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B cell–helper neutrophils stimulate the diversification and production of immunoglobulin in the marginal zone of the spleen

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Neutrophils use immunoglobulins to clear antigen, but their role in immunoglobulin production is unknown. Here we identified neutrophils around the marginal zone (MZ) of the spleen, a B cell area specialized in T cell–independent immunoglobulin responses to circulating antigen. Neutrophils colonized peri-MZ areas after postnatal mucosal colonization by microbes and enhanced their B cell–helper function after receiving reprogramming signals, including interleukin 10 (IL-10), from splenic sinusoidal endothelial cells. Splenic neutrophils induced immunoglobulin class switching, somatic hypermutation and antibody production by activating MZ B cells through a mechanism that involved the cytokines BAFF, APRIL and IL-21. Neutropenic patients had fewer and hypomutated MZ B cells and a lower abundance of preimmune immunoglobulins to T cell–independent antigens, which indicates that neutrophils generate an innate layer of antimicrobial immunoglobulin defense by interacting with MZ B cells.

Neutrophils are the first cells of the immune response to migrate to sites of infection and inflammation to eliminate microbes and necrotic cells[1]. After sensing conserved molecular signatures associated with microbes and tissue damage, neutrophils activate defensive programs that promote phagocytosis, intracellular degradation, extracellular discharge of antimicrobial factors and the formation of neutrophil extracellular traps (NETs)[2]. These structures arise after cell death and consist of decondensed chromatin embedded with granular and cytoplasmic proteins that trap and kill microbes[3]. Neutrophils also release cytokines and chemokines that recruit monocytes to optimize antigen clearance[4].

The long-held view that neutrophils function exclusively in the innate phase of the immune response has been challenged by studies

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showing that neutrophils also influence adaptive immunity by interacting with dendritic cells (DCs)[5]. These cells of the innate immune response present antigen to T cells after undergoing further maturation in response to neutrophil-derived cytokines such as tumor necrosis factor (TNF)[6]. Neutrophils also release interleukin-12 (IL-12), which promotes the polarization of naive T cells into inflammatory T helper type 1 cells that release interferon-γ (IFN-γ)[2]. In the presence of IFN-γ and other inflammatory cytokines, neutrophils also upregulate their expression of antigen-loading major histocompatibility class-II molecules to acquire DC-like antigen-presenting function[2].

Although there is growing evidence that neutrophils have an effect on the induction of T cell responses during infection, additional data have shown that neutrophils suppress T cell activation in the context of pregnancy and cancer[2]. Indeed, neutrophils are equipped with enzymatic systems such as inducible nitric oxide synthase (iNOS) and arginase that suppress T cells by generating nitrogen intermediates and depleting extracellular arginine, respectively[7]. Neutrophils would further regulate adaptive immunity by secreting IL-10 after sensing bacteria through Toll-like receptors (TLRs) and C-type lectin receptors[8]. Thus, neutrophils can either potentiate or down-modulate T cell responses in a context-dependent manner.

Neutrophils further crosstalk with the adaptive immune system by binding to B cell–derived immunoglobulin G (IgG) and IgA on opsonized microbes[9,10]. The ensuing activation of Fcγ and Fcα receptors regulates neutrophil effector functions[1]. Notably, neutrophils also produce the cytokine BAFF (BLyS) and the proliferation-inducing ligand APRIL, which are two TLR-inducible B cell–stimulating factors related to the ligand for the T cell molecule CD40 (CD40L)[11–14]. In addition to promoting the survival and differentiation of B cells and immunoglobulin-secreting plasma cells[11–14], BAFF and APRIL trigger IgM production and class switching from IgM to IgG or IgA independently of CD40L[15–18]. This T cell–independent (TI) pathway would enable antigen-sampling DCs and other cells of the innate immune response to enhance B cell responses at mucosal surfaces inhabited by commensal bacteria[19].

TI immunoglobulin responses also occur in the marginal zone (MZ) of the spleen, a B cell area positioned at the interface between the circulation and the immune system[20,21]. B cells dwelling in the MZ are in a state of active readiness that enables them to mount prompt immunoglobulin responses to blood-borne antigens via a pathway that does not require a T cell–dependent (TD) germinal-center reaction[20–22]. Although some MZ B cell responses may occur after translocation of commensal antigens across intact mucosal surfaces[20,21,23–26], others occur after systemic invasion by mucosal pathogens[20,21]. In humans, MZ B cells have a circulating counterpart, contain mutated immunoglobulin genes and express surface IgM and IgD together with the memory molecule CD27 (refs. 21,27,28). These MZ B cells are different from hypermutated IgM$^+$ memory B cells, which emerge from a canonical germinal-center reaction and express surface IgM and CD27 but not IgD[22]. The role of neutrophils in B cell activation and immunoglobulin production is unknown, but published studies show that these granulocytes home to the MZ in response to blood-borne bacteria[29].

We show here that neutrophils colonized peri-MZ areas of the spleen in the absence of infection via a noninflammatory pathway that became more prominent after postnatal mucosal colonization by bacteria. Splenic neutrophils had a phenotype distinct from that of circulating neutrophils, formed MZ B cell–interacting NET-like structures, and elicited immunoglobulin class switching, somatic hypermutation and antibody production by activating MZ B cells via a mechanism that involved BAFF and APRIL and the cytokine IL-21. Circulating neutrophils acquired B cell–helper function after exposure to splenic sinusoidal endothelial cells (SECs), releasing cytokines dependent on the transcription factor STAT3, such as IL-10, in response to microbial signals. Patients with congenital neutropenia had fewer and hypomutated MZ B cells and less production of preimmune immunoglobulins reactive to TI antigens. Thus, neutrophils may generate an innate layer of antimicrobial immunoglobulin defense by undergoing MZ B cell–helper reprogramming in the spleen.

RESULTS

Neutrophils colonize peri-MZ areas

To determine whether neutrophils interact with B cells under homeostatic conditions, we obtained lymphoid organs from people with no inflammation or infection and stained the organs for the B cell molecule CD20 and for the granulocytic enzyme myeloperoxidase by immunohistochemistry. Peripheral lymph nodes, tonsils and intestinal Peyer's patches contained no or few neutrophils, whereas we detected some neutrophils in the perifollicular area of mesenteric lymph nodes (**Fig. 1a** and **Supplementary Fig. 1**). Unexpectedly, we detected even more neutrophils in the perifollicular area of spleens that had no histological alterations (**Fig. 1a** and **Supplementary Fig. 2a**). As shown by light microscopy, fluorescence microscopy and confocal microscopy, splenic neutrophils expressed typical granulocyte molecules such as elastase, myeloperoxidase and the adhesion molecule CD15 and formed projections that interacted with outer MZ and perifollicular B cells with low expression of IgD (**Fig. 1b**,**c** and **Supplementary Fig. 2b**). Splenic neutrophils were also proximal to perifollicular SECs expressing the lectin mannose receptor and the coagulation protein von Willebrand factor, macrophages expressing the glycoprotein CD68 and the haptoglobulin receptor CD163, and DCs expressing the integrin CD11c.

Consistent with their possible role in homeostasis, we also detected perifollicular neutrophils in spleens from healthy rhesus macaques and mice (**Supplementary Fig. 3a**). Simian neutrophils expressed elastase and were positioned between MZ B cells and red-pulp SECs, whereas mouse neutrophils expressed the granulocyte molecule Ly6G and surrounded a MZ area containing B cells weakly positive for IgD and macrophages strongly positive for the sialoadhesin receptor MOMA-1. Another indication of the role of neutrophils in homeostasis was provided by analysis of spleens from patients with systemic inflammatory or infectious disorders, including systemic lupus erythematosus, hyper-IgD syndrome (an autoinflammatory disorder), infection with human immunodeficiency virus and sepsis. In these pathological spleens, neutrophils lost the selective perifollicular topography usually observed in normal spleens and instead extensively infiltrated follicular mantle and germinal-center areas populated by B cells strongly positive and negative for IgD, respectively (**Fig. 1d**,**e** and **Supplementary Fig. 3b**). The aberrant topography of splenic neutrophils in inflamed spleens was associated with partial or complete loss of the MZ. Thus, neutrophils colonized splenic peri-MZ areas but not follicular areas under homeostatic conditions.

Neutrophils have MZ B cell–helper function

Flow cytometry, light microscopy and electron microscopy did not demonstrate gross morphological and ultrastructural differences between circulating and splenic neutrophils (**Supplementary Fig. 4a**,**b**). Neutrophils amounted to 10%, 2%, 0.5% and 0.3% of total cells in spleens, mesenteric lymph nodes, peripheral lymph nodes and tonsils, respectively. MZ B cells coexpressing IgD and the memory molecule CD27 were more abundant in spleen and mesenteric lymph nodes than in other lymphoid tissues and had high expression of the

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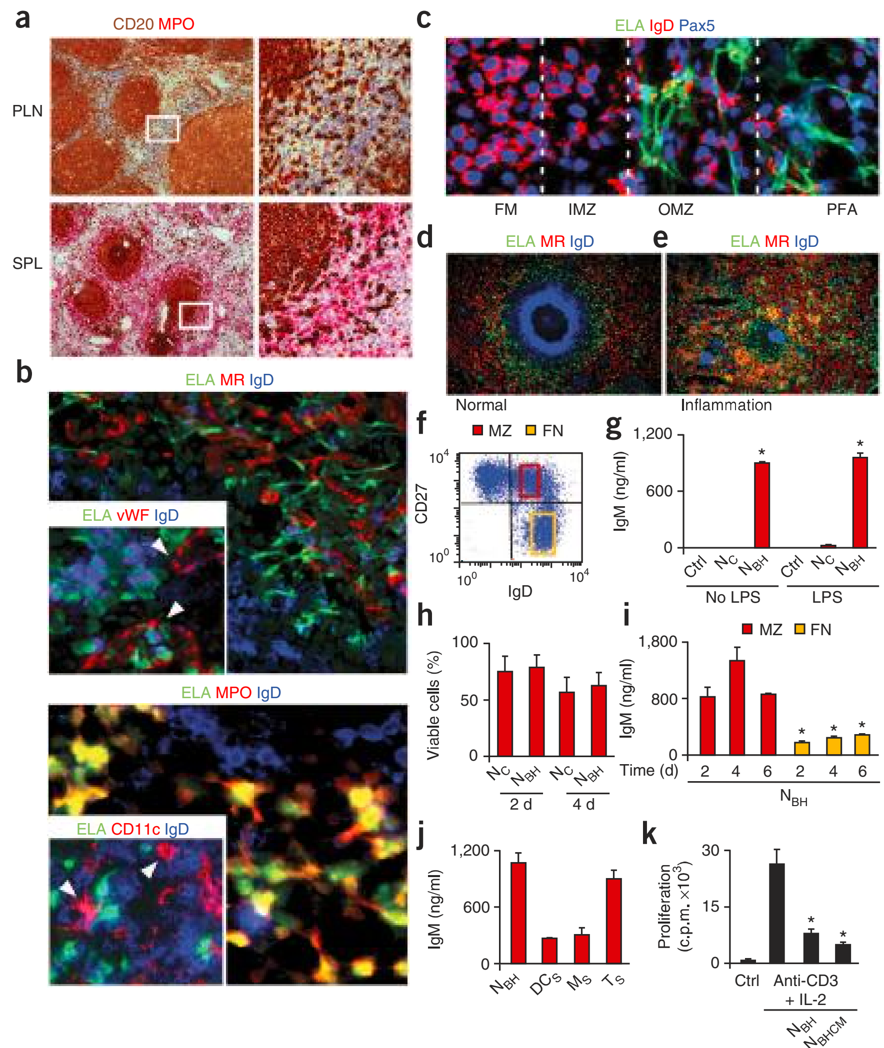

Figure 1 Neutrophils colonize the splenic MZ and function as N_{BH} cells. (**a**) Immunohistochemistry of peripheral lymph node (PLN) and spleen (SPL) stained for CD20 (brown) and myeloperoxidase (MPO; red). Outlined areas at left correspond to enlargement at right. Original magnification, ×4 (left) or ×20 (right). (**b**–**e**) Immunofluorescence of normal spleen (**b**–**d**) and inflamed spleen from a patient with hyper-IgD syndrome (**e**), stained for elastase (ELA; green); mannose receptor (MR), von Willebrand factor (vWF), myeloperoxidase, CD11c or IgD (all red); and IgD or Pax5 (both blue). Arrowheads indicate sinusoids (**b**, top inset) and DCs (**b**, bottom inset). FM, follicular mantle; IMZ, inner marginal zone; OMZ, outer marginal zone; PFA, perifollicular area. Original magnification, ×40 (**b** (main images), **c**), ×63 (**b**, insets) or ×10 (**d**,**e**). (**f**) Flow cytometry of CD27 and IgD on splenic $CD19^+$ B cells. Yellow and red outlined areas indicate gates for follicular naive (FN) $CD27^-IgD^{hi}$ B cells and MZ $CD27^+IgD^{lo}$ B cells. (**g**) ELISA of IgM from splenic MZ B cells cultured for 7 d with medium (control (Ctrl)) and from N_C or N_{BH} cells left unprimed (No LPS) or primed with LPS (LPS). (**h**) Frequency of viable splenic MZ B cells cultured for 2 or 4 d with N_C or N_{BH} cells. (**i**) ELISA of IgM from splenic MZ and FN B cells cultured for 2, 4 or 6 d with N_{BH} cells. (**j**) ELISA of IgM from splenic MZ B cells cultured for 6 d with N_{BH} cells or with splenic DCs (DC_S), macrophages (M_S) or $CD4^+$ T cells (T_S). (**k**) Proliferation of $CD4^+$ T cells (T) cultured with medium alone (Ctrl) or antibody to CD3 and IL-2 (Anti-CD3 + IL-2) in the presence or absence of N_{BH} cells or conditioned medium derived from N_{BH} cells (N_{BHCM}). c.p.m., counts per minute. *$P < 0.05$ (one-tailed unpaired Student's *t*-test). Data are from one of three experiments with similar results (**a**–**f**) or summarize three independent experiments (**g**–**k**; error bars, s.e.m.).

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complement receptor CD21 (**Fig. 1f**, **Supplementary Fig. 4c** and data not shown). Enzyme-linked immunosorbent assay (ELISA) demonstrated induction of IgM secretion by MZ B cells exposed to splenic neutrophils but not by those exposed to circulating neutrophils (**Fig. 1g**); we therefore called these 'B cell–helper neutrophils' (N_{BH} cells) and 'conventional neutrophils' (N_C cells), respectively. Priming with lipopolysaccharide (LPS) did not augment the IgM-inducing function of N_{BH} cells (**Fig. 1g**), which suggested pre-existing activation. Viability assays indicated that the enhanced IgM-inducing function of N_{BH} cells did not depend on their ability to promote better MZ B cell survival than that promoted by N_C cells (**Fig. 1h**). Accordingly, the apoptosis of N_{BH} cells was equivalent to that of N_C cells in long-term coculture despite their better survival in short-term monocultures (**Supplementary Fig. 5a**).

N_{BH} cells activated MZ B cells more effectively and more rapidly than follicular naive B cells through both contact-dependent and contact-independent mechanisms (**Fig. 1i** and **Supplementary Fig. 5b**). The functional prominence of N_{BH} cells was documented by the finding that they activated MZ B cells as effectively as splenic $CD4^+$ T cells did and more effectively than splenic macrophages or DCs did (**Fig. 1j**). The functional specificity of N_{BH} cells was emphasized by their ability to induce contact-independent suppression of the proliferation of $CD4^+$ T cells that were activated via the T cell antigen receptor molecule CD3 and the cytokine IL-2 (**Fig. 1k**). Thus, N_{BH} cells functioned as professional MZ B cell–helper cells and may suppress T cells to induce immunoglobulin responses in a TI manner.

Neutrophils induce immunoglobulin diversification

Confocal microscopy and wide-field microscopy showed that N_{BH} cells expressing elastase, CD15 and the granulocytic microbial receptor CEACAM-1 formed DNA-containing NET-like projections that were in close contact with B cells expressing IgD (**Fig. 2a** and **Supplementary Video 1**). Outer MZ and perifollicular N_{BH} cells had more abundant projections than red pulp N_{BH} cells had and often interacted with B cells expressing the DNA-editing enzyme AID (**Fig. 2b**,**c**), a hallmark of ongoing immunoglobulin gene diversification in germinal-center and some extrafollicular B cells[16,17,30,31]. Quantitative RT-PCR showed that MZ B cells had more *AICDA* mRNA (encoding AID) than naive B cells had, but had less *AICDA* than germinal center B cells had, and demonstrated that MZ B cells upregulated *AICDA* after exposure to N_{BH} cells but not after exposure to N_C cells (**Fig. 2c** and **Supplementary Fig. 6a**,**b**).

We used RT-PCR and Southern blot analysis to study class-switch recombination (CSR), a process that provides immunoglobulins with new effector functions by replacing the heavy-chain constant region of IgM with that of IgG or IgA without changing antigen specificity[30]. Consistent with their higher AID expression, some MZ B cells constitutively expressed germline γ-chain intervening region

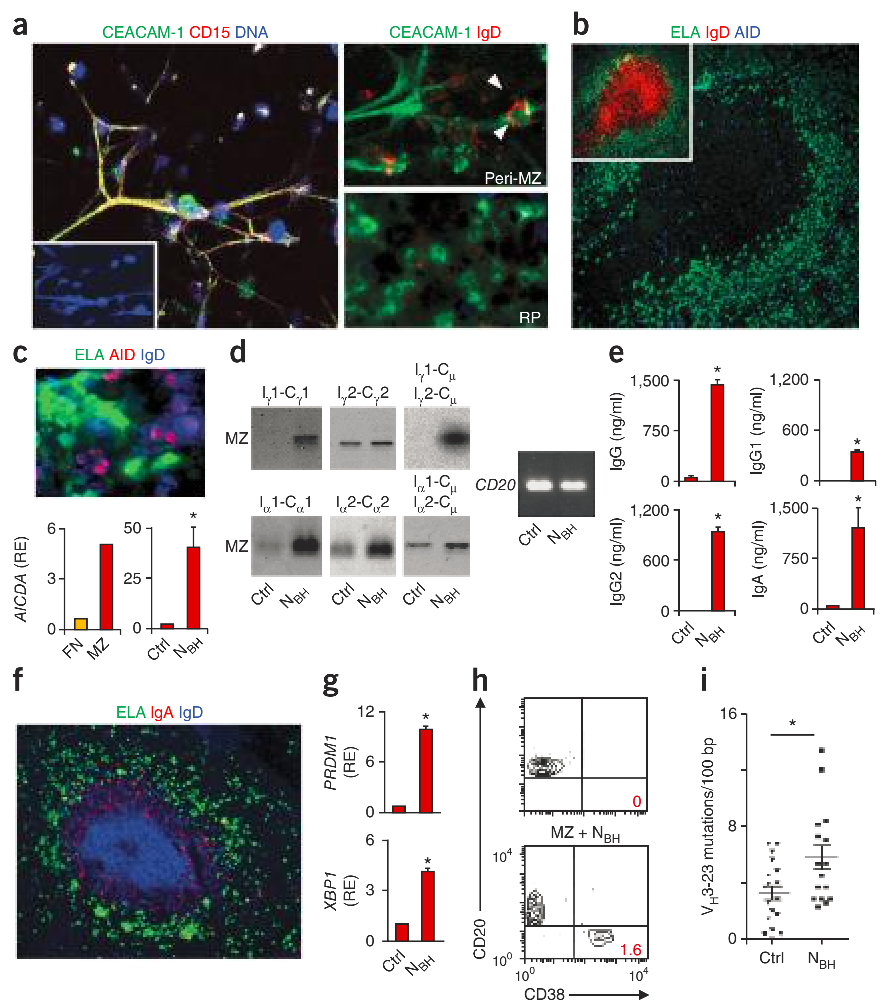

Figure 2 N_{BH} cells interact with splenic MZ B cells to induce CSR, SHM and production of immunoglobulins. (**a**) Confocal microscopy of splenic N_{BH} cell–B cell clusters stained for CEACAM-1 (green), CD15 or IgD (each red), and DNA (blue). Inset (bottom left), DNA in NET-like projections; right, enlargement of peri-MZ and red pulp (RP) N_{BH} cells. Arrowheads indicate a MZ B cell interacting with an extracellular projection from an N_{BH} cell. Original magnification, ×40 (left) or ×63 (right). (**b**) Immunofluorescence of a spleen stained for elastase (green), IgD (red) and AID (blue) with AID^+ B cells and N_{BH} cells in peri-MZ and MZ areas (main image) of an IgD^+ primary follicle (inset). Original magnification, ×10. (**c**) Immunofluorescence (top) of N_{BH} cell–B cell clusters stained for elastase (green), AID (red) and IgD (blue). Original magnification, ×63. Below, quantitative RT-PCR analysis of *AICDA* mRNA in naive splenic follicular B cells (FN) and MZ B cells (left) and in splenic MZ B cells cultured for 2 d in the presence of medium or N_{BH} cells (right); results are normalized to *PAX5* mRNA and are presented relative to the expression (RE) of cells incubated with medium alone. (**d**) Southern blot analysis of germline $I_{\gamma}1$-$C_{\gamma}1$, $I_{\gamma}2$-$C_{\gamma}2$, $I_{\alpha}1$-$C_{\alpha}1$ and $I_{\alpha}2$-$C_{\alpha}2$ transcripts and I_{γ}-C_{μ} and I_{α}-C_{μ} switch circle transcripts amplified by RT-PCR from splenic MZ B cells cultured for 4 d with medium or N_{BH} cells. *CD20* transcripts (right) serve as a B cell–specific loading control. (**e**) ELISA of IgG, IgG1, IgG2 and IgA from MZ B cells cultured for 7 d as in **d**. (**f**) Immunofluorescence of spleens stained with elastase (green), IgA (red) and IgD (blue). Original magnification, ×10. (**g**) Quantitative RT-PCR analysis of *PRDM1* and *XBP1* mRNA from MZ B cells cultured as in **e**; results are normalized to *PAX5* mRNA and are presented relative to those of cells incubated with medium. (**h**) Flow cytometry analysis of CD20 and CD38 on MZ B cells cultured as in **e**. Numbers in bottom right quadrants indicate percent $CD20^-CD38^+$ cells. (**i**) V_H3-23 gene mutations (per 100 base pairs (bp)) in MZ B cells cultured for 12 d as in **d**. Each symbol represents a cloned V_H3-23 transcript; small lines indicate the mean (±s.e.m.). $^*P < 0.05$ (one-tailed unpaired Student's *t*-test). Data are from one of three experiments with similar results (**a**–**c** (top and bottom left), **d**,**f**,**h**) or summarize three independent experiments (**c** (bottom right), **e**,**g**,**i**; error bars (**c**,**e**,**g**), s.e.m.).

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2–γ-chain constant region 2 ($I_{\gamma}2$-$C_{\gamma}2$), $I_{\alpha}1$-$C_{\alpha}1$ and $I_{\alpha}2$-$C_{\alpha}2$ transcripts (**Fig. 2d**), which are hallmarks of ongoing CSR to IgG2, IgA1 and IgA2, respectively[16–18], and contained switch circle $I_{\alpha}1$-C_{μ} and $I_{\alpha}2$-C_{μ} transcripts (**Fig. 2d**), which indicate ongoing CSR to IgA1 and IgA2, respectively[16–18]. In the presence of N_{BH} cells, MZ B cells induced the expression of $I_{\gamma}1$-$C_{\gamma}1$, $I_{\gamma}1$-C_{μ} and $I_{\gamma}2$-C_{μ} transcripts (**Fig. 2d**), which are hallmarks of early CSR to IgG1 and ongoing CSR to IgG1 and IgG2, respectively[16–18], and upregulated the expression of $I_{\gamma}2$-$C_{\gamma}2$, $I_{\alpha}1$-$C_{\alpha}1$, $I_{\alpha}2$-$C_{\alpha}2$, $I_{\alpha}1$-C_{μ} and $I_{\alpha}2$-C_{μ} transcripts (**Fig. 2d**).

As shown by ELISA, quantitative RT-PCR, flow cytometry and fluorescence microscopy, N_{BH} cells stimulated MZ B cells to produce IgA and IgG, including more IgG2 than IgG1 (**Fig. 2e**,**f**). This effect was not elicited by N_C cells, occurred more effectively in MZ B cells than naive B cells, and correlated with the induction of *PRDM1* and *XBP1* mRNA (which encode the plasma cell–associated proteins Blimp-1 and XBP-1, respectively) and with the generation of plasma cells expressing the activation molecule CD38 but not CD20 (**Fig. 2g**,**h** and **Supplementary Fig. 6c**). Finally, we used DNA cloning and sequencing to study somatic hypermutation (SHM), a process that mutates variable (V)-diversity-joining exons that encoded the antigen-binding V region of immunoglobulins[30]. Although it typically occurs in germinal-center B cells, SHM also targets MZ B cells, at least in humans[21]. In the presence of N_{BH} cells, MZ B cells accumulated more mutations in the variable heavy-chain (V_H) V_H3-23 gene (**Fig. 2i**), which is often used by MZ B cells[21]. Thus, N_{BH} cells may stimulate MZ B cells to undergo SHM in addition to CSR and plasma cell differentiation.

Neutrophils include two MZ B cell–helper subsets

We used flow cytometry, electron microscopy, light microscopy, widefield microscopy, confocal microscopy, quantitative RT-PCR arrays, ELISA, annexin V assays and TUNEL assay (terminal deoxynucleotidyl transferase dUTP nick end-labeling) to further characterize N_{BH} cells. We identified two distinct subsets of N_{BH} cells (N_{BH1} and N_{BH2}) on the basis of various parameters, including their relative expression of CD15 and CD16. Similar to N_C cells, N_{BH} cells had typical granulocytic physical features and expressed canonical granulocytic molecules such as CD15 and the Fcγ receptor CD16 (**Fig. 3a** and **Supplementary Fig. 7a**). However, whereas N_C cells had high expression of CD15 and CD16, N_{BH1} cells had intermediate expression of CD15 and CD16 and N_{BH2} cells low expression of CD15 and CD16. Relative to expression

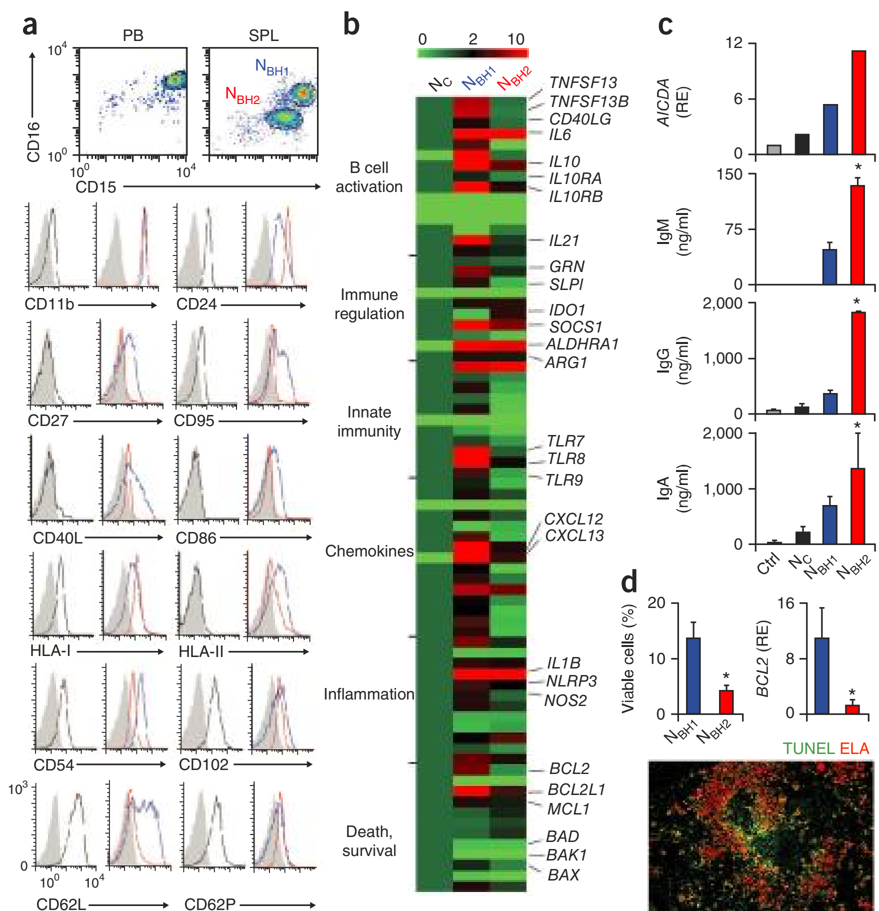

Figure 3 N_{BH} cells include N_{BH1} and N_{BH2} subsets distinct from N_C cells. (**a**) Flow cytometry of $CD15^{hi}CD16^{hi}$ N_C cells from peripheral blood (PB; black lines), or splenic $CD15^{int}CD16^{int}$ N_{BH1} cells (SPL; blue lines) and $CD15^{lo}CD16^{lo}$ N_{BH2} cells (red lines). Gray shading, isotype-matched control antibody. (**b**) Gene-expression profile of N_C, N_{BH1} and N_{BH2} cells established by customized quantitative RT-PCR arrays; results are normalized to *ACTB* mRNA (encoding β-actin) and are presented relative to expression by N_C cells. Left margin, functional mRNA clusters. Right margin, highly relevant mRNA encoding the following proteins: *TNFSF13*, APRIL; *TNFSF13B*, BAFF; *CD40LG*, CD40L; *IL10RA*, IL-10 receptor-α; *IL10RB*, IL-10 receptor-β; *GRN*, progranulin; *IDO1*, IDO; *ALDHRA1*, RALDH1; *ARG1*, arginase I; *IL1B*, IL-1β; *NLRP3*, NALP3; *NOS2*, iNOS; and *BCL2L1*, Bcl-x_L. Key (top) indicates gene expression: red, high; black, medium; green, low. (**c**) Quantitative RT-PCR analysis of *AICDA* and ELISA of IgM, IgG and IgA from MZ B cells cultured for 2 d (*AICDA*) and 4 d (immunoglobulins) with medium (Ctrl), N_C cells, N_{BH1} cells or N_{BH2} cells; results for RT-PCR are normalized to *PAX5* mRNA and are presented relative to expression of cells incubated with medium. (**d**) Frequency of viable N_{BH1} and N_{BH2} cells cultured for 18 h with medium (top left), quantitative RT-PCR of *BCL2* mRNA (top right), and immunofluorescence (bottom) of spleen stained for $TUNEL^+$ apoptotic DNA (green) and elastase (red). Results for RT-PCR are normalized to *ACTB* mRNA and are presented relative to those of N_C cells. Original magnification (bottom), ×10. *$P < 0.05$ (one-tailed unpaired Student's *t*-test). Data are from one of three experiments with similar results (**a**,**c** (RT-PCR), **d** (bottom)) or summarize three independent experiments (**b**,**c** (ELISA), **d** (top); error bars (**c**,**d**), s.e.m.).

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by N_C cells, N_{BH1} and N_{BH2} cells expressed more CD11b and CD24, which are molecules that inhibit TLR signaling[32,33], and more CD27, CD40L, CD86 (B7-2), CD95 (Fas), human leukocyte antigen I (HLA-I) and HLA-II (**Fig. 3a**), which are molecules that indicate immune activation[2,6]. In addition, N_{BH1} and N_{BH2} cells had lower expression of the adhesion molecules CD54 (ICAM-1), CD62L (L-selectin), CD62P (P-selectin) and CD102 (ICAM-2; **Fig. 3a**), a phenotype consistent with endothelial adhesion and extravasation[2,34]. Despite their morphological and ultrastructural similarity to N_{BH2} cells, N_{BH1} were more activated than N_{BH2} cells, as they had higher expression of CD27, CD40L, CD86, CD95 and HLA-II but lower expression of CD24 (**Fig. 3a** and **Supplementary Fig. 7b**,**c**). That observation correlated with the persistence of N_{BH1} cells but not N_{BH2} cells in inflamed spleens with a hypoplastic MZ (**Fig. 3a** and **Supplementary Fig. 8**).

Relative to mRNA abundance in N_C cells, N_{BH1} and N_{BH2} cells had more abundant mRNA for B cell–stimulating molecules such as BAFF, APRIL, CD40L and IL-21 (refs. 19,35,36), B cell chemoattractants such as CXCL12 and CXCL13 (ref. 19), immunoactivating receptors and cytokines such as TLR7, TLR8, IL-1β, IL-6, IL-8, IL-12 and TNF[1,2,4,6], and immunoregulatory molecules such as IL-10, the IL-10 receptor, arginase 1, RALDH1, iNOS, IDO, SOCS1, progranulin and SLPI[1,32,33,37–39] (**Fig. 3b** and **Supplementary Fig. 9**). We confirmed the presence of mRNA for CD40L in N_{BH} cells, but its expression was lower in N_{BH} cells than in splenic $CD4^+$ T cells (**Supplementary Fig. 10**). Consistent with their enhanced activation state, N_{BH1} cells had higher expression of much of the mRNA noted above than N_{BH2} cells had and had more mRNA for the antiapoptotic proteins Bcl-2, Bcl-x_L and mcl-1, but less mRNA for the proapoptotic proteins Bad and Bak1 (ref. 40).

N_{BH1} and N_{BH2} cells induced more *AICDA* mRNA and more production of IgM, IgG and IgA in MZ B cells than did N_C cells (**Fig. 3c**). Despite their worse survival, N_{BH2} cells had stronger MZ B cell–helper activity than N_{BH1} cells had (**Fig. 3c**,**d**), which was probably due to the release of more soluble BAFF, APRIL and IL-21 by N_{BH2} cells than by N_{BH1} cells (**Fig. 4a**,**b**). N_{BH2} cells may have also established firmer and more extensive interactions with MZ B cells via post-apoptotic NETs. Consistent with that possibility and with their heightened activation state, N_{BH} cells spontaneously formed NET-like projections, whereas N_C cells did not (**Supplementary Fig. 11** and **Supplementary Video 2**). Moreover, apoptotic $TUNEL^+$ N_{BH} cells, which probably corresponded to annexin V–positive N_{BH2} cells, occupied mainly perifollicular areas adjacent to MZ B cells (**Supplementary Fig. 12a**,**b**). Thus, N_{BH} cells included N_{BH1} and N_{BH2} subsets that were more activated and had more B cell–helper and regulatory properties than N_C cells had.

Neutrophils activate MZ B cells via BAFF, APRIL and IL-21

ELISA and flow cytometry showed that N_{BH1} and N_{BH2} cells expressed more surface BAFF and released more soluble BAFF, APRIL and IL-21 than N_C cells did (**Fig. 4a**,**b**). Fluorescence microscopy and quantitative RT-PCR identified N_{BH} cells that expressed BAFF and APRIL as well as MZ B cells that expressed mRNA for TACI, an immunoglobulin-inducing receptor that binds both BAFF and APRIL[18,19]

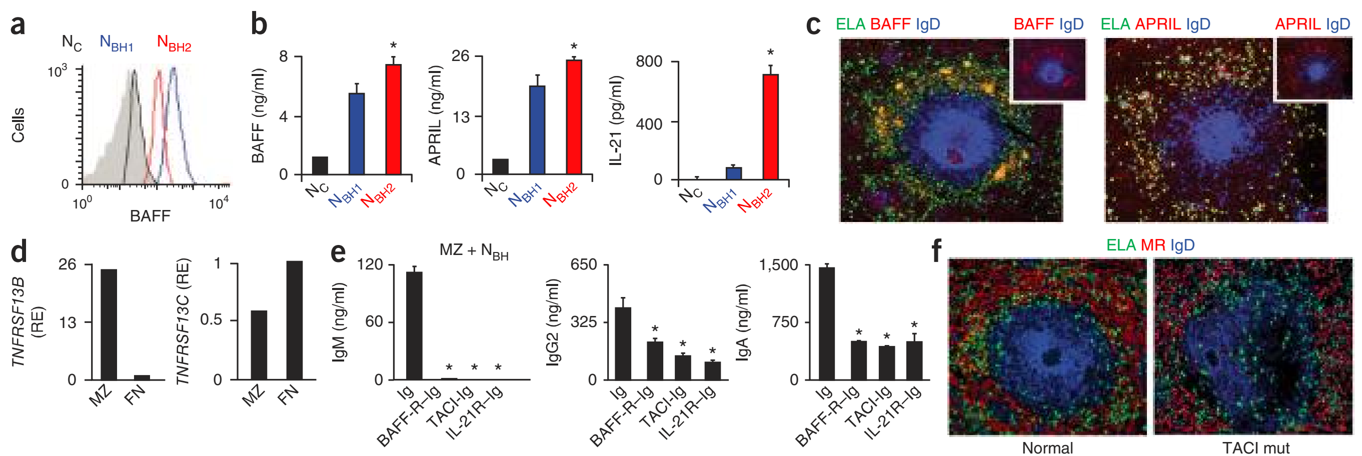

Figure 4 N_{BH} cells activate MZ B cells via BAFF, APRIL and IL-21. (**a**) Expression of BAFF on fresh N_C cells (black), N_{BH1} cells (blue) and N_{BH2} cells (red); gray shading, isotype-matched control antibody. (**b**) ELISA of BAFF, APRIL and IL-21 in N_C, N_{BH1} and N_{BH2} cells incubated for 18 h with medium. (**c**) Immunofluorescence of spleens stained for elastase (green), BAFF or APRIL (each red), and IgD (blue). Insets, staining patterns for BAFF and APRIL. Original magnification, ×10. (**d**) Quantitative RT-PCR analysis of *TNFRSF13B* (encoding TACI) and *TNFRSF13C* (encoding BAFF-R) in splenic MZ B cells and naive B cells; results are normalized to *ACTB* mRNA and are presented relative to those of naive B cells. (**e**) ELISA of IgM, IgG2 and IgA from splenic MZ B cells cultured for 6 d with conditioned medium from N_{BH} cells in the presence of control immunoglobulin (Ig), BAFF-R–immunoglobulin, TACI-immunoglobulin or IL-21R–immunoglobulin. (**f**) Immunofluorescence of elastase (green), mannose receptor (red) and IgD (blue) in spleens obtained from a healthy donor (Normal) and an immunodeficient patient with a mutation resulting in the C104R substitution associated with functional TACI deficiency (TACI mut); similar images were obtained from multiple follicles. Original magnification, ×10. (**g**) Frequency of circulating $IgD^{lo}CD28^+$ MZ B cells (calculated from total $CD19^+$ B cells) in immunodeficient patients with mutations resulting in the C104R or A181E substitution associated with functional TACI deficiency (TACI mut); immunodeficient patients with mutations resulting in the R382Q, R382W and V637M substitutions that cause functional STAT3 deficiency (STAT3 mut); and age-matched healthy donors (HD). $^*P < 0.05$ (one-tailed unpaired Student's *t*-test (**a**,**b**,**e**) or Mann-Whitney *U*-test (**g**)). Data are from one of three experiments with similar results (**a**,**c**,**d**,**f**) or summarize at least three independent experiments (**b**,**e**,**g**; error bars (**a**,**b**,**d**,**e**,**g**), s.e.m.).

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(**Fig. 4c**,**d** and **Supplementary Fig. 13**). Naive B cells had lower expression of mRNA for TACI than MZ B cells had, but had similar expression of mRNA for the BAFF receptor (BAFF-R; **Fig. 4d**), a survival-inducing receptor that binds BAFF but not APRIL[13]. Blocking BAFF and APRIL with soluble fusion protein of TACI and immunoglobulin (TACI-immunoglobulin) or IL-21 with an IL-21R–immunoglobulin fusion protein abolished IgM production and impaired the induction of IgG2 and IgA by conditioned medium from N_{BH} cells (**Fig. 4e**).

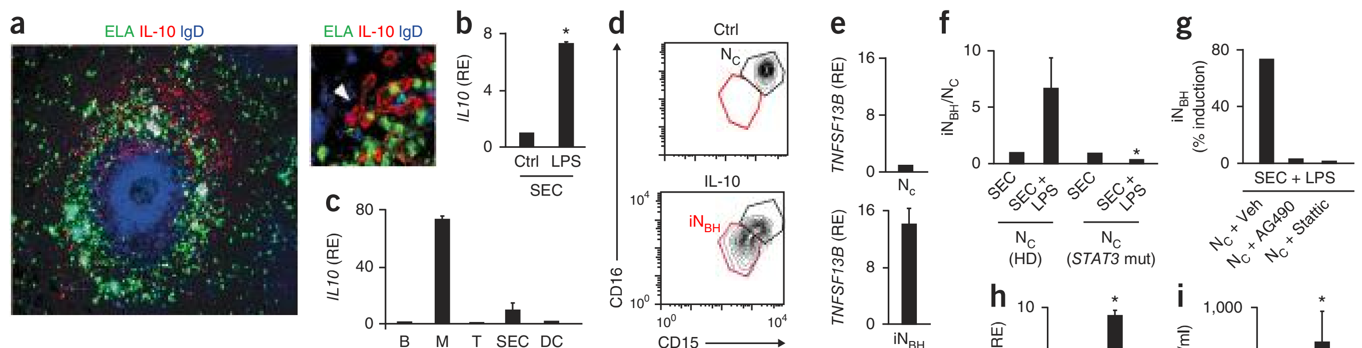

Figure 5 N_C cells acquire N_{BH} cell–like properties after exposure to SECs activated by microbial signals. (**a**) Immunofluorescence of spleens stained for elastase (green), IL-10 (red) and IgD (blue). Arrowhead indicates IL-10-expressing SECs. Original magnification, ×10 (left) or ×63 (right). (**b**,**c**) Quantitative RT-PCR analysis of *IL10* mRNA from SECs incubated for 12 h with medium (Ctrl) or LPS (**b**) or from splenic $CD19^+$ B cells (B), macrophages (M), $CD4^+$ T cells (T), SECs or DCs (**c**); results are normalized to *ACTB* mRNA and are presented relative to those of SECs cultured with medium (**b**) or with B cells (**c**). (**d**) Flow cytometry of CD15 and CD16 on N_C cells (black outline) and iN_{BH} cells (red outline) from cultures incubated for 18 h with medium (Ctrl) or IL-10. (**e**) Quantitative RT-PCR analysis of *TNFSF13B* mRNA in the N_C cells and iN_{BH} cells in **d**; results are normalized to *ACTB* mRNA and are presented relative to those of N_C cells. (**f**) Ratio of iN_{BH} cells to N_C cells after migration of N_C cells from age-matched healthy donors (HD) and people with *STAT3* mutation (*STAT3* mut) among SECs exposed for 4 h to medium (SEC) or LPS (SEC + LPS). (**g**) Induction of iN_{BH} cells by LPS-activated SECs (as in **f**) in the presence of dimethyl sulfoxide (vehicle (veh)), AG490 (Jak2 inhibitor) or Stattic (STAT3 inhibitor). (**h**) Quantitative RT-PCR of *AICDA* mRNA from circulating unswitched (IgD^+) B cells cultured for 2 d in the presence of N_C cells or SEC-induced iN_{BH} cells, normalized to *ACTB* mRNA and presented relative to results of B cells cultured with medium. (**i**) ELISA of IgG from circulating IgD^+ B cells cultured for 7 d as in **h**. $^*P < 0.05$ (one-tailed unpaired Student's *t*-test). Data are from one of three experiments with similar results (**a**,**d**,**g**) or summarize three independent experiments (**b**,**c**,**e**,**f**,**h**,**i**; error bars, s.e.m.).

Blocking BAFF with soluble BAFF-R–immunoglobulin had a similar inhibitory effect. We used N_{BH} cell–conditioned medium for these experiments because immunoglobulin-containing decoy receptors may activate N_{BH} cells via Fcγ receptors. Finally, patients with deleterious substitutions in TACI or STAT3 (a protein that drives signals from multiple cytokines, including IL-21)[35] had poor MZ development and fewer circulating MZ B cells (**Fig. 4f,g**). These data indicated that N_{BH} cells activated MZ B cells by a mechanism involving BAFF, APRIL and IL-21.

Splenic signals reprogram neutrophils

Given the close interaction of N_{BH} cells with SECs, we wondered whether N_C cells acquire B cell–helper function in response to SEC-derived signals. Such signals may include IL-10, a cytokine that confers regulatory properties to neutrophils[8]. Fluorescence microscopy, confocal microscopy and flow cytometry showed IL-10 expression by perifollicular SECs that expressed the adhesion protein CD31, the coagulation protein von Willebrand factor, the lymphoid protein CD8 and mannose receptor (**Fig. 5a** and **Supplementary Fig. 14a–c**). As shown by quantitative RT-PCR and ELISA, SECs upregulated expression of IL-10-encoding mRNA and the secretion of IL-10 in response to microbial TLR ligands such as LPS (**Fig. 5b** and **Supplementary Fig. 14d**). SECs were not the only perifollicular source of IL-10, as macrophages produced even more IL-10 (**Fig. 5c** and **Supplementary Fig. 14e**). N_C cells exposed to IL-10 became inducible N_{BH}-like cells (iN_{BH} cells) that downregulated expression of CD15 and CD16, upregulated expression of mRNA for BAFF and APRIL and activated expression of AID-encoding mRNA in B cells (**Fig. 5d,e** and **Supplementary Fig. 15**). Also, other splenic STAT3-activating stromal factors such as GM-CSF and microbial products such as LPS and zymosan induced some iN_{BH} cells (**Supplementary Fig. 15**). This induction did not strictly correlate with apoptosis but instead correlated with expression of B cell–stimulating factors.

N_C cells migrating across LPS-activated SECs acquired iN_{BH} cell properties via a process that was blocked by preexposure of N_C cells to inhibitors of the kinase Jak2 and STAT3 (**Fig. 5f,g** and **Supplementary Fig. 16**), two signal transducers activated by many cytokine receptors, including the IL-10 receptor. SEC-induced iN_{BH} cells elicited not only AID-encoding mRNA but also IgG production in MZ B cells (**Fig. 5h,i**). Finally, LPS-activated SECs produced not only IL-10 but also neutrophil-attracting chemokines such as IL-8 (CXCL8), CXCL1, CXCL2, CXCL3 and CXCL6 (**Supplementary Fig. 17a,b**), which suggested that SECs delivered both reprogramming and chemotactic signals to N_C cells.

Neutrophils regulate immunoglobulin production to TI antigens

Having shown that N_{BH} cells activated MZ B cells *in vitro*, we wondered whether N_{BH} cells also modulated MZ B cells *in vivo*. We used flow cytometry, ELISA and immunohistofluorescence to study MZ B cells

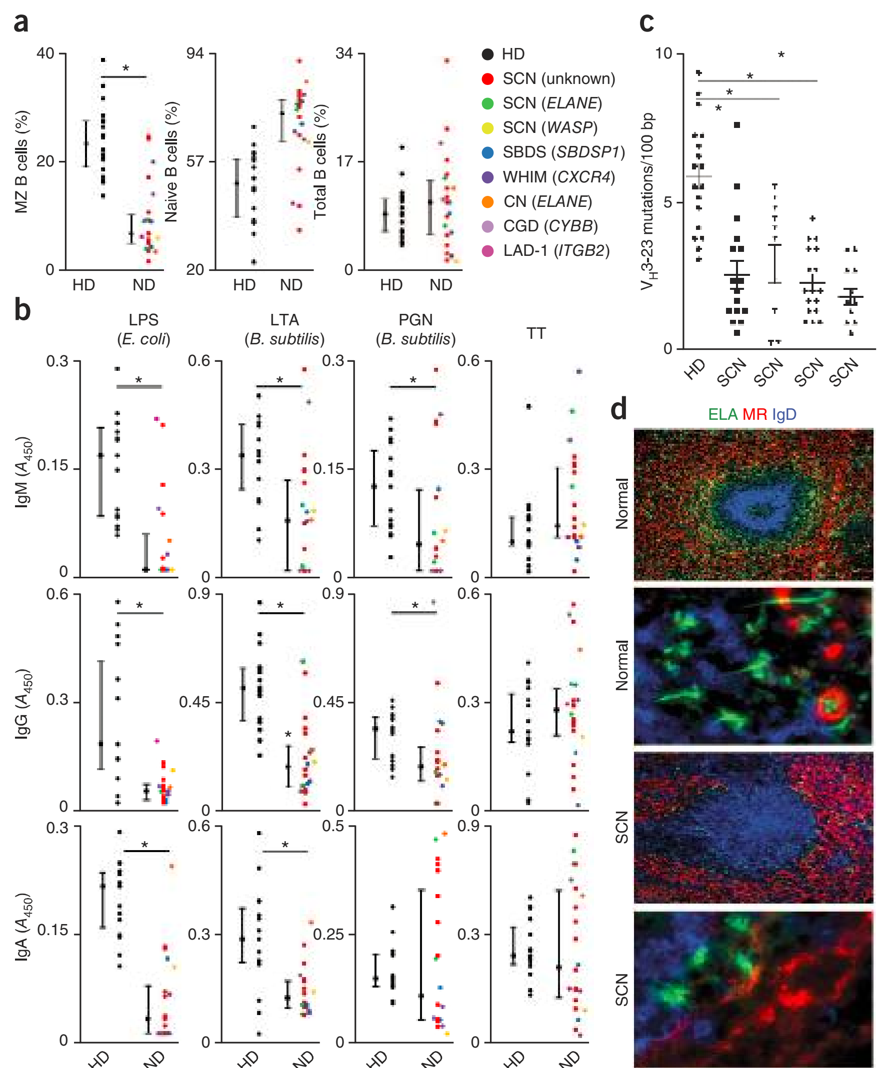

Figure 6 N_{BH} cells regulate MZ B cells and innate IgM, IgG and IgA responses to microbial TI antigens *in vivo*. (**a**) Frequency of circulating $IgD^{lo}CD27^+$ MZ B cells, $IgD^{hi}CD27^-$ naive B cells (calculated from total B cells) and $CD19^+$ B cells (calculated from total mononuclear cells) in age-matched healthy donors (HD) and patients with the following neutrophil disorders (ND): SCN, severe congenital neutropenia; SBDS, Shwachman-Bodian-Diamond syndrome; WHIM, warts-hypogammaglobulinemia-infections-myelokatexis syndrome; CN, cyclic neutropenia; CGD, chronic granulomatous disease; LAD-1, leukocyte adhesion deficiency 1. Genes (in parentheses) encode the following: *ELANE*, elastase; *WASP*, Wiskott-Aldrich syndrome protein; *SBDSP1*, SBDS protein 1; *CXCR4*, CXCR4; *CYBB*, $p91^{phox}$; and *ITGB2*, CD18. (**b**) ELISA of IgM, IgG and IgA antibodies to LPS from *Escherichia coli*, lipoteichoic acid (LTA) and peptidoglycan (PGN) from *Bacillus subtilis*, or tetanus toxin (TT), in serum of patients and age-matched healthy donors as in **a**, presented as absorbance at 450 nm (A_{450}). (**c**) V_H3-23 gene mutations (per 100 base pairs) in circulating MZ B cells from patients with SCN and an age-matched healthy donor. (**d**) Immunofluorescence of spleens from normal and patients with SCN, stained for elastase (green), mannose receptor (red) and IgD (blue). Original magnification, ×10 (first and third panels from top) or ×63 (second and fourth panels from top). Each symbol (**a**–**c**) represents an individual subject (**a**,**b**) or cloned V_H3-23 transcript (**c**); dots in vertical lines indicate the mean (lines, ± s.e.m.). $^*P < 0.05$ (Mann-Whitney *U*-test). Data are from one of three experiments with similar results (**d**) or summarize 15–24 experiments (**a**–**c**; error bars, median and percentile 25 and 75 (**a**,**b**) or s.e.m. (**c**)).

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and steady-state serum immunoglobulin titers produced in response to TI antigens in patients with severe congenital neutropenia (SCN) caused by deleterious elastase substitutions[40,41]. Additional disease models included SCN with unknown molecular defects; SCN with substitutions in the Wiskott-Aldrich syndrome protein that regulates cell motility; neutropenia in Shwachman-Bodian-Diamond syndrome caused by mutations in *SBDSP1*, which encodes an unknown protein; neutropenia in warts-hypogammaglobulinemia-infections-myelokatexis syndrome caused by substitutions in the chemokine receptor CXCR4; cyclic neutropenia caused by deleterious elastase substitutions; chronic granulomatous disease caused by deleterious substitutions in the respiratory burst protein p91-PHOX; and leukocyte adhesion deficiency 1 caused by deleterious substitutions in the adhesion protein CD18 (refs. 40,41).

Relative to B cells of age-matched healthy people, patients with neutrophil disorders had normal circulating total B cells expressing CD19 and naive B cells with high expression of IgD but not of CD27 (**Fig. 6a**). In contrast, circulating MZ B cells with expression of CD27 and low expression of IgD were less abundant in most of these patients. Notably, IgM, IgG and IgA antibodies to microbial TI antigens such as LPS, lipoteichoic acid, peptidoglycan and galactose-α-1,3-galactose were less abundant, even in patients with normal MZ B cells, whereas the abundance of IgM, IgG and IgA antibodies to TD antigens such as tetanus or diphteria toxins and protein-conjugated capsular polysaccharides was normal (**Fig. 6b** and **Supplementary Fig. 18a**,**b**). IgM, IgG and IgA antibodies to capsular polysaccharides from *Pneumococcus* types 9N, 14, 19F and 23F, but not types 1, 3, 4, 6B, 7F, 8, 9V, 12F, 18C or 19A, were also lower in abundance (**Supplementary Fig. 18b** and data not shown). We confirmed the reactivity of splenic MZ B cells to some of these TI antigens *in vitro* (**Supplementary Fig. 19**).

We also noted a lower abundance IgA and, to some extent, IgM and IgG antibodies to *Lactobacillus plantarum*, *Haemophilus influenzae* type b, *Salmonella typhimurium* and *Staphylococcus aureus* but not to *Escherichia coli* (**Supplementary Fig. 20**). Finally, patients with SCN had circulating MZ B cells with fewer V_H3-23 gene mutations, and their MZ was poorly developed and contained fewer N_{BH} cells with shorter projections (**Fig. 6c**,**d**). We found that iN_{BH} cells obtained from some SCN patients had conserved B cell–helper activity *in vitro* (**Supplementary Fig. 21**), which suggested that quantitative defects in N_{BH} cells may have been more important than functional defects in N_{BH} cells in the impairment of MZ B cell responses observed in neutropenic patients. Thus, N_{BH} cells may regulate MZ B cells and preimmune immunoglobulin responses to TI antigens *in vivo*.

Splenic neutrophils involve mucosal microbes

Systemic translocation of microbial products from mucosal surfaces influences the function of neutrophils[25]. Given the participation of microbial signals such as LPS in the reprogramming of N_C cells by SECs and the finding that N_{BH} cells enhanced innate immunoglobulin responses to microbial products that included LPS, we assessed the role of microbes in splenic colonization by N_{BH} cells. Immunohistochemistry showed that splenic colonization by N_{BH} cells began during fetal life but increased considerably as early as 2 d after birth (**Fig. 7a**), a time that coincided with mucosal colonization by bacteria. Fluorescence microscopy demonstrated that N_{BH} cell–occupied perifollicular areas contained LPS in adult spleens and mesenteric lymph nodes but not in fetal spleens or peripheral lymph nodes (**Fig. 7b**,**c**). Fluorescence *in situ* hybridization showed bacterial 16S ribosomal RNA (16S rRNA) in splenic clusters of N_{BH} cells and MZ B cells identified by means of the nuclear antigen Pax5 (**Fig. 7d**). We also

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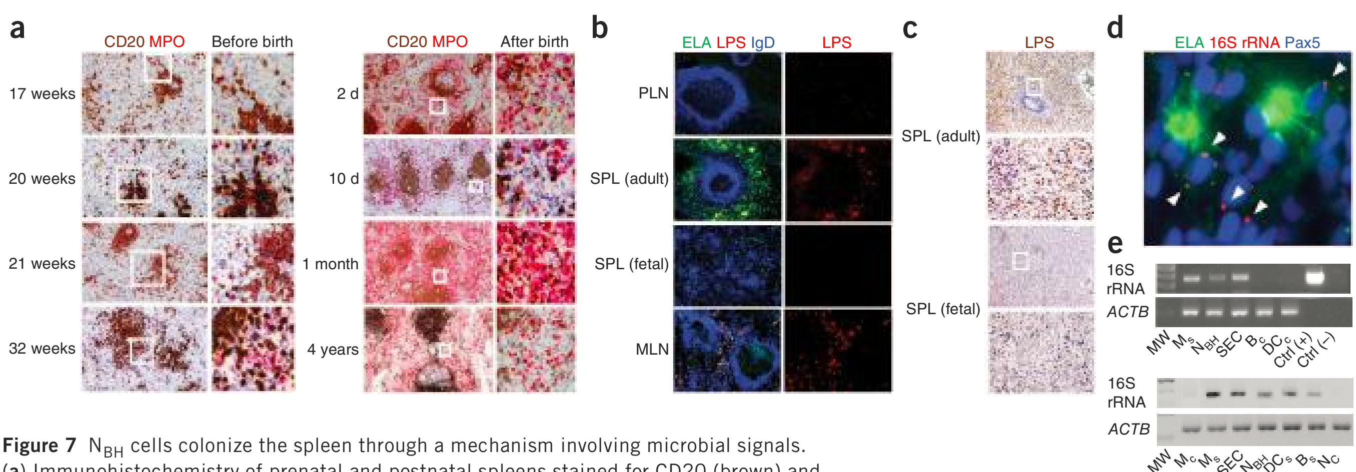

Figure 7 N_{BH} cells colonize the spleen through a mechanism involving microbial signals. (**a**) Immunohistochemistry of prenatal and postnatal spleens stained for CD20 (brown) and myeloperoxidase (red). Outlined areas (left columns) correspond to enlargement at right. Original magnification, ×4 (main images) or ×20 (enlargements). (**b**) Immunofluorescence of peripheral lymph nodes, adult or fetal spleen and mesenteric lymph node (MNL) stained for elastase (green), LPS (red) and IgD (blue). Original magnification, ×10. (**c**) Immunohistochemistry of adult or fetal spleen stained for LPS (brown). Outlined areas correspond to enlargement directly below. Original magnification, ×4 (main images) or ×20 (enlargements). (**d**) Immunofluorescence of spleens stained for elastase (green) and Pax5 (blue), with fluorescence *in situ* hybridization for bacterial 16S rRNA (red). Arrowheads indicate 16S rRNA in an N_{BH} cell–MZ B cell cluster. Original magnification, ×63. (**e**) Bacterial 16S rRNA amplified by RT-PCR from cDNA (top) or amplified by PCR from genomic DNA (bottom) of SECs and splenic ($_S$) or circulating ($_C$) monocytes (M), DCs, B cells (B), N_C cells and N_{BH} cells. Total RNA from *E. coli* and water from the PCR mixture serve as positive (+) and negative (–) controls, respectively; *ACTB* serves as a loading control. MW, molecular size marker. (**f**) Frequency and number of N_{BH} cells from the spleens of wild-type mice (WT), *Trif*$^{-/-}$*Myd88*$^{-/-}$ mice, specific pathogen–free mice (SPF) and germ-free mice (GF). $*P < 0.05$ (one-tailed unpaired Student's *t*-test). Data are from one of three experiments with similar results (**a**–**e**) or summarize three experiments with five to eight mice per group (**f**; error bars, s.e.m.).

amplified 16S rRNA by PCR from splenic N_{BH} cells but not from circulating N_{BH} cells, macrophages, DCs, SECs or B cells (**Fig. 7e**). DNA sequencing confirmed the bacterial origin of the PCR-amplified products (data not shown). Germ-free mice as well as mice lacking TLR signaling because of deletion of genes encoding the adaptor MyD88 and the TLR-associated adaptor TRIF had fewer N_{BH} cells that expressed Ly6G and CD11b (**Fig. 7f**). Thus, we propose that TLR signals from mucosal commensals enhanced the splenic recruitment and reprogramming of N_{BH} cells to enhance innate MZ B cell responses to highly conserved microbial TI antigens (**Supplementary Fig. 22**).

DISCUSSION

We have shown here that neutrophils colonized splenic peri-MZ areas via a noninflammatory pathway that became more prominent after postnatal mucosal colonization by bacteria. Splenic neutrophils had a distinct phenotype, formed MZ B cell–interacting NET-like structures and elicited class switching, somatic hypermutation and production of immunoglobulins by activating MZ B cells via a mechanism that involved BAFF, APRIL and IL-21. Patients with congenital neutropenia had fewer and hypomutated MZ B cells and their serum contained fewer preimmune immunoglobulins to TI antigens, which indicated that neutrophils interacted with MZ B cells to generate an innate layer of antimicrobial immunoglobulin defense.

Growing evidence has shown that many granulocyte subsets, such eosinophils and basophils, enhance immunoglobulin production by B cells[42,43]. We found that neutrophils occupied splenic peri-MZ areas through a noninflammatory process that accelerated after birth and involved mucosal colonization by bacteria. In contrast to circulating N_C cells, splenic N_{BH} cells included N_{BH1} and N_{BH2} subsets that expressed B cell–stimulating factors such as BAFF, APRIL and IL-21 as well as B cell–attracting chemokines such as CXCL12 and CXCL13. N_{BH} cells activated MZ B cells as efficiently as splenic T cells did via contact-dependent and contact-independent mechanisms. This MZ B cell–helper activity was associated mainly with N_{BH2} cells, which indeed secreted more BAFF, APRIL and IL-21, a cytokine with a key role in immunoglobulin production[35].

N_{BH1} cells and, to a lesser extent, N_{BH2} cells also had higher expression of TLRs, HLA-II, CD86, IL-1β, IL-6, IL-8, IL-12 and TNF than did N_C cells, which suggested *in situ* activation by splenic signals. This activation was counterbalanced by higher expression of regulatory molecules such as CD11b, CD24, SOCS1, IL-10, progranulin, SLPI, arginase, IDO and iNOS[1,32,33,37–39] and correlated with the capacity of N_{BH} cells to induce contact-independent suppression of T cells, as myeloid-derived suppressor cells do[1,7]. By exerting a dual B cell–helper and T cell–suppressor function, N_{BH} cells may maximize extrafollicular B cell responses to TI antigens while minimizing follicular B cell responses to TD antigens and inflammation. In the presence of inflammation caused by sepsis, infection with human immunodeficiency virus, hyper-IgD syndrome or systemic lupus erythematosus, N_{BH} cells became disorganized and infiltrated lymphoid follicles, possibly to present antigen to T cells[2]. Infections and autoimmune disorders may also explain follicular infiltration by neutrophils in spleens from immunodeficient patients with deleterious TACI substitutions. Of note, inflammation was associated with loss of MZ tissue and N_{BH2} cells, the N_{BH} subset with predominant MZ B cell–helper activity.

By expressing inhibitors of proteases, reactive oxygen species and TNF such as progranulin and SLPI[1,37,39], N_{BH} cells may be able to clear antigen and deliver B cell–helper signals without causing inflammatory tissue damage. In this context, N_{BH} cells occupied lymphoid sites characterized by continuous antigenic filtration, which included the presence of bacterial 16S RNA. Although largely blocked by mesenteric lymph nodes[44], some mucosal antigens undergo systemic translocation to influence the function of cells of the immune response, including neutrophils[23–25]. Accordingly, spleens and mesenteric lymph nodes contained perifollicular LPS in addition to N_{BH} cells, but LPS was detectable only after postnatal mucosal colonization by bacteria. By demonstrating fewer N_{BH} cells in mice deficient in MyD88 and TRIF and in germ-free mice, our data suggest that splenic filtration of microbial products triggers TLR-dependent recruitment, reprogramming and activation of N_C cells. Microbe-independent signals may also have a role, because some N_{BH} cells remained detectable in spleens not exposed to microbial signals.

In addition to enhancing SEC production of neutrophil-targeting chemokines such as IL-8, CXCL1, CXCL2, CXCL3 and CXCL6, microbial TLR signals facilitate the reprogramming of N_C cells into N_{BH} cells by inducing the SEC release of STAT3-inducing mediators such as IL-10, a cytokine involved in the generation of noninflammatory neutrophils[45]. IL-10 would promote reprogramming of N_C cells as they interact with SECs to reach perifollicular and outer MZ areas. Additional IL-10 originated from perifollicular macrophages positioned nearby SECs and N_{BH} cells. As suggested by published studies[46], TLR signals would target not only SECs but also MZ B cells, perhaps to enhance their responses to activation and chemotactic signals from N_{BH} cells. Consistent with that possibility, TLR ligation upregulates the BAFF and APRIL receptor TACI and the CXCL13 receptor CXCR5 on MZ B cells[18,47].

Although they had lower expression of transcripts encoding BAFF and APRIL, N_{BH2} cells released more BAFF protein and APRIL protein than N_{BH1} cells did; this may relate to the fact that BAFF and APRIL require cleavage from inactive precursors to activate B cells. Thus, an N_{BH1} 'preparatory' stage with more gene transcription may be followed by an N_{BH2} stage with more protein processing and release. Consistent with that interpretation, N_{BH2} cells stimulated MZ B cells more efficiently than N_{BH1} cells did despite having a worse survival profile. N_{BH2} cells may further amplify their MZ B cell–helper activity by forming postapoptotic NET-like projections[3,7]. These DNA-containing structures were more frequent in apoptotic peri-MZ N_{BH2} cells and might enhance TI immunoglobulin responses not only by trapping antigen but also by delivering immunostimulatory DNA to MZ B cells[48]. However, the expression of BAFF and APRIL seemed more important than apoptosis in determining the B cell–helper activity of N_{BH} cells. Accordingly, MZ B cells expressed more TACI than naive B cells did and thus responded more rapidly and effectively to N_{BH} cells.

Being strategically located at the interface with the circulation, MZ B cells are geared to rapidly respond to blood-borne antigens[20,21]. In addition to triggering rapid IgM secretion, N_{BH} cells elicited AID expression as well as CSR to IgG and IgA in MZ B cells, whereas N_C cells did not. Those findings correlated with the presence of ongoing CSR in some MZ B cells proximal to N_{BH} cells. Of note, N_{BH} cells induced CSR to IgG2, which typically occurs in B cell responses to TI antigens[20,21]. Unlike N_C cells, N_{BH} cells also triggered SHM, which could provide a mechanistic explanation for published studies showing that MZ B cells undergo SHM through an extrafollicular pathway that may not require T cells[22,27,28]. Finally, N_{BH} cells promoted plasma-cell differentiation and immunoglobulin production by a mechanism that involved BAFF, APRIL and IL-21. Consistent with those data, patients with deficiency in TACI or STAT3 had fewer MZ B cells. Of note, N_{BH} cells exerted some of their MZ B cell–helper activity via a contact-dependent mechanism that may have involved CD40L, a T cell molecule usually required for TD immunoglobulin responses in the germinal center of lymphoid folllicles[19]. Accordingly, patients

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with CD40L defects have fewer MZ B cells in addition to defective TD immunoglobulin responses[21], which supports the idea of a role for CD40L in N_{BH} cells.

The *in vivo* contribution of N_{BH} cells to innate MZ B cell responses was indicated by the observation that patients with neutropenia or functional neutrophil defects had not only fewer and hypomutated MZ B cells but also lower serum titers of IgM and IgG antibodies to TI antigens under steady-state conditions. This humoral deficiency probably did not originate from a greater microbial burden, which would instead cause polyclonal B cell activation and hypergammaglobulinemia. Accordingly, serum IgM and IgG antibodies to TD antigens were not affected by neutrophil insufficiency, which further points to specific involvement of N_{BH} cells in preimmune TI immunoglobulin responses. In agreement with the IgA-inducing activity of N_{BH} cells, neutrophil disorders were associated with lower serum titers of IgA antibodies to TI antigens and some mucosal bacteria. Although it is better known for its key role in mucosal immunity[19], IgA also enhances systemic immunity by interacting with neutrophils[9]. Thus, N_{BH} cells may engage in crosstalk with MZ B cells to generate an innate line of IgA defense as well as IgM and IgG defense against systemic invasion by microbes breaching the mucosal barrier[24,26]. This indicates that an insufficiency of N_{BH} cells could contribute to the pathogenesis of systemic infections in patients with neutrophil disorders. Conversely, harnessing N_{BH} cells with specific adjuvants may enhance vaccine-induced immunoglobulin responses to poorly immunogenic TI antigens in healthy people.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureimmunology/.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

I.P. and M.Co. designed and did research, discussed data and wrote the paper; C.M.B., B.H. and K.C. designed and did research; L.Ca., M.G., L.Co., A.Cho., M.S., W.X., G.M., A.Chi, T.B. and S.C.G. did research and discussed data; D.M.K., W.T., J.B.B., S.S., J.A.L., B.B., J.L., N.J., F.A., C.D.d.H., N.T., A.Ca., M.T., C.F., V.C., C.C., G.A.D., J.M.B., C.-M.F., G.S., C.C.-R., M.Ca., C.Du., L.D.N., V.L., A.P., J.-L.C., A.D., J.I.A., M.J., J.Y., N.M. and J.D. provided blood and tissue samples and discussed data; and A.Ce. designed research, discussed data and wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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