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HIGHLIGHTED ARTICLE

Limited role of the spleen in a mouse model of trained immunity: Impact on neutrophilia

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Abstract

Trained immunity is a de facto memory of innate immune cells, resulting in a long-term increase in innate host defense mechanisms after infection. The long-term heterologous protection conferred by trained immunity is mediated through epigenetic and functional reprogramming of hematopoietic stem and progenitor cells. Because the spleen is a reservoir of undifferentiated monocytes and is considered the prime organ for extramedullary hematopoiesis, we investigated the role of the spleen in the establishment of trained immunity. A β-glucan-induced trained immunity mouse model was performed in previously sham-operated or splenectomized animals. Removal of the spleen did not modulate the proinflammatory cytokine production of in vivo trained peritoneal cells, nor did it ablate the increased percentage of proinflammatory circulatory monocytes and natural killer cells seen in trained animals. However, spleen removal prevented neutrophilia, an important characteristic of trained immunity. These data point to a limited role of the spleen in trained immunity. The pathophysiologic relevance of the spleen in the induction of neutrophilia during trained immunity remains to be fully explored.

KEYWORDS
trained immunity, spleen, neutrophils

1 | INTRODUCTION

Immunologic memory is classically attributed to the adaptive immune system. However, a growing body of evidence in human and murine models indicates that the innate immune system has long-term memory characteristics, also termed trained immunity.[1] During induction of trained immunity after certain challenges, innate immune cells respond stronger to a secondary event, conferring protection against heterologous pathogens. Trained immunity has been described primarily in monocytes, macrophages, and natural killer (NK) cells after exposure to different stimuli, such as the bacillus Calmette-Guérin (BCG)

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vaccine and the *Candida albicans* cell wall component β-glucan.[2,3] Due to the short half-life of monocytes in the circulation, the long-lasting protection secured by innate immune memory is thought to be assured centrally with changes in the hematopoietic immune cell progenitors. Mice exposed to BCG or β-glucan show long-term change in the transcriptional and functional program of bone marrow immune progenitors with a bias toward myelopoiesis, which ultimately generates trained myeloid cells.[4,5] Similar observations were recently made also in humans after BCG vaccination.[6]

The spleen is a secondary lymphoid organ, which stores macrophages and recycles erythrocytes. The spleen serves also as a reservoir of bone marrow-derived undifferentiated monocytes that can be expanded and rapidly deployed to the circulation,[7] and is also a hematopoietic organ. Hematopoiesis is an orchestrated process in which a pool of hematopoietic stem progenitor cells (HSPCs) gives rise to multilineage blood cells. HSPCs are mostly located in the bone marrow; however, extramedullary hematopoiesis might occur under emergency conditions. HSPCs mobilized from the bone marrow seed extramedullary sites, proliferate, and mature. Specifically, proinflammatory monocytes and neutrophils have been shown to be produced by the spleen in mice models of atherosclerosis and endotoxin challenge.[8]

Trained immunity relies on HSPCs' expansion and skewing, leading to trained peripheral myeloid cells. We therefore hypothesized that the spleen, as a site of extramedullary hematopoiesis and reservoir of monocytes, is an organ that might contribute to the increased systemic responsiveness conferred by stimuli inducing trained immunity. Taking advantage of the well-established β-glucan trained immunity mouse model, we aimed to explore the role of the spleen in the induction of innate immune memory.

2 | MATERIALS AND METHODS

2.1 | Animals and ethical statement

Eight weeks old female C57BL/6JRj from Janvier Labs (Le Genest Saint Isle, France) were used for all experiments. Animals were allowed 1 wk to acclimatize to the facilities before the experiment. Mice were housed in groups of five animals per enriched type II cage on a 12 h light-dark diurnal cycle with room temperature between 21°C and 23°C. Water and food were provided ad libitum. Procedures were approved by the Central Committee on Animal Experiments, Radboud University (Nijmegen, The Netherlands; 2016-0100).

2.2 | Splenectomy and in vivo training model

Animals were randomized, and half had their spleens removed by electrocauterization (BVI Medical, Waltham, MA, USA). In sham-operated mice, the abdominal wall was opened and then closed after identifying the spleen. All surgical procedures were done under inhalation anesthesia (1% isoflurane) on a heat pad. Body temperature was moni-

tored. Pain was mitigated pre- and post-operatively. A total of 10 mg/kg Carprofen was provided in drinking water 2 d before surgery and continued until 2 d after surgery. In addition, 1 h prior to surgery 5 mg/kg Carprofen was injected s.c. To allow for the inflammation derived from the surgery to resolve, mice were housed for further 70 d without intervention. β-glucan from barley (Sigma-Aldrich, St. Louis, MO, USA) was administered i.p. to 40 mice at day 0 and at day 3. PBS was used as a vehicle control. Animals were euthanized at day 7 or at day 7 plus 3 hours after i.p. injection of 10 μg *Escherichia coli* LPS (*E. coli* LPS; serotype 055:B5, Sigma-Aldrich, St. Louis, MO, USA). Sacrifice involved deep inhalation anesthesia (5% isoflurane) followed by cervical dislocation. Two independent experiments were performed.

2.3 | Peritoneal cell isolation

Peritoneal cells were collected by peritoneal lavage. Briefly, the inner skin lining of the peritoneal cavity was exposed and 5 ml of ice-cold PBS was injected into the cavity using a 27 g needle. The peritoneum was gently massaged to dislodge attached cells. The PBS solution was then collected avoiding organs and fat tissue. Cells were kept on ice in PBS 3% FBS (Hyclone, Logan, UT, USA).

2.4 | Splenocyte isolation

Single-cell suspensions from spleen were prepared using previously described methods.[9] Briefly, spleens were cut and incubated in RPMI 1640 with 300 U/ml of grade II DNAse I (Roche, Basal, Switzerland) and 0.2 mg/ml Liberase TM (Roche, Basal, Switzerland) for 20 min at 37°C with intermittent shaking. Digested tissue was gently squeezed in a sterile 100 μm cell strainer (Fisher Scientific, Waltham, MA, USA). Red blood cells were lysed with a solution of 155 mM NH_4Cl and 10 mM $KHCO_3$.

2.5 | Flow cytometry analysis

Lysed EDTA-anticoagulated peripheral blood and 10^6 splenocytes were incubated with Fc block anti-CD16/32 (Biolegend, San Diego, CA, USA) before the addition of the antibody mix. Fluorophore-labelled anti-mouse antibodies against CD19-PE (Becton Dickinson, Franklin Lakes, NJ, USA), Ly6G-PE (Becton Dickinson, Franklin Lakes, NJ, USA), CD49b-PE (Biolegend, San Diego, CA, USA), CD90.2-PE (Becton Dickinson, Franklin Lakes, NJ, USA), CD11b-PE-Cy7 (Becton Dickinson), CD11c-APC, Cy7 (Becton Dickinson, Franklin Lakes, NJ, USA), Ly6C-APC (Becton Dickinson, Franklin Lakes, NJ, USA), MHCII-BB515 (Becton Dickinson, Franklin Lakes, NJ, USA), and CD64-BV421 (Biolegend, San Diego, CA, USA) were diluted to appropriate working concentrations as provided by the manufacturer. Live/dead fixable aqua stain (Invitrogen, Waltham, MA, USA) was added. Samples were measured in a BD LSR II Flow cytometer. Analysis was performed in FlowJo V10. The gating strategy[9] can be found in Supporting Information Figure S1.
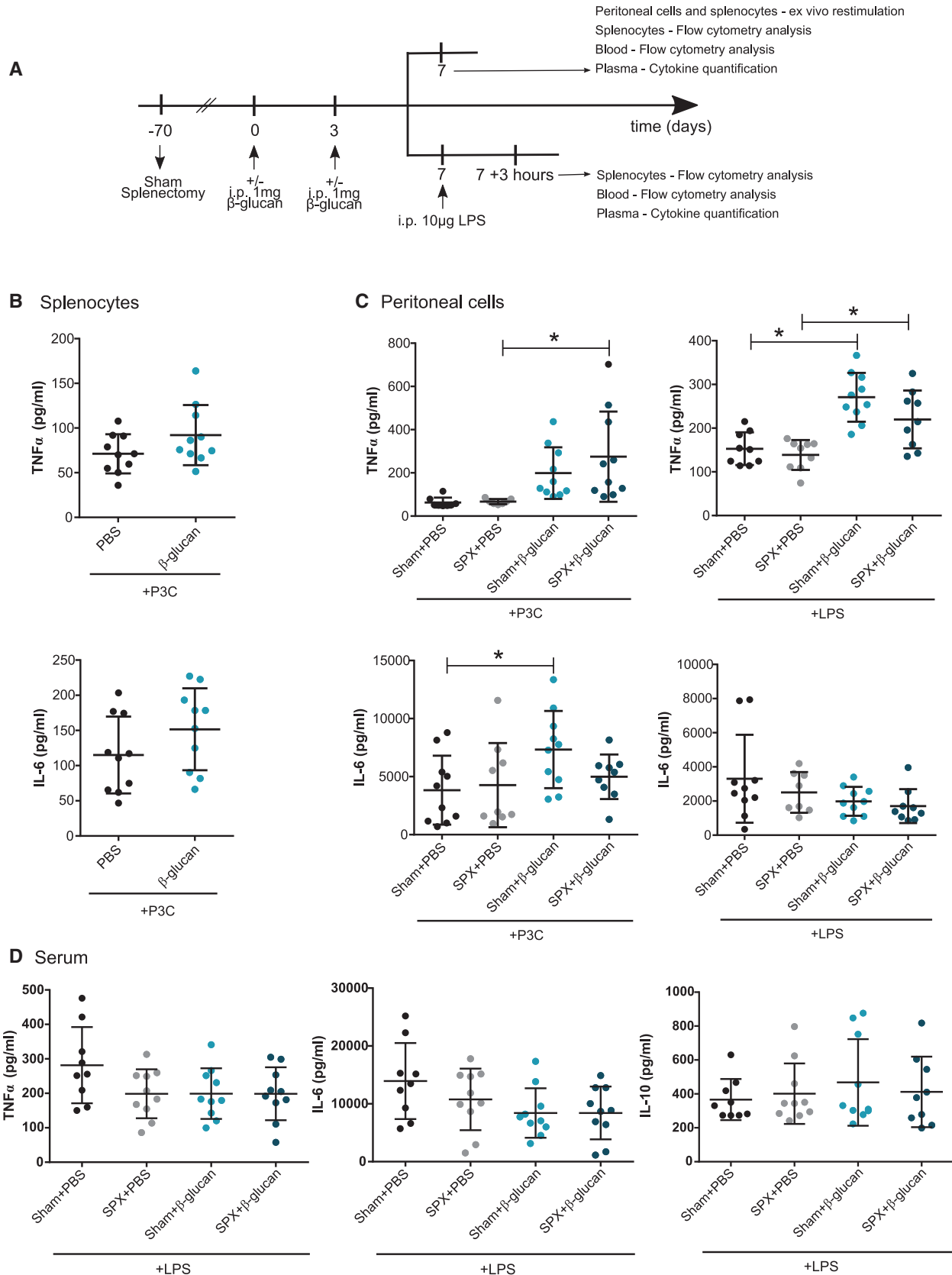
FIGURE 1 **The effect of the spleen on the long-term cytokine production.** (A) Schematic representation of the in vivo training model. (B) Splenocytes and (C) peritoneal cells were isolated 7 d after β-glucan administration. TNF-α and IL-6 secretion was determined after in vitro restimulation with LPS or/and P3C. TNF-α and IL-6 production by splenocytes restimulated by LPS were below the detection limit (*P < 0.05, 2-way ANOVA, Sidak's multiple comparisons test). (D) IL-6, TNFα, and IL-10 measured in mice plasma 3 h after LPS i.p. injection. n = 10 pooled from two independent experiments, mean ± SD. Grubbs's test was applied for outlier exclusion. (P3C Pam3Cys-SKKKK, SPX splenectomy)
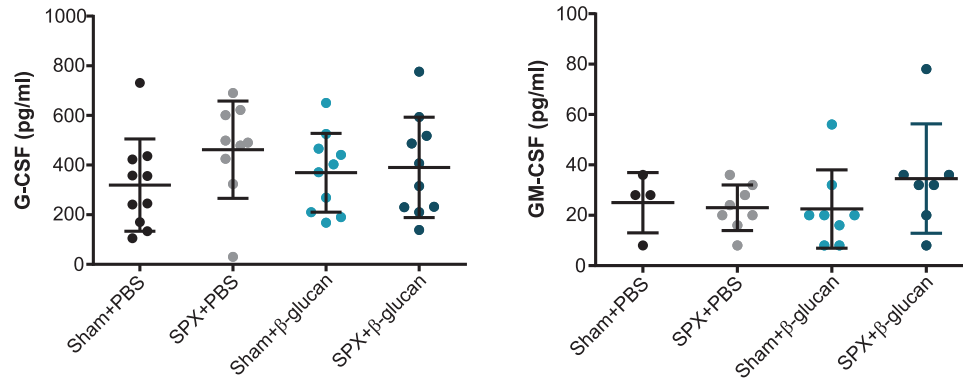
FIGURE 2   The impact of splenectomy on G-CSF and GM-CSF plasma concentrations. Levels of G-CSF and GM-CSF were determined in the serum of mice 7 d after β-glucan administration ($n = 10$ pooled from two independent experiments). GM-CSF quantification values below the limit of detection: Sham + PBS $n = 6$, SPX + PBS $n = 1$, Sham+ β-glucan $n = 2$, SPX+ β-glucan $n = 3$. (SPX splenectomy)

2.6 | Ex vivo immune cell stimulation

A total of 0.5×10^6 splenocytes or 0.5×10^6 peritoneal cells were seeded per well in round or flat bottom 96-well plates, respectively, in RPMI 1640 Dutch modified (Invitrogen, Waltham, MA, USA), 10% FCS (Hyclone), 5 μg/ml gentamicin (Centraform, Etten-Leur, the Netherlands), 2 mM L-glutamine (Gibco, Waltham, MA, USA), and 1 mM pyruvate (Gibco, Waltham, MA, USA). Peritoneal cells were left to adhere for 2 h and then washed. Cells were stimulated with 10 ng/ml LPS (*E. coli* LPS; serotype 055:B5 Sigma) or 10 μg/ml Pam3Cys-SKKKK (EMC microcollections, Tübingen, Germany).

2.7 | Measurement of cytokines

Cytokine production was determined in conditioned media and serum using IL-10, keratinocyte cell-derived chemokine (KC), TNFα, and IL-6 ELISA kits (DuoSet R&D Systems, Minneapolis, MN, USA). In mouse plasma samples, M-CSF and GM-CSF were measured by Milliplex Luminex assay (Merck Millipore, Darmstadt, Germany) according to the manufacturer's instructions.

2.8 | Statistical analysis

Statistical analysis was performed in Graph Prism 6 (USA). Data are presented as mean $\pm$ SD and analyzed using a 2-tailed Mann Whitney test, or a 2-way ANOVA test followed by Sidak's multiple comparisons test as indicated. A *P*-value below 0.05 was considered statistically significant (*$P < 0.05$).

3 | RESULTS AND DISCUSSION

To determine if the spleen plays a role in the establishment of trained immunity, mice were sham operated or splenectomized, and subse-

quently given two doses of β-glucan that has been previously shown to induce trained immunity.[10] Seven days after the first β-glucan injection, an in vivo restimulation with a nonrelated stimulus, LPS, was administered i.p. (Fig. 1A). In addition, splenocytes and peritoneal cells were isolated from control and β-glucan trained mice and restimulated ex vivo. The differences in the production of the proinflammatory cytokines TNFα and IL-6 by splenocytes collected from trained mice and ex vivo stimulated with the TLR2 agonist Pam3Cys did not reach statistical significance when compared to splenocytes isolated from PBS-treated mice (Fig. 1B). Ex vivo stimulation with Pam3Cys of in vivo trained peritoneal cells derived from sham-operated mice did not produce a significant increase in TNFα concentrations, whereas the trained splenectomized group secreted more TNFα than the splenectomized control group. Reversely, IL-6 production in β-glucan exposed sham-operated mice was increased, whereas IL-6 levels were not significantly different between the splenectomized groups (Fig. 1C). TLR4 stimulation with LPS revealed an increased production of TNFα by peritoneal cells isolated from β-glucan-treated mice, independently of splenectomy, whereas IL-6 levels were not modulated (Fig. 1C). Thus, peritoneal cells of β-glucan-treated mice generally demonstrate an increased responsiveness to stimulation, characteristic for trained cells. However, removal of the spleen did not consistently alter this enhanced responsiveness. Likewise, splenectomized and sham-operated mice show similar plasma IL-10, IL-6, and TNFα concentrations 3 h after LPS i.p. injection (Fig. 1D). However, contrary to what was previously reported,[2,11] β-glucan exposed mice do not exhibit increased cytokine levels upon LPS injection. Differences in mice substrains and housing conditions play a role in immune responses, but possibly more impactful is the surgical intervention we performed. Unlike previous studies, mice underwent sham or splenectomy operations, also including anesthesia and analgesia, which might have influenced systemic immune responses and account for the disparity in this parameter. Taken together, these data indicate that the spleen or spleen-derived myeloid cells do not modulate the capacity to mount a cytokine response against heterologous restimulation, particularly at a cellular level.
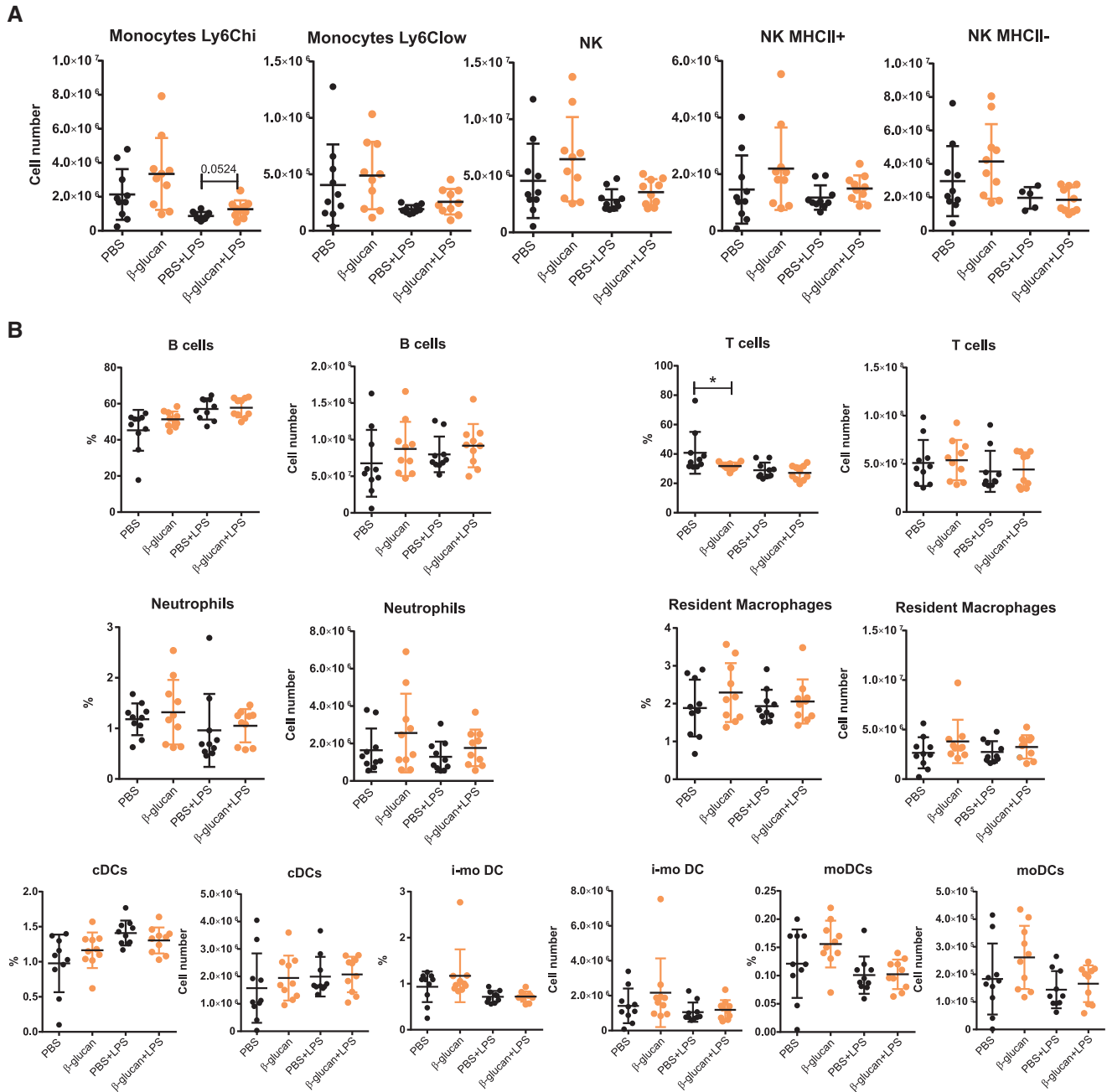
FIGURE 3 Splenocyte populations 7 d after β-glucan administration before and 3 h after LPS injection. (A) Normalized for cell number and (B) percentage and cell number ($n = 10$ pooled from two independent experiments, mean $\pm$ SD, $^*P < 0.05$ Mann Whitney test)

Another important component of the trained immunity response is represented by the skewing of the hematopoietic stem cells toward myelopoiesis and granulopoiesis, giving rise to peripheral trained myeloid cells.[4,5] This bias is associated with a GM-CSF signaling in the bone marrow.[4] G-CSF levels were not altered. In contrast, if we account for the mice that showed GM-CSF concentrations below the detection limit, there is a trend for an increase of GM-CSF concentrations in the circulation of β-glucan trained mice. However, GM-CSF concentration does not appear to be significantly influenced by splenectomy (Fig. 2).

The hematopoietic bias that contributes to systemic trained immunity is reflected in the populations represented in the peripheral blood. Thus, we assessed by flow cytometry (Supporting Information Fig. S1) whether β-glucan exposure modulates splenic and peripheral blood immune cell populations, and whether the absence of the spleen influences these changes. Our analysis was focused on cells of the innate immune system, but cells of the adaptive immune system, such as B and T cells were not influenced by splenectomy (Figs. 3 and 4). Splenic cell populations in the β-glucan exposed mice show a trend for an increased percentage of the proinflammatory Ly6C^hi monocytes, especially after in vivo LPS administration (Figs. 3A and 5Ai). The percentage of periph-
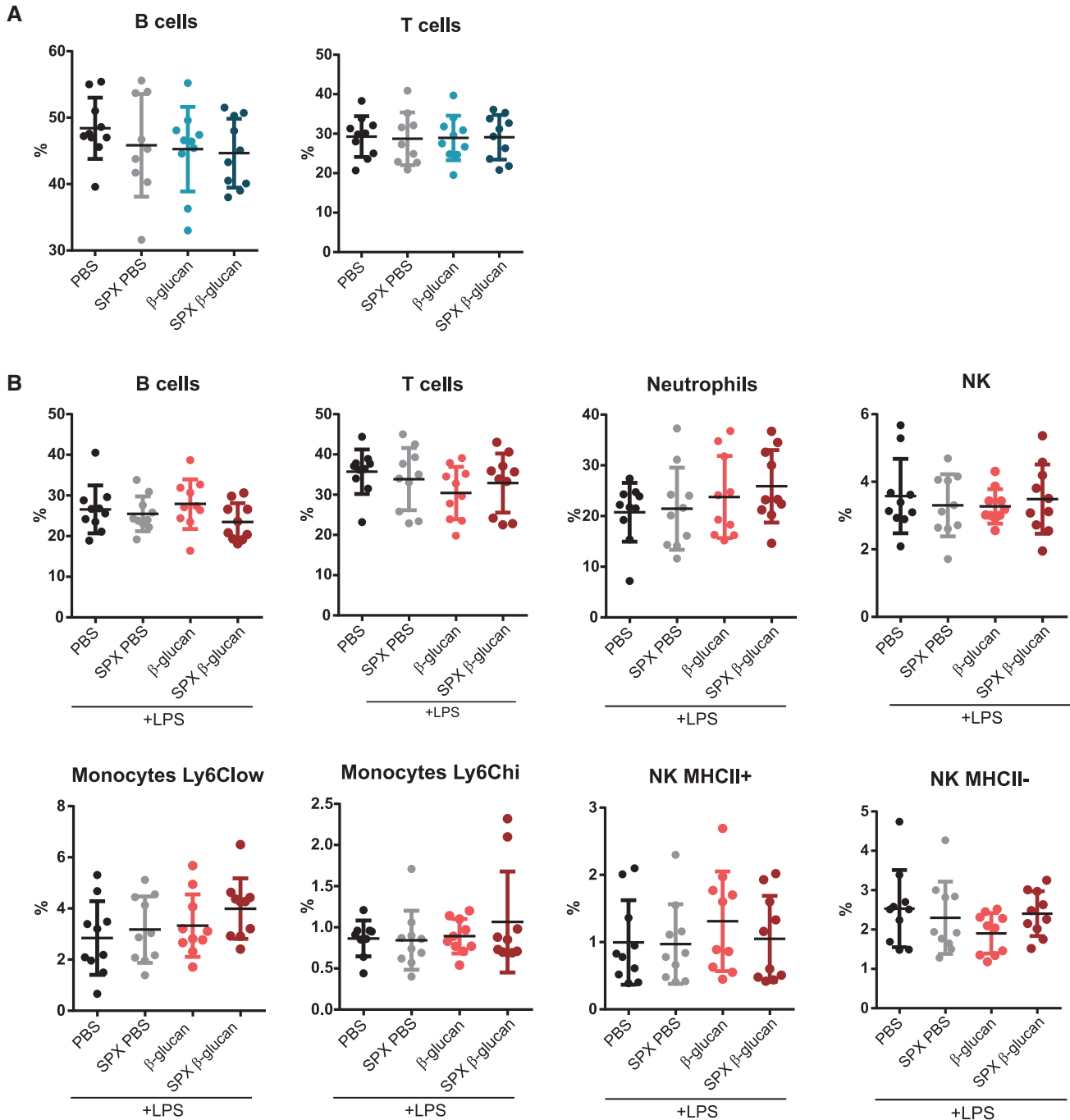
FIGURE 4 Peripheral blood populations. (A) Percentages found 7 d after β-glucan and (B) 3 h after LPS challenge ($n = 10$ pooled from two independent experiments, mean $\pm$ SD). (SPX splenectomy)

eral blood Ly6C^{hi} monocytes was also increased in the β-glucan-treated group, but only significantly in the splenectomized groups (Fig. 5Bi). In contrast, the more homeostatic Ly6C^{low} monocytic population was not modulated by β-glucan either in the spleen or in peripheral blood (Fig. 5Aii, Bii). In peripheral blood, LPS in vivo administration abrogates the distinct Ly6C^{hi} monocytes percentages induced by β-glucan training (Figs. 4B, 5Bi).

The spleen is a reservoir for undifferentiated monocytes,[7] but it does not seem to replenish the pool of circulating monocytes dur-

ing trained immunity. However, monocytes have the potential to differentiate into dendritic cells (DCs) during inflammation.[12] The monocyte-derived DCs (moDCs) and immature moDCs, characterized by decreased ability in antigen presentation, are not increased in β-glucan exposed mice (Fig. 5Biii, iv). However, trained splenectomized mice show an enhanced percentage of professional antigen-presenting cells conventional DCs (cDCs), whereas β-glucan exposure alone produces no modulation (Fig. 5Bv). However, the DC populations assessed were not modulated in the spleen of trained mice (Fig. 3B). The in
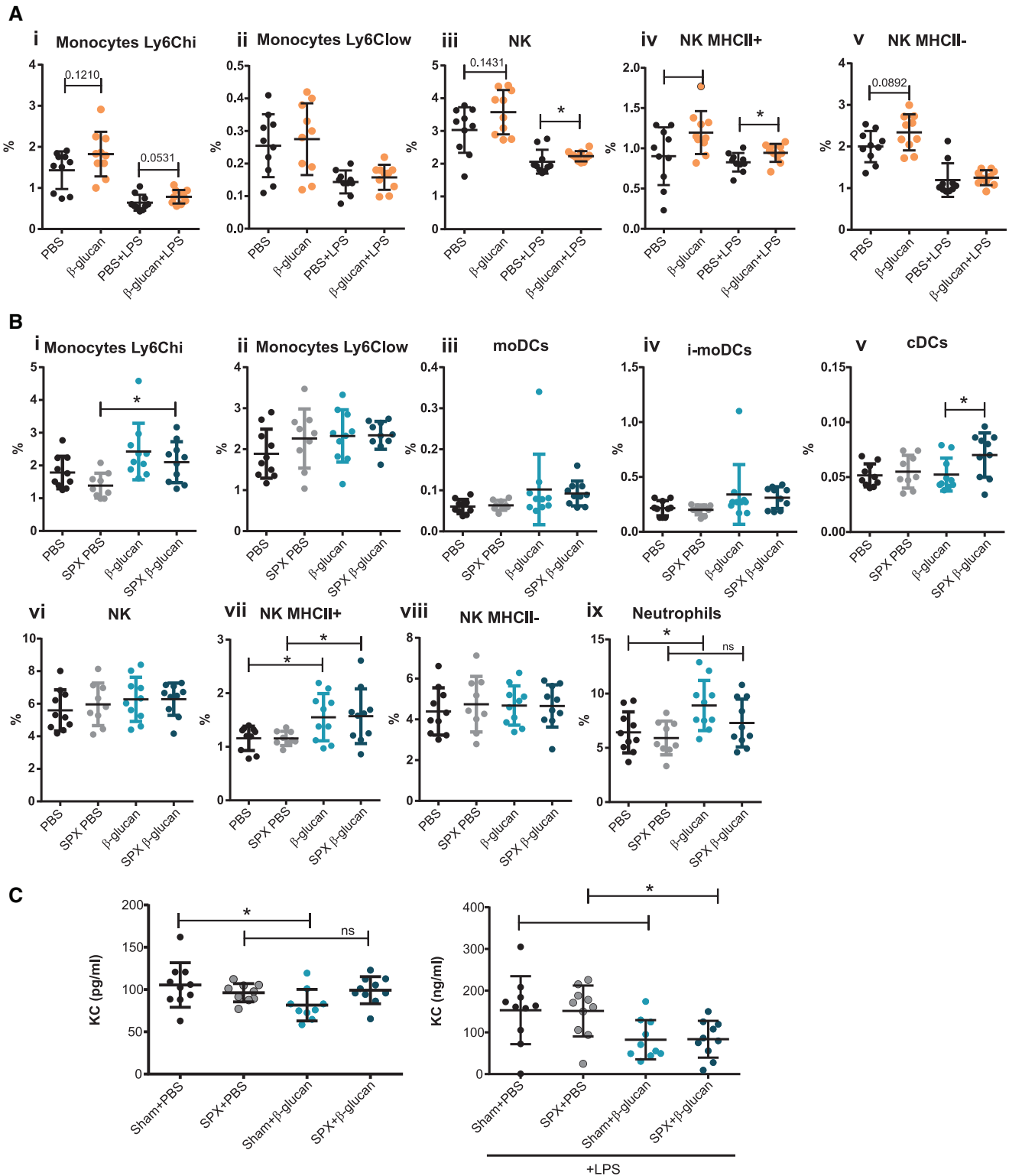
FIGURE 5 Training effect of β-glucan percentage on splenic and peripheral blood immune cell populations. (A) Splenocyte populations were assessed 7 d after β-glucan administration before and 3 h after LPS injection. Peripheral blood populations were evaluated (B) 7 d after β-glucan administration (2-way ANOVA, Sidak's multiple comparisons test). $n = 10$ pooled from two independent experiments. (C) Keratinocyte cell-derived chemokine (KC) concentration in mouse serum 7 d after β-glucan administration with and without 3 h LPS restimulation. Outliers were excluded with Grubbs's test ($n = 10$ pooled from two independent experiments). Mean ± SD, *$P < 0.05$. (A) Mann Whitney test, (B, C) 2-way ANOVA, Sidak's multiple comparisons test. (SPX splenectomy, ns nonsignificant)

vivo origins and differentiation of DCs are controversial but in vitro, DCs can be generated from monocytic precursors exposed to exogenous GM-CSF and type I IFNs, both highly secreted by activated NK cells. DCs have been shown to participate in a crosstalk with NK cells, where NK cells promote DC maturation while DCs in turn potentiate NK cell activation.[13,14] In addition, innate immune memory and adaptive immune features are also attributed to NK cells.[15,16]

As previously observed in BCG vaccinated individuals,[15] peripheral NK cell percentage was not modulated by β-glucan administration (Fig. 5Bvi). However, the more proinflammatory NK MHCII$^+$ subpopulation was significantly increased in trained animals, but this increase was not dependent on the spleen (Fig. 5Bvii). In accordance, splenic NK populations tend to be increased in trained mice and are significantly enhanced after β-glucan and LPS stimulation (Fig. 5Aiii). This augmented splenic NK percentage is likewise reflected in the increase of the NK MHCII$^+$ subpopulation (Fig. 5Aiv), whereas the NK MHC$^-$ population is not modulated after LPS (Fig. 5Av). In peripheral blood, β-glucan exposure and spleen absence do not modulate the percentage of NK cells MHC$^-$ population (Fig. 5Bviii), and these cells are also not changed in the peripheral blood of LPS restimulated mice (Fig. 5C). Overall, β-glucan training increases the systemic percentages of proinflammatory cells such as Ly6C^{hi} monocytes and NK MHC$^+$ in a spleen independent manner. In contrast, cDCs appear to be significantly overrepresented in trained animals only in the absence of the spleen, highlighting a possible role of this organ on DC differentiation during trained immunity. The increase in DC population in the blood of trained splenectomized mice could also point to the spleen as a site for inhibition of DC differentiation. For example, in a mouse model of autoimmune encephalomyelitis, spleen removal has been shown to prevent the induction of tolerance. This was attributed to the decrease of polymorphonuclear myeloid-derived suppressor cells, shown to be derived from the spleen.[17]

Another innate immune cell population that has been implicated in trained immunity is the neutrophils. A cohort of BCG vaccinated newborn infants exhibited an increase in neutrophil counts in comparison to nonvaccinated children,[6] and in adults the function of neutrophils is increased after induction of trained immunity by BCG vaccination.[18] In accordance with the previously described trained immunity enhanced granulopoiesis,[4] the percentage of neutrophils is increased in the peripheral blood of β-glucan-treated mice (Fig. 5Bix). Interestingly, β-glucan-induced neutrophilia was reduced in splenectomized trained mice, suggesting a role for the spleen in this process. However, the percentage of neutrophils in the spleen of β-glucan-treated mice is not changed (Fig. 3B), arguing against a direct role of the spleen as a reservoir of neutrophils from which they are mobilized after stimulation. Alternatively, the spleen may influence neutrophilia by modulating bone marrow granulopoiesis. Future studies are warranted to investigate these possibilities and the impact of the spleen on the function of neutrophils during induction of trained immunity. KC, also known as CXC chemokine ligand 1 (CXCL1), is a potent neutrophil chemoattractant. Contrary to the increase in circulating neutrophils, serum of β-glucan exposed mice has significant decreased KC concentrations (Fig. 5C). Circulating neutrophils and KC levels of splenec-

tomized trained mice are similar to splenectomized control mice. Upon LPS restimulation, KC concentration of trained animals is decreased, independently of the absence of spleen. These results reveal that the increase in neutrophil percentages is not correlated by an increase in recruitment to the circulation by KC, and that the decrease in circulating KC concentration depends on the presence of the spleen.

In conclusion, in vivo induction of trained immunity by β-glucan enhances the proinflammatory response of splenocytes and peritoneal cells. β-glucan exposure tends to augment circulatory GM-CSF concentrations, that might contribute to an increase in myelopoiesis and granulopoieses. However, the induction of GM-CSF is exerted in a spleen-independent manner, and splenectomized mice build a largely intact trained immunity response. Ultimately, trained animals present an increase in proinflammatory circulatory monocytes and NK cells, which does not require splenic involvement.

Our study has potential limitations. The use of a mouse model does not fully mirror human immune responses, spleen microanatomy, and its systemic role.[19] Also, our model with spleen removal prior to β-glucan exposure allows for the evaluation of the role of the spleen on the establishment and maintenance of trained immunity, but it does not permit to evaluate its contribution solely to the long-term maintenance of trained immunity. In this regard, HSPC populations were not explored in this study. However, the spleen does not appear to play a crucial role in β-glucan-induced central trained immunity, because circulating immune populations do not strongly differ in the absence of the spleen. These findings might be translated to patients who undergo splenectomy, suggesting that the establishment of innate immune memory responses will not be generally compromised.

However, here we show that a feature linked to trained immunity that was influenced by splenectomy was neutrophilia, pointing to a possible regulatory role of spleen in this process. This possible novel role of the spleen in the regulation of circulating immune cell populations gives new insights into the systemic induction of memory. Of note, trained immunity induced activation of neutrophils has been recently shown to have an important role in the anti-tumoral effects of β-glucan.[20] This unveils a possible clinical implication of the spleen in the establishment of trained immunity-derived neutrophilia, which advocates further exploration.

AUTHORSHIP

DISCLOSURES

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.