From the fetal liver to spleen and gut: the highway to natural antibody

MM Rosado[1], A Aranburu[1], F Capolunghi[1], E Giorda[1], S Cascioli[1], F Cenci[1], S Petrini[1], E Miller[2], T Leanderson[2], GF Bottazzo[1], PG Natali[3] and R Carsetti[1]

The film of sIgA lining the intestinal epithelium plays a role in the regulation of the commensal microflora and prevention of pathogen invasion. We show that, in the absence of intentional immunization, all sIgA in the gut is produced by B-1a B cells. We also show that B-1a B cells and sIgA derive from lineage-negative precursors found in the fetal liver and located in the spleen after birth. The splenic precursors do not generate B cells of the adaptive immune system in bone marrow, spleen, and lymph nodes, but efficiently replenish the cells producing the natural antibodies. Therefore, B-1a B cells with their splenic progenitors and their progeny of plasma cells fill the same function of the primordial immune system of lower vertebrates. The natural antibodies in the serum and on the intestinal epithelium may be an evolutionary ancient tool for the immediate protection against commensal and pathogenic bacteria.

INTRODUCTION

The first living organisms on our planet were bacteria. Multicellular organisms appeared 2 billions of years later, establishing symbiotic relationships with commensal bacteria and developing the innate immune system to prevent pathogen invasion. In bony fish, a primitive immune system with T and B cells evolved in the gut. Evolution led T and B cells to synergistically build what we call adaptive immunity.

In higher vertebrates, these three evolutionary subsequent layers of immunity coexist and contribute to regulate the relationship with commensal microbes, while preventing and fighting infection by pathogens.

In the mouse, two lineages of B cells have been identified: B-2 and B-1 B cells.

B2 B cells originate in the bone marrow, develop through a controlled pathway into naive B cells, populate the primary follicles of the secondary lymphoid tissues and collaborate with T cells to the germinal center reaction. The resulting high-affinity antibodies are the final product of the adaptive immune response. The two sub-populations of B-1 B cells, B-1a, and B-1b have the vital function of immediate protection against widespread pathogens.[1] B-1a B cells produce the natural antibodies that are found in the serum independently of previous antigen encounter.[2] B1-a B cells also built primary T-cell-independent (T-I) immune responses, whereas B-1b B cells are responsible for T-I memory reactions and protection against re-infection.[3,4]

We have shown that the adult asplenic and splenectomized mice lack B-1a B cells, but have normal numbers of B-1b and B-2 B cells,[5] suggesting that the spleen plays a role either in the generation or survival of B-1a B cells.

Immunoglobulin A exists as a monomer in the serum and as a dimer in mucosal secretions. The Joining (J) chain, covalently linking two monomers, is indispensable for the dimerization of IgA.[6] The study of mutant mice with targeted mutations of the J chain has shown that 1/3 of the IgA plasma cells in the gut does not express the J chain and therefore produces monomeric IgA.[7] The rest of the plasma cells synthesize the J chain and are able to assemble IgA dimers. Dimeric IgA binds to the polymeric immunoglobulin receptor (polyIg R) on the basolateral surface of mucosal epithelial cells[8] and is actively transported to the apical membrane where the external domain of the polymeric immunoglobulin receptor is cleaved generating the secretory component (SC).[9] sIgA protects against both commensal and pathogenic micro-organisms, regulates the diversity of the microflora, and prevents immune activation by enteric commensal and food antigens.[10–12]

The presence of mucosal IgA plasma cells depends on microbial colonization.[13,14] Bacterial products from the intestinal microflora can be transported by dendritic cells to the mesenteric lymph nodes.[15] Mucosal immunization induces antigen-specific responses in the organized gut-associated lymphoid tissue (GALT), including Peyer's patches and isolated

[1]Research Center, Ospedale Pediatrico Bambino Gesù (IRCCS), Roma, Italy. [2]Immunology Group, Lund University, Lund, Sweden. [3]Regina Elena Cancer Institute, Immunology Group, Roma, Italy. Correspondence: R Carsetti (Rita.Carsetti@opbg.net)

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lymphoid follicles. The germinal center reaction results in the formation of cells that migrate to the submucosa and switch to IgA.[16] It has also been shown that in mice lacking gut-associated lymphoid tissue, antigen-specific IgA responses are absent, but total intestinal IgA is maintained. Therefore, a large fraction of the IgA in the intestine does not originate from the classical adaptive immune response in the local GERMINAL CENTER. It has been recently shown that T-cell-independent IgA production may occur in isolated follicles as a result of the crosstalk between bacteria, dendritic cells, and B cells.[17]

In this paper, we show that, in the absence of intentional immunization, the film of sIgA lining the intestinal epithelium derives from B-1a B cells. B-1a B cells and sIgA differentiate from lineage negative precursors found exclusively in the spleen. These precursors migrate from the fetal liver to the spleen before birth, and they are unable to repopulate the bone marrow (BM) and the white pulp of the spleen, but efficiently generate B-1a B cells in the peritoneal cavity, develop into IgM and IgA plasma cells in the spleen, and fully replenish the pool of plasma cells producing sIgA in the intestine.

RESULTS

Hox11 asplenic mice lack sIgA in the intestine

The genetic deletion of the *Hox11* gene abrogates the formation of the splenic capsule during embryogenesis, leading to asplenia.[18] We have earlier shown that Hox11^{ko} mice lack the B-1a B cell compartment in the peritoneal cavity (**Supplementary Figure 1** online), but have normal frequencies of B-1b and B-2 B cells[5] showing that the spleen is indispensable for the generation of B-1a B cells. Because it has been shown that a large fraction of the IgA plasma cells in the intestinal mucosa is generated from B-1a B cells,[19,20] we asked the question of whether the lack of B-1a B cells reduces the frequency of IgA-secreting cells in Hox11^{ko} asplenic mice. We stained sections of the small intestine of wild–type (WT) and Hox11^{ko} mice for IgM and IgA and found that IgM-secreting cells were rare in both mouse strains (data not shown). In the intestine of WT mice most of the IgA plasma cells were collected in large groups in the axis of the villi (**Figure 1a**, control ×20, pc). In the intestinal crypts, at higher magnification, isolated plasma cells were identified under the basal membrane of epithelial cells (**Figure 1a**, control ×60, pc) and sIgA appeared distributed on the luminal side of the epithelial cells as a continuous film. The distribution of sIgA reflects the transport of dimeric IgA, which, bound to the polyIg R, is carried from the basal to the luminal surface of epithelial cells.

In Hox11^{ko} mice the number of IgA plasma cells was three times lower than that in the WT intestine (**Figure 1a,** ×20 and **1b**). Moreover, at higher magnification, the sIgA film layer was undetectable in the intestinal epithelium (**Figure 1a**, ×60).

We have shown before that in WT mice splenectomy leads to the reduction of the B-1a B cell pool (Wardemann *et al.*[5] and **Supplementary Figure 1** online) confirming that the spleen is indispensable for the homeostasis of this population. We analyzed the intestine of WT mice that were splenectomized either 1 month ($n=3$) or 1 year ($n=7$) before. In comparison to their littermates, in splenectomized mice the frequency of IgA plasma cells was reduced and sIgA was absent on the basal and apical sides and inside the epithelial cells at both time points after surgery (**Figure 1a** and **b** and **Supplementary Figure 1** online).

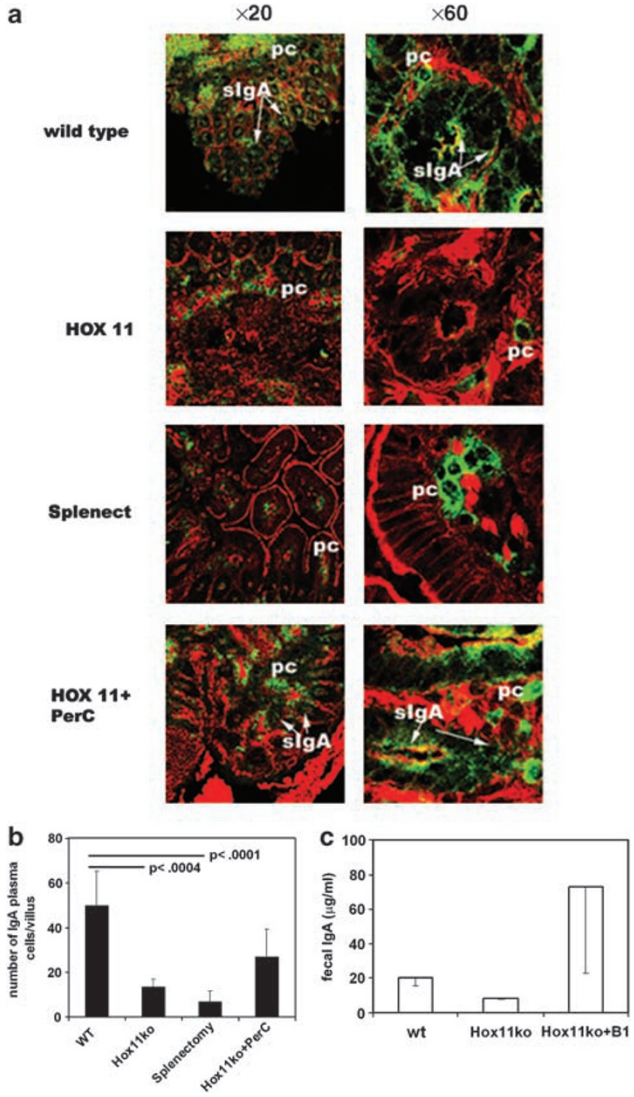

Figure 1 (**a**) Confocal images of cryosections of the intestine from wild-type, Hox11^{KO}, splenectomized wild-type mouse (1 year after surgery) and Hox11^{KO} mice injected i.p. with peritoneal cavity cells. In green IgA staining showing IgA plasma cells (pc) and secretory IgA (arrow). Sections were counterstained with phalloidin to show the structure of the intestine. Left column images at ×20 and right column at ×60 objective magnification. (**b**) The graph shows the average number of IgA plasma cells/villus (plus the s.d.) counted in 13–20 villi per slide from three different experiments including wild-type (WT, $n=7$), Hox11^{KO} ($n=7$), splenectomized ($n=7$), and Hox11^{KO} mice injected i.p with peritoneal cavity cells (Hox11^{KO}+ PerC, $n=4$). *P*, value calculated using the Student's *t*-test, is indicated only when statistically significant differences were found. (**c**) Concentration of IgA (µg/ml) in fecal extracts from wild-type (wt, $n=4$), Hox11^{KO} ($n=4$), and Hox11^{KO} mice 30 days after injection of peritoneal cavity cells (Hox11^{KO}+PerC, $n=4$).

To distinguish the relative roles of B-1a B cells and of the spleen in the generation of IgA plasma cells and sIgA, we transferred B-1a B cells into Hox11^{ko} asplenic mice. Because Hox11-deficient mice lack the B-1a subset, but have normal numbers

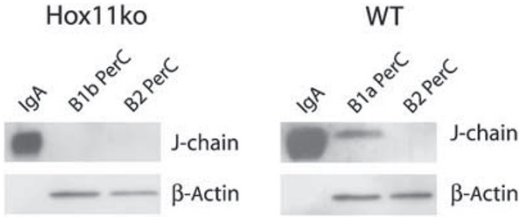

Figure 2 Western blot analysis of the J chain expression on sorted B cells from the peritoneal cavity of wild-type and Hox11-deficient animals. Cells were stained with Abs to CD5, B220, IgM, and IgD and sorted accordingly in B-1a, B-1b, and B2 B cells. In SDS gels the J chain migrates at 28 kDa approximately (theoretical molecular weight 18 kDa). β-actin was used as the lysate loading control. Panels correspond to 5 min exposure.

of B-2 and B-1b B cells in the peritoneal cavity,[5] the injection of peritoneal cavity cells from normal mice complements with B-1a B cells, the pre-existing B-cell populations.

We injected in four Hox11^{ko} mice (4.8×10^6) peritoneal cavity cells from WT mice and stained the intestine 1-month later. The frequency of IgA plasma cells increased in the villi (**Figure 1a,** ×20 and **1b**) and a thin layer of sIgA appeared lining the epithelium and crossing the epithelial cells (**Figure 1a**, ×60). Secreted IgA, which was very low in fecal extract of mutant mice, was considerably increased in mice injected with peritoneal cavity B cells (**Figure 1c**). To confirm the specific role of B-1a B cells we repeated the experiment injecting sorted B-1a B cells in three Hox11^{ko}. The results were comparable (**Supplementary Figure 1B** online).

Our results show that B-1a B cells generate part of the IgA plasma cells in the murine gut and produce sIgA. As adoptively transferred B-1a B cells can generate sIgA in asplenic mice, the spleen plays an indirect role in the maintenance of sIgA, supporting the generation or survival of B-1a B cells.

B-1a B cells constitutively express the J chain

In normal mice, the majority of the plasma cells in the intestine express the J chain and can therefore assemble dimeric IgA that bind to the polyIg R on epithelial cells and be released in the intestinal lumen as sIgA. The plasma cells lacking the J chain secrete IgA monomers, which cannot bind the polyIg R.[8] The expression of the J chain is a clonal property of B cells[21] thought to be randomly acquired.[7] We asked the question of whether the lack of sIgA and the reduced number of IgA plasma cells in asplenic and splenectomized mice could be explained by the specific depletion of the pool of plasma cells that produce the J chain, caused by the lack of B-1a B cells.

We examined the expression of the J chain by western blot[22] in B-2 and B-1a B cells sorted from the peritoneal cavity of WT mice and in B2 and B-1b B cells from Hox11^{ko} mice.

We show that the B-1a population constitutively expresses the J chain and is committed to the secretion of dimeric immunoglobulins. The J chain could not be detected either in the B-1b or B-2 B cell compartment (**Figure 2**).

We also analyzed the expression of the J chain in the intestine by confocal microscopy. We found that in WT mice the J chain was detectable in IgA plasma cells located in the axis of the villi (**Supplementary Figure 2** online) and at the basal site of epithelial cells (**Supplementary Figure 2** arrow online) in the criptae. Because the polyIg R directly binds the J chain, recognition by the specific antibody was hampered in sIgA at the luminal side. In the Hox11^{ko} mice J chain^{pos} cells were very rare. As a control for the specificity of the antibody we show the intestine of a J chain^{ko} mouse.[7]

The side population of the spleen reconstitutes spleen and peritoneal cavity

The role of the spleen for the maintenance of B-1a and sIgA has never been completely understood. We have shown that the asplenic mice lack B-1a B cells, but have normal frequencies of B-1b cells. In addition, fetal liver precursors generate B-1a B cells only in mice with an intact spleen.[5] Recently, early precursors of B-1a and B-1b B cells have been identified in the fetal BM and liver. After birth the precursors of B-1b B cells persist in the bone marrow, but those of B-1a B cells cannot be found anymore.[23,24] To reconcile all these observations, we hypothesized that the precursors of B-1a B cells may preferentially migrate to the spleen before birth[25] and persist in this organ during the adult life.

We isolated early precursors from the spleen using the side population (SP) strategy, described by Goodell *et al.*,[26] that has led to the identification of precursor cells not only from bone marrow, but also from many different physiological and pathological tissues.[27,28] As we ignore the phenotype of the hypothesized splenic precursors of B-1a B cells, we decided to use Hoechst exclusion, because this property is characteristic of cells with self-renewing potential (Preffer *et al.*[28,29] and **Figure 3a** and **b**).

We compared the repopulating potential of SP precursors isolated from either BM or spleen. SP cells were purified by cell sorting (**Figure 3c**) and injected i.v. into sublethally irradiated Rag2γ_c double-deficient mice (Rag2$^{KO}\gamma_c^{KO}$).[30] For each experiment an additional control group of animals was prepared, in which we injected total BM cells. Ten weeks later, serum immunoglobulins were measured by enzyme-linked immunosorbent assay (**Supplementary Figure 3** online), single-cell suspensions from BM, spleen, and peritoneal cavity were analyzed by flowcytometry, and sections were stained for confocal microscopy (**Figures 4** to **6**).

In all Rag2$^{KO}\gamma_c^{KO}$ reconstituted animals the concentration of serum immunoglobulins of IgM and IgG isotypes was similar to that of WT controls and serum IgA was increased (**Supplementary Figure 3A–C** online). In contrast large differences were found in the absolute numbers of splenic B220^{pos} cells (**Supplementary Figure 3D** online). Mice reconstituted with total BM had similar numbers to WT controls and only a slight reduction of B cells was observed in mice reconstituted with SP BM. Mice transplanted with SP from the spleen, notwithstanding the complete reconstitution of Ig serum levels, had 50-fold lower numbers of B220^{pos} cells.[31] We stained spleen cells with antibodies to B220, CD21, and CD23 to distinguish CD23^{pos} CD21^{pos,high} mature naive and transitional 2 B cells from the CD23^{neg} CD21^{bright} marginal

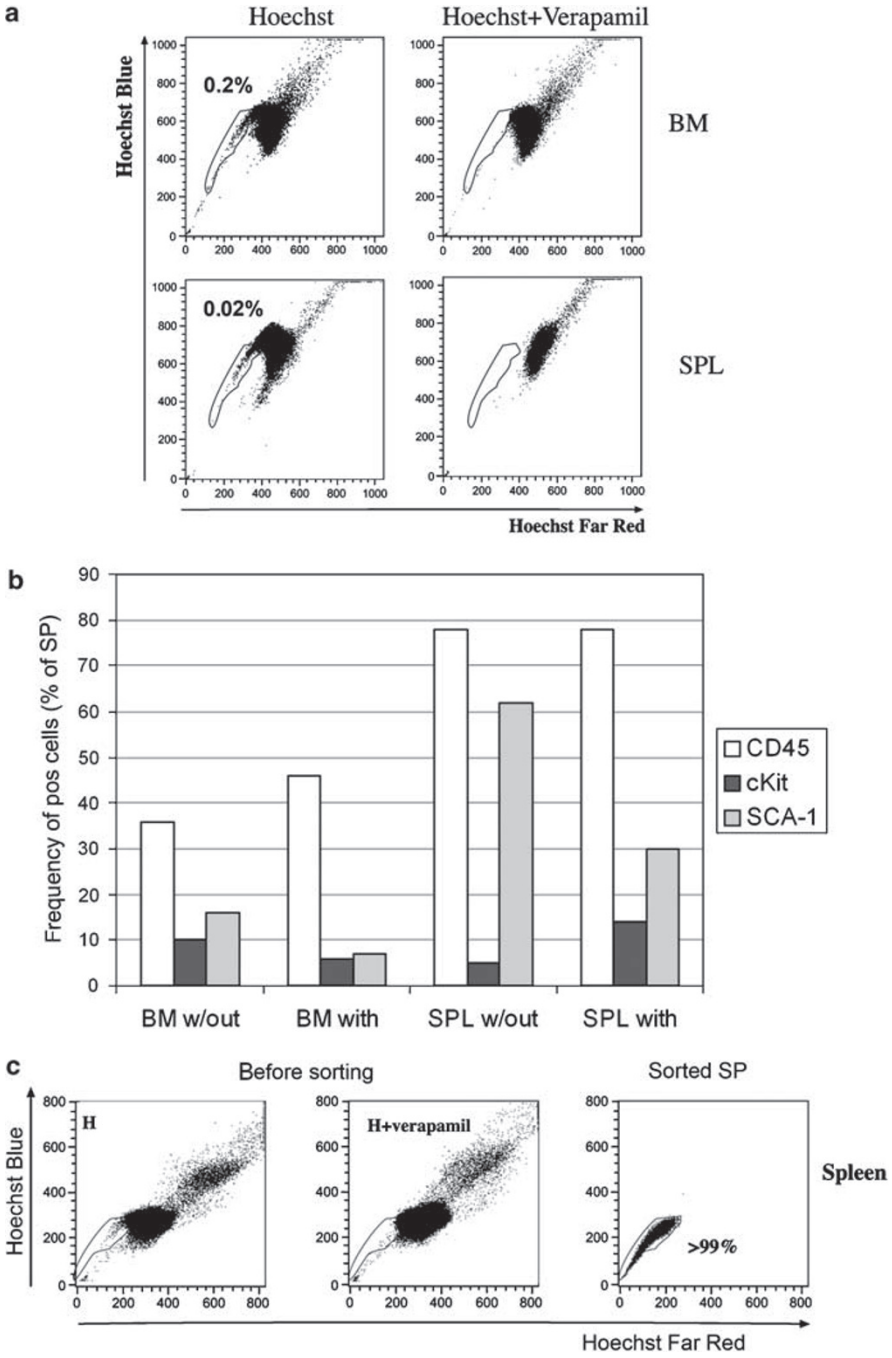

Figure 3 Hoechst 33342 emission profile of single-cell suspensions isolated from wild-type mice bone marrow and spleen. (**a**) Panels represent the Hoechst 33342 emission profile in bone marrow (upper left) and in spleen cells (lower left) incubated with Hoechst 33342 and Hoechst 33342 plus verapamil (right panels). The plots correspond to a very restricted forward/side scatter region in which death cells were excluded and small cells were selected according to size and shape (lymphocyte gate), values represent the frequency of "side population" cells. (**b**) Frequency of CD45 (white bar), c-Kit (black bar), and Sca-1 (gray bar) positive cells in "side population" (SP) cells from the bone marrow and the spleen using Hoechst 33342 staining with or without 45 min at 37°C for drug efflux. (**c**) Panels show a representative example of a splenic SP precursor cell population before and after sorting.

zone (MZ) population (**Figure 4a**). Mice reconstituted with total BM were identical to their WT controls (data not shown): mature-naive B cells were the largest population and MZ B cells were also present. Mice reconstituted with splenic SP had mature-naive B cells, but lacked B cells with the MZ phenotype (**Figure 4a**).

By confocal microscopy, we analyzed the lymphoid architecture of the spleen (**Figure 4b**). The white pulp of the spleen is constituted by physically separated B- and T-cell areas. B cells are located in the primary follicles and in the surrounding MZ and T cells in the periarteriolar lymphoid sheet.[32] By staining with IgM and Moma-1, it is possible to identify IgM^{pos} follicular B

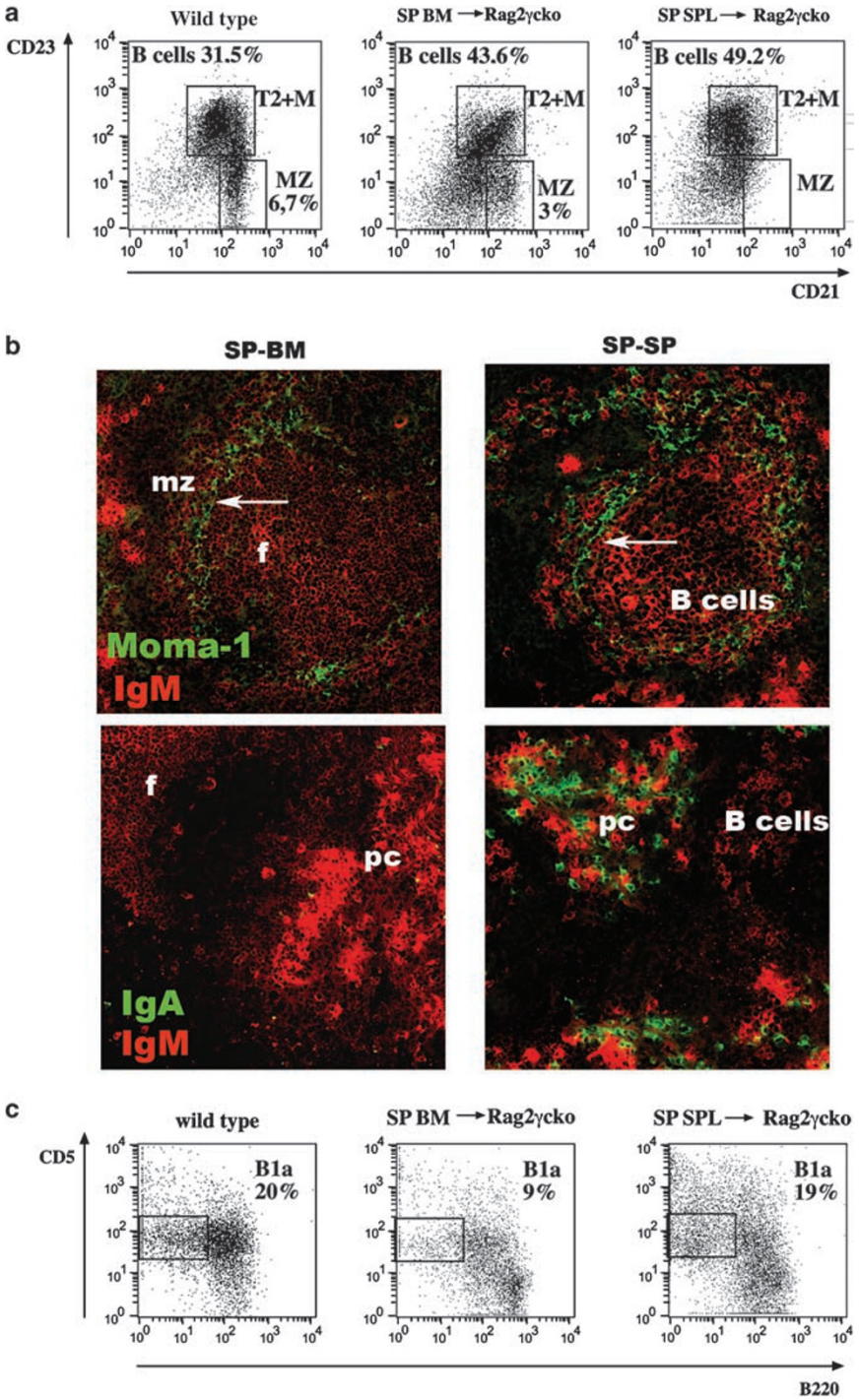

Figure 4 Phenotypic analysis of B-cell compartments in the spleen and peritoneal cavity from wild-type and Rag2γ_c double-deficient mice reconstituted with SP from BM and SP from the spleen and confocal images of spleen cryosections. (**a**) Splenic single-cell suspensions were stained with Abs to CD21, CD23, B220, and IgM and analyzed by four-colour flow-cytometry. Data are presented as dot plots. We show the staining for CD21 and CD23 in B220^{pos}IgM^{pos} gated B cells. Regions indicate marginal zone (MZ) and transitional type 2 plus mature-naive (T2+M) B cells. The frequency of total B cells and marginal zone B cells is given. (**b**) Upper panels show spleen sections stained for Moma-1 (green) and IgM (red), arrows indicate marginal zone macrophages, f- the B-cell area of the primary follicle. Lower panels show IgA (green) and IgM (red) expression, bright cells correspond to plasma cells (pc). All images were acquired at ×20 objective magnification. (**c**) Single-cell suspensions from peritoneal wash were stained for CD5, B220, IgM, and IgD. Dot plots represent CD5 vs. B220 staining on cells gated for IgM^{high} IgD^{low} and the values the frequency of B-1a (CD5^{pos}B220$^{neg/low}$).

cells, encircled by a border of Moma1^{pos} macrophages and more externally by MZ B cells. Plasma cells containing large amounts of IgM in the cytoplasm are found in the red pulp. In the mice reconstituted with SP from the BM, the lymphoid architecture was completely reconstructed and the follicles with the surrounding MZ had a normal size (**Figure 4b**). In contrast, in mice transplanted with splenic SP cells, IgM^{bright} B cells were distributed in small clusters or cords enveloping Moma-1^{pos} macrophages

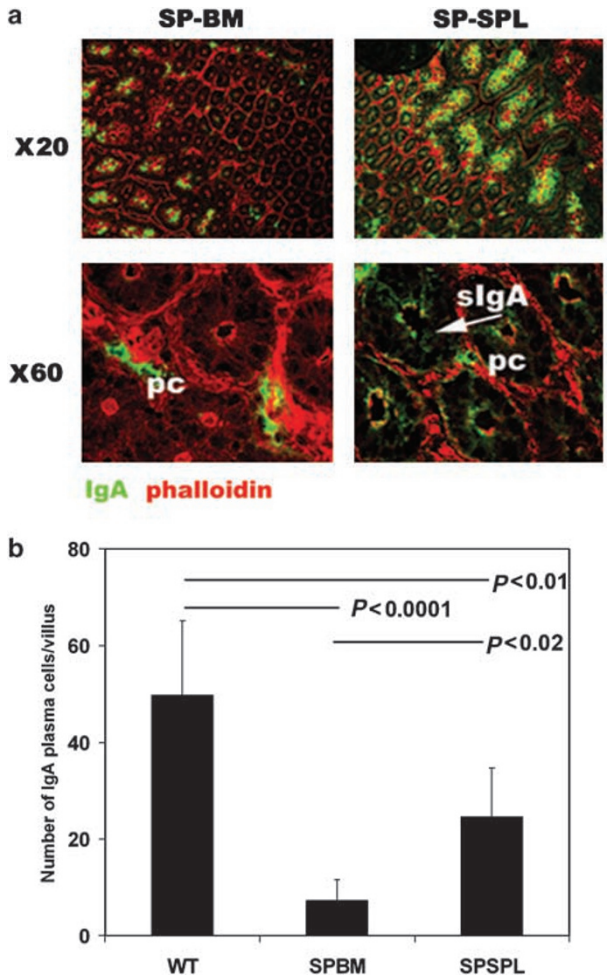

Figure 5 Confocal analysis of intestine cryosections from Rag2γ_c double-deficient mice reconstituted with SP from the bone marrow and SP from the spleen. (**a**) Panels show representative sections from the intestine stained for IgA (green) and phalloidin (red). The images were acquired with ×20 (upper panels) and ×60 objective magnification (lower panels). IgA^{bright} cells are plasma cells (pc). The arrow indicates sIgA anchored to and transported through the epithelial cell layer. (**b**) Graph indicates the average number of IgA plasma cells/villus (plus the s.d.), for each experiment 10 villi were counted from six intestine sections of different experimental mice. *P*, value calculated using the Student's *t*-test, is indicated.

(**Figure 4b**). The red pulp contained large numbers of plasma cells staining brightly for IgM or IgA. (**Figure 4b**). This was in sharp contrast with the absence of IgA plasma cells in the spleen of WT mice and mice reconstituted with BM SP (**Figure 4b**).

In the peritoneal cavity, B-1a and B-1b B cells can be distinguished by staining with CD5 and B220. B-1a B cells express CD5 and have low levels of B220, whereas B-1b B cells lack CD5 and are bright for B220 (Wardemann *et al.*[5] and **Supplementary Figure 1A** online). B-1a B cells were found at normal frequency in the peritoneal cavity of mice reconstituted with SP from the spleen, but were reduced in mice transplanted with BM SP (**Figure 4c**).

Secretory IgA is generated from precursors present in the SP of the spleen

To study the ability of SP cells to reconstitute IgA in the gut, we prepared intestine sections from: Rag2$^{KO}\gamma_c^{KO}$ mice injected with SP cells either from the BM or the spleen and stained them for IgA.

The film of sIgA, which covers the surface of the villi was reconstituted only in the mice transplanted with SP from the spleen, but not from the bone marrow. At ×60 magnification the cryptae, seen in cross-section (**Figure 5a**, arrow), appeared outlined by a thin but homogenous layer of sIgA with an inner circle corresponding to the luminal side of epithelial cells. Villi of mice reconstituted with SP from the spleen were undistinguishable from those of WT mice. In contrast, mice transplanted with BM SP cells lacked the film of sIgA both at the basal and at luminal side of intestinal epithelial cells (**Figure 5a**). The number of IgA plasma cells in the intestine was higher in mice reconstituted with SP precursors from the spleen than in the mice reconstituted with BM SP ($P < 0.02$, **Figure 5b**).

The BM is not reconstituted from the splenic SP

In the BM of control mice, the staining with the B-cell marker B220 and surface IgM can be used to distinguish IgM^{neg} pro- and pre-B cells from IgM^{pos} B cells. In Rag2$^{KO}\gamma_c^{KO}$ mice, B-cell development is blocked at the pro-B cell stage (**Figure 6,** second panel).[33] To evaluate the presence of B-lineage cells of donor origin in transplanted mice, we excluded CD43^{pos} pro-B cells from analysis and evaluated the expression of IgM and IgD on B220^{pos} cells. We could identify pre-B, transitional, and mature-naive B cells (**Figure 6,** right panels).

We found that total BM or SP cells from the BM generated pre-B, immature, and transitional B cells (**Figure 6**). In contrast, the transplantation of SP cells from the spleen did not regenerate cells of the B lineage, showing that splenic precursors are unable to restore the physiological development of B cells in the BM.

The lack of BM reconstitution by the SP of the spleen was not due to an insufficient number of injected precursors, because a 10-fold higher number of SP cells did not restore either BM development or splenic architecture (not shown).

Sorted SP cells were always more than 98% pure. However, we had to formally exclude that the puzzling results obtained with the SP from the spleen might be due to a contamination of the SP with splenic conventional B cells. We, therefore, sorted either follicular, MZ or T1 and T2 B cells and injected them in Rag2$^{KO}\gamma_c^{KO}$ mice. Ten weeks later, although B cells could be detected in the spleen and antibodies in the serum, we did not find IgA plasma cells and sIgA in the intestine of transplanted mice (**Supplementary Figure 3A–C** online). Because we injected 2–10 times more B cells than splenic SP, we can exclude that a contamination of B cells is responsible for the reconstitution of mucosal sIgA.

Segregation of BM and splenic precursors

Hematopoietic precursors able to generate B cells can be found in the fetal liver at day 13 pc. Accordingly, transplantation of fetal liver cells completely reconstitutes BM, spleen, and the B-1a B cell pool.[34,35] However, we observed that after birth, precursors of BM-derived conventional B cells segregated from those of B-1a and plasma cells producing sIgA. These results could be explained if B-cell precursors with different potentials migrated

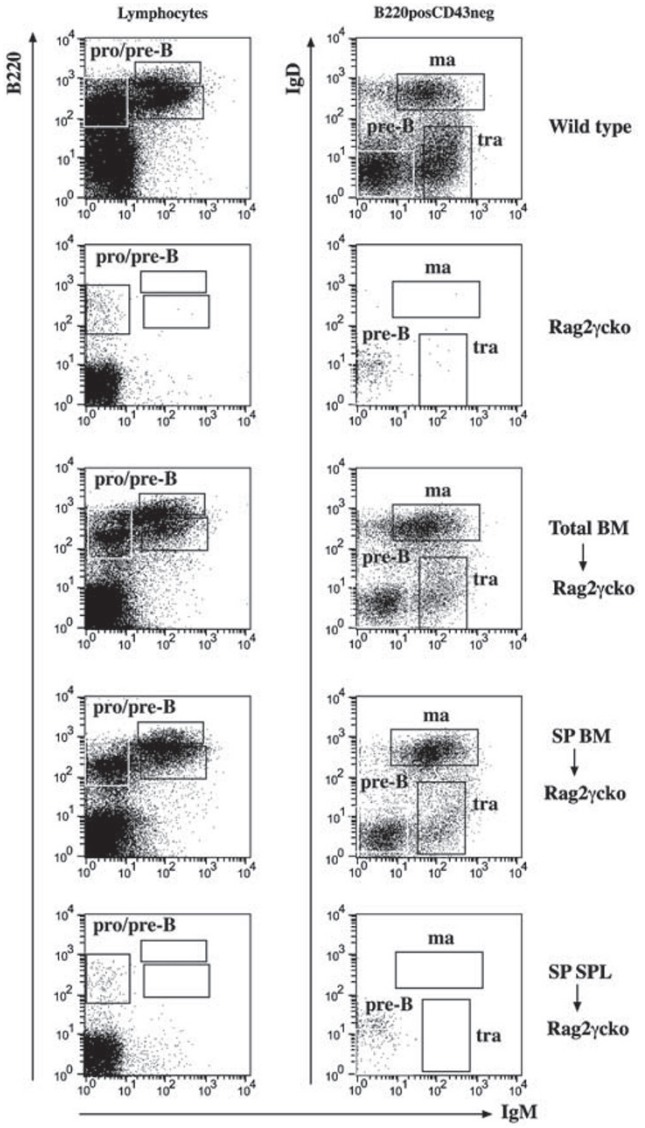

Figure 6 Phenotypic analysis of bone marrow cells from wild-type, Rag2γ_c double-deficient mice and Rag2γ_c double-deficient mice reconstituted with total BM, SP from BM (SP BM) and SP from the spleen (SP SPL). Single-cell suspensions were stained with Abs to B220, CD43, IgM, and IgD. Dot plots show the staining for B220 and IgM on cells gated according to size and shape (lymphocyte gate, left column) and IgD and IgM on B220^{pos}CD43^{neg} cells (right column). Images contain the same percentage of dots.

from their original location, the fetal liver, to different final destinations, either BM or spleen.

To prove this hypothesis, we injected 5×10^4 fetal liver cells into Rag2$^{KO}\gamma_c^{KO}$ mice. Two days later BM and spleen were removed and 7×10^6 total BM or spleen cells were injected in Rag2$^{KO}\gamma_c^{KO}$ secondary recipient mice. We did not sort SP cells because we reasoned that in Rag2$^{KO}\gamma_c^{KO}$ mice all precursors able to generate B cells derive from the injected fetal liver cells. After 10 weeks, we observed sIgA in the intestine of mice transplanted with the splenic cells, whereas the BM did not plasma cells producing sIgA (**Figure 7a**). Accordingly, the frequency of B-1a B cells was restored only after transplantation of splenic cells (**Figure 7b**).

Thus, the fetal liver at day 13 contains two different types of precursors. Injecting them in recipient mice, we forced the immediate choice of the destination to which they physiologically home before birth. The secondary transfer shows that fetal liver cells that homed to either BM or spleen have different reconstitution potentials.

DISCUSSION

The "Natural antibodies" are present in the serum independently from intentional immunization[36] and represent a very important defence against infection.[37] They collaborate with the innate immune system fighting pathogens at their entry site and facilitate the adaptive immune response trapping the infectious agent in the lymphoid organs.[1] The characteristic of natural antibodies, which makes them efficient and indispensable, is their promiscuity. The ability to bind several antigens ensures protection against any foreign antigen, which we have or not encountered before. Without this mechanism of protection, we would rarely survive long enough to display the more sophisticated defences represented by the high-affinity antibodies produced by the adaptive immune response. The need of protection against microbes starts soon after birth.[38,39] Bacteria immediately colonize airways and intestine[11,40] and in the adult 10^{14} microrganisms belonging to 1,000 different species inhabit the gastrointestinal tract.[41] This population of commensal microrganisms includes symbionts and potential pathogens. The number and quality of microrganisms is regulated by several mechanisms on the basis of the function of the innate and adaptive immune system.[42] The thin layer of sIgA covering the entire epithelial surface plays an important role in the prevention of microbial invasion. How is this film of sIgA generated? It has been shown that bacteria transported by dendritic cells to Peyer's patches and mesenteric lymph nodes induce T-dependent-specific IgA responses from conventional B-2 B cells.[43] A large fraction of mucosal IgA, however, is produced through a T-independent mechanism, triggered by the presence of microrganisms. This T-independent fraction of IgA has been suggested to represent a primitive mechanism of defence against bacteria.

In this paper, we show that in mice, that have not been intentionally immunized, most of the sIgA on the gut epithelium is produced by B-1a B-cells. Our study started from the analysis of Hox11^{KO} mice. In these mice, the congenital asplenia causes the depletion of the B-1a B cell pool, while leaving both B-1b and B-2 B cells intact. Here we show that in the gut of Hox11^{KO} mice the number of IgA plasma cells is reduced by 70%. Most importantly, no transport of IgA can be detected through the epithelial cells, and the film of sIgA on the luminal side is absent. Because lymph node structure and T-dependent reactions are normal in Hox11^{KO} mice, we hypothesized that the lack of B-1a B cells could be responsible for the defect of sIgA. Indeed, transplantation of B-1a B cells restored the film of sIgA in mutant mice.

Mucosal sIgA, similar to serum natural IgM, represents the first-line protection against infection. Both isotypes are produced by B-1a B cells, but whereas natural IgM can also be

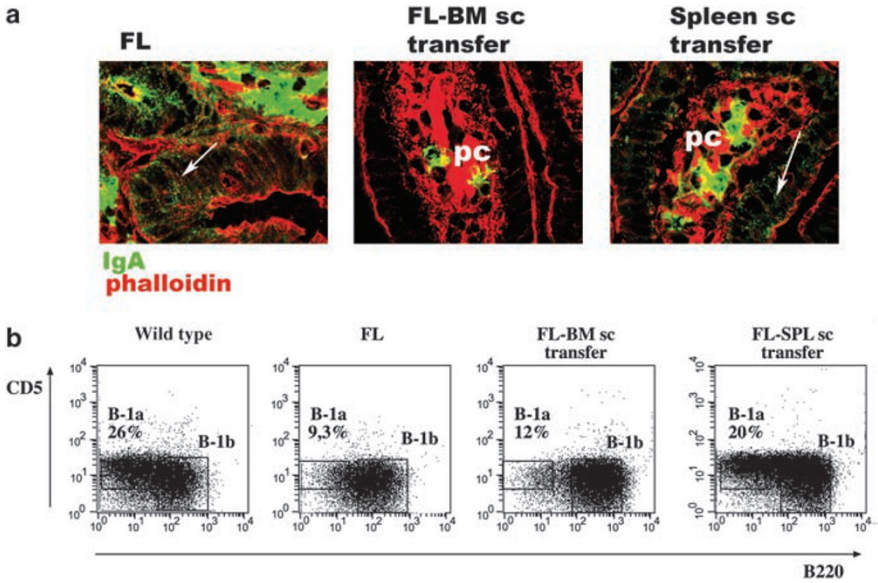

Figure 7 Confocal images of intestine cryosections and peritoneal cavity B-cell subsets from reconstituted Rag2γ_c double-deficient mice. FL mice were injected with cells from fetal liver day13 dpc, 2.5×10^{6}cells per mouse; BM sc transfer mice received total bone marrow cells (7×10^6 cells per mouse) isolated from the BM of Rag2γ_c double-deficient mice 48 h after receiving fetal liver; SPL sc transfer animals had been transplanted with total spleen (7×10^6 cells per mouse), isolated from Rag2γ_c double-deficient mice 48 h after receiving fetal liver. (**a**) Panels show representative sections from the intestine stained for IgA (green) and phalloidin (red), the IgA^{bright} B cells are plasma cells (pc) and the arrows indicate sIgA. The original objective magnification was ×60 and images were zoomed (×2). (**b**) Single-cell suspensions from peritoneal wash were stained for CD5, B220, IgM, and IgD. Dot plots represent CD5 vs. B220 staining on cells gated for IgM^{high} IgD^{low} and the values the frequency of B-1a (CD5^{pos}B220$^{neg/low}$) B cells.

detected in germ-free mice, the production of sIgA depends on bacterial colonization of the gut.[13]

The precursors of B-1a B cells can be found in the liver and BM during the fetal life and seem to disappear after birth. As B-1a B cells persist and actually increase in the adult, they are considered a self-renewing population. On the basis of our demonstration that B-1a B cells are only detectable in mice with an intact spleen, we decided to look for their precursors in this organ. To test the potential of sorted candidate precursor populations, we performed transplantation experiments in immune-deficient Rag2γ_c mice and analyzed the results 10 weeks later. We used as read-out B-1a B cells in the peritoneal cavity and sIgA in the gut. We found that none of the known B-cell populations in the spleen contained good precursors. In mice receiving T1 and T2, follicular and even MZ B cells, we found B cells in the spleen and antibodies in the serum, but neither B-1a B cells nor sIgA (**Supplementary Figure 4**). On the basis of these negative results, we reasoned that the precursors we were trying to identify might be more undifferentiated. We used the "SP" method that led to the identification and separation of stem cells and hematopoietic precursors in the BM and in many other tissues.[27,28,44,45] We sorted SP cells from BM and spleen and compared their reconstitution potential. We found that these two populations are phenotypically very similar, but generate completely different progenies. BM SP cells performed as they were expected, giving rise to all B-cell populations in the BM and to follicular, MZ B cells and to IgM plasma cells in the spleen. Few IgA plasma cells were found in the intestine, but sIgA could not be detected in transplanted mice. The SP of the spleen had a different reconstitution potential. No B-cell development was observed in the BM. In the spleen cords of B cells that lacked the typical markers of MZ B cells wrapped Moma-1^{pos} macrophages. Large numbers of plasma cells producing IgM and IgA were spread over the whole splenic tissue. B-1a B cells were detectable in the peritoneal cavity and sIgA was reconstituted in the intestine. Further studies are necessary to clarify the origin of B cells in the MZ. Although typical MZ B cells are solely reconstituted from BM SP, we show that other B cells lacking the canonical MZ markers are generated from splenic SP and surround Moma-1^{pos} macrophages. Therefore, both types of B cells produce the cytokines necessary for macrophage differentiation. We do not know whether in a normal mouse both BM- and spleen-derived B cells co-exist in the MZ. Therefore, SP cells from the spleen reconstitute B-cell populations that are different in phenotype and location from those generated by BM SP.

As all hematopoietic precursors derive from the fetal liver, we could explain the separate localization of functionally different precursors in the adult, imagining that the precursors segregated after their migration from the fetal liver. To prove this hypothesis, we forced the choice between BM and spleen injecting precursors from day 13 fetal liver into adult Rag2$^{KO}\gamma_c^{KO}$ mice recipients. Two days later, we transplanted either BM or spleen from the primary recipients into Rag2$^{KO}\gamma_c^{KO}$ secondary hosts. Again, the B-1a pool and sIgA in the intestine were replenished only in the mice receiving spleen cells.

It has been shown that the SP method identifies hematopoietic stem cells able of long-term reconstitution. In this paper, we call the SP cells of the spleen "precursors," because we only studied

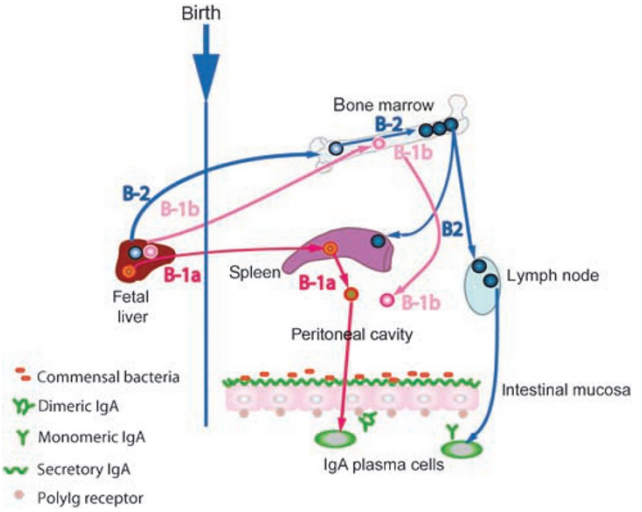

Figure 8 Proposed model depicting the origin and localization of B cells derived from different fetal liver precursors. B-2 (blue) and B-1b (pink) B-cell precursors integrate the bone marrow precursor pool and from birth they generate the most evolved immune responses in the spleen, lymph nodes, and Peyer's patches. B-1a B-cell precursors (red) migrate from the fetal liver to the spleen where they persist in adult life. Probably B-1a B cells migrate from the spleen to the intestine through the omentum and the peritoneal cavity. Their main function is to generate natural antibodies in the serum and at the mucosal sites limiting the growth of pathogens. Making this first barrier against invading pathogens, B-1a B cells and secretory Igs create a relative protected time window needed for the B-2 and B-1b B cells response and the production of high-affinity antibodies.

their progeny of B cells and we have no data on their possible "stemness." We know that the SP of the spleen also generates T cells *in vivo*, but further studies are underway to analyze the hemopoietic potential of splenic SP cells *in vitro*.

In conclusion, we show the existence of progenitor cells derived from the fetal liver and located in the spleen after birth. As shown in the model proposed in **Figure 8,** splenic SP progenitors generate B cells in a BM-independent way. These B cells mostly belong to the B-1a lineage, secrete natural antibodies of all isotypes in the serum and produce sIgA in the gut. They accomplish the function of immediate defence against pathogens at mucosal surfaces and in the circulation by the production of site-appropriate natural antibodies. In contrast, the progenitors of B-2 and probably[22] B-1b B cells also originate from the fetal liver, but migrate to the BM and persist there after birth. The adaptive immune reaction involves B-2, T and dendritic cells acting in the complex architecture of the lymphoid tissue and results in the production of high-affinity antibodies 1 week after the beginning of the infection. These antibodies efficiently and precisely eliminate the pathogen terminating the disease and remain in the serum to guarantee protection from re-infection. Part of the IgA plasma cells in the gut derives from the adaptive response to specific pathogens taking place in the regional lymph nodes and in the Peyer's patches (**Figure 8**).

An important question that still remains unanswered concerns the function of bacteria in the gut on the generation of natural antibodies. As natural antibodies can be detected in the serum of germ-free mice, but bacterial products are necessary to find IgA in the intestine, we could hypothesize that the inflammatory reaction associated with bacterial colonization is mainly needed to create the cytokine and chemokine mileau able to attract B cells committed to natural antibody production and to locally induce class switching to IgA.

In fish, B cells are generated in the head-kidney and migrate to the spleen before hatching. When the fish starts to eat, B cells and their antibodies can be found in the intestine.[46,47] It is tempting to speculate that B-1a B cells may be the remnants of the primordial immune system of lower vertebrates, evolved before the acquisition of the BM and the sophisticated structure of the adaptive immune system.

The debate over the human B-1a equivalent is still open.[48] The identification of this population may be extremely important to understand and treat B-cell immune-deficiency and could also be relevant to the issue of stem cell transplantation.

METHODS

Mice

$Rag2^{KO}\gamma_c^{KO}$, $Hox11^{KO}$, C57Bl/6 and Balb/c animals were breed and maintained in specific pathogen free conditions at the Istituto Regina Elena (Roma, Italy).

Splenectomy

C57Bl/6 mice were injected i.p. with 4 mg/20 g body weight ketamine mixed to 0.2 mg/20 g body weight xylazine and their spleen removed ($n = 11$). Animals were killed 1 year after surgery with age-matched controls.

Adoptive transfers

Peritoneal single-cell suspensions were collected from C57Bl/6 mice and injected i.p. in $Hox11^{KO}$, $4{,}8\times10^6$ cells per mouse. $Rag2^{KO}\gamma_c^{KO}$ recipient mice aged 8–14 weeks were sublethally irradiated (3 Gy) and injected intravenously 4 h later with either total BM (1×10^6cells per mouse, $n = 7$) or SP precursor cells purified from the BM (8×10^3 cells per mouse, $n = 6$) or from the spleen (2.4×10^4 cells per mouse, $n = 12$ of four independent experiments). Mice were killed and analyzed 10 weeks after injection.

Fetal liver cell transfers

Fourteen weeks old $Rag2^{KO}\gamma_c^{KO}$ recipient mice were sublethally irradiated (3 Gy) and injected intravenously with WT day 13 dpc fetal liver (FL) cells (2.5×10^6 cells per mouse). Another group of $Rag2^{KO}\gamma_c^{KO}$ recipient mice received 1×10^7 per mouse day 13 dpc FL cells and were killed after 48 h, BM and spleen were collected and re-injected in sublethally irradiated $Rag2^{KO}\gamma_c^{KO}$ recipient mice (7×10^6cells per mouse, three animals per group) and analyzed 10 weeks after transfer.

Flow cytometry analysis and cell sorting

Cells (10^6) were stained with the following mAbs: anti-IgM (clone R6-60.2), anti-IgD (clone 11.26c.2a), anti-CD5 (clones 53-7.3), anti-CD21 (clones 7G6), anti-CD23 (clones B3B4), anti-CD43 (clones S7), anti-B220 (clone RA3-6B2) all obtained from BD Biosciences (Becon and Dickinson, San Jose, CA). Dead cells were excluded from analysis by side/forward scatter gating. All analyses were performed on a FACSCalibur (Becton and Dickinson, Sunnyvale, CA) interfaced to a Macintosh CellQuest computer program. Hoechst 33342 staining was performed as described earlier.[49] Briefly, single-cell suspensions from WT spleen or BM at 10^6/ml were incubated in pre-warmed Dulbecco's modified Eagle's medium, 2% fetal calf serum, 1 mM HEPES, penicillin, streptomycin, and 5 μg/ml Hoechst 33342 (Sigma Chemical Co., St Louis, MO) for 60 min at 37°C, washed and stained for cell surface markers or left to efflux other 45 min at 37°C before the surface staining. The mAbs used for the phenotype analysis of the SP from the BM and

the spleen were anti-CD45 (clone 30-F11), anti-Sca-1 (clone E13-161.7), and anti-CD117 (c-Kit, clone2B8) all obtained from BD Biosciences. After Hoechst 33342 labeling, cells were always kept in the dark at 4°C and analyzed on a BD-LSR equipped with 325 nm He-Cd UV laser (Becton and Dickinson) interfaced to a Macintosh CellQuest computer program. Dead cells were excluded from analysis by side/forward scatter gating. Hoechst 33342low SP cells were isolated by FACS sorting using the FACSvantage SE (Becton and Dickinson). Peritoneal cavity washes from BALB/c, and Hox11^{ko} mice were sorted by FACS for B1a (IgM^{pos}IgD^{neg}B220^{low}CD5^{pos})/B2 (IgM^{pos}IgD^{pos}B220^{pos}CD5^{neg}) and B1b (IgM^{pos}IgD^{neg}B220^{inter}CD5^{neg})/B2(IgM^{pos}IgD^{pos}B220^{pos}CD5^{neg}). The sorting was performed using a FACSvantage SE (Becton and Dickinson). Cell purity was >98%.

Fecal IgA

Concentration was calculated as described for the serum (**Supplementary material** online). Equivalent volumes of intestinal content were collected into 1.5 ml Eppendorf tubes and centrifuged at 1,200 r.p.m. for 5 min at 4°C, upper liquid phases were transferred in clean tubes and used for the IgA quantification.

Western blot

Total cell extracts were prepared from sorted B1a (1×10^5cells) and B2 (2.3×10^5 cells) cells from the peritoneal cavities of five BALB/c, or from Hox11^{ko} mice (B1b, 2.5×10^5cells) (B2, 1.5×10^6) by resuspending cells in lysis buffer containing 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, and protease inhibitors (Complete Mini, Roche, Mannheim, Germany). Proteins were denatured at 95°C for 5 min in 4× Laemmli buffer[50] containing 4% β-mercaptoethanol, separated by SDS-polyacrylamide gel electrophoresis (13% acrylamide, 30:0.8 Acrylamide: Bis-acrylamide) and transferred to a nitrocellulose membrane (PROTRAN, Schleicher & Schuell, Dassel, Germany). Membranes were blocked with 5% skim milk in TBS-T (0.05% Tween-20 in TBS) o/n at 4°C, followed by an 1 h incubation with a biotinylated mouse anti-J chain antibody (1:1000 in 5% skim milk in TBS-T), or rabbit anti-actin (I-19, Santa Cruz Biotechnology, Santa Cruz, CA). After washing 3×5 min in TBS-T, membranes were incubated for 1 h with a secondary antibody, Streptavidin-peroxidase (1:3000, Jackson ImmunoResearch, West Grove, PA) or donkey anti-rabbit HRP (1:3000, GE Healthcare UK Limited, Little Chalfont, Buckinghamshire, UK), respectively, and washed 3×5 min. Detection was performed using the ECL plus western blotting system (GE Healthcare).

Confocal microscopy

Intestinal tissue and spleen samples were collected and immediately frozen in liquid nitrogen, and kept at −80°C. Multiple 5 μm cryostat sections included in cryostat-embedding medium (Bio-Optica, Milan, Italy) were fixed in cold acetone, washed with phosphate-buffered saline (Sigma Chemical Co., St Louis, MO) and incubated for 45 min with Phalloidin-TRITC (70 μM, Sigma Chemical Co., St Louis, MO), Moma-1 FITC-conjugated (Serotec Morphosys UK, Oxford, UK), FITC anti-mouse-IgA (clone C10-3) (BD Biosciences) or TRITC goat anti-mouse IgM, μ-chain specific (Jackson Immunoresearch). Sections were analyzed in a co-focal microscope (Fluoview for Olympus FV1000, Munich, Germany) and images acquired at ×20 and ×60 amplification. The frequency of IgA plasma cells was calculated using Fluoview software (Munich, Germany).

Statistic analysis

Significance was analyzed in the StatView statistical MacIntosh program (StatView Software, San Diego, CA) and determined with the paired Student's *t*-test. A level of $P<0.05$ was considered statistically significant.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/mi

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DISCLOSURE

The authors declare no conflict of interest.

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