM-CSF only) and ## $P<0.01$ and ### $P<0.001$ with respect to cytokine production by cells derived from HSPCs transiently exposed to Pam_3CSK_4.

generation of cells in a pathogen-specific manner, which are better prepared to deal with the infection.

In this study, we induced macrophage differentiation from HSPCs *in vitro* by using factors produced by the host during infection/inflammatory conditions (GM-CSF-derived control macrophages). In addition, we studied the functional consequences of the presence of PRR agonists during their differentiation. The exposure of HSPCs to TLR2, TLR4, or Dectin-1 agonists, as well as to inactivated yeasts of *C. albicans*, during the differentiation process, modified the surface expression of Ly6C and MHC class II of GM-CSF-derived macrophages. PRR signaling increased the percentage of cells that express the inflammatory monocyte marker Ly6C and are MHC class

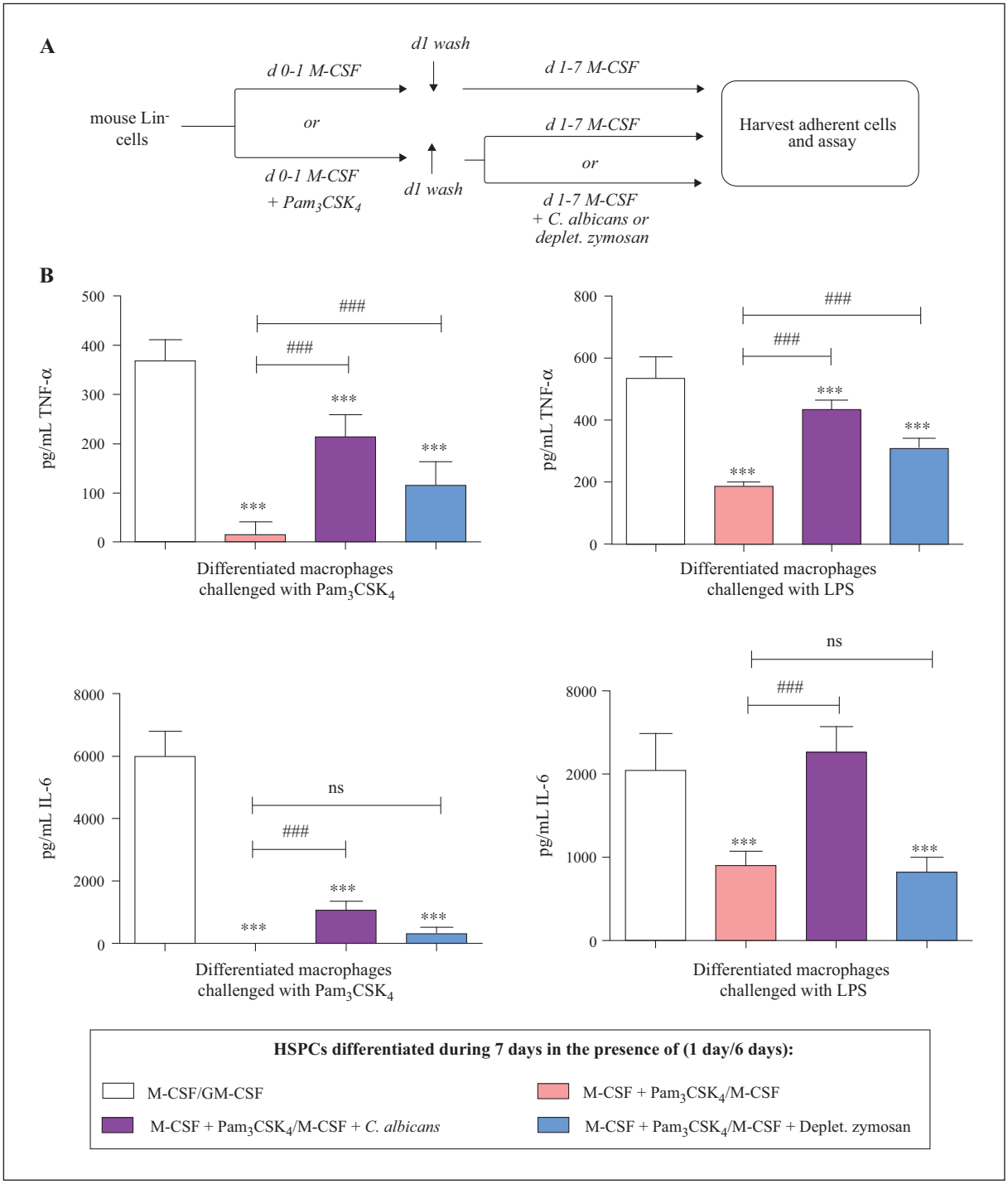

Figure 4

Cytokine production by M-CSF-derived adherent cells obtained from HSPCs under the conditions indicated in the schematic protocol (A): Lin^- HSPCs were cultured (as described in Materials and methods) on day 0 with M-CSF for 24 hours in the presence or absence of Pam_3CSK_4 (1 μg/mL). Cells were washed thoroughly on day 1 and then cultured with M-CSF for further six days in the presence or absence of depleted zymosan (10 μg/mL) or inactivated yeasts of *C. albicans* (1:7.5 murine cell:yeast ratio). (B) Adherent cells were harvested and plated at a density of 50,000 cells in 200 μL of complete cell culture medium and challenged with Pam_3CSK_4 (100 ng/mL) or LPS (100 ng/mL) for 24 hours. TNF-α and IL-6 levels in cell-free culture supernatants were measured by ELISA. Results are expressed as means ± SD of pooled data from two experiments. ns: non significant, *** $P<0.001$ with respect to cytokine production by control cells (macrophages derived from HSPCs differentiated with M-CSF only) and ### $P<0.001$ with respect to cytokine production by cells derived from HSPCs transiently exposed to Pam_3CSK_4.

II negative, a phenotype that corresponds to inflammatory macrophages that play a known role in defence against pathogens. Interestingly, transient exposure (before differentiation) to Pam_3CSK_4 induced the same phenotype clearly indicating that TLR2 signaling on HSPCs impacts the phenotype of the macrophages they produce during infection/inflammation.

Next, using the same model of HSPC differentiation, we focused on the ability to produce inflammatory cytokines by the macrophages generated in the different conditions. Here we found that GM-CSF-derived macrophages generated from HSPCs exposed to a TLR2 agonist (transiently or continuously, during differentiation) produced lower levels of inflammatory cytokines upon being challenged

with inflammatory stimuli. This result is in agreement with previous reports showing a lower production of IL-6 and TNF-α by M-CSF-derived macrophages generated from HSPCs exposed to Pam_3CSK_4 as compared with M-CSF control macrophages [12, 13]. However, the cytokine production by GM-CSF-derived macrophages was not modified by challenging HSPCs during differentiation with LPS, depleted zymosan, or *C. albicans* cells. By contrast, we have previously shown that a TLR4 ligand generates M-CSF-derived macrophages with a limited ability to produce cytokines and that HSPCs activation in response to *C. albicans* leads to the generation of macrophages that produce higher amounts of cytokines than control M-CSF macrophages [13]. These results suggest that in the absence of GM-CSF (in homeostatic conditions or very early during infection), different PRR activation of HSPCs may differentially impact the function of the macrophages they produce, while in the presence of GM-CSF (inflammatory conditions during the infection), only TLR2 activation of HSPCs determines the tolerized phenotype (lower production of inflammatory cytokines).

An increased body of evidence suggests that innate immunity can display some memory characteristics [23, 24]. After first priming, innate immune system would be altered such that upon re-exposition to the same or heterologous stimuli, it would display a trained or tolerized response. For example, exposure of monocytes or macrophages to *C. albicans* enhances their subsequent response to stimulation (trained immunity) while LPS or Pam_3CSK_4 confers to macrophages a long-lasting reduced inflammatory production (tolerance). The effect of innate memory may be even more long-lasting if exposed HSPCs continue to yield macrophages with altered function. Our data indicate that the Pam_3CSK_4-induced tolerance also occurs in HSPCs, because transient exposure of Lin^- cells to Pam_3CSK_4 results in the generation of mature macrophages (M-CSF or GM-CSF-derived) with a reduced ability to produce cytokines.

Endotoxin tolerance in macrophages is a mechanism that avoids sustained activation, which can be detrimental to the host because it can result in endotoxin shock. However, endotoxin tolerance is also partially responsible of the sepsis-induced immunosuppression [25]. In clinical studies, the magnitude and the persistent nature of this tolerized state is associated with increased mortality and nosocomial infection. In this context, it has been recently reported that Dectin-1 activation can reverse macrophage tolerance *ex vivo*, providing a potential therapeutic approach to reverse the tolerized phenotype in sepsis patients [19]. We, therefore, hypothesized that fungal ligands may be capable of reversing the tolerized phenotype of macrophages obtained from HSPCs transiently exposed to Pam_3CSK_4. However, our results showed that fungal ligands reinforced the tolerized phenotype of GM-CSF-derived macrophages but partially reversed the tolerized phenotype of M-CSF-derived macrophages. Overall, these results indicate that activation of HSPCs with *C. albicans* cells may generate macrophages with different phenotype depending on the growth factor (M-CSF or GM-CSF) that induces their differentiation, as well as on the previous stimuli the progenitors received. Therefore, it is likely that during infection the functional properties of the macrophages that have been produced depend on the specific molecular composition of the pathogen (the combination of PRRs triggered) in addition to other signals that the HSPCs receive. Although further studies will be required to define the mechanisms underlying the effects that exposing HSPCs to PAMPs has on macrophage function, epigenetic reprogramming probably underlies these effects as it has been described in several models for innate immunity memory [23, 24].

In conclusion, our data show that detection of PAMPs by HSPCs impacts the cytokine production of the macrophages they produce and that the tolerized or trained phenotype depends on the combinatorial signals they receive (PRRs and CSFs), as well as on the timing of the HSPCs activation by the different stimuli. Therefore, macrophage responses can be programmed by PRR signaling in HSPCs prior to and/or during differentiation, providing new insights in host–microbial interactions during infection, which may reveal a new potential target for anti-infection intervention.

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