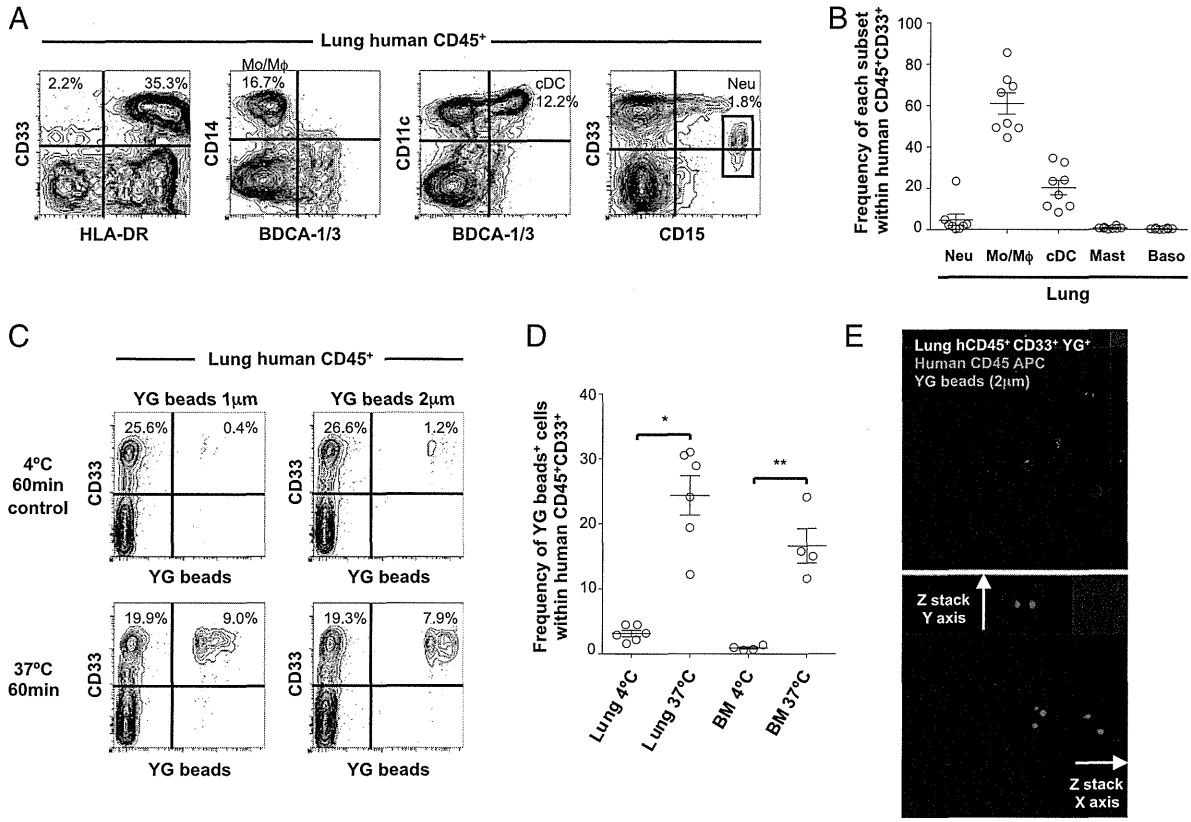

FIGURE 6. Human monocytes/macrophages developing in NSG recipient lung demonstrate phagocytosis of microparticles. (**A**) Representative contour plots demonstrating the reconstitution of human myeloid cells in the lungs of an NSG recipient. Human $CD45^+$ cells within lung cell populations were analyzed by CD33, HLA-DR, CD14, CD11c, BDCA-1/3, and CD15 to identify monocytes/macrophages, cDCs, and neutrophils. (**B**) The frequencies of human neutrophils, monocytes/macrophages, cDCs, mast cells, and basophils within $hCD45^+CD33^+$ NSG recipient lung are summarized ($n = 8$). (**C**) A set of representative flow cytometry plots demonstrating the presence of $hCD45^+CD33^+$fluorescent $beads^+$ cells. (**D**) Summary of the frequency of $hCD45^+$ $CD33^+$fluorescent $bead^+$ cells in NSG recipient lung cell populations incubated at 37°C and at 4°C (control), respectively, with fluorescent beads (lung, $n = 6$; BM, $n = 4$; $*p = 0.001$, $**p = 0.01$). (**E**) Confocal imaging of FACS-purified $hCD45^+CD33^+$fluorescent $beads^+$ cells derived from NSG recipient lung cell populations show internalization of fluorescent beads (green) within hCD45 (purple)-expressing human myeloid cells. Baso, Basophils; Mast, mast cells; Mo/Mϕ, monocytes/macrophages; Neu, neutrophils.

internalization of microparticles by human monocytes/macrophages (Fig. 6E). Taken together, these findings demonstrate the presence of human innate immunity with intact phagocytic function in the NSG recipient lung.

Humanized mouse BM-derived monocytes/macrophages exhibit IFN-γ–induced phagocytosis and killing against Salmonella typhimurium

Myeloid subsets serve essential roles in host defense against various infectious microorganisms as a part of innate immunity. Of the various myeloid subsets discussed in the current study, monocytes and macrophages display excellent phagocytic potential by phagolysosome formation, by the effects of oxidative and nitrosative stress, and by antimicrobial cationic peptides and enzymes (23). To evaluate future application of the humanized mouse system in infectious disease research, we examined the phagocytic function of human monocytes/macrophages derived from humanized NSG BM against *S. typhimurium*. We purified $mCD45^-TER119^-hCD45^+$ Lin^-CD11b^+ cells as monocytes/macrophages from the recipient BM (Fig. 7A) and cultured 10,000 purified human monocytes/macrophages with *S. typhimurium* at an MOI of 20 with or without prestimulation of human recombinant IFN-γ at 1000 U/ml. In the five in vitro experiments, stimulation of human monocytes/macrophages with rhIFN-γ resulted in the significantly potentiated phagocytosis and killing of *Salmonella* by the humanized mouse-derived monocytes/macrophages as evidenced by the decreased numbers of colony formation by *S. typhimurium* (at 3 h postinfection $p = 0.023$, at 12 h postinfection $p = 0.091$ [n.s.] compared with control versus IFN-γ stimulation by two-tailed t test) (Fig. 7B). Taken together, human monocytes that develop in the humanized NSG mice possess phagocytic activity against microbeads and bacteria and kill phagocytized bacteria presumably via signaling through cytokine receptors and TLRs.

Discussion

In vivo reconstitution of mature and functional human myeloid cells not only facilitates in vivo examination of human innate immunity but also offers a promising platform for translational research in the areas of infectious immunity and drug development. In the current study, we have aimed to clarify how functional human myeloid cells develop in NSG humanized mice.

In the NSG recipients, we found distinct levels of reconstitution of myeloid subsets in the BM and spleen. The differential myeloid reconstitution in the humanized hematopoietic organs is comparable to that seen in the human tissues, reflecting the distinct physiological roles of each hematopoietic organ in mammals. BM acts an essential reservoir of short-lived neutrophils and monocytes that readily migrate into sites of infection and inflammation. In addition, BM neutrophils function as paracrine regulators for mobi-

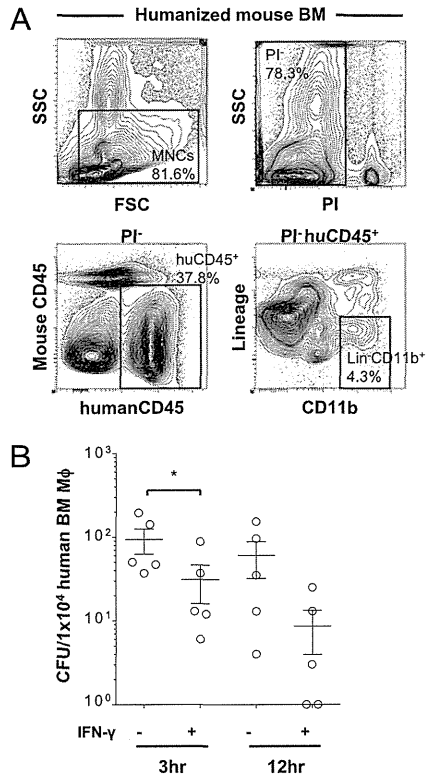

FIGURE 7. *Cytotoxicity against S. typhimurium by IFN-γ–activated human monocytes/macrophages developing in NSG recipient.* (**A**) Within mononuclear cell gate, propidium iodide$^-$ viable, hCD45$^+$Lin$^-$CD11b$^+$ cells were purified from the BM of humanized NSG recipients. Purified BM monocytes/macrophages were stimulated with or without supplementation of 1000 U/ml human IFN-γ for 24 h and then infected with *S. typhimurium* at MOI 20. (**B**) Intracellular CFU was counted at 3 and 12 h postinfection (n = 5, *p = 0.023 compared with nonstimulated).

lization of HSCs via proteases, such as matrix metalloproteinase-9 (MMP9 or gelatinase B), cathepsin G, and neutrophil elastase contained within intracellular granules. The spleen, a major secondary lymphoid organ, is not only the site of B cell maturation and APC interactions with T and B cells but also is an organ supporting the development of mast cells from their progenitors (24, 25). Cross-reactivity of murine stem cell factor with human c-Kit$^+$ cells may explain the high frequency of human mast cells observed in the recipient spleen (26).

The development of human myeloid lineages is regulated by various cytokine signals (18, 27). In the current study, we directly compared the frequencies of human myeloid subsets using humanized mouse BM and primary human BM MNCs. As to the development of human APCs, humanized mouse BM recapitulates physiological development of human monocytes and two different subtypes of cDCs. However, we could not directly compare the frequencies of human neutrophils between humanized mouse BM and primary human BM, as we have used frozen BM MNCs. According to the previous reports, the frequency of human neutrophils in the humanized mouse BM is lower than that in the primary human BM (28, 29).

Human myelopoiesis within the mouse microenvironment may occur through multiple cooperative mechanisms. First, mouse cytokines such as stem cell factor, FLT3 ligand, G-CSF, and thrombopoietin may directly stimulate human myelopoiesis by cross-reacting with their respective receptors on human hematopoietic stem and/or myeloid progenitor cells. These human myeloid cells in turn produce cytokines such as GM-CSF and IL-3, resulting in the differentiation, maturation, and maintenance of human granulocytes, monocytes, and DCs. At the same time, the cytokine milieu within the NSG recipient repopulated with human hematopoietic cells may not be completely sufficient, to support human hematopoiesis as evidenced by the relative paucity of human neutrophils in the recipient BM, spleen, and circulation that might suggest the requirement of human cytokine or adhesion molecules in the hematopoietic tissues of the recipients. Recent studies suggested that the induced expression of human cytokines in the mouse environment may lead to enhanced differentiation and maturation of human myeloid subsets including neutrophils (30–33). In the current study, however, human monocytes develop in NSG recipients despite the fact that M-CSF is exclusively produced in non-hematopoietic cells and that murine M-CSF does not cross-react with human M-CSFR. This may be attributable to the redundancy among cytokines such as M-CSF, GM-CSF, and IL-3 as demonstrated in previous studies using M-CSF–deficient mice (34).

As a measure of human myeloid cell function, we investigated cytokine responses in human neutrophils and monocytes developing in the NSG recipients. Consistent with the expression of cytokine receptors identified on the human myeloid cells, neutrophils and monocytes showed intact responses to human cytokines both in vivo and in vitro. Phosphorylation of STAT molecules represents a molecular event downstream of cytokine receptor activation. STAT1 is a key mediator of IFN-γ activation of cells and an indispensable component of IFN-γ–dependent innate defense mechanisms against infections (35). The STAT3 signaling pathway is essential for G-CSF–mediated granulopoiesis (36). Specific phosphorylation of STAT5 may be an essential molecular event enabling generation of granulocytes from myeloid progenitors and proliferation and survival of mature neutrophils (37). STAT4 and STAT6 are essential for mediating IL-12 and IL-4 signaling in Th cells (38, 39). Human myeloid cells developing in humanized NSG recipients responded to human cytokines in a specific manner, as determined by the selective activation of JAK–STAT signaling pathways to corresponding cytokines.

Similar to the analysis of the expression of cytokine receptors and signaling, we showed that human myeloid subsets developing in the NSG humanized mice expressed various TLRs at the protein level. In the analysis of TLR expression in humanized mouse BM-derived cells, specific expression of TLR2 was observed in human monocytes and BDCA1$^+$ cDCs rather than neutrophils or BDCA3$^+$ cDCs. Consistent with the expression of TLR4 in human myeloid subsets, in vivo administration of LPS provoked a potent human inflammatory response as demonstrated by the prompt elevation of plasma hIL-6, hIL-8, and hTNF levels. In addition to the examination of cytokine and TLR signaling in human myeloid cells, we investigated the function of human myeloid cells against bacteria to elucidate whether the humanized mouse system can be applied to the research for infectious immunity. As an example of bacterial infection, we chose *S. typhimurium*, a Gram-negative bacillus causing gastrointestinal infections and invasive diseases, especially in children and immunosuppressed patients (40). IFN-γ mediates signaling to activate monocytes and macrophages in phagocytosis (41, 42). In the analysis of colony formation by *S. typhimurium*, IFN-γ potentiated the phagocytosis and antimicrobial activities of humanized mouse BM-derived monocytes/macrophages against this microorganism.

We observed not only systemic reconstitution of human myeloid subsets but also development of respiratory mucosal immunity in NSG humanized mice. Recent mouse studies revealed the crucial and specific roles of mucosal immunity in immune surveillance and

immunological homeostasis in the respiratory tracts (21, 22). In the recipient lung, unlike the BM or spleen, $CD33^+CD14^+HLA\text{-}DR^+$ macrophages were the predominant myeloid population. Frequencies of human B cells, T cells, and myeloid cells in the recipient lung were distinct from those in the recipient PB, excluding the possibility that the human myeloid cells isolated from the recipient lung are contaminating PB myeloid cells. Importantly, macrophages, the predominant human myeloid subset in the recipient lung, demonstrated intact phagocytic function. Macrophages in the NSG recipients will be compared with the recently reported hGM-CSF and hIL-3 knock-in Rag2KO/IL2rγKO humanized mice showing abundant human macrophages in bronchoalveolar lavage (32). Establishment of an in vivo model of human pulmonary mucosal immunity may enable investigation of in vivo immune surveillance in the respiratory tract and in allergic pulmonary disorders and may allow evaluation of vaccines at preclinical stages (43, 44).

In this study, the reconstitution of both systemic and mucosal human innate immunity was observed in the NSG humanized mice. We performed phenotypic characterization and functional evaluation of human myeloid cells developing in the recipients, including granulocytes and APCs. Humanized mice reconstituted with both lymphoid and myeloid human lineages would facilitate in vivo investigation of interactions between the lymphoid and the myeloid compartments, allowing the dissection of the coordinated human immune response at the level of the whole organism.

Disclosures

The authors have no financial conflicts of interest.

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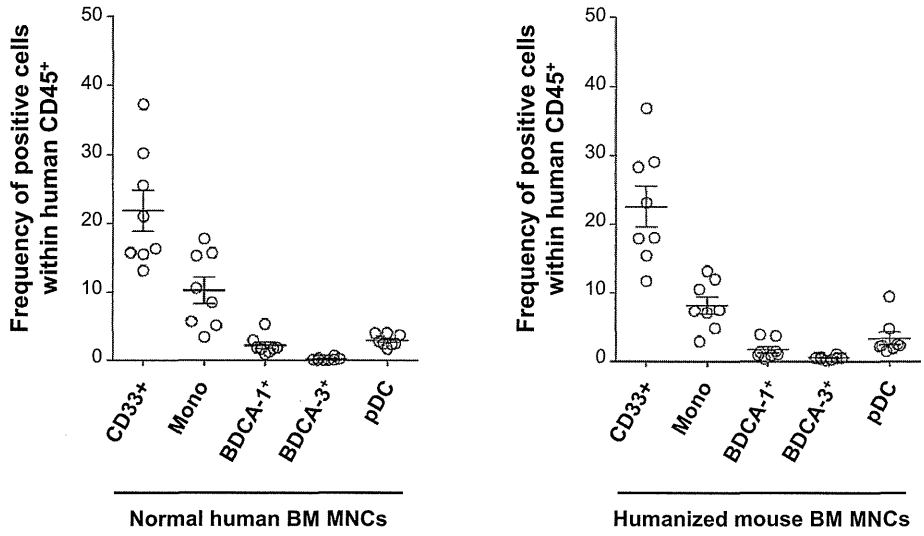
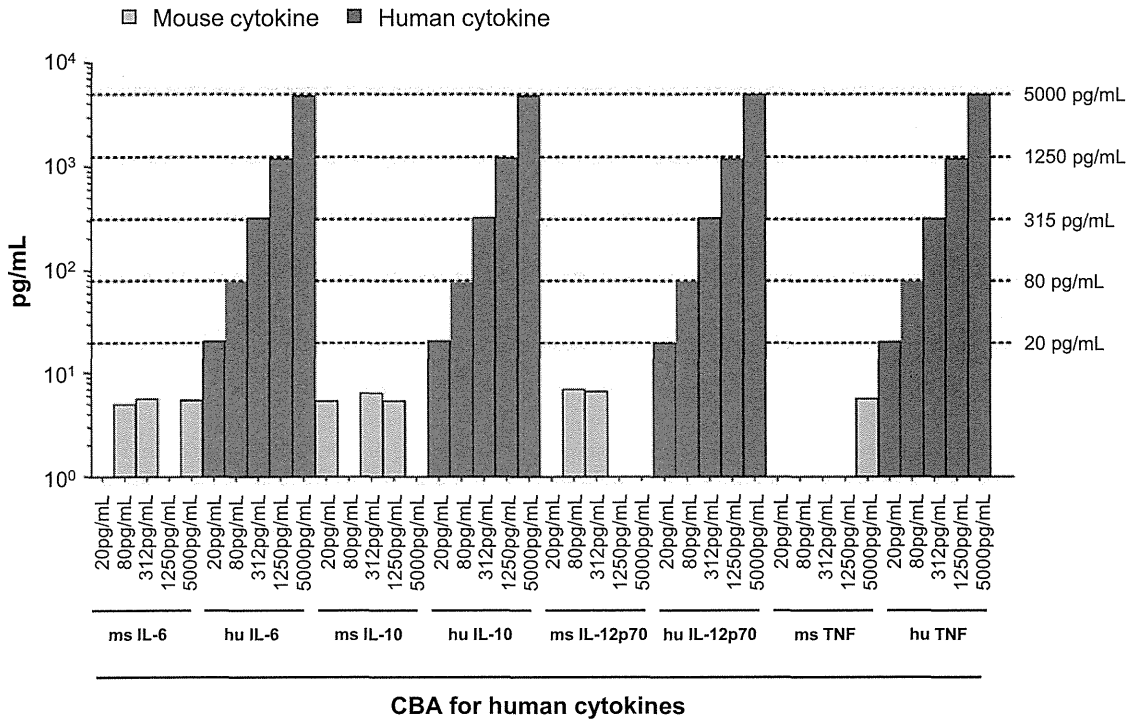
Supplementary Figure 1



SUPPLEMENTARY FIGURE 1. Frequencies of human myeloid cells in primary human BM MNCs and humanized mouse BM MNCs. Frequencies of total human $CD33^+$ myeloid subsets, monocytes, $BDCA1^+$ cDCs, $BDCA3^+$ cDCs, and pDCs out of $hCD45^+$ cells of primary human BM MNCs (left) and humanized mouse BM MNCs (right) are shown.

Supplementary Figure 2



SUPPLEMENTARY FIGURE 2. Species specificity of anti-human cytokine antibodies used in Cytometric Bead Array. Human and mouse recombinant IL-6, IL-10, IL-12p70, and TNF at 20-5000 pg/ml were used to examine whether species cross-reactivity exists in anti-human cytokine antibodies. Each anti-human cytokine antibody detected human cytokine but not mouse cytokine, in a dose-dependent manner, demonstrating species-specificity of the antibodies used.

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Immune regulation and monitoring at the epithelial surface of the intestine

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The intestinal enterocytes and other epithelial cells create physical barriers, including tight junctions and mucus layers. These cells also actively transport antibodies across the epithelium and simultaneously produce antimicrobial peptides and enzymes. These functions maintain intestinal homeostasis by allowing the selective absorption of nutrients and simultaneously preventing pathogenic infections. Recent evidence has revealed that both host-derived factors (e.g., cytokines) and gut environmental factors (e.g., commensal bacteria, dietary materials, and their metabolites) regulate the physical and immunological functions of the epithelium. Understanding the interactions between host cells and these environmental factors should help us to develop new strategies to prevent and treat immune diseases of the intestine.

The surface of the gastrointestinal tract is covered by a single layer of epithelium that separates the outside world from interstitial tissues. The intestinal epithelium is mainly composed of absorptive enterocytes (ECs) but also includes enteroendocrine, goblet, and Paneth cells [1]. Cross-communication among these cells enables the selective absorption of nutrients while simultaneously preventing the penetration of antigens and pathogens. The defense against pathogenic materials is at least partly achieved by the physical barriers of the epithelium, which include tight junctions and mucus layers. A large number of pathogens disrupt these barriers to access deeper tissues for dissemination [2,3]. The barriers also contribute to the establishment and maintenance of mucosal homeostasis. Indeed, a leaky intestinal barrier is one of the characteristics of chronic intestinal inflammatory diseases, such as inflammatory bowel disease and celiac disease [4,5].

Intestinal tissues also show intense immunological activity, and ECs contribute to the intestinal immune system by transporting and processing antibodies and associated antigens, by producing immunologically functional molecules, and by interacting with immunocompetent cells in the intestine [6]. Accumulating evidence has revealed that both host-derived factors (e.g. cytokines) and gut environmental factors (e.g. commensal bacteria, dietary materials, and their metabolites) engage in molecular crosstalk with the intestinal epithelium and affect intestinal barrier function and immune responses [7,8]. In this review, we focus on the immunological functions of ECs in the intestine and their regulation by commensal bacteria and dietary materials.

Physical barriers at the intestinal epithelium

Tight junctions

ECs provide a physical barrier to prevent the paracellular transport of luminal antigens and pathogens. Tight junctions are multifunctional complexes that are crucial for the maintenance of barrier integrity because they form a seal between adjacent ECs [9]. The tight junction regulates the absorption of nutrients, ions, and water while preventing the entry of pathogens into the host.

Tight junctions are composed of numerous interacting cellular proteins, including claudin, occludin, and zonula occludens (ZO) proteins (Fig. 1). Claudin and occludin are transmembrane proteins that seal the paracellular space between adjacent ECs. Among

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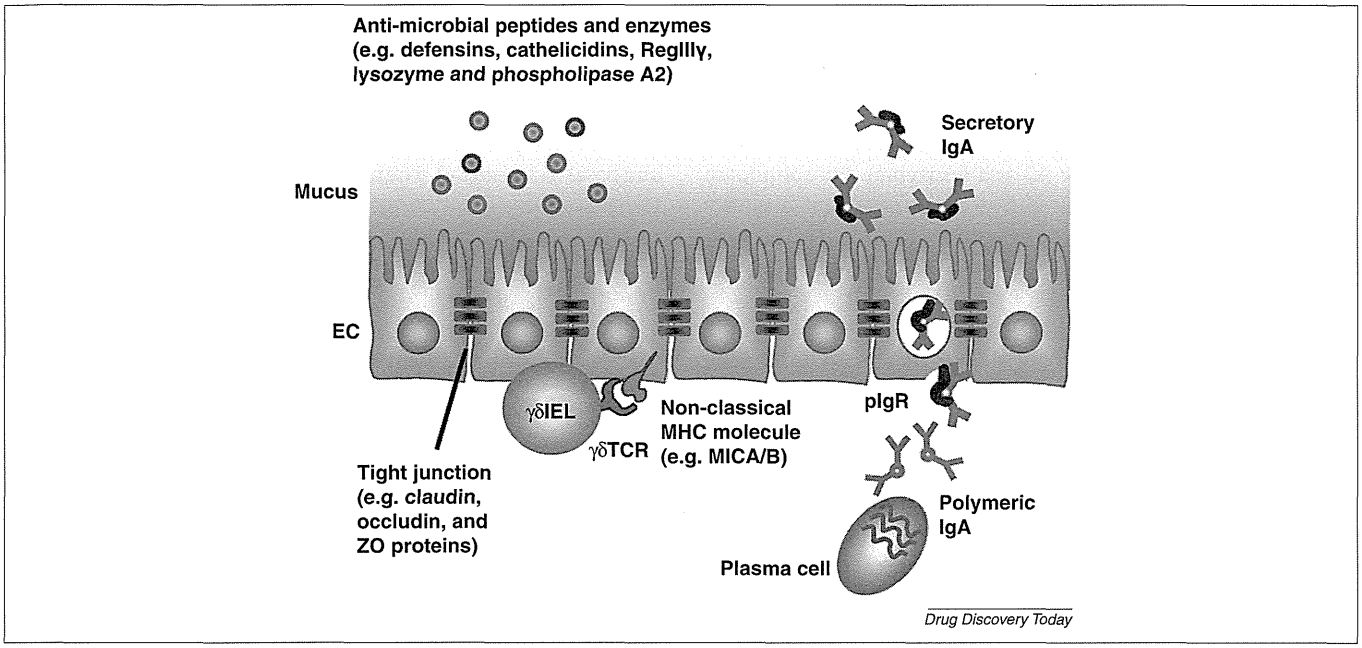

FIGURE 1

Physical and immunological barriers mediated by ECs. ECs (including Paneth cells) produce several molecules that create physical barriers in the intestine. They also produce antimicrobial peptides and enzymes, such as defensins, cathelicidins, RegIIIγ, lysozyme, and phospholipase A2 to kill the bacteria and establish a mucus layer to prevent bacterial attachment to the ECs. Tight junctions among ECs prevent bacterial penetration between the cells. ECs also have immunological functions. They express polymeric immunoglobulin receptor (pIgR), which binds and transports polymeric IgA produced from plasma cells into the intestinal lumen. ECs exposed to stresses (e.g. infection or cancer) express non-classical MHC molecules (e.g. MICA/B). MICA/B acts as a ligand for γδ T cell receptors, which are uniquely expressed on intraepithelial lymphocytes (γδIELs). *Abbreviations*: EC, enterocytes; MHC, major histocompatibility complex; ZO, zonula occludens.

the various types of claudins, claudin-1, -2, -3, -4, -5, -7, -8, -12, -15, -18, -20, and -23 are expressed in the intestinal epithelium [10,11]. ZO proteins are adaptors that connect transmembrane proteins; in particular, ZO-1 interacts with the claudin proteins and with F-actin in the intestinal ECs [12,13].

The physical barriers created by ECs are at least partly regulated by the immunological stimulation provided by commensal bacteria and dietary materials. Indeed, commensal and probiotic bacteria, their metabolites, food extracts, and dietary materials (e.g. fatty acids, polysaccharides, and flavonoids) have been shown to promote intestinal barrier integrity by increasing the expression of tight junction proteins [10].

Mucus

The mucus layer has been recognized as an important component in the intestine (Fig. 1). Mucin 2 (MUC2), a large glycoprotein characterized by variable O-linked glycans, is abundantly expressed by goblet cells located in the intestinal epithelium [14]. Generally, mucus can be divided into two layers. Although both layers have similar protein composition, the outer mucus layer is loose, whereas the inner mucus layer adheres firmly to the surface of the ECs. The firm mucus in the inner layer is an efficient barrier against pathogens [15]. In addition to the physical and biological barrier function of mucus, mucus also ensures the concentration of antimicrobial peptides and IgA antibodies at the surface of ECs. As similar to tight junctions, mucus expression is regulated by commensal bacteria, and the mucus layer of germ-free mice is thicker than that of specific pathogen-free mice [15].

Production of antimicrobial molecules at the epithelium

Antimicrobial peptides

The epithelium also secretes a variety of antimicrobial peptides [e.g. defensins, cathelicidins, and RegIIIγ (Fig. 1)]. The production of these peptides is mainly mediated by ECs and Paneth cells [16]. Paneth cells reside at the base of the crypt regions of the intestine, where they constitutively produce α-defensins. This does not require bacterial stimulation, because Paneth cells produce normal amounts of α-defensin in germ-free mice [17]. By contrast, ECs require microbial stimulation for the production of β-defensins [16]. ECs also produce cathelicidin, the expression of which is regulated by short-chain fatty acids produced when polysaccharides are metabolized by fermenting bacteria [18]. Both defensins and cathelicidin are cationic small peptides that exhibit antimicrobial activity by damaging and permeabilizing the bacterial cell membrane by pore formation [19].

RegIIIγ is a C-type lectin produced by ECs and Paneth cells in the ileum, where it kills Gram-positive bacteria by binding to surface-exposed carbohydrate moieties of peptidoglycans [20]. Commensal bacteria, especially Gram-negative bacteria, induce RegIIIγ expression on ECs, and a recent study demonstrated that MyD88 intrinsically expressed on ECs controls the production of RegIIIγ, which establishes the physical separation between the microbiota and the intestinal epithelial surface [21].

Unlike RegIIIγ, which specifically targets Gram-positive bacteria, bactericidal and/or permeability-increasing protein (BPI) shows antimicrobial activity against Gram-negative bacteria. The high affinity of BPI for lipopolysaccharide (LPS) leads to the

destabilization of the outer membrane of Gram-negative bacteria and also neutralizes LPS-induced inflammation [22].

Antimicrobial enzymes

Antimicrobial activity is also mediated by bacteriolysis enzymes (e.g. secretory phospholipase A2 and lysozyme). Phospholipase A2 is a small enzyme produced by Paneth cells that degrades bacterial phospholipids and subsequently disrupts the integrity of Gram-positive and -negative bacteria [23]. Phospholipase A2 enzyme activity is normal in the intestine of germ-free rats [24], but caloric restriction increases the gene expression of lysozyme and phospholipase A2 [25]. Therefore, it is likely that nutritional conditions rather than commensal bacteria regulate the activity of these antimicrobial enzymes in the intestine. Lysozyme is produced by Paneth cells and ECs. Its bactericidal activity derives from its cleavage of the glycosidic linkage between *N*-acetylglucosamine and *N*-acetyl muramic acid of peptidoglycan. Because Gram-positive bacteria express more peptidoglycan than Gram-negative bacteria, lysozyme acts preferentially on Gram-positive bacteria.

Transport of antibodies through ECs

IgA transport mediated by polymeric immunoglobulin receptors

One function of the epithelial immune barrier is to transport antibodies across the barrier. ECs express polymeric immunoglobulin receptors (pIgR) for the transport of polymeric forms of IgA (pIgA) and IgM (pIgM) in the basal-to-apical direction in association with an extracellular proteolytic fragment of the pIgR (known as the secretory component) [26]; together, the IgA and the secretory component form secretory immunoglobulin A (S-IgA). After S-IgA is secreted into the intestinal lumen, it inhibits adherence of pathogens to host ECs in the intestine and neutralizes pathogenic toxins by binding to their biologically active sites (Fig. 1) [27]. Additionally, IgA is able to exclude antigens and pathogens from the intestinal secretions while it is transported through ECs, and it also prevents viral replication inside ECs [28,29].

In addition to the function of S-IgA in the immunosurveillance, several lines of evidence demonstrate that S-IgA has a key role in preventing the penetration and/or growth of commensal bacteria [30]. These functions of S-IgA achieve the immune responses against commensal bacteria restricted in the intestinal but not systemic immune compartments in normal mice, while IgA-deficient mice exhibited systemic IgG responses against commensal bacteria [31–33]. A recent study also demonstrated that, in the absence of IgA, commensal bacteria-derived stimulation induced the increased expression of interferon-regulated genes in the ECs for the compensatory immunosurveillance with simultaneous reduction of lipid metabolism-related Gata4-regulated genes, which resulted in the lipid malabsorption and decreased lipid deposition [34]. Thus, S-IgA mediates the regulation between ECs and commensal bacteria, which is important not only for the maintenance of immunological homeostasis but also for metabolism [34].

Neonatal Fc receptor for IgG transport

Another receptor for immunoglobulin is the neonatal Fc receptor for IgG (FcRn). Although early studies in rodents indicated that FcRn was responsible for the passive acquisition of IgG neonatally, subsequent studies indicated that FcRn is also expressed by adult human epithelium and antigen-presenting cells in the intestine and thus is not strictly limited to neonatal life [35]. Unlike pIgR mentioned above, human FcRn binds IgG and the transport pathway is bidirectional, both apical to basal and basal to apical [36]. The bidirectional transport of IgG enables retrieval of intestinal antigens in a complex with IgG into the intestinal lamina propria, where the antigen and/or IgG complexes are subsequently taken up by antigen-presenting cells to prime T cell responses [37].

Intraepithelial T lymphocytes

The epithelium also includes lymphocytes that are commonly termed intraepithelial lymphocytes (IELs) [38]. IELs reside between the basolateral surfaces of ECs, and one IEL occurs for every 4–10 ECs in the small intestine and for every 30–50 ECs in the large intestine.

Most IELs are T cells. As similar to T cells observed at other sites (e.g. spleen and intestinal lamina propria), some portions of IELs express αβ T cell receptors and act as cytotoxic T lymphocytes by recognizing antigenic peptides presented by classical major histocompatibility complex (MHC) molecules on pathogenic ECs (e.g. microbe-infected cells) and killing them by producing cytotoxic molecules (e.g. perforin and granzymes) [38]. Other IELs express the γδ T cell receptor (and are therefore known as γδIELs) and show minimal pathogen-specific activity [38,39]. The innate immune function of γδIELs enables the rapid removal of infected ECs. To recognize the infected ECs, non-classical MHC molecules, such as MHC class I chain-related protein A/B (MICA/B) in human, act as ligands for γδIELs. MICA/B is generally not expressed on ECs, but is induced by stresses such as heat shock and microbial infections. The activated γδIELs then synthesize an array of cytokines, including interleukin (IL)-2, IL-3, IL-6, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and transforming growth factor (TGF)-β, and cytotoxic molecules, such as perforin, granzyme, and Fas ligand to kill the microbe-infected ECs [38].

Epithelium senses signals from commensal bacterial in the regulation of T cell differentiation in the intestine

The immune system requires interactions with commensal bacteria for its development. Toll-like receptors (TLRs) act as sensors of commensal bacteria although they were initially discovered as pathogen recognition receptors. ECs express several kinds of TLRs and the ligands from commensal bacteria promote immunological functions of Ecs, such as IgA transport, tight junctions, and expression of antimicrobial peptides [40]. Of note, ECs have unique expression profiles and spatially restricted distribution (apical vs. basolateral) of TLRs together with unique underlying signaling pathways, which enables the prevention of deleterious inflammatory responses in the intestine [40].

Because commensal bacteria express shared molecules which act as a ligand of TLRs, it was previously thought that unspecified commensal bacteria indiscriminately induced the development of the immune system; however, accumulating evidence has demonstrated that individual species of commensal bacteria have specific roles in the determination of immunological balance by regulating T cell differentiation in the intestine [8]. ECs have an important role in this pathway.

Segmented filamentous bacteria induce the differentiation of Th17 cells

Several groups have shown that segmented filamentous bacteria (SFB) induce components of the active immune system, including IgA-producing cells, γδIELs, and IL-17-producing T (Th17) cells [41–43]. SFB colonization on ECs results in the production of serum amyloid A, which acts on intestinal dendritic cells (DCs) to enhance the production of IL-6 and IL-23 [43]. Because these two cytokines are Th17 cell-inducing cytokines, the immunological environment mediated by SFB, ECs, and DCs results in the preferential induction of Th17 cells in the intestine.

Preferential induction of Treg cells in the colon by Clostridium *clusters IV and XIVa*

Another form of crosstalk between ECs and commensal bacteria in the regulation of T cell differentiation is mediated by *Clostridium* clusters IV and XIVa (also known as the *Clostridium leptum* and *coccoides* groups) [44]. By contrast to the effects of SFB, colonization by *Clostridium* clusters IV and XIVa induces regulatory T (Treg) cells in the colon to achieve quiescent immunity. *Clostridium* clusters IV and XIVa form a thin colonizing layer on the epithelium, where they enhance the release of the active form of TGF-β by increasing the expression of matrix metalloproteinases that convert latent TGF-β into the active form. Because TGF-β is an essential cytokine for the differentiation of Treg cells from naive T cells, colonization with these *Clostridium* species converts non-Treg cells into Treg cells locally in the colon with little effect on thymus-derived Treg cells.

Dietary metabolites regulate intestinal immunity through the epithelium

Nutritional materials also influence intestinal immunity, and commensal bacteria are involved in metabolizing indigestible dietary materials into biologically active metabolites. Dietary materials (e.g. polysaccharides, vitamins, and lipids) and their metabolites contribute to the regulation of intestinal immunity (Fig. 2).

Polysaccharides

Dietary polysaccharides and endogenous mucus in the intestine are digested and metabolized into short-chain fatty acids, such as acetate, butyrate, and propanoate, by bacterial fermentation. These short-chain fatty acids are an energy source for ECs and affect immune cell functions. For example, acetate and butyrate maintain epithelial barrier function by stimulating the release of mucin and by facilitating the maintenance of epithelial integrity [45,46]. Acetate and butyrate also regulate the proliferation of ECs and their production of cytokines [47,48]. In addition, acetate modulates the immunological function of neutrophils that express G-protein-coupled receptor 43 [GPR43, also known as free fatty acid receptor 2 (FFAR2)], a receptor for the short-chain fatty acids. Neutrophils lacking GPR43 show decreased levels of phagocytic activity and lower production of reactive oxygen species, but also are more responsive to chemoattractants such as C5a and inflammatory chemokines [49]. Consistent with these findings, intestinal inflammation is exacerbated in GPR43-deficient mice.

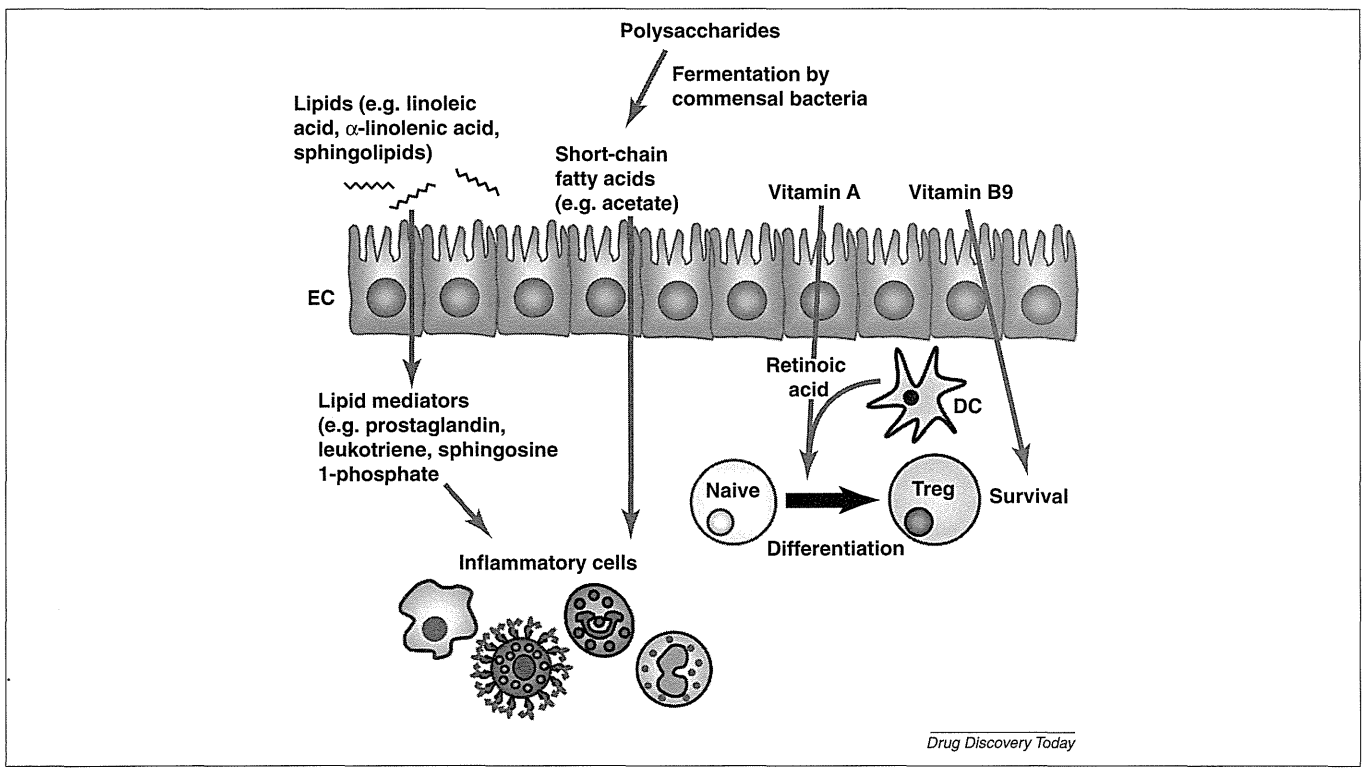

FIGURE 2

Dietary materials in the regulation of EC functions. Dietary lipids are metabolized into lipid mediators, and short-chain fatty acids are generated by fermentation of polysaccharides by commensal bacteria. These products positively or negatively regulate the functions of inflammatory cells. ECs also absorb vitamin A, and both ECs and dendritic cells (DCs) metabolize vitamin A into retinoic acid, which preferentially induces regulatory T (Treg) cells from naive T cells. The differentiated Treg cells require vitamin B9 for their survival. *Abbreviation:* EC, enterocytes.

Vitamins

Vitamins are supplied by both the diet and commensal bacteria. Several lines of evidence have shown that vitamins are involved in regulating immune responses through the epithelium. For example, retinoic acid, a metabolite of vitamin A, is involved in the preferential induction of regulatory T cells and the inhibition of Th17 cells [50]. Both ECs and DCs in the intestine are the major cell types that express retinaldehyde dehydrogenase, a key enzyme for the conversion of vitamin A into retinoic acid, suggesting that the unique gut environment mediated by ECs, DCs, and vitamin A preferentially induces Treg cells for maintaining quiescent immunity in the intestine. Because it was reported that Treg cells enhanced the differentiation of IgA^1 B cells in the intestine [51,52] and retinoic acid induced the expression of gut-homing molecules (e.g. CCR9 and $\alpha4\beta7$ integrin) on IgA-committed B cells as well as T cells [53,54], it is likely that retinoic acid directly and indirectly enhances intestinal IgA responses.

Vitamin B9 is another important vitamin in the maintenance of Treg cells. Vitamin B9 receptor (folate receptor 4) is exclusively expressed on Treg cells and can therefore be used as a cell surface marker of Treg cells [55]. We recently showed that vitamin B9 is an essential survival factor for Treg cells [56]. Indeed, Treg cells differentiate from naive T cells but fail to survive in vitamin B9-reduced conditions. Because vitamin B9 is supplied from both the diet and commensal bacteria, and dietary vitamin B9 is predominantly absorbed by ECs in the jejunum and duodenum, depletion of dietary vitamin B9 results in the reduction of Treg cells in the small intestine.

Lipids

Dietary lipids also involved in the regulation of intestinal immune responses. The ratio of omega-3 polyunsaturated fatty acids (ω-3 PUFA) to ω-6 PUFA in the diet may determine the presence and/or levels of inflammatory conditions. Dietary linoleic acid is the parent fatty acid of ω-6 PUFA which is metabolized into proinflammatory lipid mediators, whereas ω-3 PUFA, which is derived from dietary linolenic acid, is metabolized into anti-inflammatory mediators [57]. A possible molecular mechanism is that ω-3 PUFA exert anti-inflammatory effects through binding to GPR120, which is mostly expressed by macrophages, thereby inhibiting the production of inflammatory cytokines [58].

Another lipid metabolite with important immunological function is sphingosine 1-phosphate (S1P), which regulates cell trafficking, activation, and survival. Intestinal tissues contain higher levels of sphingolipids, including S1P, than other tissues and diet could be a major source of sphingolipids in the intestine, especially sphingomyelin from meat, milk, eggs, and fish [59]. Because ECs express alkaline sphingomyelinase and ceramidase to degrade dietary sphingomyelin into ceramide and sphingosine, respectively, and also express several key enzymes in the production of S1P from ceramide and sphingosine (e.g. sphingosine kinase), it is possible that ECs produce ceramide, sphingosine, and S1P for the regulation of intestinal immune responses.

Concluding remarks

ECs in the intestine have both physical and immunological barrier functions, which are achieved by immunological communication with both immunocompetent cells and gut environmental factors (e.g. commensal bacteria, dietary materials, and their metabolites). Elucidation of the complex networks established by commensal bacteria, dietary molecules, and the host immune system will provide new insights in gut environment-based mucosal immunology and should lead to new strategies to prevent and treat infectious and immune diseases in the intestine.

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Microbe-dependent CD11b$^+$ IgA$^+$ plasma cells mediate robust early-phase intestinal IgA responses in mice

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Intestinal plasma cells predominantly produce immunoglobulin (Ig) A, however, their functional diversity remains poorly characterized. Here we show that murine intestinal IgA plasma cells can be newly classified into two populations on the basis of CD11b expression, which cannot be discriminated by currently known criteria such as general plasma cell markers, B cell origin and T cell dependence. CD11b$^+$ IgA$^+$ plasma cells require the lymphoid structure of Peyer's patches, produce more IgA than CD11b$^-$ IgA$^+$ plasma cells, proliferate vigorously, and require microbial stimulation and IL-10 for their development and maintenance. These features allow CD11b$^+$ IgA$^+$ plasma cells to mediate early-phase antigen-specific intestinal IgA responses induced by oral immunization with protein antigen. These findings reveal the functional diversity of IgA$^+$ plasma cells in the murine intestine.

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Immunoglobulin (Ig) A is an antibody found predominantly in the intestinal lumen, where it protects the host against pathogenic infections[1,2]. It also has an important role in the creation and maintenance of immunological homoeostasis by shaping homeostatic communities of commensal bacteria[3–5]. Indeed, some patients with IgA deficiency show marked susceptibility to infections with pathogens such as *Giardia lamblia*, *Campylobacter*, *Clostridium*, *Salmonella* and rotavirus; they also have increased incidences of intestinal immune diseases such as coeliac disease and inflammatory bowel diseases[6].

Peyer's patches (PPs) are the major sites for the initiation of antigen-specific intestinal IgA production, mainly in a T cell-dependent manner[7]. Intestinal IgA also originates from B1 cells. B1 cells differ from B2 cells in terms of origin, surface markers (for examples, B220, IgM, IgD, CD5, CD11b and CD23), growth properties and V_H repertoire[8–10]. B1 cells are predominantly present in the peritoneal cavity (PerC) and traffic into the intestinal compartment for the production of IgA against T cell-independent antigens such as DNA and phosphatidylcholine[11]. T cell independent antigen-specific IgA responses are also initiated in the isolated lymphoid follicles (ILFs), which are small clusters of B2 cells in the intestine[12].

Upon Ig class switching from μ to α, IgA^+ B cells acquire the expression of type 1 sphingosine-1-phosphate receptor, CCR9 and α4β7 integrin, allowing them to migrate out from the PPs or PerC and traffic to the intestinal lamina propria (iLP)[11,13,14]. In the iLP, they further differentiate into IgA-secreting plasma cells (PCs) under the influence of terminal differentiation factors (for example, IL-6)[15]. As these locally produced IgA antibodies are continuously transported and secreted by epithelial cells as a form of secretory IgA into the intestinal lumen, stably high levels of IgA production are required for the maintenance of sufficient amounts of IgA; this production is determined by the generation, survival and function of IgA PCs.

Several lines of evidence have demonstrated that the function and survival of PCs in the systemic compartments (for example, spleen and bone marrow (BM)) are not only determined by intrinsic factors but are regulated by the presence of environmental niches[16]. As with systemic PCs, differentiation of IgA PCs in the iLP is regulated by exogenous factors such as IgA-enhancing cytokines (for example, interleukin (IL)-5, IL-6, IL-10, IL-15, a proliferation-inducing ligand (APRIL) and B cell activating factor (BAFF))[7,15]. In addition, microbial stimulation is required for the full effects of intestinal IgA. Indeed, germ-free (GF) mice have decreased intestinal IgA responses with immature structures of PPs and ILFs[17,18]. Previous studies in mono-associated GF mice have indicated that only a small proportion of the total amount of intestinal IgA is reactive to monoassociated bacteria; microbe-dependent IgA production is therefore mediated by polyclonal stimulation through innate immune receptors such as toll-like receptors, rather than through B cell receptors specific for microbial antigens[19,20]. Accumulating evidence has revealed the molecular and cellular pathways of IgA production mediated by innate immunity, including the involvement of myeloid differentiation primary response gene 88 (MyD88) in the regulation of tumour necrosis factor/inducible nitric oxide synthase-producing DCs in the iLP[21] and follicular DCs in the PPs[22]. However, the effects of microbial stimulation on the regulation of differentiated IgA^+ PCs remain to be investigated. Here, we identified unique microbe-dependent subsets of IgA^+ PCs, which add a new level of complexity to the intestinal IgA system of mice.

Results

Microbe dependency of intestinal IgA^+ cells. To examine the immunological elements of intestinal IgA production associated with commensal bacteria, we initially compared the IgA^+ cells of specific pathogen-free (SPF) and GF mice. Flow cytometric analysis showed that $CD11b^+$ IgA^+ cells accounted for about 30% of IgA^+ cells, and we found a lack of $CD11b^+$ IgA^+ cells in the iLP of GF mice (Fig. 1a). Similarly, the numbers of intestinal $CD11b^+$ IgA^+ cells were reduced in both antibiotic-treated SPF mice and MyD88 KO mice (Fig. 1b–d). Immunohistological analysis indicated that $CD11b^+$ IgA^+ cells were dispersed throughout the iLP of wild-type (WT) mice (Fig. 1d), although their frequency appeared lower than expected from the flow cytometric data, probably because of difference in methodological sensitivity. These findings collectively suggest that $CD11b^+$ IgA^+ cells are unique subset that requires MyD88-dependent microbial stimulation for its development and maintenance.

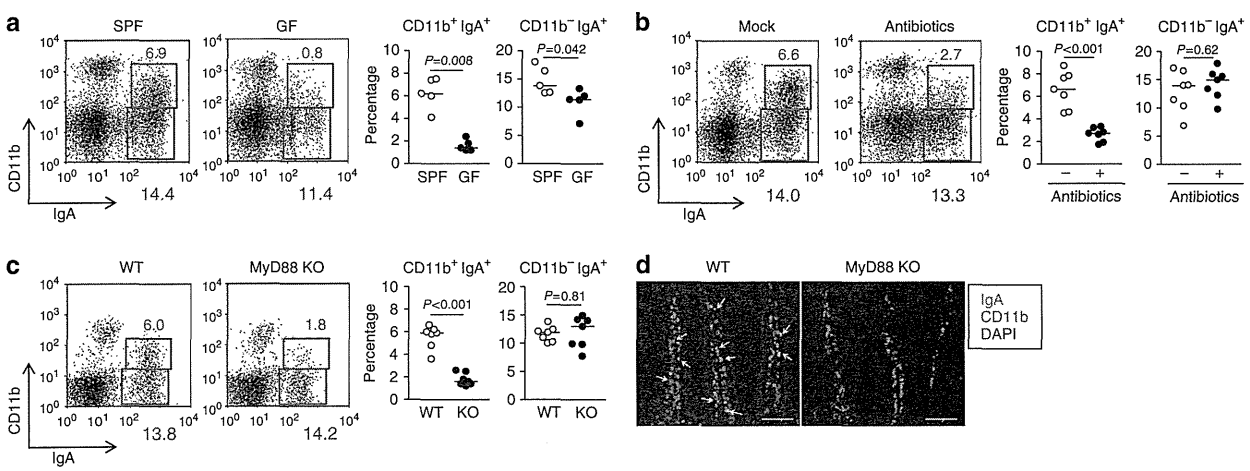

Figure 1 | Intestinal $CD11b^+$ IgA^+ cells require microbial stimulation. (**a–c**) Mononuclear cells were isolated from the small intestines of SPF or GF mice (**a**), mock- or antibiotic-treated SPF mice (**b**), or MyD88 WT or knockout (KO) mice (**c**) for analysis of IgA and CD11b expression by flow cytometry. Graphs show data from individual mice, and bars indicate median. Statistical analyses were performed with Mann–Whitney's *U*-test. (**d**) Specimens of small intestinal tissues of WT and MyD88 KO mice were stained for IgA and CD11b, and counterstained with 4′,6-diamidino-2-phenylindole. Data are representative of three independent experiments. Scale bars, 50 μm.

Intestinal CD11b$^+$ IgA$^+$ cells are PCs. We next aimed to characterize the CD11b$^+$ and CD11b$^-$ IgA$^+$ cells in the iLP. In addition to a gating strategy to exclude the possibility that the CD11b$^+$ IgA$^+$ cells detected by flow cytometry were doublets (Supplementary Fig. S1), we further performed a cytospin analysis and confirmed that both CD11b$^+$ and CD11b$^-$ IgA$^+$ cells had homogeneous morphology that was the same as that of PCs (for example, large irregular nuclei with prominent nucleoli), whereas CD11b^{hi} IgA$^-$ cells were composed of different kinds of cells, including eosinophils and macrophages (Fig. 2a). We also confirmed that both CD11b$^+$ and CD11b$^-$ IgA$^+$ cells did not express markers for macrophages (F4/80), DCs (CD11c) or eosinophils (CCR3) (Fig. 2b). Thus, CD11b$^+$ IgA$^+$ cells are neither doublets nor myeloid cells decorated by bound IgA on their surfaces.

CD11b$^+$ and CD11b$^-$ IgA$^+$ cells were identical in cell size and density, as determined by forward scatter (FSC) and side scatter (SSC), respectively, and by their surface expression patterns (CD19^{int}, B220$^-$, CD138$^+$, CD38^{hi} and CD40^{int}) (Fig. 2c). Although PCs in the systemic compartments (for example, the spleen) generally express little or no surface immunogloblin[23], we previously confirmed that CD38$^+$ CD138$^+$ cells in the iLP express IgA both on the cell surface and in the intracellular compartment (Supplementary Fig. S2)[13]. These findings indicated that both CD11b$^+$ and CD11b$^-$ IgA$^+$ cells could be classically categorized as PCs. This view was further supported by our finding that both populations expressed equal levels of Blimp1, a master transcription factor for PCs (Fig. 2c)[23].

The phenotypes of IgA$^+$ cells in the iLP differed from those of IgA$^+$ cells in the spleen. Splenic CD11b$^-$ IgA$^+$ cells exclusively had a memory phenotype (that is, B220$^+$, CD138$^-$, CD38^{int} and CD40^{hi}), whereas splenic CD11b$^+$ IgA$^+$ cells contained almost equal amounts of B220$^+$ CD138$^-$ CD38^{int} CD40^{hi} memory cells and B220$^-$ CD138$^+$ CD38^{hi} CD40^{low} PCs (Supplementary Fig. S3). These results indicated that CD11b$^+$ IgA$^+$ cells in the iLP were unique PCs that had an immunologically different status from splenic CD11b$^+$ IgA$^+$ cells.

Intestinal CD11b$^+$ IgA$^+$ PCs require PP lymphoid structure. CD11b$^+$ IgA$^+$ PCs expressed CD18 (Supplementary Fig. S4), which associates with CD11b and acts as a ligand for intercellular adhesion molecule-1 (ICAM-1)[24]. As ICAM-1 is an endothelial adhesion molecule that regulates cell trafficking[24,25], we considered that CD11b$^+$ IgA$^+$ PCs were recent emigrants from IgA-inductive tissues (for example, PPs and PerC) and had migrated into the iLP. To test this possibility, we employed FTY720 to inhibit the trafficking of IgA-committed B cells from PPs and PerC into the iLP. As we previously reported[11,13], FTY720 treatment reduced the numbers of intestinal IgA$^+$ PCs,

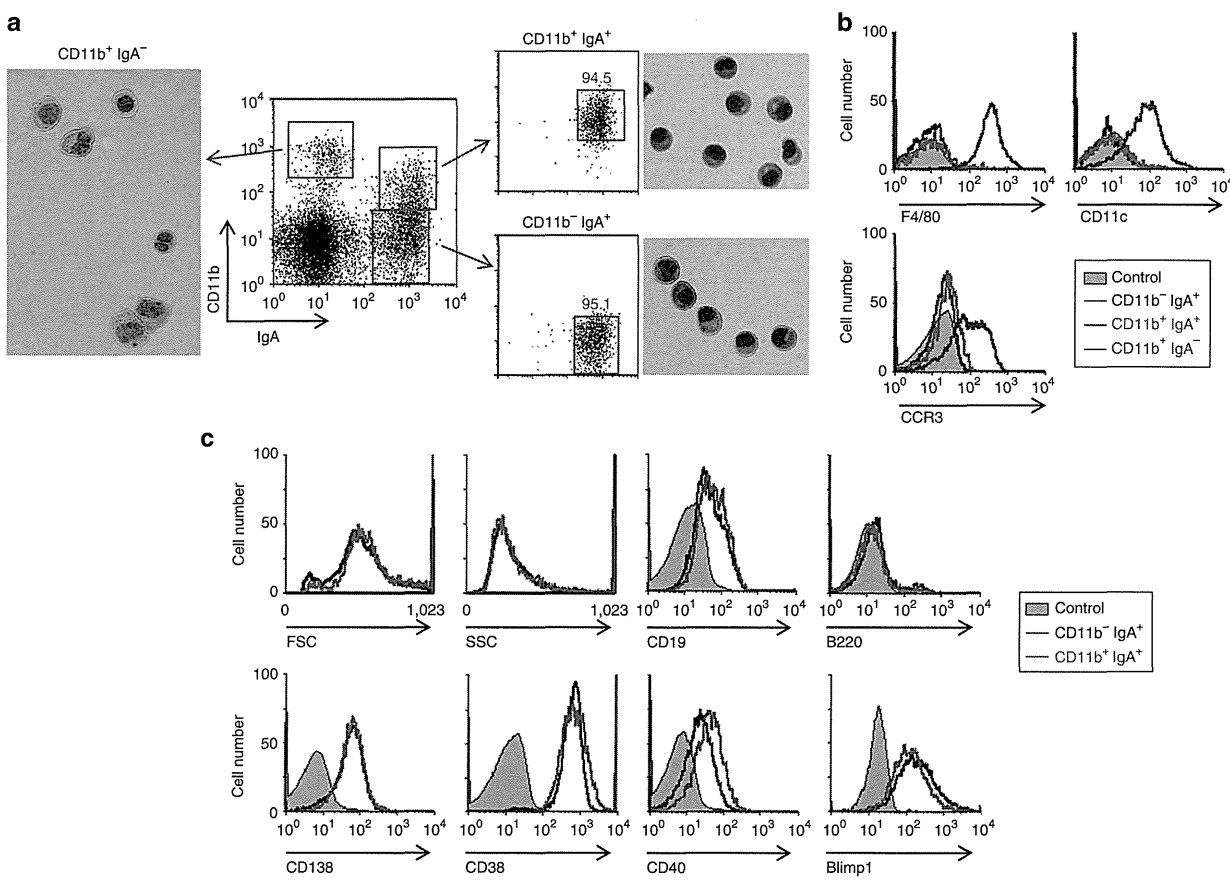

Figure 2 | Both CD11b$^+$ and CD11b$^-$ IgA$^+$ cells in the intestine are categorized as plasma cells. (**a**) Cells were purified by cell sorting from the iLP, and their morphology was examined by haematoxylin and eosin staining after cytospin. Data are representative of three independent experiments. (**b**) Cells were isolated from the iLP for the analysis of F4/80, CD11c and CCR3 expression on CD11b$^-$ IgA$^+$, CD11b$^+$ IgA$^+$ and CD11b$^+$ IgA$^-$ cells. Grey indicates isotype control. Similar results were obtained from three separate experiments. (**c**) Cells were isolated from the iLP for comparisons between CD11b$^+$ and CD11b$^-$ IgA$^+$ cells in terms of cell size (FSC) and density (SSC), and expression of CD19, B220, CD138, CD38, CD40 and Blimp1. Grey indicates isotype control. Similar results were obtained from five separate experiments.

but the effect was not specific to CD11b$^+$ IgA$^+$ PCs (Fig. 3a). These data suggested that CD11b$^+$ IgA$^+$ PCs were not recent emigrants from IgA inductive tissues (for example, PPs and PerC).

The second possibility was that CD11b$^+$ IgA$^+$ PCs originated from B1 cells, because CD11b is a marker of peritoneal B1 cells[26]. To test this possibility, peritoneal CD11b$^+$ B1 cells were purified and adoptively transferred into severe combined immunodeficiency mice. As we reported previously[11], adoptively transferred CD11b$^+$ B1 cells migrated into the intestine, where they differentiated into IgA$^+$ PCs. Although we transferred B cells expressing CD11b, they lost their CD11b expression in the iLP (Supplementary Fig. S5). Although only a few cells were detected in the iLP under these experimental conditions, CD11b expression was likely to be reversible on B cells and was thus not to be a marker of PCs originating from peritoneal CD11b$^+$ B1 cells.

As a third possibility for discriminating between CD11b$^+$ and CD11b$^-$ IgA$^+$ PCs, we examined the T cell dependency of their differentiation and IgA production. For this, we employed TCRβδ mice. Although TCRβ δ mice had decreased levels of intestinal IgA$^+$ cells, the ratio between CD11b$^+$ and CD11b$^-$ IgA$^+$ PCs did not differ between the WT mice and the TCRβ δ mice (Fig. 3b).

We also examined the production of IgA against T cell dependent and T cell independent antigens by CD11b$^+$ and CD11b$^-$ IgA$^+$ PCs. For the analysis of T cell dependent antigen, mice were orally immunized with ovalbumin (OVA) plus cholera toxin (CT). Following three oral immunizations, substantial amounts of OVA-specific IgA antibody-forming cells (AFCs) were detected in the iLP by enzyme-linked immunosorbent spot (ELISPOT) assay; this production was reduced by almost 50% when either the CD11b$^+$ IgA$^+$ or the CD11b$^-$ IgA$^+$ cells were removed before the ELISPOT assay (Fig. 3c). Similar results were obtained when we enumerated IgA AFCs against phosphorylcholine, a typical TI antigen, induced by commensal bacteria (Fig. 3c)[27]. These results collectively suggested that both CD11b$^+$ IgA$^+$ and CD11b$^-$ IgA$^+$ cells almost equally included IgA AFCs producing IgA antibodies specific for T cell dependent and T cell independent antigens.

Next, to examine the involvement of PPs, we established PP-null mice by *in utero* treatment with anti-IL-7Rα antibody[28] and found that PP-null mice had reduced numbers of CD11b$^+$ IgA$^+$ PCs in the iLP (Fig. 3d). In addition, CD11b was not expressed on IgA$^+$ B cells in the PPs (Fig. 3e). We treated mice with anti-IL-7Rα antibody only once *in utero* and confirmed that it did not affect the ILFs[28]. Although it is still possible that CD11b$^+$ IgA$^+$ PCs specifically require IL-7, the most plausible conclusion based on our current findings is that CD11b$^+$ IgA$^+$ B cells require the lymphoid structure of PPs, and CD11b$^-$ IgA$^+$ B cells acquire CD11b expression in the iLP.

As in antibiotic-treated and MyD88 KO mice (Fig. 1), the numbers of CD11b$^-$ IgA$^+$ PCs changed little in PP-null mice (Fig. 3d), suggesting that it is unlikely that CD11b$^+$ IgA$^+$ PCs differentiate back into CD11b$^-$ IgA$^+$ cells in the iLP. This view is further supported by the results of *in vitro* analysis. When purified CD11b$^+$ and CD11b$^-$ IgA$^+$ PCs were separately cultured with different kinds of stimulants (for example, phorbol 12-myristate 13-acetate plus ionomycin, or lipopolysaccharide) little change was noted in CD11b expression (Supplementary Fig. S6). Although the origin of these cells remains to be firmly established, it is plausible that CD11b$^+$ IgA$^+$ PCs act as a separate lineage once they differentiate in the iLP.

High proliferation activity of CD11b$^+$ IgA$^+$ PCs. We next performed a gene microarray analysis to assess the uniqueness of CD11b$^+$ IgA$^+$ PCs in the iLP. Gene ontology enrich-

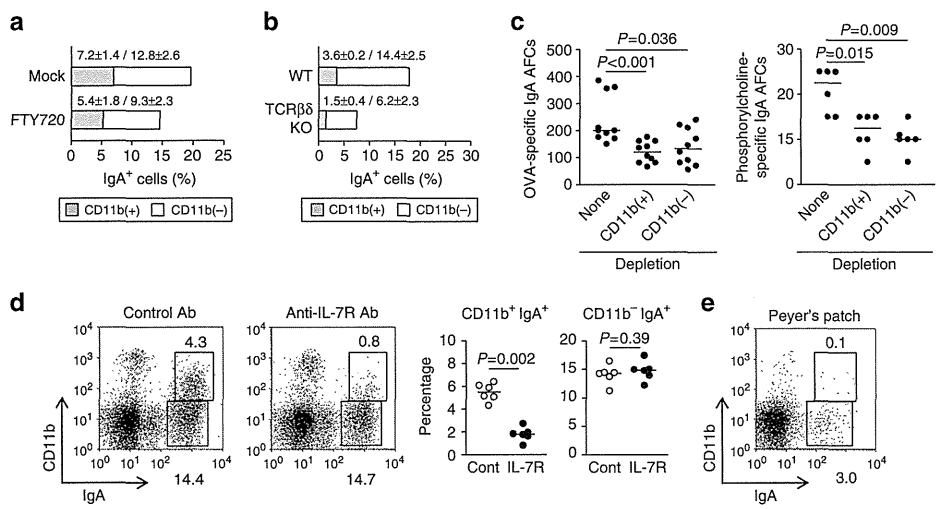

Figure 3 | CD11b$^+$ IgA$^+$ cells require the lymphoid structure of Peyer's patches. (**a**) Mice were treated with FTY720 every day for 5 days. The day after the final treatment, the proportions of CD11b$^+$ and CD11b$^-$ IgA$^+$ cells were measured by flow cytometry. Data are presented as means ± s.d. from four mice. Similar results were obtained from three separate experiments. (**b**) Proportions of CD11b$^+$ and CD11b$^-$ IgA$^+$ cells in the iLP of WT and TCRβδ KO mice were measured by flow cytometry. Data are presented as means ± s.d. from four mice. Similar results were obtained from three separate experiments. (**c**) After three oral immunizations with OVA plus cholera toxin, cells were isolated from the iLP and used in an ELISPOT assay to enumerate OVA-specific IgA AFCs. In some groups of mice, CD11b$^+$ or CD11b$^-$ IgA$^+$ cells were depleted by cell sorting before application of ELISPOT assay. Phosphorylcholine-specific IgA AFCs were measured. Graphs show data from individual mice, and bars indicate median. Statistical analyses were performed with Mann-Whitney's *U*-test. (**d**) Mononuclear cells were isolated from the iLP of Peyer's patch (PP)-normal (control Ab) and -null (anti-IL-7Rα Ab) mice for analysis of IgA and CD11b expression by flow cytometry. Graphs show data from individual mice. Statistical analyses were performed with Mann-Whitney's *U*-test. (**e**) Mononuclear cells were isolated from PPs for analysis of CD11b$^+$ and CD11b$^-$ IgA$^+$ cells by flow cytometry. Similar results were obtained from three separate experiments.

ment score computation analysis showed that the activity of cell-cycle-associated pathways was higher in CD11b$^+$ IgA$^+$ PCs than in CD11b$^-$ IgA$^+$ PCs (Supplementary Table S1). Consistent with this finding, higher expression of cell-cycle-associated genes was noted in CD11b$^+$ IgA$^+$ PCs than in CD11b$^-$ IgA$^+$ PCs; these genes included members of the cell division cycle family (Fig. 4a and Supplementary Table S2). In line with this, these cells expressed higher levels of the proliferation marker Ki67 than did CD11b$^-$ IgA$^+$ PCs (Fig. 4a and Supplementary Table S2). Additionally, CD11b$^+$ IgA$^+$ PCs showed greater uptake of bromodeoxyuridine (BrdU) than did CD11b$^-$ IgA$^+$ PCs (Fig. 4b). CD11b$^+$ IgA$^+$ PCs were preferentially removed by treatment with cyclophosphamide (CPM), which selectively targets proliferating cells (Fig. 4c). These data collectively suggested that CD11b$^+$ IgA$^+$ PCs possessed greater proliferating activity than did CD11b$^-$ IgA$^+$ PCs in the iLP.

Microarray analysis further identified CD150 (also known as signalling lymphocytic activation molecule family member 1, SLAMF1)[29], β1 integrin and CD168 (also known as hyaluronan-mediated motility receptor)[30] as possible candidates uniquely expressed on CD11b$^+$ IgA$^+$ PCs (Supplementary Table S3). Flow cytometric analysis confirmed that CD11b$^+$ IgA$^+$ PCs expressed higher levels of CD150 than did CD11b$^-$ IgA$^+$ PCs, whereas CD11b$^+$ IgA$^+$ and CD11b$^-$ IgA$^+$ PCs identically expressed β1 integrin and no CD168 (Supplementary Fig. S7).

IL-10 is essential for intestinal CD11b$^+$ IgA$^+$ cells. We next aimed to identify key molecules for inducing and maintaining CD11b$^+$ IgA$^+$ PCs in the iLP. As CD11b$^+$ IgA$^+$ PC numbers were reduced in MyD88 mice (Fig. 1c), and MyD88 is expressed in not only hematopoietic cells, including B cells, but also non-hematopoietic cells, including epithelial cells[31], we performed BM chimeric experiments to determine whether MyD88 in non-hematopoietic cells, hematopoietic cells, or both, was required for the generation of CD11b$^+$ IgA$^+$ cells. Similar levels of CD11b$^+$ IgA$^+$ cells were observed in irradiated WT mice receiving WT or MyD88 BM cells and in irradiated MyD88 mice receiving WT BM cells (Supplementary Fig. S8), suggesting that MyD88-dependent molecules commonly expressed in both non-hematopoietic and hematopoietic cells are involved in the microbe-dependent induction of CD11b$^+$ IgA$^+$ PCs.

We then examined the involvement of cytokines known to enhance IgA responses. Among several IgA-enhancing cytokines (for example, IL-5, IL-6, IL-10 and APRIL/BAFF)[7,15], we found that neutralization of IL-10 resulted in preferential reduction in CD11b$^+$ IgA$^+$ PCs, whereas blocking of other cytokines induced a reduction in IgA$^+$ cell numbers regardless of CD11b expression (Fig. 5a). Additionally, CD11b$^+$ IgA$^+$ cell numbers were preferentially reduced in IL-10 KO mice (Fig. 5b). As normal differentiation into IgA$^+$ B cells was observed in the PPs and PerC of IL-10 KO mice (Supplementary Fig. S9), it is plausible that IL-10 targets the maintenance of CD11b$^+$ IgA$^+$ cells in the iLP, but not the induction of IgA$^+$ cells in inductive tissues such as PPs and PerC.

Early-phase robust IgA responses by proliferating IgA$^+$ PCs. To examine the immunological importance of proliferating IgA$^+$ PCs present mainly in CD11b$^+$ IgA$^+$ PCs, mice were orally immunized with OVA plus CT. In this assay, one group received CPM treatment during immunization and the second group received CPM treatment 4 days after the final immunization (Fig. 6a). Because of the high cell-proliferation activity, CPM treatment during oral immunization resulted in efficient killing of peanut agglutinin (PNA^{hi}) B220$^+$ GC B cells and thus a reduction in the numbers of IgA$^+$ IgM$^-$ plasmablasts in the PPs (Supplementary Fig. S10). Thus, treatment with CPM during oral immunization led to an ~90% reduction in the numbers of OVA-specific IgA AFCs (Fig. 6b); this was associated with almost complete disappearance of faecal IgA produced against OVA (Fig. 6c). On the other hand, when mice were treated with CPM 4 days after the final immunization to remove proliferating cells mainly present in CD11b$^+$ IgA$^+$ cells in the iLP, the reduction in numbers of OVA-specific IgA AFCs in the iLP was only about 50% (Fig. 6b). This finding was consistent with our current finding that CD11b$^+$ IgA$^+$ PCs accounted for half the number of OVA-specific IgA AFCs (Fig. 3c). Thus, CPM treatment after the last immunization preferentially depleted CD11b$^+$ IgA$^+$ cells, with little influence on CD11b$^-$

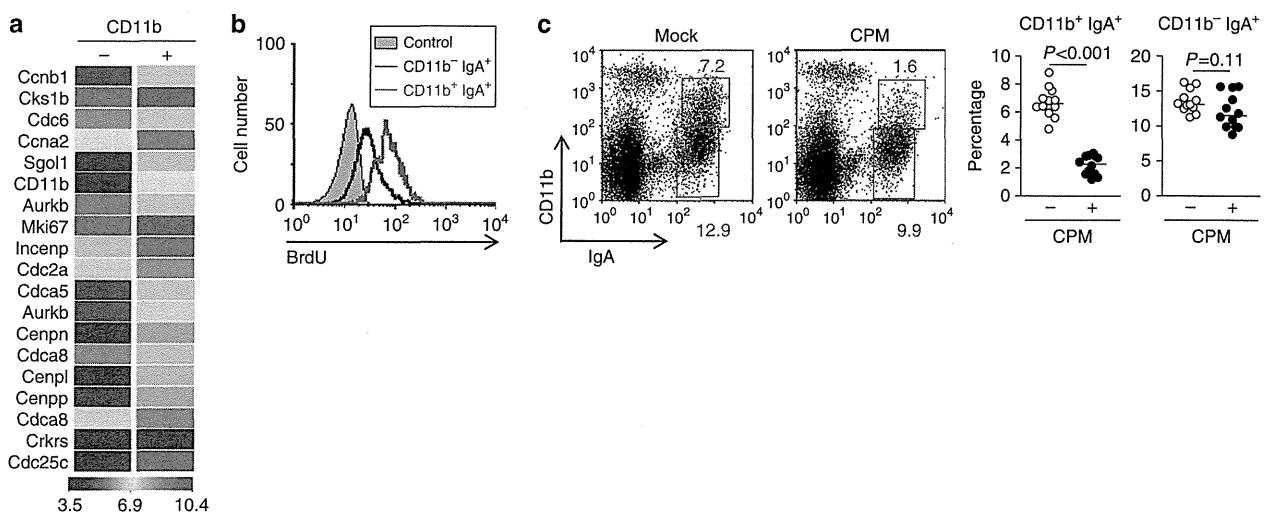

Figure 4 | CD11b$^+$ IgA$^+$ cells are proliferating cells. (**a**) mRNA was purified from small intestinal CD11b$^+$ and CD11b$^-$ IgA$^+$ cells and used for microarray analysis. Data related to the cell cycle and proliferation are shown. Data are representative of two independent experiments. (**b**) Mice were treated with BrdU, and uptake of BrdU by CD11b$^+$ and CD11b$^-$ IgA$^+$ cells was determined by flow cytometry. Data are representative of four independent experiments. (**c**) Cells were isolated from the intestinal lamina propria of mice receiving CPM to analyse CD11b$^+$ IgA$^+$ cells. Similar results were obtained from four separate experiments. Graphs show data from individual mice. Statistical analyses were performed with Mann–Whitney's *U*-test.

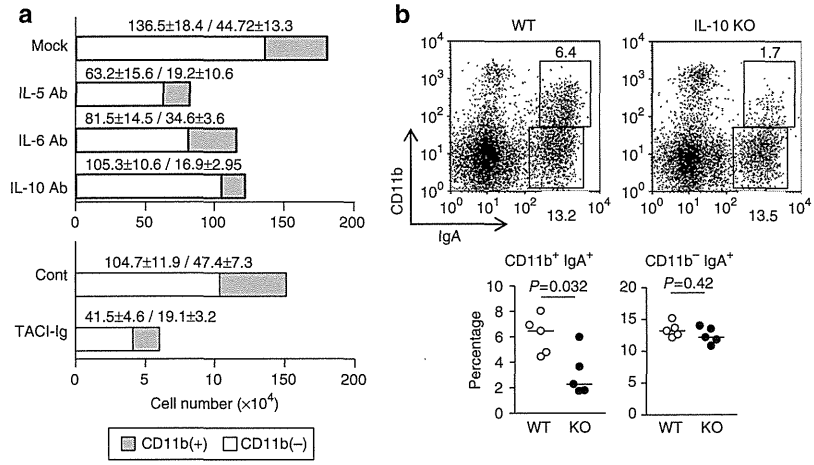

Figure 5 | Role of IL-10 in the maintenance of CD11b$^+$ IgA$^+$ cells in the iLP. (**a**) Mice were treated with antibodies to block IL-5, IL-6, IL-10 or antagonistic TACI-immunoglobulin (TACI-Ig) fusion protein. Mononuclear cells were isolated from the iLP and used for analysis of CD11b$^+$ and CD11b$^-$ IgA$^+$ cells by flow cytometry. Data are presented as means ± s.d. ($n=4$). (**b**) Mononuclear cells were isolated from the iLP of WT or IL-10 KO mice for analysis of IgA and CD11b expression by flow cytometry. Graphs show data from individual mice. Statistical analyses were performed with Mann–Whitney's *U*-test.

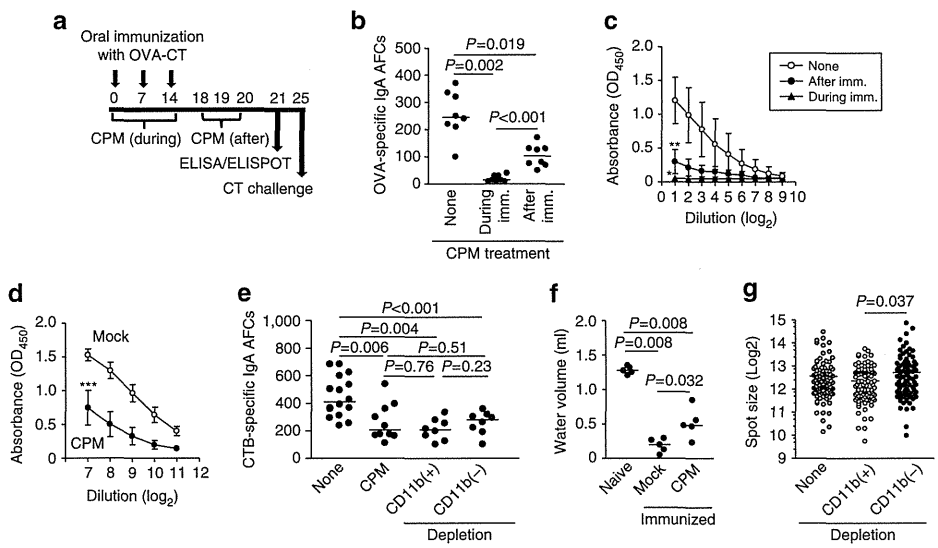

Figure 6 | Proliferating IgA$^+$ cells mediate early-phase IgA responses to oral antigen. (**a**) Experimental schedule for oral immunization and CPM treatment. Mice were orally immunized with OVA plus CT on days 0, 7 and 14. One group received CPM during oral immunization (days 0, 7 and 14) and another received CPM after the last immunization (days 18, 19 and 20). (**b**,**c**) One week after the final immunization (day 21), mononuclear cells were isolated from the iLP to quantify OVA-specific IgA-forming cells by ELISPOT (**b**). Simultaneously, faeces (**c**,**d**) were collected and were used for the detection of the (**c**) OVA-or (**d**) B subunit of CT (CTB)-specific IgA by enzyme-linked immunosorbent assay. Data are from individual mice and bars indicate median (**b**) and represent means ± s.d. ($n=10$) from two separate experiments (**c**,**d**). *$P<0.001$, **$P<0.01$, ***$P<0.05$ (two tailed unpaired *t*-test). (**e**) Mononuclear cells were isolated from the iLP of mock- or CPM-treated mice 1 week after the final immunization to quantify CTB-specific IgA-forming cells by ELISPOT. In some groups of mock-treated mice, CD11b$^+$ or CD11b$^-$ IgA$^+$ cells were depleted by cell sorting before application of ELISPOT assay. Graphs show data from individual mice, and bars indicate median. (**f**) On day 21, mice were orally challenged with 100 μg CT. After 15 h, the volume of intestinal fluid was measured. Graphs show data from individual mice, and bars indicate median. Similar results were obtained from two separate experiments. (**g**) Spot sizes of CTB-specific IgA AFCs were measured by Zeiss KS ELISPOT software. Graphs show data from individual mice, and bars indicate median. Statistical analyses were performed with Mann–Whitney's *U*-test (**e**–**g**).

IgA$^+$ cells. Of note, these mice showed ~90% reduction in OVA-specific IgA content in the faeces compared with mice not treated with CPM (Fig. 6c). We also confirmed that CPM treatment 4 days after final immunization induced a reduction in the production of IgA specific to the B subunit of CT (that is, CTB), which was associated with the halving of the abundance of CTB-specific IgA AFCs in the intestine (Fig. 6d,e). Like OVA-specific IgA responses (Figs. 3c and 6b), similar levels of reduction of CTB-specific IgA AFCs were noted when CD11b$^+$ IgA$^+$ cells were depleted before ELISPOT assay (Fig. 6e). These mice showed reduced resistance to oral challenge with CT and developed watery diarrhoea (Fig. 6f and Supplementary Fig. S11).

These findings led us to hypothesize that CD11b$^+$ IgA$^+$ PCs are capable of producing more IgA than are CD11b$^-$ IgA$^+$ PCs. To test this hypothesis, we measured the size of each spot in CTB-specific IgA AFCs in an ELISPOT assay. The cells in the CD11b$^+$ IgA$^+$ cell-enriched fraction (depletion of CD11b$^-$ IgA$^+$ cells) were bigger than those in the CD11b$^-$ IgA$^+$ cell-enriched fraction (depletion of CD11b$^+$ IgA$^+$ cells) (Fig. 6g). Furthermore, an adoptive transfer experiment demonstrated higher intestinal IgA production in severe combined immunodeficiency mice receiving CD11b$^+$ IgA$^+$ PCs than in those receiving CD11b$^-$ IgA$^+$ PCs (Supplementary Fig. S12), presumably because of both high IgA production and proliferating activity of CD11b$^+$ IgA$^+$ PCs. Although some possibilities (for example, proliferation and CD11b expression of IgA$^+$ cells might be changed during immunization) cannot be excluded, it is plausible that the actual production of IgA secreted into the intestinal lumen was derived mainly from CD11b$^+$ IgA$^+$ PCs in the early phase of the IgA response against orally immunized antigen.

Discussion

PCs could secrete antibodies to provide antigen-specific humoral immune responses in both systemic and mucosal tissues. Here, we demonstrated that intestinal IgA$^+$ PCs in mice could be categorized into two populations on the basis of CD11b expression. CD11b is an integrin αM that non-covalently associates with CD18 to form αMβ2 integrin (Mac-1) and binds to ICAM-1 (ref. 24). We therefore expected that CD11b$^+$ IgA$^+$ PCs were newly migrating cells whose migration was mediated by endothelial cells expressing ICAM-1, but in fact they were not. We also found no uptake of opsonized bacteria in either CD11b$^+$ or CD11b$^-$ IgA$^+$ cells (Supplementary Table S4 and Supplementary Fig. S13a), although CD11b is a receptor for complement (iC3b)[24]. In addition, unlike in human CD11b$^+$ B cells, which stimulate T cells strongly[32], major histocompatibility complex (MHC) class II (I-A^d) and costimulatory molecules (for example, CD80) were identically expressed on both CD11b$^+$ and CD11b$^-$ IgA$^+$ cells (Supplementary Table S4 and Supplementary Fig. S13b).

A similar subset of CD11b$^+$ IgA$^+$ cells was observed in the systemic murine compartments (for example, spleen), but the immunological characteristics of these cells differed from those of the cells in the intestine. Indeed, intestinal CD11b$^+$ IgA$^+$ cells consisted exclusively of PCs, but not memory B cells, whereas splenic CD11b$^+$ IgA$^+$ cells included both PCs and memory B cells. We further found that CD11b could not be used as a marker of B1 cells in the intestine. Our current findings show for the first time that CD11b could be a specific marker for discriminating IgA$^+$ PCs that require microbial stimulation and IL-10, and presumably contribute to the early phase of the intestinal IgA response in mice.

We have identified unique CD11b$^+$ IgA$^+$ PCs in mice; the next question is whether or not the same population of IgA$^+$ PCs exists in humans. Our preliminary experiments have shown that no human intestinal IgA$^+$ cells express CD11b, but that some IgA$^+$ cells express Ki67, a marker of proliferating cells (unpublished data). One possible explanation for this difference between human and mice is difference in the composition of commensal bacteria. In this regard, we examined the involvement of segmented filamentous bacteria (SFB), which are a known major IgA stimulus in mice, but has not yet been confirmed as part of the human microbiota[19]. As expected, SFB stimulated IgA production following colonization of SFB-deficient C57BL/6 mice from the Jackson laboratory (JAX mice) with bacterial suspensions from SFB-monoassociated mice (JAX + SFB mice)[33]; however, we found that CD11b is expressed on IgA$^+$ cells independently of SFB colonization (Supplementary Fig. S14). It is possible that other commensal bacteria such as *Lactobacillus* (abundant in mice) and *Bifidobacterium* (abundant in human) are responsible for the species-specific expression of CD11b on IgA$^+$ cells. It is important to recognize the differences between the mouse and human immune systems, but it is obvious that proliferating IgA$^+$ cells are present in the iLP of both mouse and human. The immunological function of human proliferating IgA$^+$ cells in the intestine will therefore be the subject of our next study.

In the initial step of the antibody response to T cell dependent antigens, B cells are activated by antigens and form GCs in the lymph nodes[7]. As depleting antigen-specific GC B cells by CPM treatment during oral immunization resulted in complete loss of the IgA response to orally immunized antigen, it is likely that both CD11b$^+$ and CD11b$^-$ IgA$^+$ PCs against T cell dependent antigen are derived from GC B cells. We also found that depletion of proliferating CD11b$^+$ IgA$^+$ PCs by CPM treatment after final immunization led to a decrease in the early-phase IgA response, although it is possible that proliferation activity and/or CD11b expression on IgA$^+$ cells might be wobble during immunization. Our *in vivo* findings indicated that the reduction in CD11b$^+$ IgA$^+$ PC numbers in MyD88 KO, IL-10 KO and PP-null mice did not affect the numbers of CD11b$^-$ IgA$^+$ PCs (Figs 1c, 3d and 5b). These findings, together with our *in vitro* data (Supplementary Fig. S6), indicate that it is likely that CD11b$^+$ IgA$^+$ PCs act as a separate lineage once they differentiate in the iLP.

Proliferating CD11b$^+$ IgA$^+$ PCs required microbial stimulation in the intestine. As proliferation is one of the characteristics of plasmablasts, it was possible that CD11b$^+$ IgA$^+$ cells have been recently committed to the PC fate. Notably, intestinal IgA$^+$ cells expressed MHC class II molecules; this expression is one of the unique characteristics of plasmablasts. Therefore, it is likely that intestinal IgA$^+$ PCs partly retain their plasmablast features. However, our findings indicated that CD11b$^+$ and CD11b$^-$ IgA$^+$ cells expressed identical levels of Blimp-1 and MHC class II. In addition, similar reduction was noted in CD11b$^+$ and CD11b$^-$ IgA$^+$ cells when cell trafficking from IgA inductive tissues (for example, PPs and the PerC) into the iLP was inhibited by treatment with FTY720. Thus, our findings suggest that CD11b$^+$ IgA$^+$ cells uniquely exhibit high proliferating and IgA-producing activity, although their other immunological features as PCs are similar to those of CD11b$^-$ IgA$^+$ PCs.

Proliferating CD138$^+$ PCs have been detected in the spleens of NZB/W mice with signs of systemic lupus erythematosus, but not in naive mice[34]. In contrast, the number of non-proliferating CD138$^+$ PCs is unchanged in the intestines of GF mice, as it is in the spleens of NZB/W mice[34]. These findings suggest that MyD88-dependent homeostatic stimulation of commensal bacteria determines the fate of proliferating CD11b$^+$ IgA$^+$ CD138$^+$ PCs in the intestine. Several lines of evidence have revealed the cellular and molecular mechanisms of microbe-dependent initiation of IgA responses. B cells express several toll-like receptors, and B cell-intrinsic MyD88-mediated signalling has been implicated in enhanced antibody production in some studies[35,36]. However, our current findings indicated that MyD88-mediated signalling in hematopoietic cells, including B cells, was not essential for intestinal CD11b$^+$ IgA$^+$ PC production. Additionally, we found IL-10 as a key molecule inducing CD11b$^+$ IgA$^+$ PC production. Previous studies have demonstrated that IL-10 promotes the proliferation of activated B cells and subsequent IgA production *in vitro*[37,38], which are consistent with our current findings of high-level proliferation of, and IgA production by, CD11b$^+$ IgA$^+$ PCs. Thus, our current findings proved that IL-10 functions in IgA production *in vivo* and that CD11b$^+$ IgA$^+$ PCs are the main targets in this

pathway. Despite these findings, our preliminary study demonstrated that treatment of CD11b$^+$ or CD11b$^-$ IgA$^+$ PCs with IL-10 alone did not induce their reciprocal differentiation into each other, and IL-10 KO mice with colitis possessed CD11b$^+$ IgA$^+$ PCs (J.K., unpublished data). Thus, IL-10 is redundant in some cases and additional factors are required for the maintenance of CD11b$^+$ IgA$^+$ PCs. Our current findings identified CD150 as a surface molecule that is highly expressed on CD11b$^+$ IgA$^+$ PCs. CD150 is a 70-kDa glycoprotein expressed on some B and T cells, thymocytes and macrophages[29]. Homophilic interaction of CD150 induces proliferation of, and antibody synthesis by, B cells[39], and notably IL-10 synergistically enhances CD150-mediated B cell proliferation[39]. Thus, it is likely that, at least partly, IL-10 and CD150 determine the unique features (for example, proliferation and high IgA production) of CD11b$^+$ IgA$^+$ PCs in the iLP. In addition, accumulating evidence has revealed an important immunological function of stromal cells as survival niches for PCs in the BM[40] and intestine[41,42]. It is possible that complex immunological communications among commensal flora, epithelial and stromal cells, and the cells involved in innate and acquired immunity determine the differentiation and maintenance of IgA PCs in the intestine.

Taken together, our results provide new insights into the nature of IgA$^+$ PCs in the murine intestine, and especially into the regulation of the early-phase IgA responses to intestinal antigens and requirement of microbe-dependent stimulation, IL-10, and the PP lymphoid structure. These findings add a new level of complexity to the intestinal IgA system of mice.

Methods

Mice. SPF and GF Balb/c mice were obtained from Japan CLEA (Tokyo, Japan). MyD88 KO mice, IL-10 mice (Balb/c background) and TCR$\beta\delta$ mice (C57/BL6 background) were maintained under SPF conditions at the Experimental Animal Facility, The Institute of Medical Science, The University of Tokyo, and WT littermates were used as controls. To deplete gut commensal bacteria, mice received broad-spectrum antibiotics, namely ampicillin (1 g l^{-1}; Sigma-Aldrich, St Louis, MO), vancomycin (500 mg l^{-1}; Shionogi, Osaka, Japan), neomycin sulphate (1 g l^{-1}; Sigma-Aldrich) and metronidazole (1 g l^{-1}; Sigma-Aldrich), in their drinking water for 4 weeks[43]. To establish BM chimeric mice, we injected γ-irradiated (960 rad, Gammacell 40, Atomic Energy of Canada Limited, Ontario, Canada) recipient mice with 5×10^6 BM cells through the tail vein and used them in experiments 8 weeks after injection. Under our experimental conditions, the reconstitution efficacy was about 90–95%. To obtain PP-null mice, pregnant BALB/c mice were injected intravenously and subcutaneously with 1 mg anti-IL-7Rα antibody (A7R34, BioLegend, San Diego, CA) at 14.5 days post coitus, as described previously[28]. We confirmed the disruption of organized PPs and the existence of ILFs in the offspring, as described previously[28]. To neutralize cytokines, mice were treated intraperitoneally with 250 µg of monoclonal antibodies specific for IL-5 (TRFK5), IL-6 receptor (D7715A7) or IL-10 (JES5.16E3) (BioLegend, San Diego, CA); control antibody (Rat IgG2b); or 100 µg of soluble TACI-Fc fusion protein (R&D Systems, Minneapolis, MN) every second day for 2 weeks[44,45]. For assessing the role of SFB, mice purchased from the Jackson laboratory were orally inoculated with bacterial suspensions obtained by homogenizing faecal pellets from SFB-monoassociated mice in water. SFB colonization was confirmed by quantitative PCR[33] and CD11b$^+$ IgA$^+$ cells were analysed in the small intestine 2 weeks post gavage by flow cytometry. All experiments followed the guidelines of the Animal Care and Use Committee, The University of Tokyo and Columbia University.

Oral immunization. Mice were given sodium bicarbonate solution to neutralize stomach acid[11,13]. Thirty minutes later, the mice were orally immunized with 1 mg OVA (Sigma-Aldrich) and 10 µg CT (List Biological Laboratories, Campbell, CA). This procedure was conducted on days 0, 7 and 14. In some groups, mice were intraperitoneally given CPM (35 mg kg^{-1} each time, Sigma-Aldrich). One week after the final immunization, faecal samples and mononuclear cells from the iLP were collected for enumeration of OVA-specific antibody responses by enzyme-linked immunosorbent assay and ELISPOT, respectively[13]. *In vivo* CT challenge was performed by oral challenge of naive or immunized mice with 100 µg of CT as previously described[46].

Cell isolation. To isolate mononuclear cells from PPs, we stirred the tissues in RPMI-1640 medium containing 2% fetal calf serum plus 0.5 mg ml^{-1} collagenase (Wako, Osaka, Japan)[11,13]. To isolate mononuclear cells from the iLP, PPs were carefully removed and the remaining intestines including ILFs were opened longitudinally, washed with RPMI-1640, cut into 2-cm pieces and stirred for 20 min at 37 °C into RPMI-1640 containing 0.5 mM EDTA and 2% fetal calf serum to remove epithelial cells and intraepithelial lymphocytes[11,13]. The tissues were then stirred three times in 0.5 mg ml^{-1} collagenase for 20 min before undergoing discontinuous Percoll gradient centrifugation (40 and 75%). Peritoneal cells were obtained by peritoneal flushing with 8 ml ice-cold phosphate-buffered saline (PBS)[11,13].

Flow cytometry and cell sorting. Mononuclear cells were preincubated with 10 µg ml^{-1} anti-CD16/32 antibody (BD Biosciences, San Diego, CA). They were then reacted with the following antibodies: Pacific blue-rat anti-mouse CD45R (B220) (RA3-6B2, 0.8 µg ml^{-1}), phycoerythrin (PE)-rat anti-mouse CD11b (M1/70, 0.1 µg ml^{-1}), PE-Cy7-hamster anti-mouse CD11c (HL3, 0.4 µg ml^{-1}), PE-rat anti-mouse CD18 (C71/16, 0.8 µg ml^{-1}), PE-rat anti-mouse CD19 (1D3, 0.8 µg ml^{-1}), PE-rat anti-mouse CD38 (90, 0.13 µg ml^{-1}), FITC-rat anti-mouse IgA (C10-3, 2 µg ml^{-1}), PE-Cy7-rat anti-mouse IgM (R6-60.2, 1 µg ml^{-1}), PE-anti-mouse I-A^d (AMS-32.1, 0.4 µg ml^{-1}), APC-Cy7-rat anti-mouse CD11b (M1/70, 1 µg ml^{-1}), APC-Cy7-anti-mouse β1-integrin (HMβ1-1, 4 µg ml^{-1}), APC-anti-mouse CD40 (3/23, 2 µg ml^{-1}), Pacific blue-anti-mouse CD11b (M1/70, 1 µg ml^{-1}), PE-Cy7-anti-mouse F4/80 (BM8, 0.4 µg ml^{-1}) and biotin mouse anti-CD138 (281-2, 10 µg ml^{-1}) (all antibodies from BD Biosciences) followed by incubation with streptavidin-APC (1 µg ml^{-1}, BD Biosciences), PE-anti-mouse CD150 (TC15-12F12.2, 0.1 µg ml^{-1}), Alexa Fluor 647-anti-mouse CD80 (16-10A1, 1 µg ml^{-1}) (BioLegend, San Diego, CA), anti-mouse CD267 (TACI) (8F10-3, 4 µg ml^{-1}) (eBioscience, San Diego, CA), PE-mouse CCR3 (83101, 0.5 µg ml^{-1}) (R&D Systems) or biotinylated anti-peanut agglutinin lectin (1 µg ml^{-1}, Vector Laboratories, Burlingame, CA), followed by staining with streptavidin PE (1 µg ml^{-1}, BD Biosciences). For staining for Blimp-1, cells were fixed and permeabilized with a Cytofix/Cytoperm kit (BD Biosciences) and stained with PE-conjugated anti-Blimp1 goat polyclonal IgG (0.4 µg ml^{-1}, Santa Cruz Biotechnology, Santa Cruz, CA). FSC-H and FSC-A discrimination was used to exclude doublet cells, and ViaProbe cell-viability solution (BD Biosciences) was used to discriminate between dead and living cells. To detect proliferating cells, mice received 1 mg BrdU intraperitoneally 24 h before analysis; the BrdU signal was detected with the manufacturer's protocol (BD Biosciences). Concentration-matched isotype antibodies were used as negative controls. Flow-cytometric analysis and cell sorting were performed with FACSCanto II and FACSAria (BD Biosciences), respectively. We confirmed that cell purity was about 95% (Fig. 2a).

Immunohistological analysis. Intestines were fixed in 4% paraformaldehyde for 15 h at 4 °C, washed with PBS and treated sequentially in 10 and 20% sucrose for 12 h at 4 °C[13]. The tissues were embedded in OCT compound (Sakura Fine-technical Co., Tokyo, Japan). Cryostat sections (7 µm) were pre-blocked with anti-CD16 and CD32 antibody for 15 min at room temperature and stained for 15 h at 4 °C with FITC-rat anti-mouse IgA (C10-3, 2 µg ml^{-1}) and biotin anti-mouse CD11b antibody (M1/70, 1 µg ml^{-1}). This was followed by incubation with horseradish peroxidase (HRP)-conjugated streptavidin (Pierce, Rockford, IL) for 30 min at 4 °C and amplification of the fluorescent signal with Cy3-tyramide (TSA-Direct kit; PerkinElmer, Waltham, MA)[13]. We confirmed that no signal was detected when the specimens were stained with the concentration-matched isotype antibodies. They were then counterstained with 4′,6-diamidino-2-phenylindole (Sigma-Aldrich). Deconvoluted fluorescence images of specimens were obtained by fluorescence microscopy (BZ9000, Keyence, Osaka, Japan).

Detection of antibody responses by enzyme-linked immunosorbent assay and ELISPOT. To measure OVA- or CTB-specific IgA levels in faecal extracts, faeces were homogenized in PBS by vigorous vortexing[11,13]. After centrifugation of the extracts (9,000*g* for 15 min) the supernatants were used as faecal extracts. Plates were coated with 1 mg ml^{-1} OVA or 2 µg ml^{-1} CTB in PBS; this was followed by blocking for 1 h at room temperature with 200 µl PBS containing 1% (w/v) bovine serum albumin. After extensive washing of the plates with PBS containing 0.05% Tween 20, serial sample dilutions were added for incubation overnight at 4 °C. Samples were then incubated for 1 h at room temperature with optimally diluted HRP-conjugated goat anti-mouse IgA (SouthernBiotech, Birmingham, AL). After sample washing, the colour reaction was developed at room temperature with 3,3′,5,5′-tetramethylbenzidine (Moss, Pasadena, MD) and terminated by adding 0.5 M HCl. The colour reaction was measured as the optical density (wavelength 450 nm).

ELISPOT assay was used to enumerate IgA-producing AFCs in the iLP[11,13]. Briefly, various concentrations of mononuclear cells were cultured at 37 °C for 4 h in 96-well nitrocellulose membrane plates (Millititer HA; Millipore, Bedford, MA) coated with 1 mg ml^{-1} OVA and 5 µg ml^{-1} bovine serum albumin-conjugated phosphorylcholine (Biosearch Technologies, Novato, CA). After vigorous washing of the plates with PBS and PBS containing 0.05% Tween 20, HRP-conjugated goat anti-mouse IgA was added; the plates were then incubated overnight at 4 °C. Spots of AFCs were developed with 2-amino-9-ethylcarbazole