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Prominent Role for Plasmacytoid Dendritic Cells in Mucosal T Cell-Independent IgA Induction

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SUMMARY

Although both conventional dendritic cells (cDCs) and plasmacytoid dendritic cells (pDCs) are present in the gut-associated lymphoid tissues (GALT), the roles of pDCs in the gut remain largely unknown. Here we show a critical role for pDCs in T cell-independent (TI) IgA production by B cells in the GALT. When pDCs of the mesenteric lymph nodes (MLNs) and Peyer's patches (PPs) (which are representative GALT) were cultured with naive B cells to induce TI IgA class switch recombination (CSR), IgA production was substantially higher than in cocultures of these cells with cDCs. IgA production was dependent on APRIL and BAFF production by pDCs. Importantly, pDC expression of APRIL and BAFF was dependent on stromal cell-derived type I IFN signaling under steady-state conditions. Our findings provide insight into the molecular basis of pDC conditioning to induce mucosal TI IgA production, which may lead to improvements in vaccination strategies and treatment for mucosal-related disorders.

INTRODUCTION

In mammals, IgA is the most abundant immunoglobulin isotype in the body under steady-state conditions. It is synthesized mainly as a secretory form in gut-associated lymphoid tissues (GALT) (Cerutti, 2008). Secretory IgA prevents mucosally transmitted pathogens and commensal bacteria from binding to epithelial cells and neutralizes their toxins to maintain homeostasis at mucosal surfaces. IgA class-switch recombination (CSR) is induced by both T cell-dependent (TD) and -independent (TI) pathways, with the former predominating at Peyer's patches (PPs) (Butcher et al., 1982; Casola et al., 2004). The latter may occur in the lamina propria (LP) (Fagarasan et al., 2001; He et al., 2007) and isolated lymphoid follicles (ILFs) (Tsuji et al., 2008). In TD IgA CSR, antigen (Ag)-specific naive B cells differentiate into IgA^+-committed B cells upon stimulation with CD40L on activated T cells and TGF-β1 expressed by multiple cell types, such as intestinal epithelial cells (IECs), dendritic cells (DCs), stromal cells (SCs), and T cells, leading to production of Ag-specific high-affinity IgA, which neutralizes pathogens and their toxins (Cerutti, 2008). Alternatively, TI IgA CSR is induced in polyclonal naive B cells by DC- and IEC-derived molecules: a proliferation-inducing ligand (APRIL) and B cell-activating factor of the tumor necrosis factor family (BAFF) through their receptor transmembrane activator and cyclophilin ligand interactor (TACI) and retinoic acid (RA). TI IgA CSR contributes to production of polyclonal low-affinity IgA, which presumably limit the adhesion of commensal bacteria to IECs (Mora et al., 2006; Tezuka et al., 2007; He et al., 2007; Xu et al., 2007; Massacand et al., 2008). B-1 cells also participate in mucosal TI IgA production (Bos et al., 1996).

DCs are composed of two phenotypically and functionally distinct subpopulations, conventional DCs (cDCs) and plasmacytoid DCs (pDCs), both of which are present in the GALT (Iwasaki, 2007). cDCs have been well characterized by their distributions in the GALT and their critical functions in the maintenance of gut homeostasis, immune responses against gut infection, and inflammatory bowel diseases (Iwasaki, 2007). In contrast, although pDCs are also found in the GALT, e.g., in the subepithelial dome (SED) and interfollicular regions of the PPs and LP (Contractor et al., 2007; Wendland et al., 2007), little is known about their function in the maintenance of gut homeostasis.

In the present study, we found that MLN and PP pDCs rather than MLN and PP cDCs express high amounts of APRIL and BAFF and that their induction of TI IgA CSR is APRIL and BAFF dependent. In addition, type I IFN-producing stromal cells (SCs) were preferentially present in the MLNs and PPs under steady-state conditions. Thus, SC-derived type I IFNs are likely to upregulate the expression of APRIL and BAFF by pDCs, leading to TI IgA production in the GALT.

RESULTS

T Cell-Independent IgA Production Is Dominant in the MLNs

IgA CSR takes place in the MLNs, a representative GALT (Bos et al., 1996; Yamamoto et al., 2000, 2004; Macpherson and Uhr, 2004; Bergqvist et al., 2006). By using WT and T cell-deficient $Tcrb^{-/-}Tcrd^{-/-}$ mice, we first examined the relative contribution of TD versus TI IgA CSR in the MLNs (Figure 1A). As

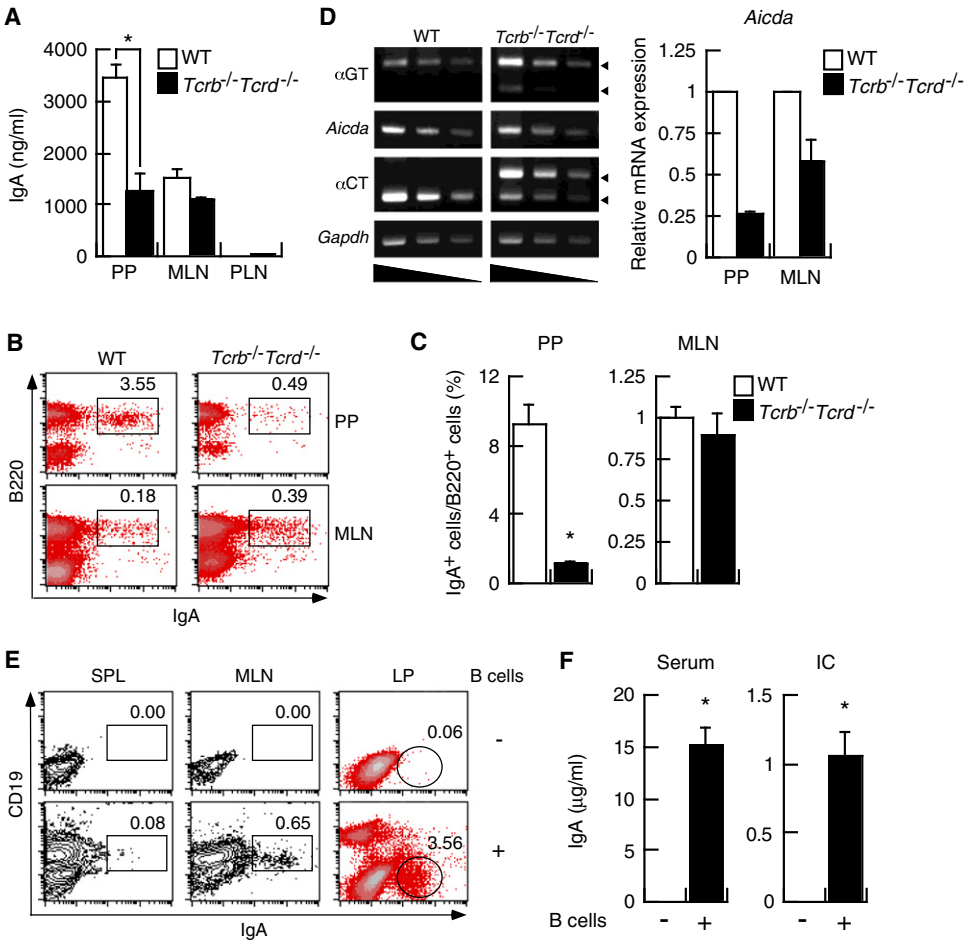

Figure 1. MLN Is an Inductive Site for T Cell-Independent IgA Production

(A) IgA production by MNCs from PPs, MLNs, and PLNs from WT and *Tcrb*$^{-/-}$*Tcrd*$^{-/-}$ mice cultured for 3 days. Data are representative of four independent experiments.

(B) MNCs from PPs and MLNs from the indicated mice stained for IgA and B220.

(C) Percentage of IgA^{+} cells per total B220^{+} cells in the PP and MLN of WT and *Tcrb* $^{-/-}$*Tcrd*$^{-/-}$ mice. Numbers in FACS plots indicate the proportion of the cells. Each group includes four mice.

(D) *Aicda* expression in PP and MLN B cells, determined by qPCR (left), and α-germline transcripts (αGT), *Aicda*, and α-circle transcripts (αCT) expression in MLN CD19^{+} B cells, determined by RT-PCR.

(E and F) Naive B cells from the *Tcrb*$^{-/-}$*Tcrd*$^{-/-}$ mice were transferred into *Il2rg*$^{-/-}$*Rag2*$^{-/-}$ mice, and 2 weeks later, MNCs from the indicated organs were stained for IgA and B220 (E), and the IgA amounts in the sera and intestinal contents (IC) was measured (F).

Numbers in FACS plots indicate the proportion of the cells. Data are representative of three independent experiments with three mice per group. *$p < 0.05$. Error bars indicate the SEM.

expected from previous studies (Butcher et al., 1982; Casola et al., 2004), the mononuclear cells (MNCs) isolated from the PPs of *Tcrb*$^{-/-}$*Tcrd*$^{-/-}$ mice produced substantially lower amounts of IgA than those of WT mice, indicating that the TD IgA CSR is dominant in the PPs. In contrast, the MNCs from the MLNs of the *Tcrb*$^{-/-}$*Tcrd*$^{-/-}$ mice produced IgA at similar titers to those of WT mice, implying that TI IgA production is relatively dominant in the MLNs. Nonmucosal PLN cells failed to produce IgA in either strain. Indeed, compared with WT mice, both the number of postswitch IgA^{+} B cells and the B cell expression of *Aicda*, a gene encoding activation-induced cytidine deaminase (AID), a critical enzyme for CSR, were reduced in the PP B cells of the *Tcrb*$^{-/-}$*Tcrd*$^{-/-}$ mice, whereas there was much less effect on the MLN B cells of these mice (Figures 1B–1D). Consistent with these results, α-germline transcripts and circle transcripts were detected in MLN B cells (Figures 1D). The MLNs in WT mice contained postswitch IgA^{+} B cells that aggregated within germinal centers (GCs) and were scattered in B cell follicles (Figures S1A and S1B available online), whereas in the MLNs of *Tcrb*$^{-/-}$*Tcrd*$^{-/-}$ mice that lack GCs (Tsuji et al., 2008), all IgA^{+} B cells were scattered in B cell follicles (Figures S1C and S1D). In addition, naive B cells, which were isolated from *Tcrb*$^{-/-}$*Tcrd*$^{-/-}$ mice and injected into cytokine receptor common γ chain (*Il2rg*)$^{-/-}$*Rag2*$^{-/-}$ mice, which lack PPs, ILFs, and most LNs except for MLNs (Cao et al., 1995), differentiated into a small but substantial number of postswitch IgA^{+} B cells in the MLNs but not in the spleen (Figure 1E). Consistent with these results, IgA^{+} plasma cells in the LP as well as IgA

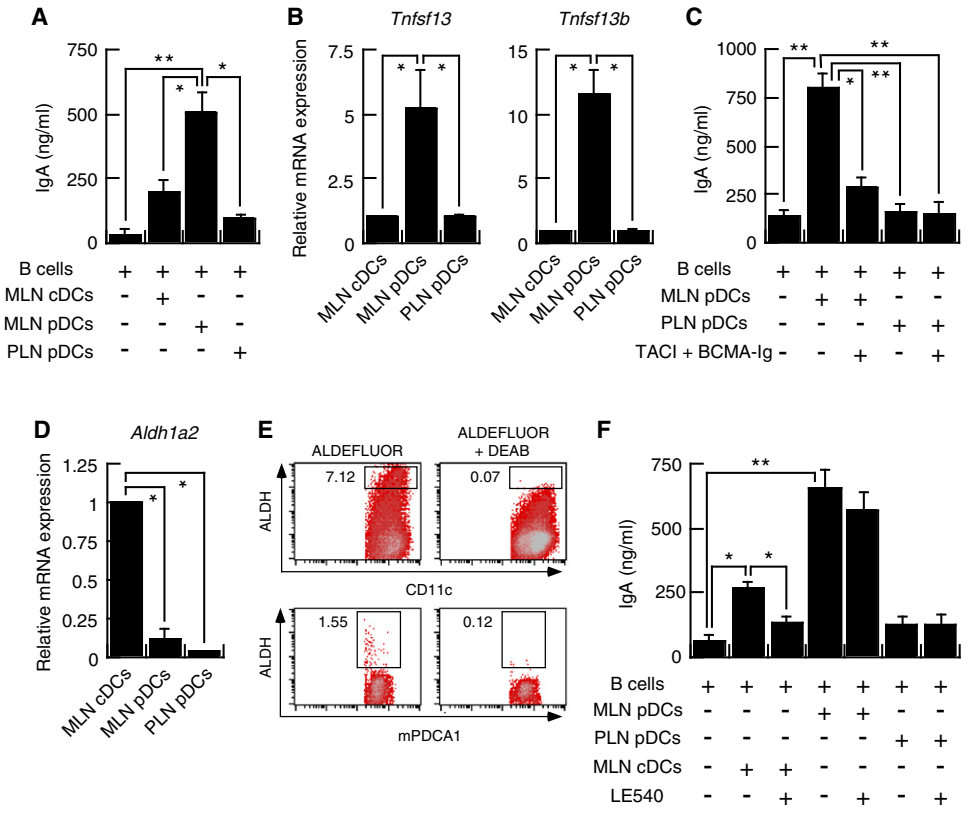

Figure 2. MLN pDCs Induce IgA Production in an APRIL- and BAFF-Dependent Manner

(A) IgA level produced by naive B cells cocultured with MLN pDCs, MLN cDCs, or PLN pDCs for 7 days, evaluated by ELISA. Data are representative of four independent experiments.

(B and D) *Tnfsf13*, *Tnfsf13b* (B), and *Aldh1a2* (D) expression in MLN cDCs or pDCs, and PLN pDCs, examined by qPCR. Data are representative of three independent experiments.

(C and F) IgA level produced by naive B cells cocultured with the indicated DC subsets in the presence or absence of TACI- and BCMA-Ig (C) and LE540 (F) for 7 days, evaluated by ELISA. Data are representative of five independent experiments.

(E) MLN cells were incubated with ALDEFLUOR (ALDH) in the presence or absence of DEAB, a competitive inhibitor of ALDH, then stained for CD11c or mPDCA1. Numbers in FACS plots indicate the proportion of the cells and data are representative of four independent experiments. $^{**}p < 0.01$; $^{*}p < 0.05$. Error bars indicate the SEM.

production in the serum and intestinal contents (IC) were detected in *Il2rg*$^{-/-}$*Rag2*$^{-/-}$ mice that had received the naive B cells from the *Tcrb*$^{-/-}$*Tcrd*$^{-/-}$ mice (Figures 1E and 1F). These results indicate that TI IgA production is dominant in the MLNs.

Prominent Role of Mucosal pDCs in TI IgA Induction

Previous studies have shown that DCs of the GALT, possibly including both cDCs and pDCs, can induce naive or activated B cells to differentiate into IgA-producing plasma cells in the absence of T cells in vitro (Mora et al., 2006; Tezuka et al., 2007; Massacand et al., 2008). However, little is known about the distinct features of cDCs and pDCs in the induction of TI IgA CSR. To compare the induction of TI IgA CSR by cDCs and pDCs, each DC subset was isolated from the MLNs and PPs (Figures S2A and S2B) and cultured with naive B cells without additional stimulation. Although both DC subsets from these tissues induced IgA production from naive B cells, the pDCs induced substantially higher amounts of IgA production than the cDCs, which was coincident with the pDC-dependent induction of α-circle transcripts in B cells (Figure 2A; Figures S2C and S2D). In addition, PLN pDCs induced little IgA (Figure 2A), indicating that pDCs residing in the MLNs and PPs play a pivotal role in the TI IgA CSR in B cells. Supporting this notion, although pDCs represent only a minor population (15%–20%) of the total DC compartment in WT mice, the contribution of the MLN and PP pDCs to TI IgA production reached 40%–45% of the total induced by MLN and PP DCs (Figures S2E and S2F).

Mucosal DCs are a main producer of APRIL and BAFF, critical cytokines for TI IgA CSR (Massacand et al., 2008). Because MLN and PP pDCs were the prominent inducer of TI IgA production, we asked whether this unique and distinct property of pDCs was due to their differential production of APRIL and BAFF, via quantitative PCR (qPCR). Importantly, the freshly isolated MLN and PP pDCs expressed *Tnfsf13* and *Tnfsf13b*, genes encoding APRIL and BAFF, respectively, at substantially higher amounts than the MLN and PP cDCs or PLN pDCs (Figure 2B; Figure S2G), and the results correlated well with the ability of each DC subset to induce TI IgA production (Figure 2A; Figure S2C). Interestingly, the IgA titers induced by pDCs were comparable to those induced by around 400 ng/ml BAFF or APRIL (Figure S2H).

To examine whether the MLN and PP pDC-derived APRIL and BAFF were responsible for the TI IgA induction, naive B cells were cocultured with the pDCs in the presence of BCMA-, TACI-, and BAFFR-Ig, which block the binding of APRIL and BAFF to their corresponding receptors. Notably, although BAFFR was originally reported to be a BAFF-specific receptor (Thompson et al., 2001), a comprehensive survey of ligand-receptor interactions revealed that mouse APRIL binds substantially to mouse BAFFR, although with weaker affinity than to mouse TACI or BCMA (Bossen et al., 2006). The MLN and PP pDC-induced IgA production was markedly lower in the presence of BCMA-, TACI-, and BAFFR-Ig compared with control Ig, suggesting that the effects of BCMA-, TACI-, and BAFFR-Ig are specific and that the IgA-inducing capacity of MLN and PP pDCs is indeed due to their prominent expression of APRIL and BAFF (Figure 2C; Figures S2I and S2L). Compared with that of MLN pDCs, the PLN pDC-induced IgA production was much lower and was unaffected by BCMA- and TACI-Ig (Figure 2C). Consistent with these results, some pDCs were in close contact with B cells, including IgA^+ B cells, in B cell follicles of the MLNs of WT and $Tcrb^{-/-}Tcrd^{-/-}$ mice (Figures S1A–S1D).

Involvement of Other Factors in pDC-Mediated TI IgA Induction

We further tested whether TGF-β, IL-6, or IL-10 was required for the pDC-mediated TI IgA production, because these cytokines are known to be important in the APRIL and BAFF-dependent IgA production in human and mouse (Cerutti, 2008; Tsuji et al., 2008). Culturing naive B cells with MLN and PP pDCs in the presence or absence of control Ab or with a neutralizing Ab against TGF-β, IL-6, or IL-10 revealed that IgA production was unaffected by the anti-TGF-β (Figures S2J and S2M) and was partially reduced by anti-IL-6 or anti-IL-10 (Figures S2K and S2N), suggesting that IL-6 and IL-10, but not TGF-β, are involved in the pDC-mediated IgA production, at least in this experimental setting. In line with the results, MLN and/or PP pDCs produced both IL-6 and IL-10 without stimulation, whereas they expressed *Tgfb1* and *Itgb8*, a component of αvβ8 that mediates the conversion of latent TGF-β to active one, at substantially lower levels compared with cDCs (Figures S2O and S2P).

In the GALT, vitamin A (also known as retinol), which is obtained from the diet, is oxidized to retinal by alcohol dehydrogenases (ADH) and then to retinoic acid (RA) by aldehyde dehydrogenase 1A (ALDH1A) (Iwata et al., 2004; Mora et al., 2008). RA from MLN DCs functions synergistically with IL-6 and IL-5 to induce IgA production (Mora et al., 2006). To determine whether pDCs contribute substantially to RA production, we compared mRNA expression and enzymatic activity of ALDH1A2, an isoform of ALDH, between the cDCs and pDCs in MLNs. As reported previously, the MLN cDCs expressed *Aldh1a2* and showed ALDH activity at substantial levels (Figures 2D and 2E; Coombes et al., 2007; Yokota et al., 2009). In contrast, the MLN and PLN pDCs expressed little *Aldh1a2*, if any, and exhibited low ALDH activity (Figures 2D and 2E). Consistent with these results, the MLN cDC-induced IgA production was significantly inhibited, whereas the MLN pDC-induced IgA production was minimally affected by the addition of LE540, an RA-receptor antagonist (Figure 2F). These results indicated that the MLN pDC-induced IgA production is largely dependent on APRIL and BAFF and independent of RA. In contrast, MLN cDC-mediated IgA production is significantly dependent on RA, although some contribution of APRIL and BAFF cannot be completely excluded.

Unique Crosstalk between pDCs and B Cells Resulting in IgA Production

APRIL and BAFF can exist in soluble or membrane-bound forms (Mackay et al., 2003). To examine whether soluble and/or membrane-bound APRIL and BAFF were critical for the MLN pDC-induced TI IgA production, we cultured MLN DC subsets and naive B cells in a transwell system in which the DCs were unable to contact the B cells, thereby restricting their access to all but diffusible factors, and measured the IgA production. In this system, MLN cDCs induced IgA to a similar degree to that achieved in conventional culture (Figure 3A). In contrast, the MLN pDC-mediated IgA production was substantially inhibited by blocking the cell-to-cell interaction via the transwell system (Figure 3A). Irradiation blocks synthesis of soluble proteins but not expression of membrane-bound molecules. Consistent with these results via a transwell system, irradiated MLN pDCs were able to induce IgA to a similar degree as untreated MLN pDCs and failed to induce IgA in the presence of BCMA- and TACI-Ig (Figure 3B), suggesting that membrane-bound APRIL and BAFF on MLN pDCs are biologically active and responsible for TI IgA production. In contrast, irradiated MLN cDCs failed to induce TI IgA production. Supporting this notion, membrane-bound APRIL and BAFF were indeed detectable on some MLN and PP pDCs but not on MLN cDCs, in the absence or presence of CpG stimulation, an efficient activator of DCs (Figure 3C; Figure S3A). In this context, the pDCs did not express BCMA, TACI, or BAFFR, excluding the possibility that soluble APRIL and BAFF act by binding to their receptors expressed on pDCs (Figure S3B).

In addition, the expression of furin convertase, an enzyme that cleaves the membrane-bound form of APRIL and BAFF to release them as soluble ligands (Mackay et al., 2003), was substantially lower in MLN pDCs than in MLN cDCs (Figure 3D). Interestingly, upon CpG stimulation, the expression of furin was enhanced in cDCs but not in pDCs (Figure S3C), implying that APRIL and BAFF largely remained membrane-bound to pDCs, even after their activation. Consistent with these results, CpG-stimulated MLN pDCs remained unable to secrete detectable amounts of soluble BAFF (data not shown), and MLN pDC- and cDC-induced IgA production was further enhanced in the presence of a furin inhibitor (Figure S3D). These results collectively indicated that MLN pDCs uniquely express membrane-bound APRIL and BAFF for IgA induction and thus require some cell-to-cell contact with B cells to carry out this function.

pDC Expression of APRIL and BAFF Is Dependent on Type I IFN Signaling

Given the important and unique role of MLN and PP pDCs in TI IgA induction, and the requirement for high amounts of *Tnfsf13* and *Tnfsf13b* expression, we investigated how *Tnfsf13* and *Tnfsf13b* expression is regulated in these cells. Previous work had established that type I IFNs regulate Ig production (Le Bon et al., 2001; Jego et al., 2003) and are potent inducers of APRIL and BAFF in human DCs (Litinskiy et al., 2002), and we therefore

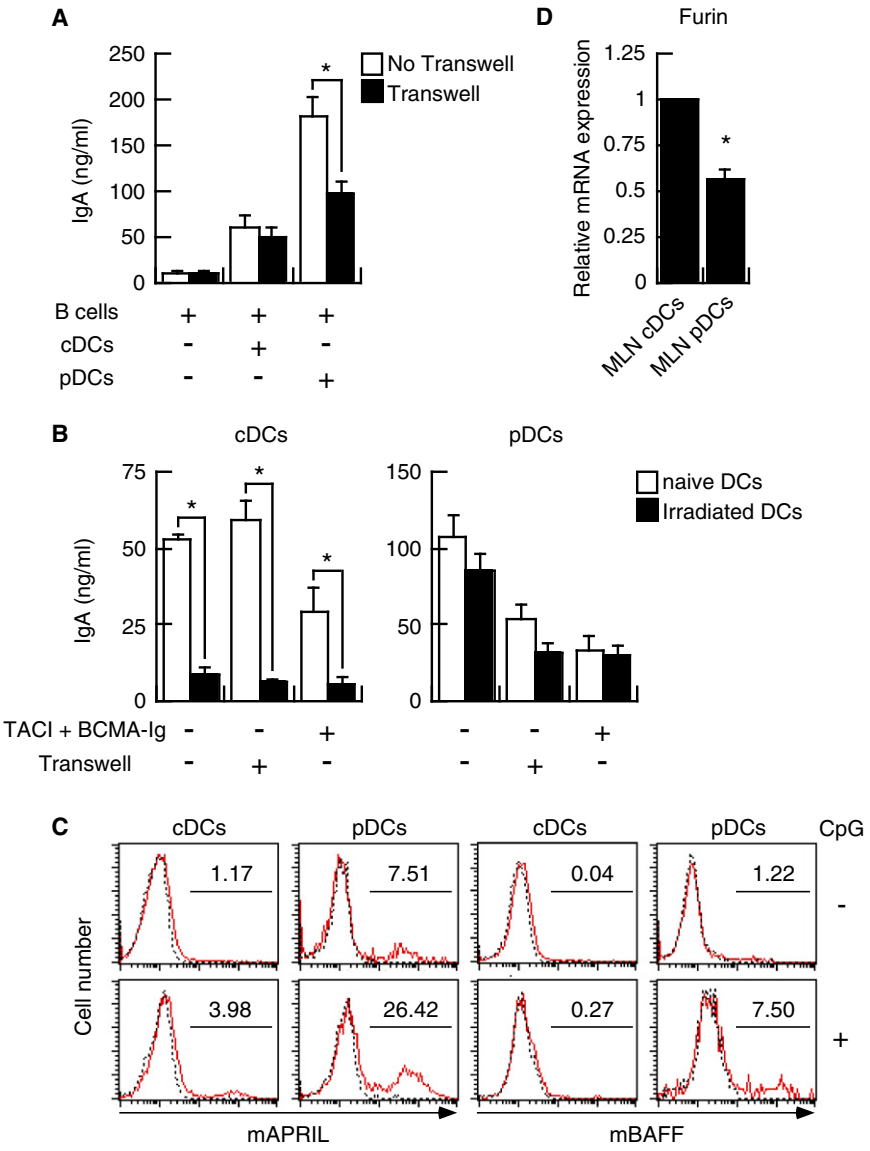

Figure 3. Unique Crosstalk between pDCs and B Cells Leading to IgA Production

(A and B) IgA level produced by naive B cells cultured with untreated (A) or irradiated (B) DC subsets isolated from MLNs in a Transwell system or in conventional culture for 7 days, and evaluated by ELISA. Data are representative of three or four independent experiments.

(C) The expression of membrane-bound APRIL and BAFF on untreated and CpG-stimulated MLN pDCs. Solid line, APRIL and BAFF staining; dashed line, isotype control staining. Numbers in histograms indicate percentages of the cells.

(D) MLN cDCs and pDCs were examined for furin expression by qPCR. Data are representative of two to four independent experiments.

*$p < 0.05$. Error bars indicate the SEM.

hypothesized that type I IFNs play a critical role in TI IgA synthesis by upregulating the pDC expression of *Tnfsf13* and *Tnfsf13b*. To test this idea, we cultured PLN pDCs, which express neither *Tnfsf13* nor *Tnfsf13b*, in the presence of IFN-α. Notably, the PLN pDCs acquired the expression of *Tnfsf13* and *Tnfsf13b* at substantial amounts (Figure S4A), implying that the high amounts of these molecules in MLN pDCs are due to physiologically produced type I IFNs in the GALT. In fact, under steady-state and specific-pathogen-free (SPF) conditions, type I IFNs are produced constitutively, although at low amounts, by a variety of cell types and tissues (Taniguchi and Takaoka, 2001).

To further study the role of type I IFNs in TI IgA CSR, we quantified the IgA levels in the sera and fecal pellets of mice deficient for IFNAR1, a component of type I IFN-receptor, or IFN-β, and found that these IgA titers were substantially lower than in wild-type mice (Figure 4A; Figure S4B). However, upon stimulation with APRIL or BAFF in vitro, naive *Ifnar1*$^{-/-}$ B cells produced IgA to a degree similar to naive WT B cells, suggesting that *Ifnar1*$^{-/-}$ B cells are competent for TI IgA synthesis (Figure 4B). Therefore, we investigated whether *Ifnar1*$^{-/-}$ pDCs could induce TI IgA production by culturing either WT or *Ifnar1*$^{-/-}$ MLN pDCs with naive WT B cells. We found that the WT MLN pDC-induced IgA production was significantly reduced in the presence of BCMA- and TACI-Ig (Figure 4C), confirming the involvement of pDC-derived APRIL and BAFF in TI IgA production. Furthermore, the *Ifnar1*$^{-/-}$ MLN pDC-induced IgA production was also substantially reduced, to the amount of the WT MLN pDCs cultured with BCMA- and TACI-Ig, which correlated well with the impaired expression of *Tnfsf13* and *Tnfsf13b* in these cells (Figures 4C and 4D). Finally, the *Ifnar1*$^{-/-}$ MLN pDC-induced IgA production was restored by the addition of APRIL or BAFF (Figure 4C).

We previously showed that IgA production is severely impaired in *Nos2*$^{-/-}$ mice and that nitric oxide (NO) derived from naturally occurring TNF, iNOS (double)-producing DCs (Tip-DCs) plays a critical role in expression of *Tnfsf13* and *Tnfsf13b* in GALT DCs (Tezuka et al., 2007). As expected, the *Nos2*$^{-/-}$ pDCs isolated from MLNs showed a reduced capacity to induce TI IgA, with impaired *Tnfsf13* and *Tnfsf13b* expression (Figures S4C and S4D), and the expression of *Nos2* was significantly impaired in *Ifnar1*$^{-/-}$ CD11c^{+} cells in the MLNs (Figure S4E), implying that, under steady-state conditions, type I IFNs induce Tip-DCs, and their product NO acts on pDCs and, together with type I IFN signaling, effectively enhances BAFF-APRIL expression by pDCs. Supporting this hypothesis, iNOS-expressing cells in the GALT are Tip-DCs and do not include pDCs (B220^{+}CD11c^{int}) (Tezuka et al., 2007) in vivo. An impaired expression of iNOS is also evident in *Ifnar1*$^{-/-}$ macrophages (Vadiveloo et al., 2000).

To confirm the critical role of pDCs in mucosal TI IgA production in vivo, we carried out the adoptive transfer of 1 × 10^{6} pDCs,

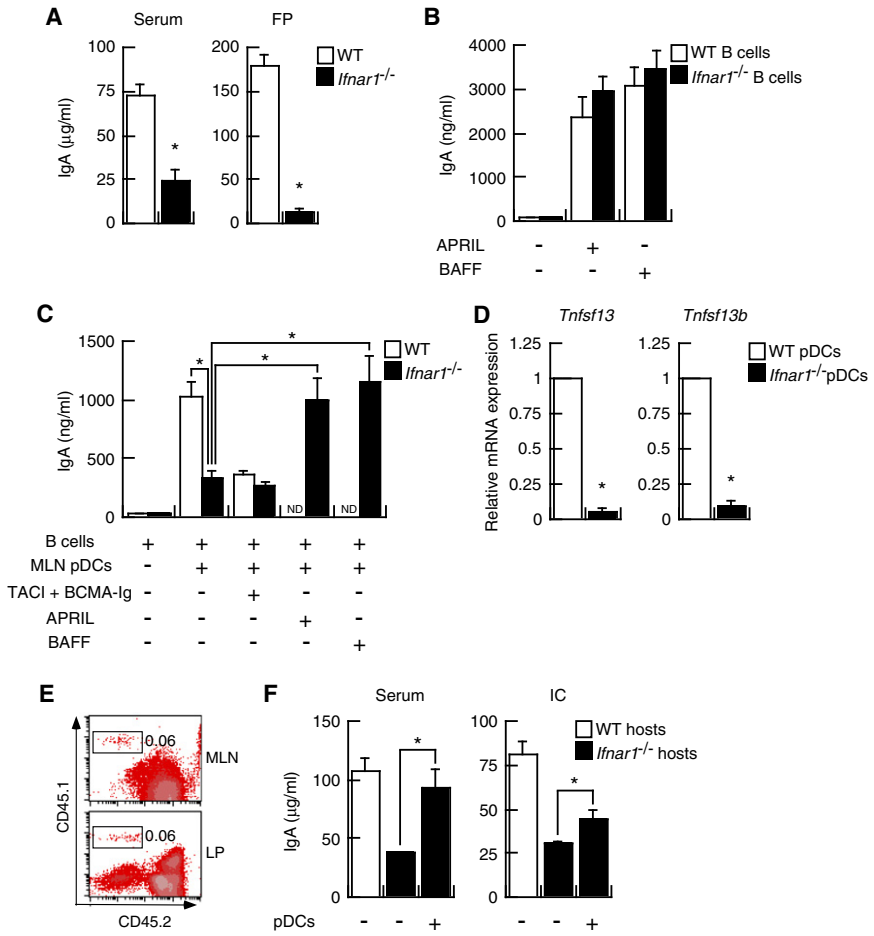

Figure 4. Impaired APRIL and BAFF Expression and TI IgA Induction by pDCs Lacking the Type I IFN Receptor

(A) IgA level in the serum and fecal pellets (FP) of *Ifnar1*$^{-/-}$ mice. Representative data from two independent experiments with four mice per group are shown.

(B) IgA level produced by naive B cells from WT and *Ifnar1*$^{-/-}$ mice stimulated with APRIL or BAFF for 7 days.

(C) IgA level produced by naive B cells cocultured with MLN pDCs of WT and *Ifnar1*$^{-/-}$ mice in the presence or absence of APRIL or BAFF for 7 days. ND, not determined. Data are representative of four independent experiments.

(D) *Tnfsf13* and *Tnfsf13b* expressed by WT and *Ifnar1*$^{-/-}$ pDCs, determined by qPCR. Data are representative of three independent experiments.

(E and F) PLN pDCs of B6.SJL mice (CD45.1^{+}) were transferred into *Ifnar1*$^{-/-}$ hosts (CD45.2^{+}). The number of CD45.1^{+} cells in the MLN/LP 16 hr after the transfer (E) and the IgA level in the serum and IC (F) 2 weeks after the transfer.

Numbers in FACS plots indicate the proportion of the cells, and representative data from two independent experiments with four mice per group are shown. IgA levels were determined by ELISA. *$p < 0.05$. Error bars indicate the SEM.

isolated from the PLNs, into *Ifnar1*$^{-/-}$ mice. Importantly, the transferred PLN pDCs, which express CCR9, a representative gut-homing receptor, migrated into both the MLNs and intestinal LP to some extent and significantly restored IgA production in the sera and intestinal contents (Figures 4E and 4F). Taken together, these results indicate that type I IFN signaling is critical for mucosal pDC expression of APRIL and BAFF.

Identification of Type I IFN-Producing Cells and Evidence of Type I IFN Signature on Mucosal pDCs

Our results implied that physiological type I IFN-producing cells are present in the MLNs of WT mice under SPF conditions. In this context, we found that, in the MLNs, numerous CD45^{-}ER-TR7^{+} stromal cells (SCs) expressed IFN-α and IFN-β, which were located at or near the edge of the cytoplasm (Figures 5A and 5B for IFN-α, Figures 5D and 5E for IFN-β). In contrast, few SCs in the PLNs expressed type I IFNs (Figures S5A and S5B for IFN-α, Figures S5C and S5D for IFN-β). Importantly, the SC expression of type I IFNs was impaired in germ-free and *Myd88*$^{-/-}$*Trif*$^{-/-}$ mice (Figures 5C and 5F; Figures S5E and S5F), suggesting that the TLR-dependent recognition of commensal bacteria is required for the SC production of type I IFNs, leading to the activation of pDCs to make APRIL and BAFF.

We next investigated the expression of various genes in the type I IFN pathway and obtained supporting evidence of the influence of type I IFNs on pDCs in the MLNs. First, mRNA expression of both IFN-α and IFN-β in the MLN and PP SCs was higher than in the MLN pDCs and splenic SCs (Figure 5G; Figures S5G and S5H). Second, expression of the mRNA for protein kinase receptor (PKR), a type I IFN-inducible gene essential for the expression of APRIL and BAFF (Hardenberg et al., 2007), was higher in the MLN pDCs of WT mice than in the MLN cDCs of WT mice or the MLN pDCs of *Ifnar1*$^{-/-}$ mice (Figure 5H), suggesting that MLN pDCs constitutively receive type I IFN signaling under steady-state and SPF conditions. Importantly, the expression level of type I IFN receptor in pDCs was significantly higher than that in cDCs (Figure 5I), which was likely to lead to their different responses to type I IFNs in the MLNs. In addition, both pDCs and cDCs were in close contact with SCs in the extrafollicular area beside the B cell follicles in the MLNs (Figures S5I–S5L), implying that after pDCs are conditioned at this site, they migrate into the B cell follicles to induce TI IgA CSR. Supporting this scenario, the colocalization of naive B cells and pDCs was detected predominantly in the B cell follicle (Figures S1A–S1D), whereas little colocalization of naive B cells and pDCs with SCs was observed (data not shown). Third, type I IFNs mediate the upregulation of CD69 expression on pDCs during viral infections and even under steady-state conditions (Gao et al., 2009). We also found that WT MLN pDCs expressed CD69 at a substantially higher level than WT PLN pDCs, and *Ifnar1*$^{-/-}$ pDCs expressed little CD69 (Figure 5J), suggesting the preferential expression of type I IFNs in the MLNs. Collectively, these results suggest that, under steady-state SPF conditions, type I IFNs are produced constitutively,

albeit at low levels, by MLN SCs, and that they act on pDCs to induce the expression of APRIL and BAFF in the MLNs.

DISCUSSION

PPs are a major inductive site for TD IgA CSR. In contrast, TI IgA CSR is preferentially induced in ILFs where few T cells are found (Tsuji et al., 2008). IgA CSR is also induced in the MLNs (Bos et al., 1996; Macpherson and Uhr, 2004; Bergqvist et al., 2006), although the frequency of IgA CSR in the MLNs is lower than in PPs. Furthermore, antigen-specific IgA CSR takes place in the MLNs of PP-null mice (Yamamoto et al., 2000). Although *Ltb*$^{-/-}$ mice lack PPs and ILFs, most individuals have MLNs, albeit smaller than normal, and some secreted IgA is produced (Alimzhanov et al., 1997; Fletcher et al., 2006). Given this background, we examined the contribution of TD and TI IgA induction and the role of DC subsets in IgA induction in the MLNs, and we noted that TI IgA CSR is relatively prominent in this tissue. Among the DC subsets, the pDCs played a pivotal role in the TI IgA CSR by their predominant expression of APRIL and BAFF. Moreover, the SC-derived type I IFNs induced the expression of APRIL and BAFF by pDCs in the MLNs under steady-state conditions. Notably, in the MLNs, pDCs were located in B cell follicles as well as extrafollicular area where SCs are present, implying that pDCs are conditioned by SCs in the extrafollicular area and subsequently migrate into the B cell follicles to induce TI IgA CSR.

TGF-β is essential for T cell (CD40L)-dependent IgA production (Cerutti, 2008). Regarding the TI IgA production by mouse B cells, SC-mediated TI IgA production requires TGF-β, APRIL, and BAFF (Tsuji et al., 2008), whereas APRIL and BAFF can induce IgA CSR without TGF-β in naive B cells (Castigli et al., 2005), implying that the requirement of TGF-β for TI IgA production depends on the cell type and experimental system used. In this context, GALT pDCs might express an unidentified molecule(s) in addition to membrane-bound APRIL and BAFF, which, instead of TGF-β, mediates Cα transcription. The mechanisms of Cα transcription remain to be clarified in future studies.

Unlike the ILFs, MLNs contain distinct T cell zones populated by a large number of T cells. Nevertheless, TI IgA CSR is preferentially induced in the MLNs. The MLNs are also the key site for tolerance induction to food proteins and commensal bacteria (Macpherson and Smith, 2006). DCs transport food and commensal antigens from the intestinal surface into the MLNs through the afferent lymphatics, present the antigens to T cells, and induce T cell tolerization, which may explain why the MLNs play only a minor role in TD IgA CSR.

Type I IFNs are induced to high amounts by infectious stimuli, including viral and bacterial infections (Asselin-Paturel et al., 2001). Under basal conditions, in the absence of infection, as in mice kept under SPF conditions, type I IFNs are produced constitutively at low levels by multiple cell types, including DCs, macrophages, Paneth cells (defensin- and lysozyme-secreting intestinal epithelial cells), and thymic epithelial cells (Taniguchi and Takaoka, 2001; Montoya et al., 2002; Munakata et al., 2008; Lienenklaus et al., 2009). The functional importance of this basal expression of type I IFN production is demonstrated by the finding that *Ifnar1*$^{-/-}$ mice kept under SPF conditions spontaneously develop osteoporosis caused by a lack of physiological type I IFN signaling, which limits osteoclast differentiation (Takayanagi et al., 2002).

In line with these results, we found that type I IFNs in the MLNs are exclusively expressed by the stromal cells in the MLNs under steady-state SPF conditions and that the TLR-dependent recognition of commensal bacteria is required for this process. Although large amounts of type I IFNs are produced by pDCs in response to viral infections, we did not detect the production of type I IFNs in the pDCs of the MLNs under steady-state conditions. Related to this, pDCs of the PPs fail to produce type I IFNs upon CpG stimulation (Contractor et al., 2007), implying the presence of regulatory mechanisms in the GALT, e.g., prostaglandin E_2, IL-10, and TGF-β signaling, that prevent harmful immune responses and maintain tolerogenic conditions.

Accumulating evidence suggests that SCs function not only to provide migratory guidance for immune cells (Bajénoff et al., 2006) but also as immune regulatory cells in the GALT. For example, the SCs of the MLNs express ALDH enzymes to induce gut-homing tropism by T cells (Hammerschmidt et al., 2008), and the SCs of the LPs produce TGF-β to induce IgA CSR (Fagarasan et al., 2001). In addition, the formation of ILFs requires interactions between the lymphoid-tissue inducer cells and SCs (Tsuji et al., 2008). Given that SC-derived type I IFNs act on pDCs to induce APRIL and BAFF, it appears that SCs play a critical role in the construction and maintenance of immune homeostasis in the GALT.

Here we showed that, under steady-state conditions, type I IFNs are critical for the induction of APRIL and BAFF in pDCs but not cDCs, and pDCs uniquely express a membrane-bound form of APRIL and BAFF, raising a couple of questions. First, regarding the critical function of SC-derived type I IFNs in APRIL and BAFF induction in the GALT, why do only pDCs, but not cDCs, express these molecules at high amounts? Related to this, we unexpectedly found that type I IFNR, i.e., IFNAR1, is expressed predominantly on pDCs but minimally on cDCs, indicating that pDCs but not cDCs are capable of a potent response to the SC-derived type I IFNs under steady-state conditions. Supporting our findings, the pDCs of the MLNs express high levels of PKR and CD69, type I IFN-inducible genes. Second, is there any functional difference between soluble and membrane-bound APRIL and BAFF? In this context, effective signaling via TACI, a critical receptor for APRIL- and BAFF-mediating IgA CSR, requires multimerization of BAFF, heparan sulfate proteoglycans (HSPGs) as a platform for APRIL, or membrane-bound ligands, but not soluble ligands (Mackay and Schneider, 2009). In addition, membrane-bound IL-15 is more potent, in terms of induction of T cell proliferation, than its soluble form (Musso et al., 1999). Under the background, we speculate that, compared with soluble APRIL and BAFF, which are randomly diffused and have limited chance to display their function, membrane-bound APRIL and BAFF firmly bind, even with relatively low doses, their corresponding receptors on B cells, effectively inducing IgA CSR in B cells.

Under steady-state SPF conditions, a variety of cytokines condition immune cells to maintain gut homeostasis. For example, IECs express thymic stromal lymphopoietin (TSLP) to limit the production of proinflammatory cytokines, including DC-derived IL-12 (Zaph et al., 2007; Rimoldi et al., 2005). Granulocyte-macrophage colony-stimulating factor (GM-CSF) produced by MLN

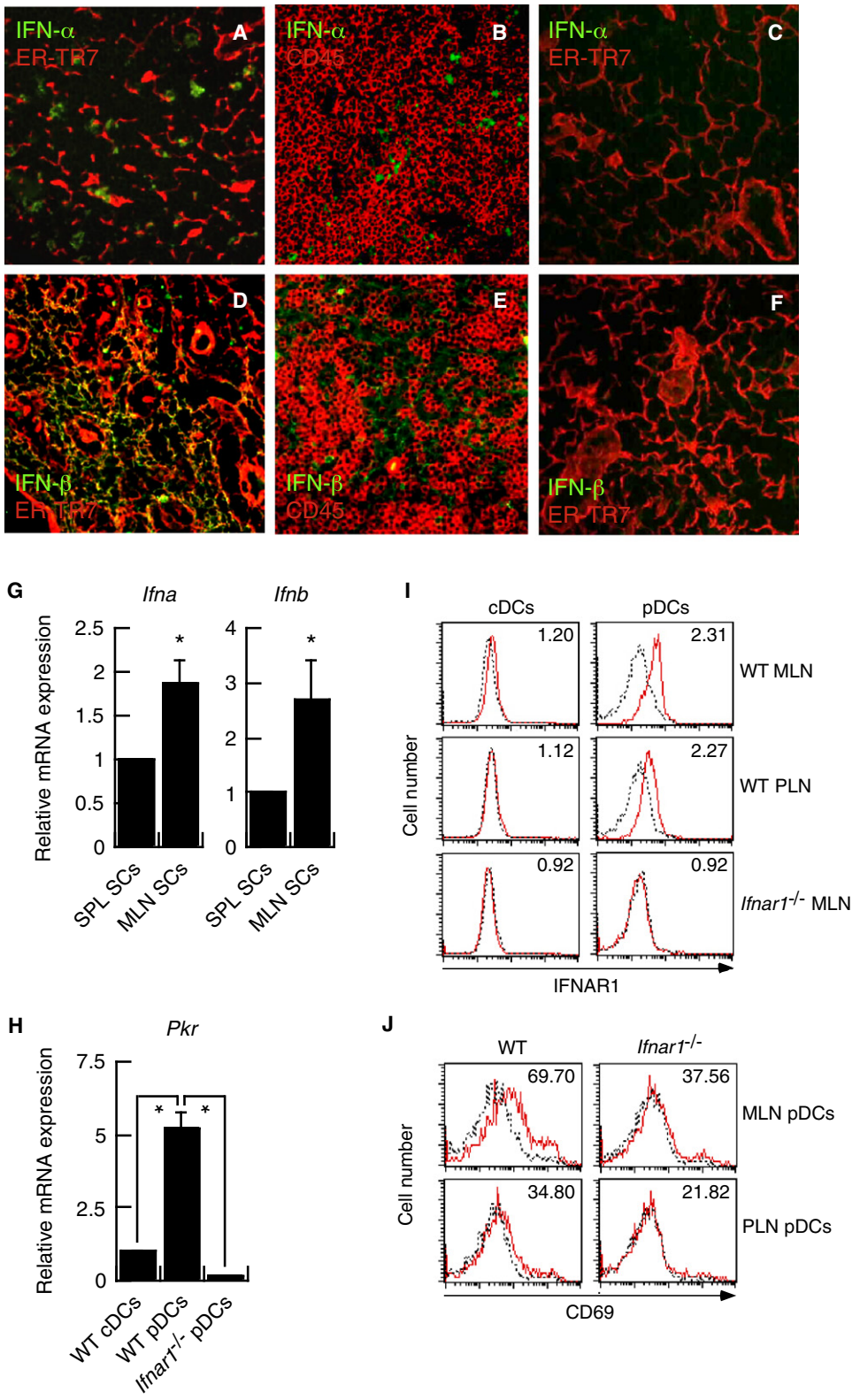

Figure 5. Identification of Type I IFN-Producing Cells and Type I IFN Signature in Mucosal pDCs

(A–F) Frozen sections of MLNs from WT mice (A, B, D, E) and germ-free mice (C, F) fixed and stained for IFN-α or IFN-β (green) and ER-TR7 or CD45.2 (red). Original magnification ×200.

(G and H) *Ifna* and *Ifnb* expression in stromal cells (SCs) (G) and *Pkr* expression in MLN DCs (H) determined by qPCR.

macrophages conditions cDCs to express ALDH1A2, an enzyme involved in generating RA (Yokota et al., 2009) and critical for the imprinting of gut-homing specificity on lymphocytes and the differentiation of Foxp3$^+$ iTreg cells. Our data indicated that type I IFNs derived from MLN SCs condition pDCs to express APRIL and BAFF once peripheral pDCs have migrated into the GALT. Given that both APRIL and BAFF are important for B cell maturation and survival and that their overproduction is associated with cancer and autoimmunity (Mackay and Schneider, 2009), our findings suggest a possible molecular basis for the control of gut homeostasis and related disorders.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6 (B6), B6.*Rag2*$^{-/-}$, B6.*Ifnar1*$^{-/-}$, and B6.germ-free mice were obtained from Japan CLEA, Central Laboratories for Experimental Animals, B&K Universal, and Sankyo Labo Service, respectively. B6.*Tcrb*$^{-/-}$*Tcrd*$^{-/-}$, B6.*Ifnb*$^{-/-}$, and B6.*Myd88*$^{-/-}$*Trif*$^{-/-}$ mice have been described (Nishiyama et al., 2002; Takaoka et al., 2000; Atarashi et al., 2008). All mice were maintained in our SPF animal facility with the Micro-VENT system (Allentown Caging Equipment Company), and all animal experiments were done with the approval of the Institutional Animal Care Committee of the Tokyo Medical and Dental University.

Cell Preparation

PPs collected from small intestines were shaken for 45 min at 37°C in PBS containing 2 mM EDTA to remove epithelial cells. Collagenase (type IV, Sigma)-digested PP, MLN, and PLN (axillary, brachial, and inguinal LNs) cells were applied to a discontinuous 40% and 70% Percoll density gradient (GE Health Care), and the cells at the interface were used as MNCs. To prepare naive B cells, PP cells were labeled with JF05-1C2.4.1 MicroBeads (anti-mPDCA-1, Miltenyi Biotec) and Biotin-281-2 (anti-CD138, BD Bioscience) followed by anti-Biotin MicroBeads (Miltenyi Biotec). The negative fraction was labeled with PE-11-26c (anti-IgD, eBioscience) and subsequently with anti-PE MicroBeads (Miltenyi Biotec). IgD$^+$ B cells were isolated with an AutoMACS Pro Separator (Miltenyi Biotec) and used as naive B cells (>97% IgD$^+$). To prepare cDCs, cells from collagenase-digested MLNs, PPs, and PLNs (axillary, brachial, and inguinal LNs) were treated with FcR blocking reagent (Miltenyi Biotec), which blocks the nonspecific binding of Abs to FcR. The cells were labeled with FITC-145-2C11 (anti-CD3ε), FITC-eBio1D3 (anti-CD19), FITC-C10-3 (anti-IgA), FITC-II/41 (anti-IgM), PE-281-2 (anti-CD138), FITC-RA3-6B2 (anti-B220), FITC-1A8 (anti-Ly6G), FITC-DX5 (anti-DX5), PE-TER119 (anti-TER119), mPDCA1 MicroBeads, and subsequently with anti-FITC MicroBeads and anti-PE MicroBeads. The negative fraction was labeled with N418-MicroBeads (CD11c) and isolated with the AutoMACS Pro. The positive fraction was used as cDCs (>95% CD11c^{hi}I-A$^+$mPDCA1$^-$). pDCs were purified with the pDC isolation kit II (Miltenyi Biotec) with Biotin-281-2 (anti-CD138). The negative fraction was labeled with N418-MicroBeads (anti-CD11c) and sorted with the AutoMACS Pro (>95% CD11c^{int}B220$^+$mPDCA1$^+$).

Cell Culture and Measurement of Immunoglobulins and Cytokines

To evaluate TI IgA production, naive B cells (2 × 10^5) were either directly stimulated with APRIL or BAFF (50–500 ng/ml, R&D Systems) or cocultured with cDCs (0.5–2 × 10^5) or pDCs (0.5–2 × 10^5) in the presence or absence of APRIL (100 ng/ml) or BAFF (100 ng/ml) and TACI-Ig, BCMA-Ig, BAFFR-Ig, control-Ig (human IgG1-Ig) (5 μg/ml each, R&D Systems), LE540 (1 μM, Wako), anti-IL-6 (MP5-20F3), anti-IL-10 (JES5-2A5), rat IgG1 isotype control (10 μg/ml each, BioLegend), anti-TGF-β1, β2, β3 (25 μg/ml, 1D11, R&D systems), mouse IgG1 isotype control (25 μg/ml, BioLegend), and decanoyl-Arg-Val-Lys-Arg-chloromethylketone (CMK, 50 μM, Alexis) for 7 days. To block cell-to-cell contact, the same numbers of B cells and DCs were cultured in the separate wells of a transwell device (Corning). In some experiments, MLN cDCs and pDCs that had been irradiated at 25 Gy were cocultured with naive B cells for 7 days. The level of IgA in the culture supernatants was measured by ELISA as described previously (Nishiyama et al., 2002; Tezuka et al., 2007). To examine the effect of type I IFNs on the expression of APRIL and BAFF, PLN pDCs were stimulated with recombinant mouse IFN-α (100 U/ml, RDI) for 18 hr. To determine membrane-bound forms of APRIL and BAFF, MLN cells were cultured with or without CpG (2 μM, ODN1668 [5′-TCCATGACGTTCCTGATGCT-3′], Hokkaido System Science) for 16 hr. The levels of IL-6 and IL-10 in 2-day culture supernatants of DC subsets were measured by ELISA (BioLegend and eBioscience, respectively).

Adoptive Transfer

To examine the trafficking and IgA induction ability of pDCs, pDCs (1 × 10^6) purified from the MLN and PLN of WT mice (CD45.1$^+$) were intravenously transferred into *Ifnar1*$^{-/-}$ hosts (CD45.2$^+$). Sixteen hours after the transfer, MLN and LP cells isolated from the hosts were stained with FITC-104 (anti-CD45.2) and PE-A20 (anti-CD45.1) and analyzed on a FACSCalibur with the CELLQuest program (BD Bioscience). Two weeks after the transfer, the IgA levels of the serum and intestinal contents (IC) were determined by ELISA. To examine whether the MLNs were the site of IgA CSR, splenic naive B cells (1 × 10^7) from the *Tcrb*$^{-/-}$*Tcrd*$^{-/-}$ mice were transferred into BALB/c *Il2rg*$^{-/-}$*Rag2*$^{-/-}$ hosts. Three weeks after the transfer, LP cells isolated from the hosts were stained with FITC-C10-3 (anti-IgA) or PE-mA-6E1 (anti-IgA) and FITC- or PE-eBio1D3 (anti-CD19), and the level of serum IgA was determined by ELISA.

Flow Cytometric Analysis

Cells were pretreated with FcR blocking reagent (Miltenyi Biotec) and/or normal mouse serum and then stained with the following mAbs: APC-N418 (anti-CD11c), PE-JF05-1C2.4.1 (anti-mPDCA1), FITC-RA3-B2 (anti-B220), PE-A3D8 (anti-APRIL), PE-121808 (anti-BAFF), PE-MER1-5A3 (anti-IFNAR1), Biotin-H1.2F3 (anti-CD69), PE-8F10 (anti-TACI), FITC-161616 (anti-BCMA), PE-7H22-E16 (anti-BAFF-R), PE-rat IgG2a isotype control, PE-mouse IgG1 isotype control, PE-hamster IgG isotype control, or Biotin-hamster IgG isotype control. To examine the ALDH activity, cells were stained with an ALDEFLUOR staining kit (StemCell Technologies) with or without the ALDH inhibitor diethylaminobenzaldehyde (DEAB, 100 μM, Sigma Aldrich) as described previously (Yokota et al., 2009).

PCR

Total RNA was extracted from isolated DCs with the RNeasy Mini Kit (QIAGEN). The preparation and amplification of cDNA were performed with RT-PCR high and ReverTra Ace qPCR RT Kit (Toyobo). Specific primer pairs for RT-PCR were as follows: *Aicda* sense (5′-ATATGGACAGCCTTCTGATGAAGC-3′) and *Aicda* antisense (5′-TCAAAATCCCAACATACGAAATGC-3′); *αGT* sense (IαF, 5′- CCAGGCATGGTTGAGATAGAGATAG-3′) and *αGT* antisense (CαR, 5′-GAGCTGGTGGGAGTGTCAGTG-3′); *αCT* sense (IαF, 5′-CCAGGCATGGTTGAGATAGAGATAG-3′) and *αCT* antisense (CμR, 5′-AATGGTGCTGGGCAGGAAGT-3′); and *Gapdh* sense (5′-ACCACAGTCCATGCCATCAC-3′) and *Gapdh* antisense (5′-TCCACCACCCTGTTGCTGTA-3′). Specific primers for nested PCR (for αCT) were as follows: sense (5′-ACCCTGGATGACTTCAGTGT-3′) and antisense (5′-CATCTGGACTCCTCTGCTCA-3′). Real-time PCR analysis was performed with a LightCycler 480 (Roche) to measure SYBR green I (Roche) incorporation. Specific primer pairs (Hokkaido System Science Co) used for real-time PCR were as follows: *Aicda* sense (5′-AACCCAATTTTCAGATCGCG-3′) and *Aicda* antisense (5′-AGCGGTTCCTGGCTATGATAAC-3′); *Tnfsf13* sense (5′-TCACAATGGGTCAGGTGGTATC-3′) and *Tnfsf13* antisense (5′-TGTAAATGAAAGACACCTGCACTGT-3′); *Tnfsf13b* sense (5′-TGCTATGGGTCATGTCATCCA-3′) and *Tnfsf13b* antisense (5′-GGCAGTGTTTTGGGCA

(I and J) Expression of IFNAR1 (I) and CD69 (J) on cDCs or pDCs from MLNs or PLNs (inguinal LNs). Solid line, IFNAR1 or CD69 staining; dashed line, isotype control staining. Numbers in histograms indicate mean fluorescence intensity (MFI) (J) and fold-change of MFI (IFNAR1 staining/isotype control staining) (I). Data are representative of three independent experiments. *$p < 0.05$. Error bars indicate the SEM.

TATTC-3′); *Furin* sense (5′-CAGAAGCATGGCTTCCACAAC-3′) and *Furin* antisense (5′-TGTCACTGCTCTGTGCCAGAA-3′); *Aldh1a2* sense (5′-GACTTGTAG CAGCTGTCTTCACT-3′) and *Aldh1a2* antisense (5′-TCACCCATTTCTCTCC CATTTCC-3′); *Ifna* (all genes) sense (5′-TCCTGGCGGTGATGAGCTA-3′) and *Ifna* (all genes) antisense (5′-AGTCTGAGGCAGGTCACATCCT-3′); *Ifnb* sense (5′-CCACCACAGCCCTCTCCATCAACTAT-3′) and *Ifnb* antisense (5′-CAAGTG GAGAGCAGTTGAGGACATC-3′); *Tgfb1* sense (5′-GCAACATGTGGAACTCT ACCAGA-3′) and *Tgfb1* antisense (5′-GACGTCAAAAGACAGCCACTCA-3′); *Itgb8* sense (5′-CATTCTTGATCGGGTTGCTT-3′) and *Itgb8* antisense (5′-CA GGCTTTTCTCGTCGGTAG-3′); *Nos2* sense (5′-CTGCCTCATGCCATTGAGT T-3′) and *Nos2* antisense (5′-TGAGCTGGTAGGTTCCTGTTG-3′); and *Gapdh* sense (5′-TGTGTCCGTCGTGGATCTGA-3′) and *Gapdh* antisense (5′-CCTG CTTCACCACCTTCTTGA-3′). The relative expression levels of the mRNA were normalized to the *Gapdh* mRNA level in each sample.

Immunohistochemical Analysis

LNs and PPs were embedded in Tissue Tek OCT compound and then frozen in liquid nitrogen. Frozen tissue sections (6 μm) were prepared with a cryostat (Leica), mounted on microslides, and fixed in methanol. To detect pDCs, the frozen sections were blocked with Block Ace (Yukijirushi) containing FcR blocking reagent (Miltenyi Biotec) with or without 5% normal goat or donkey serum, and then stained with Alexa Fluor 488-N418 (anti-CD11c), Alexa Fluor 488-1D3 (anti-CD19) (Biolegend), FITC-anti-IgA (goat polyclonal, Cappel), PE-ER-TR7 (Santa Cruz) or PE-11-26c (anti-IgD), and Alexa Fluor 647-927 (anti-PDCA1, Biolegend). To detect IFN-α^+ and IFN-β^+ cells, the frozen sections were blocked with Block Ace containing 5% normal donkey serum and Avidin D/biotin solutions (Vector Laboratories, Burlingame, CA) and stained with anti-mouse IFN-α or anti-mouse IFN-β (RMMA-1 and RMMB-1, respectively; PBL Biomedical Laboratories), followed by staining with biotinylated anti-rat IgG (eBioscience). IFN-α^+ and IFN-β^+ cells were visualized with avidin-conjugated FITC and PE-104 (anti-CD45) (both from eBioscience) or PE-ER-TR7. Stained sections were analyzed by fluorescence microscopy (Leica) and confocal microscopy (Olympus).

Statistical Analysis

The statistical significance of the obtained values was evaluated by Student's t test. A p value < 0.05 was considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and can be found with this article online at doi:10.1016/j.immuni.2011.02.002.

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