Innate lymphoid cells integrate stromal and immunological signals to enhance antibody production by splenic marginal zone B cells

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Innate lymphoid cells (ILCs) regulate stromal cells, epithelial cells and cells of the immune system, but their effect on B cells remains unclear. Here we identified RORγt+ ILCs near the marginal zone (MZ), a splenic compartment that contains innate-like B cells highly responsive to circulating T cell–independent (TI) antigens. Splenic ILCs established bidirectional crosstalk with MAdCAM-1+ marginal reticular cells by providing tumor-necrosis factor (TNF) and lymphotoxin, and they innately activate MZ B cells via B cell–activation factor (BAFF), the ligand of the costimulatory receptor CD40 (CD40L) and the Notch ligand Delta-like 1 (DLL1). Splenic ILCs further helped MZ B cells and their plasma-cell progeny by coopting neutrophils through release of the cytokine GM-CSF. Consequently, depletion of ILCs impaired both pre- and post-immune TI antibody responses. Thus, ILCs integrate stromal and myeloid signals to orchestrate innate-like antibody production at the interface between the immune system and circulatory system.

The spleen is a highly perfused organ specialized in host defense against blood-borne antigens. Interspersed between the follicles of the splenic white pulp and the circulation, the marginal zone (MZ) contains B cells enmeshed with macrophages and dendritic cells (DCs) in a stromal reticular cell network1–3. All of those cells provide efficient immunosurveillance of the circulatory system by readily interacting with circulating antigens owing to the slow flow rate of the blood passing through the MZ4. Following antigen capture, macrophages, DCs and possibly neutrophils of the innate immune system expose antigen to MZ B cells, a unique subset of antibody-producing lymphocytes that develop from transitional B cells in response to signals from the Notch2 signaling receptor5.

Lymphoid sites positioned between the host and the environment contain innate-like B cells and T cells that belong to the adaptive immune system but share several properties with effector cells of the innate immune system. Mucosal and serosal membranes include innate-like B-1 cells that generate a first line of protection through the early production of low-affinity immunoglobulin M (IgM) in response to bacteria6. When microbes breach the mucosal barrier and enter the general circulation, innate-like MZ B cells provide a second line of protection via low-affinity IgM and IgG that bridge the temporal gap required for the slower production of high-affinity IgG by follicular (FO) B cells4.

Similar to B-1 cells, MZ B cells express clonally distributed and somatically recombined but rather nonspecific B cell antigen receptors (BCRs) encoded by poorly diversified immunoglobulin genes4,6. MZ B cells also express nonclonally distributed and germline-encoded Toll-like receptors (TLRs)7, a subfamily of nonspecific microbial sensors generally known as ‘pattern-recognition receptors’. Typically expressed by effector cells of the innate immune system, TLRs activate MZ B cells after recognizing conserved microbial molecular signatures in cooperation with BCRs8. The activation of MZ B cells is further enhanced by B cell–stimulating cytokines released by DCs, macrophages and neutrophils9,10.

In addition to containing innate-like lymphocytes, mucosal surfaces have innate lymphoid cells (ILCs) that express neither somatically recombined antigen receptors nor phenotypical markers of myeloid cells11. Those ILCs require the transcriptional repressor Id2 and the cytokine interleukin 7 (IL-7) for their development, and they generate cytokine-secretion patterns that mirror those of helper T cells of the adaptive immune system11,12,13. Similar to proinflammatory T helper type 1 cells, ILCs of group 1 release interferon-γ (IFN-γ) and require

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the transcription factor T-bet for their development, as do natural killer (NK) cells of the innate immune system. ILCs of group 2, which include natural helper cells and muocytes, secrete IL-5 and IL-13 and require the transcription factor GATA-3 and thus resemble proinflammatory T helper type 2 cells. Finally, ILCs of group 3 (ILC3 cells) require the transcription factors RORγt and AhR and include not only mucosal 'NK-22' cells, which secrete IL-22 and thus mimic noninflammatory cells of the T H22 subset of helper T cells, but also fetal and mucosal lymphoid tissue–inducer cells (LTIs), which produce IL-22 and IL-17 and thus resemble inflammatory ILCs of the T H22 subset of helper T cells. While NK-22 cells express natural cytotoxicity receptors (NCRs) usually associated with NK cells and mediate mucosal homeostasis by targeting epithelial cells via IL-22 (refs. 25–27), LTIs lack NCRs and promote fetal lymphoid organogenesis and postnatal mucosal immunity by targeting stromal cells via lymphotixin and tumor-necrosis factor (TNF)

Mucosal NK-22 cells, also defined as 'NCR+ ILC3 cells' to distinguish them from inflammatory NCR− ILC3 cells characterized by the constitutive production of IL-17 and IL-22 and activation-induced production of IFN-γ, express B cell–activating factor (BAFF)18, a cytokine of the TNF family used by DCs, macrophages and neutrophils to help MZ B cells and plasma cells in a T cell–independent (TI) manner. BAFF and its homolog APRIL are related to the ligand of the costimulatory receptor CD40 (CD40L), a member of the

Figure 1 Human splenic ILCs have a mucosa-like ILC3 phenotype and occupy MZ and perifollicular areas. (a) Expression of CD4, CD56, Nkp44, Nkp46, CD96, CD161, CD25, CD69 and CCR6 (red lines) on splenic Lin−CD117+CD127+ ILCs (red outlined area, top left), assessed by flow cytometry. Gray shaded curves, negative control. (b) Quantitative RT-PCR analysis of mRNA encoding RORγt, AhR, IL-22 (IL22), TNF (TNF), LT-α (LTA), LT-β (LTK), perforin-1 (PRF1) and IFN-γ (IFNG) in freshly isolated splenic or tonsillar ILCs and NK cells, assessed by flow cytometry. Each symbol represents an individual sample; small horizontal lines indicate the mean (± s.e.m.). (c) Viability of splenic and tonsillar ILCs cultured for 72 h with medium alone (Med), IL-1β, IL-7 or IL-23, assessed by flow cytometry. Numbers in plots indicate percent viable ILCs (annexin V negative, propidium iodide (PI) negative; bottom left quadrant). (d) ELISA of IL-22 in splenic and tonsillar ILCs cultured as in c. (e) IFAs of spleen stained for the mannose receptor (MR; purple), RORγt (green) and DNA (with the DNA-binding dye DAPI; blue); circles indicate perimeters of spleen regions. RP, red pulp; PFZ, perifollicular zone; FO, center of the follicle. Original magnification ×10. (f) Immunohistochemical quantification of CD117+ tryptase-negative ILCs from MZ–perifollicular zone and red pulp areas in fields with an original magnification of ×20. (g) Immunohistochemical quantification of CD117+ MR+ ILCs from MZ–perifollicular zone and red pulp areas in fields with an original magnification of ×20. (h) Immunohistochemical quantification of CD117+ MR+ ILCs from MZ–perifollicular zone and red pulp areas in fields with an original magnification of ×20.
TNF family used by follicular helper T cells to activate FO B cells. Given their involvement in the mucosal production of TI antibodies, ILCs might also regulate humoral immunity in the MZ, a lymphoid area that is continually exposed to antigen, as are mucosal membranes.

Here we identified ILCs with mucosa-like properties in the MZ and perifollicular zones of the spleen. These ILCs required survival signals from marginal reticular cells (MRCs), a MZ subset of stromal cells that responded to TNF and lymphotoxic from ILCs. In addition to stimulating MZ B cells and plasma cells via BAFF, APRIL, CD40L and the Notch2 ligand Delta-like 1 (DLL1), splenic ILCs coopted MZ B cell–helper neutrophils via the cytokine GM-CSF (CSF2). Consequently, depletion of ILCs hampered the production of antibodies by MZ B cells in response to TI antigens.

**RESULTS**

**Splenic ILCs have type 3 mucosal features**

The MZ is continually exposed to blood-borne antigens and may thus require homeostatic signals from ILCs, as mucosal membranes do. Flow cytometry showed that histologically normal adult human

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**Figure 2** Human splenic ILCs establish bidirectional crosstalk with MRCs. (a) IFA of spleens stained for MAdCAM-1 (green or red), IgD (blue), von Willebrand factor (vWF; red) and/or RORγt (green). Original magnification, ×40 (top row, main images), ×10 (top row, inset) or ×63 (bottom left), with a 2× enlargement of the image at left (bottom right). (b) Expression of CD31 (PECAM-1), CD34, CD45, Thy-1, CD141, ICAM-1, VCAM-1, TLR3, TLR4 and TLR9 (red lines) in MRC populations expanded ex vivo, assessed by flow cytometry. Gray shading, negative control. (c) Quantitative RT-PCR analysis (bottom) of mRNA encoding IL-1β (IL1B), IL-7 (IL7), IL-23 (IL23), CCL20 (CCL20), ICAM-1 (ICAM1) and MAdCAM-1 (MADCAM1) in samples microdissected from the immunohistochemically stained splenic tissue above: MAdCAM-1+ areas enriched for MRCs (red outlined area, top left), MAdCAM-1−, mannos receptor–positive areas enriched for sinusoid-lining cells (blue outlined area, top right; MR+) and a control mannos receptor–negative area (black outlined area, top right; Ctrl MR−); results are normalized to those of mRNA encoding β-actin (ACTB) and are presented relative to those of mannos receptor–negative areas. Numbers adjacent to bars indicate values above top value along vertical axis. Original magnification (top), ×20. (d) Expression of integrins α3 and β7 on freshly isolated splenic ILCs (left) or ILCs cultured for 72 h with IL-1β and IL-7 (right), assessed by flow cytometry. (e) Expression of ICAM-1 and VCAM-1 on MRCs incubated for 72 h alone (far left) or with ILCs (middle) or MZ B cells (right), assessed by flow cytometry. (f) Expression of ICAM-1 on MRCs cultured for 72 h alone (left) or with ILCs in the presence of no antibody (No Ab) or control antibody (IgG1), anti-TNF, anti–LT-αβ or anti-TNF plus anti–LT-αβ, assessed by flow cytometry. Numbers in plots indicate mean fluorescence intensity. (g) Frequency of viable ILCs among splenic ILCs cultured for 72 h alone (left) or with MRCs (right) (presented as in Fig. 1e). (h) Frequency of viable ILCs among splenic ILCs cultured for 72 h with MRCs in the presence (+) or absence (−) of a Transwell (left) or with a Transwell and no antibody, the control antibody IgG1 or anti–IL-7 (right). *P < 0.05 (two-tailed unpaired Student’s t-test). Data are from one experiment of four experiments with similar results (a,b,d–g) or from three experiments with one donor in each (c,h; error bars, s.e.m.).
spleens contained cells that lacked common molecules associated with the lymphoid and myeloid lineage (Lin−) but expressed the receptor for IL-7 (CD127) and the receptor for stem cell growth factor (CD117) (Fig. 1a and Supplementary Fig. 1a). These ILCs resembled mucosal NCR+ ILC3 cells19,20, because they expressed the NK cell–related molecules CD56, CD96, CD161, NKp44 and the NK cell–related molecules CD56, CD96, CD161, NKp44 and NKp46 than NK cells had (Supplementary Fig. 1c). Splenic ILCs expressed neither the coreceptor CD8, which is expressed by some mucosal lymphocytes, and did not express the tissue IFA, IHC and flow cytometry. We detected fibroblast-like cells like ILCs that were different from NK cells and were positioned near the periarteriolar lymphoid sheath (data not shown). Thus, the human spleen contained mucosal LTi cells.

Analysis of gene expression by quantitative RT-PCR showed that splenic ILCs expressed the transcription factors RORγt, AhR and Idd2, the cytokines IL-22, IL-26, lymphotoxin-α (LT-α), LT-β and TNF and the receptor for IL-23, whereas splenic NK cells, T cells and macrophages did not, except that B cells had abundant expression of LT-β and TNF and lower expression of IL-22 and higher expression of LT-α, LT-β and TNF (Fig. 1b and Supplementary Fig. 1d). Compared with mucosal ILCs from tonsils, splenic ILCs had similar expression of RORγt and AhR, but lower expression of IL-22 and higher expression of LT-α, LT-β and TNF (Fig. 1b). Those last three cytokines, together with IL-17, are hallmarks of LTi cells. However, splenic ILCs lacked expression of IL-17, which we detected instead in T cells (Supplementary Fig. 2a–d). Some ILCs were also present in the periarteriolar lymphoid sheath (data not shown). Thus, the human spleen contained mucosa-like ILCs that were different from NK cells and were positioned near MZ B cells.

Splenic ILCs receive survival signals from MRCs

Given that LTi cells interact with stromal cells via lymphotoxin and TNF, we sought to identify stromal cells in the human MZ by tissue IFA, IHC and flow cytometry. We detected fibroblast-like cells that expressed the integrin αβ ligand MadCAM-1 in the MZ near

Figure 3 Human splenic ILCs express the MZ B cell–helper factors BAFF, CD40L and DLL1 and activate MZ B cells in cooperation with MRCs. (a) IHC of spleen stained for RORγt (brown) and CD20 (magenta); arrowheads indicate RORγt+ cells. Original magnification, ×40. (b) Quantitative RT-PCR analysis of mRNA encoding BAFF (TNFSF13B), APRIL (TNFSF13), CD40L (CD40LG) and DLL1 (DLL1) in splenic or tonsilar ILCs and NK cells and splenic macrophages, B cells and T cells (presented as in Fig. 1a). (c) Expression of BAFF, APRIL, CD40L and DLL1 (red lines) on splenic ILCs exposed for 72 h to IL-1β plus IL-7, assessed by flow cytometry. Gray shading, negative control. (d) ELISA of soluble (s) BAFF and APRIL from splenic ILCs and NK cells cultured as in c, as well as from macrophages cultured with medium alone. (e) Frequency of viable splenic MZ B cells and FO B cells (top; presented as in Fig. 1e) and their divided counterparts (bottom; identified by a low concentration of the cytosolic dye CFSE) after incubation for 5 d with medium alone or ILCs, assessed by flow cytometry. (f) ELISA of IgM, IgG and IgA from splenic MZ B cells incubated for 5 d with or without ILCs, CpG and/or MRCs (below graphs). (g) Flow cytometry of splenic MZ B cells cultured for 5 d with MRCs (bottom row) or without MRCs (top row), in the presence or absence of ILCs and CpG (above plots). Numbers below outlined areas indicate percent CD27+/CD38+ plasmablasts.

*P < 0.05 (two-tailed unpaired Student’s t–test). Data are from one experiment of three experiments with similar results (a,c,e–g; error bars, s.e.m.) or three pooled experiments with one donor in each (b,d; error bars, s.e.m. of triplicates).
IgD⁺ B cells and some RORγt⁺ ILCs (Fig. 2a and Supplementary Fig. 3a). Those MadCAM-1⁺ cells did not express the coagulation protein von Willebrand factor, which characterizes endothelial cells, whereas MadCAM-1⁻ sinusoid-lineating cells from the red pulp did (Fig. 2a). MadCAM-1⁺ cells also lacked the endothelial molecules CD31 (PECAM-1) and CD34, as well as the leukocyte molecule CD45, that included IL-7 (θ-μ, CD90), CD141 (thrombomodulin), ICAM-1, VCAM-1 and α-smooth muscle actin (Fig. 2b and Supplementary Fig. 3b,c), and thus we considered them equivalent to mouse MRCs.

Human MRCs expressed TLR3 (which binds double-stranded RNA), TLR4 (which binds lipopolysaccharide (LPS)) and TLR9 (which binds unmethylated CpG–rich DNA) (Fig. 2b). This was indicative of a role for microbial products in the regulation of MRCs. Laser-capture microdissection followed by quantitative RT-PCR demonstrated that MadCAM-1⁺ tissue enriched for MRCs contained more ILC survival factors (such as IL-1β, IL-7 and IL-23) and more ILC-recruiting factors (such as CCL20, a ligand for CCR6) than did tissue enriched for sinusoid-lineating cells and positive for the endocytic mannose receptor or control tissue negative for the mannose receptor (Fig. 2c).

As shown by flow cytometry, splenic ILCs upregulated expression of the MadCAM-1⁻binding integrin α6β1 in response to IL-7 and IL-1β (Fig. 2d), which indicated a role for the MadCAM-1–α6β1 interaction in the perifollicular positioning of ILCs. Conversely, MRCs upregulated expression of ICAM-1 and VCAM-1 in response to splenic ILCs or a combination of lymphotoxin and TNF or LPS (Fig. 2e and Supplementary Fig. 3d,e). Blocking experiments and quantitative RT-PCR indicated that ILCs activated MRCs via lymphotoxin and TNF (Fig. 2f and Supplementary Fig. 3f), whereas MRCs upregulated expression of IL-7, CCL20 and MadCAM-1 in response to lymphotoxin and TNF (Supplementary Fig. 3g). Of note, Transwell assays showed that MRCs supported the survival of ILCs via contact-dependent and contact-independent signals that included IL-7 (Fig. 2g,h). Finally, splenic macrophages and DCs had higher expression of IL-1β and IL-23 than did MRCs (Supplementary Fig. 3h), which suggested that ILCs established bidirectional interaction with MRCs in splenic niches that may have also entailed cells of the innate immune system.

**Splenic ILCs and MRCs deliver B cell–helper signals**

Given their MZ and perifollicular location, human splenic ILCs may regulate MZ B cells. As shown by tissue IFA and quantitative RT-PCR, splenic RORγt⁺ ILCs were positioned near CD20⁺ B cells and had higher expression of BAFF, CD40L and DLL1, three major factors for the provision of help to B cells and plasma cells, than that of other splenic leukocyte subsets or mucosal ILCs (Fig. 3a,b).
Unlike splenic macrophages, splenic ILCs had little or no expression of APRIL (Fig. 3b), a BAFF-related plasma cell–survival factor.

As shown by flow cytometry and ELISA, splenic ILCs had surface expression of BAFF, CD40L and DLL1 and secreted BAFF, whereas APRIL was secreted mainly by macrophages (Fig. 3c,d). Accordingly, splenic ILCs stimulated the survival, proliferation, IgM secretion and plasmablast differentiation of IgD^hi^CD27^−^ MZ B cells but not of IgD^lo^CD27^+^ FO B cells (Fig. 3e–g). Having shown that MRCs provided survival and activation signals to ILCs, we investigated whether MRCs augmented the B cell–helper function of ILCs. MZ B cells exposed to both ILCs and MRCs not only underwent more IgM secretion and plasmablast differentiation than did MZ B cells exposed to ILCs or MRCs alone but also acquired the ability to release IgG and IgA, particularly in the presence of CpG DNA (Fig. 3f,g). Thus, human splenic ILCs may integrate stromal signaling and signaling of the innate immune system to orchestrate antibody production.

**Splenic ILCs help MZ B cells via BAFF, CD40L and DLL1**

Next we used flow cytometry and ELISA to determine the mechanism by which human splenic ILCs help MZ B cells. BAFF–R–Ig, a soluble decoy receptor that blocks the binding of BAFF to B cells, attenuated the survival of MZ B cells exposed to ILCs (Fig. 4a). Of note, MZ B cells underwent plasmablast differentiation and IgM secretion in response to ILCs but not in response to ILC-conditioned medium (Fig. 4b,c), which suggested the involvement of contact-dependent signals from membrane-bound BAFF, CD40L and/or DLL1. Accordingly, BAFF–R–Ig, CD40–Ig (a decoy receptor that blocks the binding of CD40L to B cells) or an inhibitor of Notch signaling mitigated the ILC-induced secretion of IgM, particularly when we used Notch inhibitors in combination (Fig. 4d).

Although it is required for the early development of MZ B cells, Notch2 continues to be expressed on mature MZ B cells and may thus modulate their function. Indeed, when combined with BAFF, DLL1-expressing OP9 stromal cells stimulated plasma-cell differentiation and IgM secretion (through Notch signaling) in MZ B cells (Fig. 4e–g). In contrast, BAFF alone or combined with DLL1-negative OP9 cells had little or no stimulatory effect (Fig. 4e–g).

Thus, in addition to eliciting the survival and activation of MZ B cells, human splenic ILCs promoted plasmablast differentiation and survival via BAFF, CD40L and DLL1.

**Splenic ILCs activate perifollicular neutrophils via GM-CSF**

In human spleens, MZ B cells interact with neutrophils (‘NBH cells’) that possibly originate from circulating precursors (‘NC cells’). Given
the involvement of RORγt in GM-CSF expression by T H 17 cells and the importance of GM-CSF in the activation and survival of neutrophils, we sought to determine whether splenic ILCs expressed GM-CSF. Tissue IHC showed CD117 + ILCs near CD66 + (CEACAM-1 +) N B H cells positioned around the MZ (Fig. 5a). Quantitative RT-PCR, ELISA, and flow cytometry demonstrated abundant expression of GM-CSF in splenic ILCs but not in other leukocyte subsets (Fig. 5b,c). Splenic ILCs further expressed the neutrophil-recruiting chemokine IL-8 (CXCL8), which was also produced by macrophages and NK cells, albeit in smaller amounts (Fig. 5b,c).

Given that GM-CSF enhances the B cell–helper function of neutrophils, we used flow cytometry, ELISA, IFA, and quantitative RT-PCR to establish whether GM-CSF–producing splenic ILCs induced N B H cells to upregulate expression of APRIL and DLL1, which are antigen-trapping projections that often emanate from neutrophils via IL-17 and GM-CSF. Flow cytometry showed that Rorc −/− mice had fewer splenic Lin + CD117 + CD127 + ILCs than did Rorc +/+ (wild-type) mice (Fig. 6a). Flow cytometry and quantitative ELISA of total IgG3 demonstrated that ILC-conditioned medium augmented the capacity of N B H cells to induce IgM, IgG, and IgA in MZ B cells (Fig. 5f,g), and when incubated with splenic ILCs or their conditioned medium, N B H cells upregulated expression of APRIL and thus stimulated IgA production in MZ B cells (Fig. 5f,g). ILC-conditioned medium also augmented the capacity of N B H cells to induce IgM and IgA in MZ B cells (Fig. 5f,g). Similar to GM-CSF or LPS, ILC-conditioned medium stimulated N C cells to form neutrophil extracellular traps (Fig. 5i), which are antigen-trapping projections that often emanate from N B H cells. Thus, human splenic ILCs may amplify the responses of MZ B cells by stimulating perifollicular neutrophils via GM-CSF.
RT-PCR demonstrated that in those wild-type mice, splenic ILCs included both CD4⁺ and CD4⁻ subsets that had higher expression of RORγt, IL-22, LT-α and TNF than that of other splenic leucocytes (Fig. 6b and Supplementary Fig. 4a). In contrast to human splenic ILCs, mouse splenic ILCs lacked expression of BAFF and CD40L but expressed APRIL and DLL1 (Fig. 6b).

We then investigated whether Rorc⁻/⁻ mice had an altered MZ. As shown by tissue IFA, Rorc⁻/⁻ mice had no perturbation of MOMA-1⁺ metallophilic macrophages, ER-TR9⁺ (SIGN-R1⁺) MZ macrophages or ICAM-1⁺ marginal sinus cells from the MZ, IgM⁺ B cells from the MZ and white pulp, or F4/80⁺ macrophages or VCAM-1⁺ endothelial cells from the red pulp (Supplementary Fig. 4b). Flow cytometry, tissue IFA and ELISA demonstrated that spleens from Rorc⁻/⁻ mice that were not immunized had conserved B cells but fewer plasmablasts and plasma cells that expressed IgG3, an antibody produced by MZ B cells, including MZ B cells 40.

In agreement with the MZ nature of preimmune IgG3 production, Rorc⁺/⁺ and Rorc⁻/⁻ mice were used to determine serum concentrations of preimmune IgG3 naturally reactive to the bacterial TI antigen phosphorylcholine (Supplementary Fig. 5b-f). To ascertain the relative contribution of ILCs and T cells to preimmune IgG3 responses, we crossed Rorc⁻/⁻ mice with Rorc⁻/⁻ mice with T cell–deficient Cd3e⁻/⁻ mice. In agreement with the T cell nature of preimmune IgG3 production, Rorc⁻/⁻ mice that were not immunized had a normal abundance of splenic B cells, plasma cells and plasmablasts that expressed IgG3 (Fig. 6g).

Unlike the preimmune serum concentrations of IgG3, those of IgM were augmented in Rorc⁻/⁻ mice, together with splenic IgMhB220⁻/⁻Cd3e⁻/⁺CD5⁺ B-1 cells but not B220⁺CD21⁺CD23⁺ FO B cells or B220⁺CD21⁺CD23⁺ MZ B cells (Supplementary Fig. 5b, c). The population expansion of IgM-producing B-1 cells in Rorc⁻/⁻ mice may have resulted from enhanced migration of B-1 cells from the peritoneal cavity to the spleen due to the lack of gut-associated lymphoid tissue and increased systemic translocation of gut bacteria 22,28,34,42. Nonetheless, Rorc⁻/⁻ mice had lower preimmune serum concentrations of IgM naturally reactive to phosphorylcholine than did their Rorc⁻/⁺ counterparts (Supplementary Fig. 5d), which suggested a role for ILCs in at least some TI IgM responses.

To delineate the MZ B cell–helper function of ILCs in a more physiological model, we used an antibody to Thy-1.2 (anti-Thy-1.2) to achieve depletion of ILCs in Thy-1-disparate Rag⁻/⁻ chimeras generated as described 43. Mice treated with anti-Thy-1.2 had fewer splenic ILCs, fewer IgG3-expressing plasmablasts and plasma cells and lower preimmune serum concentrations of IgG3 than did mice treated with a control antibody (Fig. 7a-c and Supplementary Fig. 5e). Furthermore, mice treated with anti-Thy-1.2 had impaired post-immune IgG3 responses to TNP-Ficoll (the hapten TNP (2,4,6-trinitrophenyl) linked to the hydrophilic polysaccharide Ficoll) (Fig. 7d), a TI antigen that induces antibody production in innate-like B cells, including MZ B cells 40.

We also demonstrated the involvement of splenic ILCs in the TI production of antibodies in mixed chimeras generated by the reconstitution of lethally irradiated Rorc⁻/⁻ mice with a mixture of bone marrow cells from Rag⁻/⁻ mice (which generate ILCs but not T cells or B cells) and Rorc⁻/⁻ mice (which generate T cells and B cells but not ILCs). ILC-sufficient (ILC⁺) mixed chimeras developed splenic ILCs, whereas ILC⁻ control chimeras (generated by the reconstitution of irradiated Rorc⁻/⁻ mice with bone marrow cells from Rorc⁻/⁻ mice) did not (Fig. 7e). ILC⁻ control chimeras had fewer splenic
plasmablasts and plasma cells that expressed IgG3 and lower preimmune serum concentrations of IgG3 than did ILC+ mixed chimeras (Fig. 7fg). In all mouse models, IgM responses were affected only marginally (data not shown), which indicated that splenic ILCs helped mainly IgG3-expressing plasmablasts and plasma cells emerging from MZ B cell responses to TI antigens.

**Splenical ILCs regulate neutrophil homeostasis**

We next investigated the role of mouse splenic ILCs in the homeostasis of NRGκ cells. Tissue IFA and flow cytometry showed that ILC- control chimeras had fewer splenic Ly6G+CD11b+ neutrophils and extrafollicular IgG3-producing plasma cells than did ILC+ mixed chimeras (Fig. 8ab). Rorc+ mice on either a T cell–sufficient back- ground or a T cell–deficient background also had fewer splenic neutrophils than did Rorc+ mice (Supplementary Fig. 6ac), which indicated that splenic ILCs provided helper signals to neutrophils independently of T cells. To ascertain the contribution of splenic neutrophils to preimmune IgG3 production, we depleted ILC+ mixed chimeras of splenic neutrophils with anti-Ly6G (Fig. 8c). Those mice had a normal abundance of splenic ILCs and IgG3-expressing B cells and plasmablasts but fewer splenic IgG3-expressing plasma cells and lower preimmune serum concentrations of IgG3 than did their ILC+ mixed counterparts treated with a control antibody (Fig. 8df). Thus, unlike ILCs, neutrophils may control terminally differentiated IgG3-secreting cells.

Finally, we determined whether splenic ILCs regulate neutrophils via GM-CSF. As shown by quantitative RT-PCR, splenic ILCs from wild-type mice had higher expression of GM-CSF than that of other leukocyte subsets, including macrophages and NK cells (Fig. 8g). GM-CSF-deficient (Csf2−/−) mice had lower preimmune serum concentrations of IgM antibodies to bacterial phosphorylcholine and fewer splenic neutrophils than did wild-type mice, and those neutrophils increased in abundance after inoculation of the Csf2−/− mice with B16 melanoma cells expressing GM-CSF (Fig. 8hi and Supplementary Fig. 6d). Also, Rag1−/− Il2rg−/− mice, which lack the IL-2 receptor (common γ-chain) mandatory for the IL-7-dependent development of ILCs13, had fewer splenic neutrophils than did their Rag1−/− Il2rg−/− control counterparts (Fig. 8i). Moreover, adoptive transfer of ILCs from Csf2+/+ increased the abundance of splenic neutrophils in Rag1−/− Il2rg−/− mice, but adoptive transfer of ILCs from Csf2−/− mice did not (Fig. 8i). Thus, in addition to helping MZ B cells and plasma cells through BAFF (or APRIL), CD40L and DLL1, splenic ILCs coopted neutrophils via GM-CSF to enhance the TI production of antibodies (Supplementary Fig. 7ab).
DISCUSSION

We have shown that the MZ and perifollicular zone of the spleen contained mucosa-like ILC3 cells that released lymphotoxin and TNF to establish bidirectional crosstalk with stromal MRCs. Splenic ILCs stimulated MZ B cells and plasma cells through BAFF, APRIL, CD40L and DLL1. They also coopted neutrophils with MZ B cell– and plasma cell–helper functions through GM-CSF and thereby sustained the TI production of antibodies.

Mucosal and serosal membranes contain evolutionarily primitive lymphocyte subsets that include B–1 cells, γδ T cells, invariant NKT cells and mucosa-associated invariant T cells.44 By sensing conserved microbial signatures through somatically recombined and germline-encoded receptors, those innate-like lymphocytes rapidly activate protective programs that cross over the conventional boundaries between the innate immune system and adaptive immune system.44 Mucosal membranes also contain ILCs that lack somatically recombined receptors and yet mount prompt T cell–like responses.1,3 Here we found that splenic ILCs enhanced antibody production in response to TI antigens by activating innate-like B cells positioned at the interface between the immune system and circulatory system.

Similar to mucosal ILC3 cells,19,20 human splenic ILCs expressed both RORγt and AhR, as well as NCRs and various activation molecules, and secreted IL-22 in response to IL-23. In addition, human splenic ILCs activated MZ-based stromal MRCs via lymphotoxin and TNF and thus also resembled fetal or mucosal LTi cells.28–30,45 However, unlike LTi cells,23,30 human splenic ILCs did not express IL-17, which raises questions about the ontogenetic relationships between these ILC subsets. Mouse splenic ILCs had a phenotype and gene-expression profile similar to those of human splenic ILCs but expressed CD4 and thus showed closer affiliation with LTi cells.

Human splenic ILCs not only expressed the gut-homing receptor α4β7, but also interacted with MRCs equipped with MadCAM-1, an α4β7 ligand usually associated with gut endothelial cells.34 Given their additional expression of the chemokine CCL20 and the presence of its receptor CCR6 on splenic ILCs, MRCs might be involved in the positioning of ILCs within and around the MZ. Consistent with their stromal phenotype and reticular morphology, MRCs lacked endothelial molecules and exhibited strong responsiveness to lymphotoxin and TNF from ILCs and thus resembled the MadCAM-1+ stromal cells that line the mouse marginal sinus.46 Conversely, MRCs delivered both contact-dependent survival signals and contact-independent survival signals, including IL-7, to ILCs. Notably, MRCs increased their IL-7 expression in response to lymphotoxin and TNF, which indicated the presence of bidirectional functional crosstalk between MRCs and ILCs in the MZ and perifollicular zone of the spleen. Additional ILC survival factors, such as IL-1β and IL-23, were expressed mainly by splenic DCs and macrophages, which suggested that ILCs inhabit a splenic niche that generates ILC survival effect of soluble BAFF on plasmablasts.35 Given that DLL1 also acts together with BAFF to stimulate the early development of MZ B cells, it is possible that DLL1 on ILCs operates at both early and late stages of MZ B cell differentiation with distinct signaling programs. Of note, splenic ILCs further helped MZ B cells by coopting Nbh cells through GM-CSF. That cytokine may also enhance the MZ B cell–stimulating function of DCs and possibly macrophages,4,14 which may explain why a relatively small number of ILCs was sufficient to enhance TI antibody responses.

Despite expressing CD40L, a class switch–inducing factor typically detected on follicular helper T cells,33 human splenic ILCs did not trigger the production of IgG or IgA in MZ B cells. Nonetheless, MZ B cells induced the production of IgA and IgG when exposed to ILCs in the presence of MRCs and microbial TLR ligands. Accordingly, small amounts of those bacterial components can be detected in splenic perifollicular areas, together with plasma cells that release IgM, IgG or IgA.10 Thus, similar to gut LTi cells, splenic ILCs may integrate stromal signals and signals from the innate immune system to rapidly generate antibodies for antimicrobial protection.

Although they lacked both CD40L and BAFF, mouse splenic ILCs expressed APRIL, a BAFF-related molecule that enhances plasma-cell survival.45 By also expressing DLL1, mouse splenic ILCs would sustain the differentiation and/or survival of plasmablasts and plasma cells that emerge during IgG3 responses to TI antigens. In mice, deple- tion of ILCs did not impair IgM production, which suggests that ILCs enhance mainly MZ B cell–derived IgG3 responses rather than B-1 cell–derived IgM responses. Nonetheless, splenic ILCs sustained preimmune production of IgM in response to bacterial phosphorylcholine, which indicated the involvement of ILCs in IgM responses to some but not all TI antigens.

In ILC-deficient mice, impaired TI production of IgG3 was associated with fewer Nbh cells, which help MZ B cells via BAFF and APRIL.10 Accordingly, human splenic ILCs enhanced the survival and B cell–helper activity of Nbh cells through GM-CSF. That cytokine also induced structures similar to neutrophil extracellular traps, which might enable Nbh cells to capture blood-borne antigens transiting through the MZ.4,10 Human splenic ILCs also released IL-8, which may contribute to the recruitment of Nbh cells. Mice do not express IL-8, but their splenic ILCs may attract Nbh cells via alternative factors, including GM-CSF. Notably, splenic ‘innate response–activator’ B cells also express GM-CSF,49 which could explain the persistence of some Nbh cells in mice that lack ILCs.

GM-CSF further derives from TγH17 cells,37 but our analysis of T cell–deficient mice suggested that ILCs can regulate the homeostasis of Nbh cells independently of T cells. In mice, splenic ILCs sustained both IgG3+ plasmablasts and IgG3+ plasma cells, whereas mouse Nbh cells helped mainly IgG3+ plasma cells, which suggested that multiple cell subsets of the innate immune system orchestrate TI IgG3 responses in a hierarchical manner. Of note, IgG3 may provide protection against certain pathogens following its interaction with ficolins, a family of soluble pattern-recognition receptors released by hepatocytes and various cells of the innate immune system.41 In summary, our studies have indicated that splenic ILCs orchestrate a stromal cell–innate cell network that fosters the TI production of antibodies by MZ B cells. Harnessing this network with adjuvants may enhance protective humoral responses to poorly immunogenic TI antigens,4,50 including viral glycoproteins and bacterial carbohydrates.

METHODS

Methods and any associated references are available in the online version of the paper.
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AUTHOR CONTRIBUTIONS
G.M. and M. Miyajima designed and did research, discussed data and wrote the paper; B.S., A.M., I.P. and A. Chudnovskiy designed and did research; L. Cassis, C.M.B., L. Comerma, M.G., D.L. and M.C. did research; S.S., I.I.A, M.J. and J.Y. provided blood and tissue samples and discussed data; S.F. and M. Merad designed research, provided reagents and discussed data; and A. Cerutti designed research, discussed data and wrote the paper.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

Human samples. Mononuclear cells and neutrophils were isolated from the peripheral blood of healthy volunteers or from histologically normal spleens from deceased organ donors or trauma patients without clinical signs of infection or inflammation that had undergone splenectomy. Tonsils were from people with follicular hyperplasia. The use of blood and tissues was approved by the Ethical Committee for Clinical Investigation of the Institut Hospital del Mar d’Investigacions Mèdiques (CEIC-IMIM 2011/4494/1). Tissue samples were collected by the Department of Pathology of Hospital del Mar. Prior to sample collection, signed informed consent was obtained from the patient or his or her parent or guardians. All blood and tissue samples were assigned coded identifiers and relevant clinical information remained concealed.

Mice. Rorc−/− (Rorc[µ/µ]), Cd3e−/−, Rag1−/−, Rag1−/−I12rg−/− and Csf2−/− mice on the C57BL/6 background have been described and C57BL/6 CD90.1+ mice were from the Jackson Laboratory. All mice were housed in specific pathogen-free conditions. Male and female mice were used at 8–12 weeks of age unless specified otherwise in the text. All experiments involving mice were in accordance with approved protocols from the Institutional Animal Care at RIKEN and Icahn School of Medicine at Mount Sinai.

Mouse chimeras and depletion of neutrophils. 4 × 10^7 bone marrow cells from Rag1−/− mice were mixed with 1 × 10^7 bone marrow cells from Rorc−/− mice and the mixture was injected intravenously into irradiated (10 Gy) 2-month-old Rorc−/− mice. After transplantation, mice were given 500 mg/l ampicillin (Sigma) and 1 g/l neomycin (Sigma) in their drinking water for 2 weeks. For the depletion of neutrophils, control monoclonal antibody (mAb) RTK2758 to keyhole limpet hemocyanin (Supplementary Table 2) or neutrophil-depleting mAb I A8 to Ly6G (Biolegend) were administered intraperitoneally every 5 d at a dose of 250 µg per mouse for a month. Thy-1−disparate Rag1−/− chimeras were generated and depleted of ILCs with mAbs 30–H12 to Thy-1.2 (BioXCell) injected intraperitoneally every 3 d at a dose of 500 µg per mouse for a month, as published. mAb LTF-2 to keyhole limpet hemocyanin (BioXCell) served as control (Supplementary Table 2).

Adoptive transfer. 2 × 10^6 to 3 × 10^6 ILCs from the small intestine of Csf2−/− or Csf2−/− mice were adoptively transferred into Rag1−/−I12rg−/− mice via retro-orbital injection as described and splenic neutrophils were analyzed 21 d later. 1 × 10^6 B16-562 melanoma cells, which overexpress GM-CSF (from G. Dranoff), were injected into Csf2−/− mice, and splenic neutrophils were analyzed 12–14 d later.

Immunization. Serum TNP-specific antibody titers were determined by ELISA and after intraperitoneal injection of 50 µg TNP-Ficoll.

Cells. Human splenocytes or tonsillar mononuclear cells were obtained from fresh tissue samples as reported in published studies. Human ILCs, NK cells, naive B cells, MZ B cells, macrophages, T cells, N C cells and N BH cells were sorted by flow cytometry as described and splenic neutrophils were analyzed 21 d later. 1 × 10^5 B16-562 melanoma cells, which overexpress GM-CSF (from G. Dranoff), were injected into Csf2−/− mice, and splenic neutrophils were analyzed 12–14 d later.

Cultures and reagents. Human splenic ILCs (5 × 10^4 per well) were plated in 96-well U-bottomed plates and were cultured in complete RPMI medium supplemented with 10% fetal bovine serum (FBS), penicillin and streptomycin (10 U/ml), or with or without 50 ng/ml IL-7, 50 ng/ml IL-1β and/or 50 ng/ml IL-23 (Peprotech). Conditioned medium was obtained by culture of splenic ILCs for 24 h in complete RPMI medium. In coculture experiments, populations of splenic ILCs were first expanded for 3–8 d with IL-7 and IL-1β as published. ILCs were cultured for 72 h with confluent human MRCs in flat-bottomed plates. To avoid cell-to-cell contact, some cells were cocultured in a 24-well Transwell system (Corning). Human ILCs were also cultured with human MZ B cells, N C cells and/or N BH cells at a ratio of 1:10. Some cocultures were supplemented with 500 ng/ml RAFF (Alexis), 0.25 µg/ml CpG ODN-2006 (InvivoGen), 30 µg/ml BAFF–R-Ig, 5 µg/ml CD40-Ig, 10 µg/ml neutralizing mAb 3209 to human GM-CSF (R&D Systems) or 10 µg/ml isotopematched IgG1 control mAb 11711 (R&D System). For inhibition of Notch2, human MZ B cells were incubated with 25, 5 or 2.5 µM DAPT (N-[N-(3,5-difluorophenacetyl)-l-t-allyl]-S-phenylglycine t-buty1 ester, Sigma), which interferes with the cleavage of Notch proteins by g-secretase. In some experiments, human MRCs were stimulated with 100 ng/ml LT-αβ1 and 10 ng/ml TNF (R&D Systems) or with ILCs in the presence or absence of 10 µg/ml mAb 6401 to TNF (R&D Systems) or 10 µg/ml mAb 135125 to LT-α1β1 and LT-α1β2 (R&D Systems). Human N C cells were stimulated with 50 ng/ml GM-CSF (Peprotech) or 100 ng/ml LPS (InvivoGen).

Flow cytometry. Cells were incubated at 4 °C with Fc-blocking reagent (Milenyi Biotec) before the addition of the appropriate ‘cocktails’ of fluorochrome-labeled mAbs (Supplementary Tables 1 and 2). Dead cells were excluded through the use of DAPI (4′,6-diamidino-2′-phenylindole) as indicated by the manufacturer (Boehringer Mannheim). For analysis of intracellular transcription factors, human ILCs or NK cells were stained with mAbs to specific surface molecules, fixed, permeabilized using the Cytofix/Cytoperm kit (BD Pharmingen) and labeled with mAb to human RORg or mAb to T-bet (each in Supplementary Table 1). For intracellular staining of cytokines, human ILCs were cultured for 4 h in complete RPMI medium with GolgiStop (BD Pharmingen) in the presence or absence of 100 nM phorbol myristate acetate and 0.5 µg/ml ionomycin (Sigma). Those cells were stained with mAbs to specific surface molecules, fixed, permeabilized using the Cytofix/Cytoperm kit (BD Pharmingen), and finally incubated with mAb to human GM-CSF or IL-8 (each in Supplementary Table 1). To stain intracellular IgG3, mouse splenocytes were stained with mAbs to specific surface molecules (Supplementary Table 2), permeabilized, fixed with the Cytofix/Cytoperm kit (BD Pharmingen), and labeled with mAb R40-82 to mouse IgG3 (Supplementary Table 2). Cells were acquired with a FACSort, FACSARIA II, LSR II or LSRFortessa (BD Biosciences) and data were further analyzed with FlowJo software (TreeStar).

Sorting of cells by flow cytometry. Human CD3+CD14+CD19+CD11+CD27+ ILCs, CD3+CD14+CD19+CD117+CD127+CD56+ NK cells, CD19+IgG4CD27− naive B cells, CD19+IgD+CD27+ MZ B cells, CD14+ macrophages and CD3+ T cells, as well as mouse CD3+ T cells, B220+ B cells, CD11c+ DCs, CD11b+ macrophages, CD49b+ NK cells, Ly6G+ neutrophils, CD4+CD45+CD117+CD127+Lin− (CD3−B220−CD11b−CD11c−Ly6G+) ILCs and CD4+CD45+CD117+CD127+Lin− ILCs were stained with the appropriate ‘cocktails’ of fluorochrome-labeled mAbs (Supplementary Tables 1 and 2) and were sorted with a FACSARIA II (BD Biosciences) after exclusion of dead cells through DAPI staining. The purity of cells sorted this way was consistently >95%.

Viability and proliferation assays. Cell survival was measured with Annexin-V Apoptosis Detection Kit II (BD Pharmingen). Gates and quadrants were drawn to give ≤1% total positive cells in samples incubated with isotype-matched control mAbs. Cell proliferation was assessed by staining with CFSE (carboxyfluorescein diacetate succinimidyl ester) through the use of CellTrace CFSE Cell Proliferation Kit (Invitrogen).

Laser-capture microdissection. For microdissection, fresh human splenic tissues were frozen in optimal cutting temperature compound with the ArcturusXT Laser Capture Microdissection System according to the manufacturer’s instructions (Life Technologies). Then, tissue slices 10 µm in thickness were isolated on membrane slides (Life Technologies) and were
fixed for 10 s in cold acetone. Immunohistochemistry with specific mAbs (Supplementary Table 1) was followed by tissue dehydration and fixation with xylene. Groups of cells from completely dried tissues were acquired by microdissection and RNA was extracted with an Arcturus PicoPure RNA Extraction Kit (Life Technologies).

Immunofluorescence analysis. Frozen tissues and cells from humans or mice were fixed as published10 and stained with various combinations of antibodies (Supplementary Tables 1 and 2). Biotinylated antibodies were detected with horseradish peroxidase–conjugated streptavidin followed by tetramethylrhodamine from a Tyramide Signal Amplification Kit (PerkinElmer Life Sciences) or streptavidin–Alexa Fluor conjugates. Nuclear DNA was stained with DAPI. Coverslips were applied with FluorSave reagent (Calbiochem). Images were obtained with an Axioplan2 microscope (Carl Zeiss) and were further analyzed with Axiovision software (Carl Zeiss). For analysis of the formation of neutrophil extracellular traps, human circulating neutrophils were seeded on poly-D-lysine–coated glass coverslips and were stimulated for 3 h with either human GM-CSF or human splenic ILC–conditioned medium. Cells were fixed with 4% paraformaldehyde and stained with mAb ab21595 to elastase (Abcam) and DAPI.

Immunohistochemistry. Formalin-fixed and paraffin-embedded tissue sections 5 µm in thickness were stained with various mAbs (Supplementary Table 1) with the EnVision + Dual Link System-HRP (DAB+) kit for single staining (Dako) and the EnVision G/2 Doublestain System Rabbit/Mouse (DAB+/Permanent Red) kit for double staining (Dako). Sections were counterstained with hematoxylin.

ELISA. Total human IgM, IgG and IgA were measured as published10. BAFF Soluble (human) Matched Pair Detection Set (Adipogen), LEGEND MAX Human APRIL/TNFSF13 ELISA Kit (Biolegend), Human GM-CSF ELISA Development Kit and Human IL-8 ELISA Development Kit (Peprotech) were used for the measurement of human BAFF, APRIL, GM-CSF and IL-8, respectively. A human Th1/Th2/Th9/Th17/Th22 13plex FlowCytomix Multiplex system (eBioscience) was used for the measurement of human IL-22. The concentration of IgM, IgG3 and IgA in mouse serum was determined with a Mouse ELISA Quantification Set (Bethyl Laboratories). For the detection of mouse TNF-specific IgG3, ELISA plates coated with 5 µg/ml bovine serum albumin (BSA)-conjugated TNP (BioSearch Technologies) were sequentially incubated with mouse serum and horseradish peroxidase–conjugated goat polyclonal antibody to mouse IgG3 (A90-101P; Bethyl Laboratories). For the detection of mouse phosphorylcholine-specific IgM, ELISA plates coated with 5 µg/ml BSA-conjugated phosphorylcholine were sequentially incubated with serum and horseradish peroxidase–conjugated goat polyclonal antibody to IgM (A90-101P; Bethyl Laboratories). BSA-conjugated phosphorylcholine was synthesized as follows: 1 mg of BSA was coupled to 1 mg p-aminophenylphosphorylcholine in 0.1 M 2-(N-morpholino)ethanesulfonic buffer (Sigma) at a pH of 4.5, supplemented with 2 mg 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride in a final volume of 1.6 ml. That mixture was incubated for 2 h at 20 °C. Conjugated BSA was dialyzed against PBS at a pH of 7.4 and 4 °C.

Quantitative RT-PCR. For the quantification of human gene products, total RNA was extracted and reverse-transcribed into cDNA as reported10. Quantitative RT-PCR was done as reported10 with specific primer pairs (Supplementary Table 3). For the quantification of mouse gene products, total RNA was extracted with TRIzol reagent (Gibco BRL). After treatment of RNA with DNase I (Invitrogen), random hexamers (Invitrogen) were used for first-strand cDNA synthesis. Quantitative RT-PCR was done in 96-well plates with a LightCycler 480 real-time PCR system (Roche Diagnostics) with a LightCycler 480 SYBR Green I Master kit (Roche Diagnostics) and specific primer pairs (Supplementary Table 4). Gene expression was normalized to that of the gene encoding GAPDH (glyceraldehyde-3-phosphate dehydrogenase) for each sample.

Statistical analysis. Statistical significance was assessed with a two-tailed or one-tailed unpaired Student’s t-test unless specified otherwise. A Mann-Whitney U-test or Wilcoxon matched-pairs signed-rank test was used for analysis of non-parametric data. Results were analyzed with Prism software (Graph Pad) and P values of less than 0.05 were considered significant. In animal experiments, littermates (a minimum of three mice per group) were randomly distributed into the treatment groups so that all groups were age matched and sex matched. No specific randomization or blinding protocol was used, and no animals were excluded from analysis.

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