B-1a transitional cells are phenotypically distinct and are lacking in mice deficient in IκBNS

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T cells are central in the immune response to infections. After pathogen encounter, B cell responses to protein-based antigens are induced via help from T cells, whereas polysaccharide and/or particulate antigens can stimulate B cells to produce antibodies in a T cell-independent (TI) fashion, giving rise to a more immediate response. Antibodies to T cell-dependent (TD) antigens are mainly produced by follicular B cells, whereas marginal zone B (MZB) cells, B-1a cells, and B-1b cells, collectively referred to as innate-like B cells, facilitate rapid responses to TI antigens found on the surface of many classes of pathogens. These innate B cells play distinct, although sometimes overlapping, roles in pathogen confinement and presentation. In particular, MZB cells and B-1a cells both contribute to protection against Gram-negative bacteria by responding to LPSs (1, 2), whereas B-1b cells and MZB cells are required for optimal recall responses to TI antigens found on the surface of many classes of pathogens. These innate B cells play distinct, although sometimes overlapping, roles in pathogen confinement and presentation.

Conventional B (B-2) cells are replenished throughout life from a common precursor in the bone marrow. Differentiation into mature naive B cells takes place in the periphery upon exit of immature B cells from the bone marrow. The cells then migrate to the spleen, where they undergo transition and are subjected to selection (5). MZB and follicular B cells diverge at this B-cell transitional stage, dependent on the strength of signals mediated by the B-cell receptor (BCR), the B-cell–activating factor (BAFF) receptor, and Notch2, all of which involve the NF-κB pathway (6). Less is known about the development of B-1 cells, but it is well established that B-1 cells, in contrast to B-2 cells, are generated more abundantly from fetal liver than from the bone marrow and are maintained by self-renewal throughout the life span of the individual (7, 8). Studies on the early stages of the development of B-1 cells have identified B-1 progenitors (B-1p cells; Lin−CD93+CD19+IκBNS−) in fetal liver but also, at a lower frequency, in the bone marrow and spleen of neonatal as well as adult mice (9, 10). Recently, Montecino-Rodriguez and Dorshkind (11) proposed that B-1 cells develop through a transitional (CD93+IgM+CD23+) splenic intermediate population similar to that described for B-2 cells, with the exception that the transitional window of B-1 cells is limited to the neonatal stage. However, these studies did not provide information on how neonatal transitional B-1 (TrB-1) cells differ phenotypically or functionally from their TrB-2 counterparts.

TI antigens have traditionally been classified based on whether they induce antibodies in mice with a mutation in the gene coding for Bruton’s tyrosine kinase (xid/Btk) (TI-1) or not (TI-2) (12). Later, it was discovered that Btk interacting with phospholipase Cγ2 is needed for activation of the NF-κB transcription factor upon BCR ligation (13) and that NF-κB signaling also regulates Btk expression. This finding may partially explain why some of the immunological defects described for xid mice are also observed in mice where regulators of the NF-κB pathway are ablated. The NF-κB family includes NF-κB1 (p50), NF-κB2 (p52), RelA (p65), c-Rel, and RelB, which interact to form functional homo- or heterodimer complexes. The interplay between these components is regulated by IkB proteins, including IkBa, IkBβ, and IkBe, which are characterized by their ankyrin repeat structure (14). NF-κB activation is mediated by the p50, RelA, c-Rel, and NF-κB essential modulator (NEMO)-dependent classical pathway.

Significance

A subpopulation of antibody-secreting cells, B-1 cells, provides early protection against several types of pathogens. Both the development and function differ between B-1 cells and the better known B-2 cells, and exclusively B-1 cells are lacking in mice deficient for the nuclear inhibitory xB protein, IκBNS. B-1 cells mature similar to B-2 cells via a transitional stage. We demonstrate here the existence of a phenotypically distinct B-1 transitional B (TrB)-cell population in the neonatal spleen of wild-type mice. This TrB-1a–cell subset was lost in the absence of IκBNS, thus revealing a requirement for intact NF-κB signaling via IκBNS during this stage of the development of B-1 cells. Learning more about the development of B-1 cells may reveal new targets for therapeutic intervention.


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Data deposition: The bumble strain (C57BL/6J-Nfkbid−/−) (13) is available from the Mouse Mutant Regional Resource Centers.

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or by the alternative pathway, which involves p52/RelB complexes. Classical and alternative NF-κB pathways are implicated in multiple stages of B-cell life. Furthermore, function and survival signals mediated through the BAFF receptor and/or BCIR require functional NF-κB signaling (15).

The development of B-cell subsets is differentially dependent on intact NF-κB pathways. Thus, B-1 cells and MZB cells were severely reduced in mice where classical NF-κB signaling components, including CARMA1, Bcl10, Malt1, and NF-κB1, were ablated, whereas follicular B-cell development and function were less affected under these conditions (15–17). In contrast, compound deficiencies in NF-κB1/NF-κB2 or c-Rel/RelA impair development of all mature B-cell subsets (18, 19). Also, the more recently identified atypical IκB proteins are involved in B-cell development (20–22). The atypical IκBs interact with NF-κB transcription factors in the nucleus rather than in the cytoplasm and, in contrast to traditional IκBs, are not only inhibitory but may either augment or repress transcriptional activity of target genes, depending on the cell type and conditions of activation that are studied (23). We previously identified a mouse strain with genes, depending on the cell type and conditions of activation may either augment or repress transcriptional activity of target genes.

In the present study, we used the bumble mice to investigate at which point in the development of B-1 cells NF-κB signaling via IκBNS is required. We demonstrate that bumble mice have largely normal frequencies of fetal liver B-1p and splenic neonatal transitional B cells, both of which have previously been described to give rise to B-1 cells. However, upon close examination of the splenic neonatal Trb cells, we found that they can be phenotypically divided into at least two sublineages, of which one predominantly gives rise to B-1a cells and the other to B-2 cells. We show here that bumble mice only harbor the latter population, and thus propose that the development of B-1a cells in the absence of IκBNS is blocked before this splenic transitional stage in the neonate. This study advances our understanding of the ontogeny of B-1 cells and identifies IκBNS as a central component required for the development of B-1 cells.

**Results**

**Bumble Mice Lack B-1 Cells and Display Reduced Antibody Responses to TI Antigens.** Mice with ablated IκBNS expression (bumble) were previously found to display normal development of pro/pre-, immature, and mature follicular B cells, whereas peritoneal B-1a cells were completely absent and the frequencies of B-1b cells were severely reduced (21, 22). There could be several possible reasons for the lack of peritoneal B-1 cells in bumble, including impaired B-1 development or maintenance, unsupportive microenvironment, and/or migrational defects. To investigate if the deficiency of B-1 cells in bumble was limited to the peritoneal cavity, we stained for B-1 cells in the major lymphoid organs. Similar to the case in the peritoneal cavity (Fig. 1A), no B-1a and very few B-1b cells were detected in the spleen and bone marrow of bumble mice (Fig. 1B), indicating that the lack of peritoneal B-1 cells in IκBNS-deficient mice is not simply due to a migration defect. B-1 cells produce most of the steady-state circulating natural IgM, and bumble mice displayed a severe reduction in total serum IgM (Fig. 1C), as well as IgM-secreting cells, in the spleen and bone marrow (Fig. 1D). B-1 cells are important for TI antibody responses, and bumble mice did not produce specific IgM when challenged with the TI type II antigen nitrophenylacetyl (NP)-Ficoll (22), and the IgG response was similarly reduced (Fig. 1E). The bumble mice also failed to produce a trinitrophenyl (TNP)-specific IgM antibody response but maintained the ability to generate TNP-specific IgG after administration of the TI type I antigen TNP-LPS (Fig. 1F).

**Transfer of Wild-Type Peritoneal Cavity Cells to Bumble Reconstitutes the B-1 Natural IgM Levels.** To investigate if bumble mice were capable of providing a supportive environment for the survival of mature B-1 cells, we transferred peritoneal cavity cells from wild-type (wt) mice into 3-wk-old nonirradiated bumble mice. We chose this age to minimize the risk of graft rejection. Because

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**Fig. 1.** Bumble mice have impaired frequencies of B-1 cells and responses to TI antigens. The wt and bumble (bmb) tissues were stained for B-1 cells identified as CD19+ B220+ CD43+ (B-1a) and CD19+ B220+ CD43+ CD5– (B-1b). (A) Representative plots showing the gating strategy for identification of peritoneal B-1a and B-1b cells. Peritoneal cells were isolated by flushing with 5 mL of PBS plus 1% fetal bovine serum (FBS). (B) Peritoneal, spleen, and bone marrow cells were isolated and stained for B-1 cells, and the absolute number of cells is shown as mean ± SD. (C) Total serum IgM was determined by ELISA. (D) IgM antibody secreting cells from spleen (Left) and bone marrow (Right) were examined by ELISpot. Plates were coated with anti-IgM, and the indicated numbers of wt and bumble cells were added in triplicate. IgM producing cells were determined 17 h later. Each picture shows one well from an ELISpot plate of standard size (96-wells, diameter: 6.4 mm). (E) Mice were evaluated for NP-specific antibodies by ELISA 10 d postadministration with 50 μg NP-Ficoll. (F) Mice were evaluated for TNP-specific antibodies by ELISA 10 d after TNP-LPS administration (10 μg). Figures represent 8- to 14-wk-old mice with three to seven mice per group, and results are representative of at least two independent experiments. Graphs display mean ± SD. Statistically significant differences between bumble and wt mice are indicated by denoting *P < 0.05, **P < 0.01, or ***P < 0.001 as determined by an unpaired t test. **
B-1 cells are major producers of natural IgM antibodies, we monitored serum IgM as an indication of successful transfer. Serum IgM levels of *bumble* peritoneal wt cell recipients reached wt levels at 6–7 wk posttransfer (Fig. 2A). Both B-1a and B-1b cells from the wt cell donors were recovered in the peritoneal cavity, and at 14 d posttransfer, the levels of B-1a and B-1b cells were 30% and 50%, respectively, of those levels in wt mice (Fig. 2B).

To confirm the donor origin of the B-1 cells, we transferred wt peritoneal cells expressing the CD45.1 allotype. Recipient *bumble* mice (CD45.2) were analyzed 2 mo posttransfer. As expected, all B-1a cells and the majority of B-1b cells in the peritoneal cavity of reconstituted *bumble* mice were donor-derived (Fig. 2C). The donor B-1 cells were distributed to the spleen (Fig. 2D), indicating that mature B-1 cells migrate normally in *bumble* mice. In contrast to B-1 cells, few B-2 cells of donor origin were found in the peritoneum or spleen of *bumble* recipients (Fig. 2C and D), as would be expected due to their inability to self-renew. These transfer experiments indicated that *bumble* mice support survival of mature wt B-1 cells.

**Impaired Antibody Response to TI Antigens in *Bumble* Can Be Partially Attributed to the Lack of B-1 Cells.** B-1 and MZB cells respond in concert to challenge with TI antigens. We investigated if the lack of antibody response to the TI-2 antigen NP-Ficoll was restored after transfer of peritoneal wt cells to *bumble* mice. We chose to immunize *bumble* mice 50 d posttransfer, when the IgM levels had reached the levels observed in wt mice. Notably, transfer of wt peritoneal cells partially restored the NP-specific IgM and IgG response in *bumble* mice (Fig. 2E); however, the response did not reach the levels observed in NP-Ficoll–injected wt mice (*P* < 0.05). Similar to the case in wt mice, NP-specific antibodies of the IgG3 subclass dominated the response in the *bumble* mice that had received wt peritoneal cells. Reconstitution of the B-1 compartment in *bumble* mice completely restored the IgM response to the TI-1 antigen TNP-LPS (Fig. 2E). These results suggest that the absence of TI antibody responses in *bumble* can be attributed, at least in part, to their lack of B-1 cells.

**Lack of B-1 Cells in *Bumble* Mice Is Due to a Cell-Intrinsic Defect.** We next investigated whether the lack of B-1 cells in *bumble* was due to B cell-autonomous defects or alterations in the stromal microenvironment. As noted previously, initial studies showed that B-1 cells were generated from adult wt bone marrow when transferred into immunodeficient hosts (24–27). To distinguish between extrinsic and intrinsic contributions to the *bumble* phenotype, we mixed bone marrow from *bumble* (CD45.2) and wt (CD45.1) mice and transferred it into irradiated RAG1−/− mice. Control animals were RAG1−/− mice receiving *bumble* only or wt (CD45.1)/wt (CD45.2) cells. Fig. 3 shows the number of *bumble* or wt donor-derived CD45.2-expressing cells of the different B-cell subsets in the presence or absence of supplementing wt CD45.1 cells. The *bumble* bone marrow transferred alone gave rise to B-2 cells of similar numbers as those numbers observed in RAG1−/− mice that had received only wt cells. In the mixed *bumble*/wt chimeras, although *bumble* bone marrow generated nearly similar B-2 cell frequencies and numbers as the cotransferred wt counterpart, only the wt bone marrow gave rise to B-1 cells in the peritoneal cavity and spleen (Fig. 3 A and B).

Because B-1 cells are predominantly generated from the fetal liver, we next tested if B-1 cells would develop from *bumble* fetal liver progenitors in the presence of wt fetal liver cells. Analogous to the bone marrow transfers, fetal liver chimeras were made by mixing equal numbers of wt (CD45.1) and *bumble* (CD45.2) cells and transferring these into irradiated RAG1−/− recipient mice. Confirming the results in bone marrow chimeras, all B-1a and B-1b cells in the fetal liver chimeras were wt-derived (Fig. 3 C and D). The *bumble* B-2 cell numbers were also reduced in the fetal liver chimeras, although the difference was not statistically significant (*P* = 0.07). In summary, supplementation of wt cells did not restore the deficiency in *bumble* bone marrow or fetal liver precursor cells to differentiate into B-1 cells, illustrating that the reduction in *bumble* B-1 cells is due to a cell-intrinsic defect.

**Neonatal Bumble Mice Lack B-1 Cells.** Most B-1 cells are generated at the neonatal stage and are then maintained by self-renewal. It was therefore possible that the lack of B-1 cells in adult *bumble* mice was due to a limited capacity for mature B-1 cells to self-renew. We therefore tested in a kinetic study whether *bumble* B-1 cells were present at the neonatal stage and wane with increasing age (Fig. 4A). Only very low frequencies of B-1 cells...
were severely reduced. B-1p cells were not generated in adult mice. The frequency of B-1a cells increased in adult mice. The neonatal wt splenocytes displayed a higher frequency of B-1a cells than adult mice (Fig. 4 C). Neonatal wt mice had a distinct CD19hiB220lo peritoneal population of B-1 cells, as previously described (21, 22). We then investigated if peritoneal B-1 cells develop from neonatal splenic TrB cells by transferring sorted IgM+CD93+ cells into nonirradiated 3-wk-old bumble recipients and analyzing for donor-derived B-1 cells 5–7 d later. The transferred wt CD45.1 neonatal TrB cells predominantly generated B-1a cells, but B-2 cells and a few B-1b cells were also observed in the peritoneum of reconstituted bumble mice (Fig. 5C). The similar frequencies of neonatal transitional IgM+CD93+ B cells in bumble and wt mice, coupled with the finding that most neonatal TrB cells give rise to B-1 cells, suggested that bxBNS is dispensable for the development of B-1 cells until after the B transitional stage.

**Transitional B Cells from Neonatal Mice Can Be Divided into Subsets Displaying B-1a or B-2 Markers, and Only the Latter Are Found in Bumble Mice.** B-1p cells, as well as mature B-1 cells, express low levels of B220. Curiously, we observed that similar to mature CD19- B cells, CD93+ IgM+ CD19+ TrB cells could be divided

were detected in the peritoneal cavity of wt mice at 1 wk of age (<2% of cells in the lymphocyte gate). In contrast, 2-wk-old wt mice had a distinct CD19hiB220lo peritoneal population of B-1 cells, of which most were CD43+CD5+ B-1a cells. The frequency of B-1a cells peaked at 5 wk of age and then declined, whereas B-1b cells were maintained at similar frequencies from 8 to 16 wk of age. No peritoneal B-1a cells and only a few B-1b cells were detected in bumble mice at any time point. In wt mice, B-1a cells appeared earlier in the spleen than in the peritoneal cavity, because B-1 cells, mostly of the CD43+CD5+ B-1a phenotype, were already readily detected 1 wk after birth (Fig. 4B). Neonatal wt splenocytes displayed a higher frequency of B-1a cells than adult mice (Fig. 4C). Conversely, the frequency of B-2 cells was greatly increased in adult mice. The bumble mice did not have any detectable splenic B-1 cells at 1 wk of age (Fig. 4B and C), similar to adult bumble mice. These data suggest that rather than being lost due to an incapability of self-renewal, mature B-1a cells were not generated in bumble mice and B-1b cells were severely reduced.

**Fig. 4.** B-1 cells are impaired already in neonatal bumble mice. The wt and bumble mice of different ages were stained for B-1 and B-2 cells using the same gating strategy as for Fig. 1A. (A) Peritoneal cells were isolated by flushing with 1–10 mL of PBS plus 1% FBS, and the frequencies of B-1a (Left) and B-1b cells (Right) are shown. (B) Representative plot of splenocytes from 1-wk-old mice stained for B-1 cells. (C) Frequencies of splenic B-1a and B-2 cells in 1-wk-old and 10-wk-old wt and bumble mice. The mean frequencies of cells in the lymphocyte gate ± SEM are shown for three mice per group. Results are representative of two independent experiments.
into B220+ and B220lo populations and that the B220lo cells expressed CD5. In 1-wk-old wt mice the fraction of B220loCD5+ cells was 7–12% of TrB cells (Fig. 6A). Strikingly, a CD93+IgM+ CD19+B220+B220loCD5+ cell population could not be detected in bumble mice (Fig. 6A). This finding led us to suggest that transitional cells giving rise to B-1a cells may be phenotypically distinct from transitional cells giving rise to B-2 cells and that these two B-cell populations may be distinguished based on B220 and CD5 expression similar to other cells of B-1a/B-2 lineage. Furthermore, the lack of B220loCD5+ TrB cells in bumble mice indicates that the development of B-1a cells in IkBNS-deficient mice is blocked before this transitional stage of B-1a cells. We defined the neonatal splenic B220loCD5+ TrB cells as TrB-1a cells and characterized these cells further by comparing their surface marker expression with that of CD93+IgM+CD19+B220+B220loCD5+ TrB cells and mature B-1 and B-2 cells (Fig. 6B). Both TrB-1a and TrB cells were CD24hi. In contrast to TrB cells, the TrB-1a population also expressed the common B-1 marker CD43 (Fig. 6B). B-2 cells undergo transitional stages expressing CD3 and various levels of IgM and CD23. Notably, only the TrB cells could be separated into CD23+ populations corresponding to T1 and T2 B cells, respectively, whereas TrB-1a cells were CD23−. In contrast, TrB-1a and TrB cells expressed similar levels of IgD, which were higher than in mature B-1 cells and lower than in mature B-2 cells.

Transfer of Neonatal Transitional Cells Reveals at Least Two Subsets That Give Rise to B-1 Cells. To gain more insight into which neonatal TrB-cell population gives rise to B-1a, B-1b, and B-2 cells, we repeated the transfer of TrB cells into bumble mice, this time sorting cells based on TrB-cell markers in addition to CD5, which, in mice, is exclusively expressed by B-1a cells and not by other cells of the B lineage (Fig. 6C). The IgM+CD93+ cells from neonatal wt mice were purified into CD5+ and CD5− populations, transferred into bumble mice, and analyzed 4–6 d later. The purity of the sorted populations was 95–97%. As expected, transfer of TrB-1a (IgM+CD93+CD5+) cells almost exclusively gave rise to CD19+B220loCD43+CD5−B-1a cells, which were recovered in the peritoneal cavity of recipient bumble mice (Fig. 6C). Transfer of IgM+CD93+CD5+ neonatal transitional cells yielded mostly B-2 cells. However, donor-derived peritoneal B-1a and B-1b cells could also be observed in the bumble recipients (Fig. 6D). These data suggest the existence of at least two different subsets of TrB-1 cells in the neonatal spleen and suggest that expression of CD5, the hallmark surface marker for B-1a cells, is initiated at the TrB-cell stage. Spontaneous secretion of natural IgM antibodies is a hallmark function of B-1a cells. In contrast, adult TrB cells do not spontaneously secrete IgM antibodies. Interestingly, both wt neonatal TrB-1a and TrB cells spontaneously produced IgM antibodies after 17 h of incubation, although the number of IgM+ antibody-secreting cells in the TrB-1a population was twice as high compared with TrB cells (Fig. 7A). Another phenotypic characteristic of B-1a cells is increased λ-light chain use compared with B-2 cells (28). Neonatal TrB-1a cells and, to a lesser extent, TrB cells displayed
Neonatal TrB cells spontaneously secrete IgM and display increased λ light chain use compared with adult TrB cells. (A and B) Transitional IgM+ CD93+ cells were sorted by FACS into CD5− (TrB-1a) or CD5+ (TrB) cells and examined for antibody secreting cells (ASCs) spontaneously producing IgM by ELispot by incubating 2 × 10^6 cells for 17 h. Samples were pooled from one litter of wt mice. (C) Representative plot of TrB-1a and TrB cells expressing λ or κ light chains from neonatal wt mice. (D) Ratio of λ/κ using neonatal or adult TrB cells. The λ/κ ratio was determined in three independent experiments with three to four wt mice. Statistically significant differences are indicated *P < 0.05, **P < 0.01, and ***P < 0.001, as determined by an unpaired t test.

Discussion

A principal component of B cell activation and antibody production is specific recognition of antigen by the BCR, combined with signals received through interaction with T cells. With the discovery of Toll-like receptors and other pathogen recognition receptors on the surface of B cells, it became increasingly clear how activation signals and antibody production may also be stimulated by innate immune system activation in the absence of T-cell help. However, the different B cell subsets differ in terms of responsiveness to TI signals, with the innate-like B cells, MZB cells, and B-1 cells, being most responsive (29). Pathogenic microorganisms are complex, expressing both TD and TI antigens, requiring both the innate and adaptive arms of the immune systems to act in concert to facilitate a protective immune response. B-1 cells constitute an important first line of defense by recognizing TI antigens found on the surface of pathogens and rapidly responding by secreting antibodies to confine the infectious agent and activate other arms of the immune system. These events are illustrated upon infection with influenza viruses (30).

Although the development of B-2 cells and MZB cells has been investigated extensively, less is known about the development of B-1 cells. Recent years have witnessed an increased interest in this area, particularly with the identification of B-1p cells in murine fetal liver, bone marrow, and spleen (9, 10). The present study advances our knowledge of the later steps in the development of B-1 cells and establishes that IκBNS is required for lymphopoiesis of B-1a cells rather than for maintenance of mature B-1 cells. We would like to emphasize the following points. First, the development of B-1a cells occurs via a splenic transitional intermediate, which is phenotypically distinct but shares some characteristics with the well-established splenic TrB cells that predominantly give rise to B-2 cells. Second, mice deficient in the atypical nuclear IκB protein IκBNS lack B-1 cells due to a cell-intrinsic mechanism, and the lack of B-1 cells explains, at least in part, the impaired antibody responses to TI antigens. Finally, IκBNS is required for the development of B-1a cells after the B-1p stage but before the herein identified B-1a transitional stage. The possibility that B-1 cells develop through a transitional stage was only recently demonstrated by Montecino-Rodriguez and Dorshkind (11), who showed that splenic TrB cells (IgM<sup>CD93^CD23^+</sup>) from neonatal but not adult mice differentiated into B-1a and B-1b cells. We have confirmed and extended this finding by identifying phenotypic characteristics, which can be used to distinguish transitional cells with B-1a potential from their B-2 counterparts. In line with studies that demonstrated B-1a cells to be generated as an early wave (8), TrB cells of B-1a lineage were found in the spleen of neonatal rather than adult mice. Similar to adult B-2 transitional cells, the B-1a–specified TrB-1a cells expressed CD93 and IgM on the surface; however, they were unique in expressing CD43 and only low levels of B220. In addition, the TrB-1a cells expressed CD5, and the majority of mature peritoneal B-1 cells generated from these precursors were B-1a cells.

Neonatal wt CD93<sup>+</sup>IgM<sup>+</sup>CD5<sup>−</sup> TrB cells predominantly matured into B-2 cells when transferred to bumble mice. Interestingly, this population also gave rise to some B-1b cells and even B-1a cells, illustrating an unappreciated heterogeneity of the neonatal TrB-cell population. Interestingly, neonatal TrB cells spontaneously secreted IgM and displayed increased λ-light chain use compared with their adult counterparts. These features are characteristics of B-1a cells, further suggesting the potential of TrB cells to develop into B-1a cells. The finding that both CD5<sup>−</sup> and CD5<sup>+</sup> TrB cells give rise to B-1a cells suggests that CD5 expression is initiated at the immature/TrB-cell stage. This observation is also in line with the finding that B-1p cells are CD5<sup>−</sup>. Following from these data, it seems likely that the identified CD5<sup>+</sup> TrB-1a cells constitute a direct descendant of CD5<sup>−</sup> TrB cells, and thus represent a late transitional stage in the development of B-1a cells. However, it remains possible that different developmental pathways may lead to the generation of cells with a B-1a phenotype. In this regard, it was recently shown that both Lin<sup>−</sup>CD19<sup>+</sup>B220<sup>+</sup> and Lin<sup>−</sup>CD19<sup>+</sup>B220<sup>+</sup> fetal liver B-1p cells generate B-1a cells but that B-1a progeny differed functionally in terms of N-nucleotide addition patterns (31). The reduced frequency of B-1 cells in several NF-xB-deficient mouse strains has long been a conundrum. The finding that B-1 cells develop from neonatal TrB cells, coupled with the fact that the latter are found in similar frequencies in wt and NF-xB–deficient mice, led to the hypothesis that signaling via the classical NF-xB pathway is not needed for the development of B-1 cells but rather for the maintained self-renewal capability of mature B-1 cells (11). Interestingly, we observed that the transitional cells in neonatal wt mice could be phenotypically separated into at least one additional subset (IgM<sup>CD93^CD23^B220^CD43^CD5^</sup>) in addition to the previously established T1, T2, and T3 cells and that bumble mice were devoid of this newly identified subset of TrB-1a cells. This finding suggests that IκBNS is required during the development of B-1a cells before the identified TrB-1a stage. The lack of IgM<sup>CD93^B220^CD43^CD5^</sup> TrB-1a cells that we demonstrate for IκBNS–deficient mice might also hold true for other strains deficient in classical or alternative NF-xB signaling, which is of interest to examine. It should also be noted that although our findings demonstrate that IκBNS is required at the B-1a developmental stage, IκBNS might also be required for the maintenance of mature B-1 cells.

B-1a and B-1b cells share many phenotypic features but have largely different functions. The bumble mice were deficient in generating B-1a cells but were not completely devoid of peritoneal B-1b cells, with ~15–20% of wt levels consistently observed. Whether B-1a and B-1b cells have a different requirement for
because transfer of wt peritoneal cells to has previously been demonstrated (37). Stored antibody responses to NP-Ficoll, our data illustrate that the development of B-1 cells clearly demonstrated that the development of B-1 cells explains the impaired TI antibody responses in organs, we propose the following developmental pathway for a time point where we failed to detect a clear population of B-1a cells in the spleen of 1-wk-old neonatal mice, (33, 34). The finding that final maturation of B-1 cells occurs in Hox11-null mice lacking a spleen and in splenectomized wt mice drastically reduced frequencies of peritoneal B-1a cells in intact spleen. This assumption is supported by the finding of B-2 cells were observed in the mixed wt/bumble chimeras, indicating a competitive disadvantage for bumble B-1b cells.

The identification of splenic TrB-1a cells indicates that development of B-1a cells, similar to that of B-2 cells, requires an intact spleen. This assumption is supported by the finding of dramatically reduced frequencies of peritoneal B-1a cells in Hox11-null mice lacking a spleen and in splenectomized wt mice (33, 34). The finding that final maturation of B-1a cells occurs in the spleen is also consistent with our observation of a clear population of B-1a cells in the spleen of 1-wk-old neonatal mice, a time point where we failed to detect a clear population of B-1a cells in the peritoneal cavity (Fig. 4). Based on the current study and on recent findings of B-1p cells in various lymphoid organs, we propose the following developmental pathway for B-1 cells, as indicated in Fig. 8.

B-1 cells are important responders to TI antigens, but the contribution of these cells vs. MZB cells for the response to polysaccharides from encapsulated bacteria, as well as other TI antigens, is not completely clear. Preponderant evidence supports MZB cells as the major responder to the TI antigen NP-Ficoll (35, 36). The lack of both of MZB and B-1 cells may explain the impaired TI antibody responses in bumble mice, but because transfer of wt peritoneal cells to bumble partially restored antibody responses to NP-Ficoll, our data illustrate that B-1 cells mediate at least some of the response to this antigen, as has previously been demonstrated (37).

In humans, the existence or phenotype of B-1 cells is still a matter of debate, although recent reports suggest the existence of a cell type that at least shares many of the functions attributed to B-1b cells in mice (38). Our study may therefore assist future studies aimed at identifying human B-1 cells by prompting evaluation of neonatal TrB cells for the proposed human B-1 markers. In sum, we show that B-1a cells develop via a transitional intermediate, which can be phenotypically distinguished from that giving rise mainly to B-2 cells. Furthermore, we demonstrate that IxBNS-deficient mice are devoid of these B-1a transitional cells, thus revealing a requirement for this factor for normal development of B-1a cells before the B-1a transitional stage.

Materials and Methods

Mice. Mice were housed and bred at the animal research facility of the Department of Microbiology, Tumor, and Cell Biology, Karolinska Institutet. IxBNS-deficient bumble mice, generated by ENU mutagenesis of C57BL/6J mice, and their wt C57BL/6J counterparts were described previously (22). CD45.1 and RAG1−/− mice on a C57BL/6 background were bred locally. Mice were studied at 8–14 wk of age or at the indicated age. All animal studies were conducted with the approval of the Committee for Animal Ethics (Stockholms Norra djurförsöksnämnd.)

Tissue Preparation. Single-cell suspensions of splenocytes and fetal livers were prepared by a 70-μm cell strainer using a syringe plunger. Peritoneal cells were isolated by flushing with cold PBS plus 1% FBS (1–10 mL, depending on mouse age). Peritoneal cells were discarded if contaminated with blood. Femurs and tibias were flushed with a 26-gauge needle. Cell suspensions were diluted in RPMI 1640 supplemented with 2 mM l-glutamine, penicillin (100 IU)-streptomycin (100 μg/mL), and 10% FBS (complete RPMI). Cell suspensions were washed once in Ca2+-free, Mg2+-free PBS and treated with RBC lysis buffer before further processing. RBC lysis buffer was omitted for peritoneal cells and when preparing cells for bone marrow or fetal liver chimeras.

Immunization. Mice were injected i.p. with 10–20 μg of 2,4,6, Trinitrophenyl hapten conjugated to LPS or TNP (10–LPS (0111:B4)) or with 50 μg of NP (40)-Ficoll (Biosearch Technologies) in 100 μL of PBS.

ELISA. Antigen-specific ELISA was performed by coating ELISA plates (Nunc) with 500 ng per well of NP (25) or TNP (20)-BSA conjugated with BSA (Biosearch Technologies) and incubated overnight (Ach). Following washing (PBS plus 2% Tween 20) and blocking for 1 h with PBS containing 2% (wt/vol) dry milk, serum was added in threefold serial dilutions in blocking buffer and incubated for 1.5 h at room temperature (RT) before addition of secondary antibody HRP-coupled IgM, IgG1, IgG2b, IgG2c, or IgG3 (all from Southern Biotech). The assay was developed with 3,3′,5′-tetramethylbenzidine substrate (KPL) followed by 1 M H2SO4, and the OD at 450 nm was read using an Asys Expert 96 ELISA reader (Biochrom).}

Enzyme-Linked Immunosorbent Spot Assay for Detection of Antibody-Secreting Cells. Detection of total IgM-producing cells was performed using an enzyme-linked immunosorbent spot (ELISpot) assay. Multiscreen-IP filter plates (Millipore) were pretreated with 70% ethanol and washed in sterile PBS. Plates were coated with 5 μg/mL anti-mouse IgM (Southern Biotech) diluted in PBS and incubated overnight at 4 °C. Following washing (PBS plus 2% Tween 20) and blocking for 1 h with PBS containing 2% (wt/vol) dry milk, serum was added in threefold serial dilutions in blocking buffer and incubated for 1.5 h at room temperature (RT) before addition of secondary antibody HRP-coupled IgM, IgG1, IgG2b, IgG2c, or IgG3 (all from Southern Biotech). The assay was developed with 3,3′,5′-tetramethylbenzidine substrate (KPL) followed by 1 M H2SO4, and the OD at 450 nm was read using an Asys Expert 96 ELISA reader (Biochrom).

Flow Cytometry and Cell Sorting. Bone marrow and splenic RBCs were lysed before Fc blockade (anti-CD16/32; Becton Dickinson), washing in PBS plus 1% FBS, and staining with fluorochrome or biotin-conjugated monoclonal antibodies in washing buffer. The antibodies used were as follows: CD19 phycoerythrin (PE), B220 peridinin-chlorophyll protein (PERCP), CD23 biotin, CD24 PE, CD43 allopolycoyinin (APC), CD45.1 APC-Cy7, CD45.2 V450, λ chain-light chain, and κ-light chain PerCP-Cy5.5 (all from Becton Dickinson);
B220 APC–Fluor 780, IgM eFluor 450, CD39 APC, and IgG1 FITC (all from eBioscience), IgM FITC (Southern Biotech); and CD5 biotin (Biolegend). In panels containing biotin-coupled antibodies, cells were washed and stained again with streptavidin conjugated to AF488 (Invitrogen) or PerCP-Cy5.5 (eBioscience). Before staining for fetal liver B-1p cells, fetal liver cells were prepared using LymphoPrep (Axis-Shield), and non-B-lineage cells were depleted by means of the B-cell isolation kit (STEMCELL Technologies) according to the manufacturer’s instructions. Cells were analyzed on a Becton Dickinson FACSAria-Calibur or LSRII instrument. Cell sorting was performed on a Moflo XDP instrument (Beckman Coulter). To obtain highly pure populations, the sorted cells were resuspended in PBS plus 1% FBS and resorted. The purity of the sorted populations constituted 95–97% as determined by a presorted sample run in parallel and reanalysis of the sorted populations. Data were analyzed in FlowJo version 9.6.4 or version X.0.7 (TreeStar). Flow cytometry plots depict log10 fluorescence.

Adoptive Cell Transfer. Peritoneal cells (4 × 10^6 cells) from adult C57BL/6J CD45.1 or CD45.2 mice were resuspended in 100 μL of PBS and transferred i.p. into 3-wk-old nonirradiated bumble recipient mice. Mice were killed at different time points posttransfer and analyzed for successful grafting. To evaluate the TI-2 antibody response in grafted bumble mice, these mice were immunized with NP-Ficoll 50 d after cell transfer. For transfer of neonatal TrB cells, 1–3 × 10^7 C57BL/6J CD45.1 cells from 1-wk-old mice were sorted as CD3−IgM+ and transferred i.p. into 3-wk-old bumble mice. In some studies, the TrB cells were further sorted into CD5− subsets and 2–3 × 10^6 cells were transferred. The bumble mice that had received sorted TrB cells were analyzed 4–6 d posttransfer. No signs of graft-versus-host disease were observed in recipient mice.

Bone Marrow and Fetal Liver Chimeras. Bone marrow and fetal liver chimeras were generated by transferring cells from CD45.1 wt mice mixed 1:1 with CD45.2 wt or bumble mice i.v. into nonlethally irradiated RAG1−/− mice (600 rad, 133Cs source). For bone marrow and fetal liver chimeras, 50 × 10^6 and 3 × 10^6 cells, respectively, were transferred via each mouse strain. The recipient mice were given antibiotics in the drinking water for 21 d. Chimeras were analyzed 8–12 wk after reconstitution.

Statistics. Differences between groups were analyzed by a two-tailed unpaired t test (Prism version 6.0d; GraphPad).

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