Gastrointestinal, Hepatobiliary and Pancreatic Pathology

CC Chemokine Receptor 6 Expression by B Lymphocytes Is Essential for the Development of Isolated Lymphoid Follicles

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Isolated lymphoid follicles (ILFs) are organized lymphoid structures that facilitate the efficient interaction of antigen, antigen-presenting cells, and lymphocytes to generate controlled adaptive immune responses within the intestine. Because CC chemokine receptor 6 (CCR6) deficiency affects the generation of mucosal immune responses, we evaluated a potential role for CCR6 in the development of ILFs. We observed that CCR6 and its ligand CCL20 are highly expressed within ILFs and that B lymphocytes are the largest CCR6-expressing population within ILFs. ILF development was profoundly arrested in the absence of CCR6. Concordant with a block in ILF development at a stage corresponding to the influx of B lymphocytes, we observed that CCR6-deficient mice had a diminished population of intestinal B lymphocytes. Bone marrow reconstitution studies demonstrated that ILF development is dependent on CCR6-sufficient B lymphocytes, and adoptive transfers demonstrated that CCR6^{-/-} B lymphocytes were inefficient at localizing to intestinal lymphoid structures. Paralleling these findings, we observed that CCR6-deficient mice had a reduced proportion of Peyer's patch B lymphocytes and an associated reduction in the number and size of Peyer's patch follicular domes. These observations define an essential role for CCR6 expression by B lymphocytes in localizing to intestinal lymphoid structures and in ILF development. (*Am J Pathol* 2007, 170:1229–1240; DOI: 10.2353/ajpath.2007.060817)

The mucosal immune system is a complex network of lymphoid compartments generating immune responses that both protect the host and mitigate potential damage due to uncontrolled inflammation. In the gastrointestinal tract this system includes diffuse effector sites, such as the intestinal lamina propria, as well as organized lymphoid structures that collectively are referred to as the gastrointestinal-associated lymphoid tissues. Organized lymphoid structures provide sites for the efficient interactions of antigens, antigen-presenting cells, and lymphocytes in a controlled environment. These structures are essential for the initiation of primary immune responses and the regulated environment they provide is felt to be necessary to prevent inappropriate immune responses. Isolated lymphoid follicles (ILFs) have only recently become appreciated as distinct members of the gastrointestinal-associated lymphoid tissues. ILFs resemble Peyer's patches (PPs) in their architecture, cellular composition, and ability to act as inductive sites for mucosal immune responses.^{1–5} Our understanding of how these organized structures develop is rapidly evolving. PP formation, like lymph node formation, is developmentally driven, with the early vital events leading to PP formation occurring only during embryogenesis.⁶ On the other hand, ILF formation occurs after birth and may be induced or augmented by luminal stimuli, including normal intestinal flora.^{1,2} Further delineation of this process indicates that in the normal animal ILFs are a spectrum of lymphoid aggregates in various stages of formation and that cryptopatches (CPs) are the precursor lymphoid aggregates giving rise to ILFs.^{2,7–9}

Deficiency of CC chemokine receptor 6 (CCR6) has been demonstrated to adversely affect the development of mucosal immune responses,¹⁰ but the mechanisms

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resulting in this defect are still being elucidated.^{11–14} In contrast to most chemokine receptors, CCR6 pairs monogamously with its ligand, CCL20.¹⁵ β -Defensins have also been identified as potential ligands for CCR6; however, these ligands have a significantly reduced affinity for binding CCR6 and a reduced ability to induce CCR6-dependent chemotaxis when compared with CCL20.¹⁶ CCL20 is preferentially produced at mucosal surfaces by a variety of cell types including monocytes, endothelial cells, dendritic cells, fibroblasts, and epithelial cells in the follicle-associated epithelium (FAE).¹⁷ Multiple cell types express CCR6, including immature dendritic cells, memory T lymphocytes, and B lymphocytes.^{15,18,19} Most investigations into the role of CCR6 in mucosal immune responses have revolved around alterations in the function of PP dendritic cells in CCR6-deficient mice.^{11–14} The contributions of other cell types and the effects of CCR6 deficiency on the development of ILFs have not been previously addressed.

In this study, we evaluated the role for CCR6 in the development of ILFs. We observed elevated expression of CCR6 by ILF B lymphocytes when compared with B lymphocytes from other tissues and elevated expression of CCL20 within ILFs. We found that CCR6^{-/-} mice have significantly reduced numbers of ILFs, but the formation of CPs, as well as CPs containing a large population of dendritic cells, was unaffected. Consistent with this block in ILF formation at a stage before the influx of B lymphocytes, we observed that CCR6^{-/-} mice had a significantly reduced population of non-PP intestinal B lymphocytes, and this defect was most pronounced in the distal small intestine where we have observed the majority of ILFs in wild-type mice. Bone marrow reconstitution demonstrated that CCR6-sufficient B lymphocytes are required for the formation of ILFs, and adoptive transfer studies confirmed that CCR6^{-/-} B lymphocytes have a deficiency in their ability to localize to wild-type PPs and CP/ILFs. Expanding previous reports, and paralleling our observations with ILFs, we observed that CCR6^{-/-} mice have a reduced proportion of B lymphocytes within the PP with an associated decrease in the number of follicles within each PP and a decreased surface area of each PP follicular dome. These findings demonstrate a role for CCR6 expression by B lymphocytes in localizing these cells to organized intestinal lymphoid structures and suggest this requirement is particularly important for ILF development. The profound defect we observed in ILF development in CCR6^{-/-} mice, coupled with the inflammatory nature of CCL20 expression, further highlights the dynamic nature of ILF development and suggests that CCL20 expression facilitates the transition of CPs into ILFs.

Materials and Methods

Mice

Mice used for this study were housed in a specific pathogen-free facility and fed routine chow diet. Animal procedures and protocols were performed in accordance with

the institutional review board at Washington University School of Medicine. C57BL/6 mice, C57BL/6 congenic mice (B6.Cg-IgHa Thy1a Gpi1a/J), and B-cell-deficient JH^{-/-} mice²⁰ on the C57BL/6 background were purchased from The Jackson Laboratory, Bar Harbor, ME. Lymphotoxin (LT) α -deficient mice²¹ (a gift from Dr. Dave Chaplin, University of Alabama, Birmingham, AL) were bred onto the C57BL/6 background for more than 10 generations before use in experiments. CCR6-deficient mice²² were bred on to the C57BL/6 background for four generations before use in experiments. Timed pregnant C57BL/6 female mice and CCR6^{-/-} mice for use in experiments involving the injection of lymphotoxin β receptor (LT β R)-Ig fusion protein, were generated by matings with C57BL/6 male mice or CCR6^{-/-} male mice, respectively. Six- to 10-week-old LT α ^{-/-} mice were used as recipients for bone marrow transfers.

Bone Marrow Transfers

Bone marrow transfers were performed after lethal irradiation as previously described.²³ A total of 1×10^7 T-lymphocyte-depleted bone marrow cells from gender-matched donors were injected intravenously into recipients on the second day of irradiation. Mice receiving bone marrow from multiple donors (C57BL/6 and JH^{-/-} or CCR6^{-/-} and JH^{-/-}; see Figure 4) received 5×10^6 cells from each donor. Mice were allowed 12 weeks for reconstitution with donor bone marrow before use in experiments. Flow cytometric analysis was performed on splenocytes from recipients at the time of sacrifice to document appropriate B- and T-lymphocyte reconstitution.

LT β R-Ig Treatment

LTR β -Ig was purified from supernatants generated by a Chinese hamster ovary cell line producing the LTR β -Ig fusion protein (a gift from Dr. W. Yokoyama, Washington University School of Medicine, St. Louis, MO) as previously described.²³ Timed pregnant female mice were injected with 100 μ g of LT β R-Ig or 100 μ g of human Ig (Bayer Corporation, Elkhart, IN) via tail vein on day 16 postcoitus. Mice receiving LT β R-Ig or human Ig *in utero* were analyzed for the presence of ILFs at 7 weeks of age (see Figure 2).

Whole Mounts of Small Intestine

Small intestines were removed intact, flushed with ice-cold phosphate-buffered saline (PBS), and opened along the mesenteric border. Intestines were mounted, lumen facing up, and fixed with ice-cold 10% phosphate-buffered formal saline (Fisher Scientific, Pittsburgh, PA) for 1 hour at 4°C. Intestines were washed three times in ice-cold PBS, incubated in a solution of 20 mmol/L dithiothreitol, 150 mmol/L Tris, and 20% ethanol at room temperature for 45 minutes, washed three times in ice-cold PBS, and incubated in a solution of 1% H₂O₂ for 15 minutes at room temperature to block endogenous peroxidases. In-

testines were washed three times in PBS, followed by incubation in PBS containing 1% bovine serum albumin (BSA) and 0.3% Triton X-100 for 30 minutes. Intestines were incubated with horseradish peroxidase-conjugated lectin from *Ulex europaeus* (UEA-I; Sigma-Aldrich, St. Louis, MO) in PBS, BSA, and Triton X-100 solution overnight at 4°C to facilitate the identification of PPs and mature ILFs (mILFs). The following day intestines were washed three times in PBS, incubated in DAB metal peroxide substrate (Pierce Chemical Co., Rockford, IL) for 15 minutes, rinsed twice in distilled water, and returned to PBS for further analysis. Investigators unaware of the treatment groups determined the presence of mILF. Under low-power microscopy (25 to 65×) previously established criteria were used to determine the presence of mILF²: 1) presence of a nodular structure with size equal to or greater than the width of one villus; 2i) nodular structure possessing an overlying dome resembling the FAE of PP; and 3) nodular structures occurring singly or in groups of two.

For the analysis of PPs, whole mounts of intestines were stained with UEA-I as above. The following criteria were used for the purpose of enumerating PPs. PPs were defined as nodular structures more than five villi wide, located along the anti-mesenteric border of the small intestine. These criteria overlap with the criteria used to enumerate mILFs but were necessary because some of the PPs of the CCR6^{-/-} mice contained only one follicle. PPs were identified using a dissecting microscope at 32× magnification and photographs obtained of each PP. Photographs of PPs and a scale were enlarged to 10 × 12 inches, and the diameter of each dome was measured. The surface area of each dome was calculated using the following formula: surface area = π (diameter/2).²

For anti-B220 and anti-CD11c staining of whole mounts to determine the numbers of iILFs and CD11c⁺ clusters, intestines were removed intact, flushed with PBS, opened along the mesenteric border, and mounted as above. Intestines were then incubated three times in Hanks' balanced salt solution (BioWhittaker, Walkersville, MD) containing 5 mmol/L EDTA at 37°C with shaking for 10 minutes to remove epithelial cells. Intestines were then fixed in 10% phosphate-buffered formal saline and treated with 1% H₂O₂ for 15 minutes at room temperature as above. Intestines were incubated in a solution of 50 mmol/L Tris, pH 7.2, 150 mmol/L NaCl, 0.6% Triton X-100, and 0.1% BSA for 1 hour at 4°C to block nonspecific antibody binding and then incubated with rat anti-mouse B220 antibody (PharMingen, San Diego, CA) or biotin-conjugated hamster anti-mouse CD11c (eBiosciences, San Diego, CA) diluted in the above solution overnight at 4°C. Intestines were washed three times in the above solution and incubated with a horseradish peroxidase-conjugated goat anti-rat IgG antibody or streptavidin-conjugated horseradish peroxidase (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted in the above solution at room temperature for 1 hour. Intestines were washed three times and incubated in DAB metal peroxide substrate as above. Intestine whole mounts were examined under a dissecting microscope at 25 to

65×. Immature ILFs (iILFs) were counted as clusters of B220⁺ cells occurring at the base of villi and not containing an overlying dome. Dendritic cell clusters were counted as clusters of CD11c⁺ cells occurring at the base of villi.

Cell Isolation from Spleen, PPs, and mILFs

Spleens and PPs were removed from unmanipulated C57BL/6 mice and disrupted by mechanical dissociation. Intestines were removed from C57BL/6 mice receiving LT β R-Ig *in utero*, flushed with ice-cold PBS, opened along the mesenteric border, and mounted with the lumen facing up in cold PBS, as described above. Using the dissecting microscope and a blunt-end 26-gauge needle and syringe, multiple mILFs were aspirated and placed in cold PBS. Red blood cells were lysed from cellular suspensions and then used for flow cytometric analysis as described below. Average yield of viable mononuclear ILF cells ranged from 3 to 7 × 10⁵ cells/intestine.

Flow Cytometric Analysis

Single-cell suspensions were obtained as above and flow cytometric analysis performed as previously described.²³ Reagents used for analysis were fluorescein isothiocyanate-conjugated or phycoerythrin-conjugated rat anti-mouse CD19, fluorescein isothiocyanate-conjugated anti-mouse CD45, streptavidin-conjugated phycoerythrin, appropriate isotype control antibodies (all from BD Biosciences, San Diego, CA), and rat anti-mouse CCR6 (R&D Systems, Minneapolis, MN). Dead cells were excluded based on forward and side light scatter. Gates for positive staining were defined such that 1% of the analyzed population stained positive with the appropriate isotype control antibody. Flow cytometric analysis for the expression of CCR6 was performed using directly conjugated anti-CCR6 antibodies in some replicates as well as anti-CCR6 antibodies with an anti-rat IgG secondary antibody (eBiosciences) to augment the fluorescence intensity in some replicates (Figure 1, c and d); both methods gave equivalent results.

Immunohistochemistry

Paraffin-embedded sections containing PPs from whole-mount intestines (performed as described above) were deparaffinized, treated with antigen-unmasking solution (Vector Laboratories, Burlingame, CA), treated with avidin/biotin blocking kit (Vector Laboratories), washed three times in PBS, and blocked for 15 minutes at room temperature in PBS containing 1% BSA and 0.1% Triton X-100. Sections were then incubated with biotin-conjugated lectin from *Arachis hypogaea* (PNA) (Sigma-Aldrich) or biotin-conjugated anti-B220 antibody (BD Biosciences) diluted in PBS containing 1% BSA and 0.1% Triton X-100 overnight at 4°C. Biotinyl-tyramide signal amplification (DuPont/NEN, Boston, MA) followed by incubation with streptavidin-conjugated cyanine 2 dye (Jackson ImmunoResearch) was used for detection of

Figure 1. CCR6 and CCL20 are expressed within ILFs. RNA was isolated from non-PP, non-mILF-bearing small intestine, PPs, and mILFs and used to analyze the expression of CCR6 and CCL20 as described in Materials and Methods (**a** and **b**). Cellular populations were isolated from spleen, PPs, and mILFs and used to examine cell surface expression of CCR6 by flow cytometry as described in Materials and Methods (**c** and **d**). ILFs demonstrated elevated expression of CCR6 when compared with adjacent non-PP, non-ILF-bearing intestine (**a**). A large population of ILF cells express cell surface CCR6, and the majority of CCR6⁺ ILF cells are B lymphocytes (CD19⁺); gates are set such that less than 1% of the cellular population stains positive with the isotype control antibody (**c**). In side-by-side comparisons, we observed that CCR6 expression was higher in mILF and PP B lymphocytes when compared with splenic B lymphocytes (**d**). We observed that CCL20 expression was significantly higher in PPs and mILFs when compared with non-PP, non-mILF bearing small intestine (**b**). Numeric values in parentheses (**d**) represent the mean fluorescence intensity for each population; the mean fluorescence intensity for the isotype control staining of the CD19⁺ cells from the PPs and mILFs in **d** were 94.6 and 67.3, respectively. Data in **a** and **b** represent the mean $\pm$ 1 SD of each of two independent experiments. Data in **c** and **d** are representative of one of four independent experiments. **P* < 0.05 for each replicate when compared with non-PP non-ILF-bearing intestine in **a** and **b**.

PNA staining. Anti-B220 staining was detected using streptavidin-conjugated cyanine 3 dye (Jackson ImmunoResearch). Sections were counterstained with Hoescht dye (Sigma-Aldrich) to visualize nuclei.

Immunohistochemistry on frozen sections of intestine was used to enumerate CD90⁺ cellular clusters and to evaluate the localization of adoptively transferred B lymphocytes. Intestines were embedded in OCT compound and serial 8- μ m sections were obtained from each block. Slides were fixed with a 1:1 solution of acetone and methanol for 15 minutes, dried, rehydrated, and blocked with PBS containing 1% BSA. Sections were incubated with primary antibodies overnight at 4°C, washed with PBS, and incubated with fluorescently labeled secondary reagents for 1 hour at room temperature. Sections were stained with Hoescht dye (Sigma-Aldrich) to visualize nuclei.

Quantification of CD90⁺ Cellular Clusters

Segments of small intestine of 1.5 cm were embedded in OCT compound, frozen, and cut at an axis perpendicular

to the villi into 8- μ m sections. The segments comprised more than one-half of the entire intestine, and identical areas of the intestine were obtained from each animal evaluated. Two serial sections of small intestine were stained with anti-CD90 (eBioscience) and anti-CD3 (eBioscience) as above using two-color immunofluorescence. The serial sections were examined at a magnification of 100 $\times$ or higher, and the number of CD90⁺CD3⁺ cellular clusters falling within the crypt area on each section was counted and averaged between the serial sections. Additional sections were stained with anti-CD90 and anti-c-kit to confirm that the CD90 clusters are also c-kit⁺, stained with anti-B220 to assess the numbers of B lymphocytes associating with the CD90 clusters (Figure 2, d and e), and stained with anti-CD11c (BD Biosciences) to assess the clustering of dendritic cells associated with the CD90 clusters. Photomicrographs of the sections taken with a 25 $\times$ objective were assembled, and the crypt area of each section was determined using AxioVision software (Carl Zeiss MicroImaging GmbH, Gottingen, Germany). The average number of CD90⁺ clusters/mm² of crypt surface area was then determined.

Figure 2. ILF formation is arrested at a stage corresponding to the influx of B lymphocytes in the absence of CCR6. Intestines from CCR6^{-/-} mice and wild-type mice were evaluated for the presence of CD90⁺ clusters (**a**), corresponding to all CP and ILF lymphoid aggregates, CD11c⁺ clusters (**b**), corresponding to CPs with a substantial proportion of dendritic cells and ILFs, B220⁺ clusters (**c**), corresponding to immature ILFs, and mature ILFs (**c**) as outlined in Materials and Methods. There were no significant differences in the numbers of CD90⁺ clusters (**a**) or CD11c⁺ clusters (**b**) between the two groups. There was a significant difference between the groups with respect to the numbers of B220⁺ clusters or iILFs (**c**). The defect in the development of ILFs in the CCR6^{-/-} mice could not be overcome by treatment with LTβR-Ig *in utero*, a manipulation that augments the numbers of ILFs in wild-type mice; treatment with control (human) Ig did not augment ILF numbers (**c**). CD90⁺ clusters from wild-type mice (**d**) and CCR6^{-/-} mice (**e**) were of equivalent size; however, the CD90⁺ clusters from CCR6^{-/-} mice had fewer B lymphocytes (**d** and **e**). Red, anti-CD90; green, anti-B220; blue, Hoechst dye to visualize nuclei. Original magnification, ×400. The defect in the development of ILFs in the CCR6^{-/-} mice correlated with a lack of B lymphocytes in the diffuse lamina propria of the intestine, which contains CPs and ILFs (**f**). This defect was most pronounced in the distal small intestine, the region in which the majority of ILFs are located in wild-type mice in our colony (**f**). Data are displayed as the mean ± SD for **a**, **c**, and **f**. The data from **a** are generated from two mice from each group. The data from **c** and **f** were generated from three or more mice from each treatment group. **P* < 0.05 when comparing C57BL/6 and CCR6^{-/-} mice using a standard Student's *t*-test for unmanipulated animals and using a one-way analysis of variance to compare LTβR-Ig- and control Ig-treated animals.

RNA Isolation and Real-Time Polymerase Chain Reaction

PPs were removed from unmanipulated C57BL/6 mice. Mature ILFs were isolated from C57BL/6 mice receiving LTβR-Ig *in utero* using a dissection microscope and 26-gauge needle as described above. Non-PP, non-mILF-bearing intestine from the distal small intestine of C57BL/6 mice was identified using a dissecting microscope and removed. The PP and mILF tissue contained the overlying FAE, stromal elements, and mononuclear cells.

RNA was isolated using Trizol (Invitrogen, Carlsbad, CA) and treated with DNase I (Ambion, Austin, TX) to remove contaminating DNA, and cDNA was synthesized from 2 μg of total RNA using Superscript II RNase H⁻ reverse transcriptase (Invitrogen). Expression of targets was detected by real-time polymerase chain reaction using ABI prism 7700 sequence detection system and SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). The following primers were used for detection of the targets, forward primers are listed first, followed by reverse primers: 18S 5'-CGGCTACCACATCC-AAGGAA-3' and 5'-GCTGGAATTACCGCGGCT-3', β-actin 5'-GCTTCTTTCAGCTCCTTCGT-3' and 5'-ATAT-CGTCTATCCATGGCGAAC-3', CCL20 5'-TGATGCTTTT-TGGGATGGAA-3' and 5'-AGCCTTCAACCCAGCTGT-3', and CCR6 5'-TGTTCTGCTATCTGTTTCATTATCAAG-A-3' and 5'-CACGACTCGGATGGCTCTGT-3'. Samples were measured in triplicate. Relative quantitation of target expression using 18S or β-actin as a housekeeping gene was determined using the comparative crossing threshold method as described in the ABI Prism 7700 sequence detection system user bulletin.

Adoptive Transfers

To assess the ability of wild-type and CCR6^{-/-} B lymphocytes to localize to the organized intestinal lymphoid tissues, splenocytes isolated from CCR6^{-/-}, C57BL/6 congenic mice (expressing the IgH^a allotype) were injected intravenously into JH^{-/-} (B lymphocyte-deficient) recipients. Intestinal B lymphocytes could not be used for these studies because the CCR6^{-/-} mice had few intestinal B lymphocytes (Figure 2f). Recipients received a total 3 × 10⁷ cells from either CCR6^{-/-} mice, C57BL/6 congenic mice, or combined CCR6^{-/-} and C57BL/6 congenic mice. Flow cytometric analysis was performed on the cellular populations at the time of transfer to confirm that equivalent numbers of B lymphocytes from each donor were injected into recipients. Mice were sacrificed 1 week later and frozen sections from PPs and small intestines were evaluated for the presence of transferred cellular populations using immunohistochemistry with anti-IgM^b (BioLegend, San Diego, CA), anti-IgM^a (BD Biosciences), and anti-B220 antibodies. In co-transfer experiments, serial sections of intestine were stained with either IgM^a or IgM^b. In experiments involving separately transferred populations, intestinal sections were stained with anti-CD11c and anti-B220. Sections were examined

with fluorescence microscopy at a magnification of 200 $\times$ or higher. The number of IgM^a- or IgM^b-positive cells in each PP serial section or the number of B220⁺ cells in each CD11c⁺ cluster was determined by an individual unaware of the treatment groups.

Statistical Analysis

Data analysis using Student's *t*-test and a one-way analysis of variance with a Dunnett's multiple comparison post-test was performed using GraphPad Prism (GraphPad Software Inc., San Diego, CA).

Results

CCL20 Is Highly Expressed within PPs and ILFs, and CCR6 Is Highly Expressed by ILF and PP B Lymphocytes

To assess the role of CCR6 in ILF formation, we examined the mRNA expression of CCR6 in ILFs using real-time polymerase chain reaction. We noted that CCR6 expression was significantly higher in ILFs when compared with non-PP, non-ILF-bearing intestine (Figure 1a). To evaluate CCR6 protein expression and to determine the cell types expressing CCR6, we examined ILF cellular populations for CCR6 expression by flow cytometry. We found that a large proportion of the ILF cellular population expresses CCR6; gates for positive staining were set such that less than 1% of cells stain positive for the isotype control (Figure 1c). The majority of cells falling within the CCR6⁺ gate are B lymphocytes (CD19⁺) (Figure 1c). Directly comparing B-lymphocyte populations from the spleen, PPs, and ILFs, we observed that ILF B lymphocytes displayed greater cell surface expression of CCR6 when compared with splenic B lymphocytes and Peyer's patch B lymphocytes (Figure 1d). We observed that T lymphocytes (CD3⁺) and dendritic cells (CD11c⁺) displayed staining consistent with CCR6⁺ and CCR6⁻ populations, which comprised approximately 3% and 2% of the CCR6⁺ ILF cellular population, respectively; the remaining CCR6⁺ population of the ILFs was predominantly comprised of lineage c-kit⁺ cells (data not shown). In attempts to understand further the significance of CCR6 in ILF and PP formation, we examined the expression of CCL20 in the spleen, PPs, and ILFs. We found that both ILFs and PPs displayed CCL20 mRNA expression that was significantly greater than that seen in the non-PP, non-mILF-bearing intestine (Figure 1b).

ILF Development Is Arrested at a Stage Corresponding to the Influx of B Lymphocytes in the Absence of CCR6

To determine whether CCR6 played an important role in the formation of CPs and ILFs, we examined the intestines of CCR6^{-/-} and C57BL/6 mice for the presence of CPs and ILFs. CPs are clusters of lineage marker-nega-

tive c-kit-positive cells randomly distributed throughout the small intestine.²⁴ These CP cells also express other markers such as CD90. CPs can only be identified using immunohistochemistry on frozen sections of intestine cut on an axis perpendicular to the villi. We observed that anti-CD90 is a useful marker for enumerating CPs, as the anti-CD90 staining is robust and CPs can easily be identified using 100 $\times$ magnification. In contrast, anti-c-kit staining is relatively weak and therefore less useful to enumerate CPs. We observed that CCR6^{-/-} mice had an equivalent number of CD90⁺ clusters when compared with wild-type mice (Figure 2a). CPs and ILFs also contain a surrounding population of CD11c⁺ dendritic cells. In some CPs, this CD11c⁺ population becomes prominent. We can enumerate these CPs containing a large population of CD11c⁺ cells as well as CD11c⁺ clusters within ILFs using whole mounts with anti-CD11c staining. We observed no differences in the numbers of large CD11c⁺ clusters when comparing CCR6^{-/-} mice to wild-type mice (Figure 2b).

The transition of CPs to ILFs is thought to occur when a subset of CPs are infiltrated by B lymphocytes. Initially, B lymphocytes cluster in the CP at the base of the villi and form a loosely organized structure that we have termed the iILFs. A subset of these immature structures continue to accumulate B lymphocytes and develop a follicle-associated epithelium and a loosely organized germinal center; we have termed these structures mILFs. Using whole mounts of the intestine to enumerate ILFs, we observed that CCR6^{-/-} have a near absence of both iILFs and mILFs, and the reduced number of iILFs was significantly different from wild-type mice (Figure 2c). Mature ILFs are rare in C57BL/6 mice in our colony, making it difficult to assess a defect in mILF development by comparing wild-type and CCR6^{-/-} mice. In addition, underdeveloped PPs in the CCR6^{-/-} mice would be counted as mILFs in the current scoring system, further complicating this assessment. However, the numbers of mILFs can be augmented by LT β R-Ig treatment *in utero*. *In utero* LT β R-Ig treatment on day 16 of gestation ablates PP development and results in a transient IgA deficiency in the progeny, which is subsequently associated with augmented development of ILFs.²⁵ Therefore, to identify a defect in mILF development, mice were given LT β R-Ig *in utero* and examined for the presence of ILFs. Treatment of C57BL/6 mice with LT β R-Ig *in utero* augmented the development of both iILFs and mILFs (Figure 2c). CCR6^{-/-} mice showed a modest increase in the numbers of iILFs following LT β R-Ig treatment; however, the numbers of iILFs formed were still significantly less than unmanipulated C57BL/6 mice (Figure 2c). This indicates that in the absence of CCR6 other pathways can facilitate iILF formation, but these pathways are less effective. Treatment of CCR6^{-/-} mice with LT β R-Ig *in utero* failed to induce the formation of mILFs (Figure 2c). Consistent with a block in the ability of B lymphocytes to infiltrate the CP in CCR6^{-/-} mice, we observed few B220⁺ cells within CD90⁺ clusters in CCR6^{-/-} mice (Figure 2, d and e). In C57BL/6 mice in our colony, ILFs are located preferentially in the distal small intestine.² Consistent with the loss of ILFs and the inability of CCR6^{-/-} CP to become infil-

trated with B lymphocytes, we observed that CCR6^{-/-} mice have a diminished population of B lymphocytes in their diffuse lamina propria, which contains the CPs and ILFs (Figure 2f). This defect was most pronounced in the distal small intestine where the majority of ILFs are located in the wild-type mice (Figure 2f). These findings indicate that in the absence of CCR6 the development of ILFs is blocked at a stage of B-lymphocyte infiltration into CPs, or the formation of iILFs.

PPs Have a Reduced B-Lymphocyte Population and a Reduced FAE in the Absence of CCR6

To determine whether CCR6 deficiency affected the B-lymphocyte population in other intestinal organized lymphoid structures, we examined the PPs of the CCR6^{-/-} mice. The initial observations regarding the PP phenotype in the CCR6^{-/-} mice was unclear, with one group reporting normal PP development and a second group reporting that the CCR6^{-/-} PPs contained fewer domes and had reduced total cellularity.^{10,13} We analyzed the numbers of PPs, the numbers of domes, and the surface area of each dome in CCR6^{-/-} and wild-type mice. We observed that CCR6^{-/-} mice had an equal number of PPs when compared with wild-type mice (data not shown); however, each PP in the CCR6^{-/-} mice contained significantly fewer domes when compared with wild-type mice (Figure 3, a, e, and f). Moreover, the average surface area of each PP in the CCR6^{-/-} mice was significantly less than that seen in the wild-type mice (Figure 3, b, e, f, g, and h). Consistent with these observations, we observed significantly decreased total cellularity of PPs from CCR6^{-/-} mice when compared with wild-type mice (Figure 3c), and we observed a decrease in the B-lymphocyte population in the PPs of CCR6^{-/-} mice when compared with wild-type mice (Figure 3d). We did not observe any differences in the proportion of CD4⁺ or CD8⁺ T lymphocytes or in the proportion of dendritic cells (MH-CII⁺, CD11c⁺) within the PP when comparing CCR6^{-/-} and wild-type mice (data not shown). The architecture and organization of the PP from CCR6^{-/-} mice and wild-type mice were similar. Each contained a large population of B220⁺ cells with a central lucent area with PNA⁺ staining consistent with the presence of germinal centers (Figure 3, g–l). These findings confirm and extend earlier findings regarding the PPs of CCR6^{-/-} mice and demonstrate that although CCR6 deficiency affects B-lymphocyte populations in both types of organized intestinal lymphoid structures, this deficiency more dramatically effects ILF development.

CCR6-Sufficient B Lymphocytes Are Essential for the Formation of ILFs

Given the above findings, we wished to determine whether CCR6-sufficient B lymphocytes were required for the development of ILFs and to examine the stage of ILF development for which a requirement for CCR6-sufficient B lymphocytes might exist. LT α ^{-/-} mice lack CPs, iILFs, and mILFs, but the formation of ILFs can be reliably induced in LT α ^{-/-}

Figure 3. CCR6^{-/-} mice have a decreased population of PP B lymphocytes and an associated decrease in the size of the PP FAE. Intestines from CCR6^{-/-} and C57BL/6 mice were analyzed for the numbers of PPs, the numbers of domes per PP, the surface area of each dome, PP cellularity, and PP cellular composition as described in Materials and Methods. Panels **e**, **g**, **i**, and **k** are representative photomicrographs of PPs from C57BL/6 mice. Panels **f**, **h**, **j**, and **l** are representative photomicrographs of PPs from CCR6^{-/-} mice. Panels **e** and **f** demonstrate the appearance of PPs on UEA-I-stained whole mounts from the small intestine. Panels **g** and **h** demonstrate the appearance of PPs on H&E-stained sections. Panels **i** and **j** and panels **k** and **l** demonstrate the appearance of PPs on anti-B220-stained (red) and PNA-stained (green) sections, respectively. Nuclei are identified by blue staining with Hoechst dye in **i–l**. CCR6^{-/-} mice had normal numbers of PPs (not shown), but PPs from CCR6^{-/-} mice had a significantly reduced number of domes (**a**, **e**, and **f**). In addition, the surface area of each dome was reduced in PPs from CCR6^{-/-} mice (**b**, **g**, and **h**; arrows in **g** and **h** denote the FAE in cross section). Consistent with the reduced number of domes we noted a reduced overall cellularity of PPs from CCR6^{-/-} mice (**c**). Analysis of cellular composition revealed a reduced population of B lymphocytes in PPs from CCR6^{-/-} mice (**d**). The size of the PP follicles from CCR6^{-/-} and C57BL/6 mice were comparable (**g** and **h**), and both contained a population of B lymphocytes (B220⁺ cells staining red, **i** and **j**) with lucent areas consistent with the presence of germinal centers (PNA⁺ cells staining green, **k** and **l**). Data were generated from nine animals of each genotype. **P* < 0.05.

Figure 4. CCR6-sufficient B lymphocytes are essential for the development of ILFs. CP and ILF formation can be restored in LT $\alpha^{-/-}$ mice following the transfer of LT-sufficient bone marrow. To assess a requirement for CCR6 on B lymphocytes in ILF development, intestines from lethally irradiated LT $\alpha^{-/-}$ mice reconstituted with CCR6 $^{-/-}$ bone marrow, a combination of CCR6 $^{-/-}$ and JH $^{-/-}$ bone marrow, such that the recipients would be selectively deficient in CCR6 expressing B lymphocytes, or a combination of C57BL/6 and JH $^{-/-}$ bone marrow were examined for the presence of iILFs and mILFs. Mice reconstituted with a combination of C57BL/6 and JH $^{-/-}$ bone marrow developed the expected numbers of iILFs and mILFs, whereas mice reconstituted with CCR6 $^{-/-}$ bone marrow or a combination of CCR6 $^{-/-}$ and JH $^{-/-}$ bone marrow developed few iILFs or mILFs (**a**). Flow cytometric analysis of splenocytes from recipients was used to confirm appropriate reconstitution of CCR6-sufficient and -deficient lineages (data not shown). Representative photomicrographs of intestinal whole mounts from LT $\alpha^{-/-}$ mice receiving CCR6 $^{-/-}$ bone marrow (**b** and **e**), LT $\alpha^{-/-}$ mice receiving CCR6 $^{-/-}$ and JH $^{-/-}$ bone marrow (**c** and **f**), or LT $\alpha^{-/-}$ mice receiving C57BL/6 and JH $^{-/-}$ bone marrow (**d** and **g**) stained with UEA-I (**b–d**) to visualize mature ILFs, or with anti-B220 (**e–g**) to visualize immature ILFs, demonstrate the requirement for CCR6 $^{-/-}$ B lymphocytes in ILF formation. Mature ILFs appear as white domes on the background of nonspecific staining (**d**); UEA-I-positive M-cells in the FAE cannot be visualized at this magnification. Immature ILFs appear as clusters of brown (B220 $^{+}$) cells (**f** and **g**). Recipients of CCR6 $^{-/-}$ bone marrow failed to develop iILFs or mILFs (**b** and **e**), recipients of a combination of JH $^{-/-}$ and CCR6 $^{-/-}$ bone marrow failed to develop mILFs (**c**), but developed rare iILFs (**f**, arrow), whereas recipients of a combination of wild-type and JH $^{-/-}$ bone marrow developed mILFs (**d**, arrowheads) and iILFs (**g**, arrows). Data are displayed as the mean $\pm$ SD, data for **a** was generated from five animals in each treatment group. Original magnification for **b–g**, $\times 20$. * $P < 0.05$ using a one-way analysis of variance.

mice following the transfer of LT-sufficient bone marrow reconstituting the LT/LT β R, as well as the LT/TNFR and LT/HVEM axes.^{2,25} This provides a useful model to study the requirements of bone marrow-derived cell populations on ILF formation. LT $\alpha^{-/-}$ mice reconstituted with CCR6 $^{-/-}$ bone marrow, a combination of CCR6 $^{-/-}$ and JH $^{-/-}$ bone marrow, such that all B lymphocytes are CCR6-deficient, or a combination of C57BL/6 and JH $^{-/-}$ bone marrow were examined for the presence of iILFs and mILFs. LT $\alpha^{-/-}$ recipients of CCR6 $^{-/-}$ bone marrow and recipients of a combination of CCR6 $^{-/-}$ and JH $^{-/-}$ bone marrow were deficient in the formation of iILFs and mILFs when compared with recipients of a combination of C57BL/6 and JH $^{-/-}$ bone marrow (Figure 4), thus demonstrating a requirement for CCR6-sufficient B lymphocytes in the development of ILFs.

CCR6 $^{-/-}$ B Lymphocytes Have a Diminished Ability to Localize to Organized Gastrointestinal-Associated Lymphoid Tissues

The above findings suggest that CCR6 $^{-/-}$ B lymphocytes have a diminished capacity to localize to intestinal lymphoid structures. To confirm this defect we injected equal numbers of CCR6 $^{-/-}$ (IgH^b allotype) and wild-type (IgH^a allotype) splenic B lymphocytes into B-cell-deficient JH $^{-/-}$ mice and looked for their presence in organized intestinal lymphoid structures. When equal numbers of CCR6 $^{-/-}$ and wild-type B lymphocytes were injected into the same JH $^{-/-}$ recipient, we observed that wild-type B lymphocytes have a threefold increased ability to localize to PPs as determined by the number of IgM^{bright} cells (Figure 5, a–c). We also observed a larger number of wild-type IgM^{dull} cells when compared with CCR6 $^{-/-}$ IgM^{dull} cells in the recipients' PPs; however, definitive quantification of these IgM^{dull} cells was not feasible due to the intensity of staining. We were unable to identify many donor B lymphocytes within CD90 $^{+}$ or CD11c $^{+}$ clusters when using IgM allotype staining, despite being able to identify B220 $^{+}$ CD11c $^{-}$ cells within these clusters. We feel this is due to the relatively lower expression of IgM by the majority of ILF B lymphocytes, making their allotype-specific identification more difficult. To examine the ability of CCR6 $^{-/-}$ B lymphocytes to localize to CD11c $^{+}$ clusters, we transferred equivalent numbers of wild-type or CCR6 $^{-/-}$ splenic B lymphocytes to individual recipients and examined the recipients for the presence of B220 $^{+}$ CD11c $^{-}$ cells within the clusters. We observed that wild-type B lymphocytes had approximately a two-fold better ability to localize to CD11c $^{+}$ clusters when compared with CCR6 $^{-/-}$ B lymphocytes (Figure 5, d–f). These findings demonstrate that CCR6 $^{-/-}$ B lymphocytes have an impaired ability to localize to organized intestinal lymphoid structures.

Discussion

A primary function of the mucosal immune system is to protect the host from invading pathogens while avoiding uncontrolled, damaging immune responses. Organized lymphoid structures within the intestine, including PPs and ILFs, play a key role in this function. They provide an environment allowing for the efficient interaction of antigen, antigen-presenting cells, and lymphocytes to facilitate the development of protective adaptive immune responses. A key distinction between PPs and ILFs are the pathways leading to their formation. The formation of PPs is developmentally driven, with the initial steps in PP formation occurring only during embryogenesis,⁶ whereas the development of ILFs occurs after birth and can be influenced by luminal stimuli, including normal intestinal flora.^{1,2,26} Recently, CPs have been suggested to give rise to ILFs.^{7–9} Therefore, CP and ILF development may be closely linked, and events affecting CP development may subsequently affect ILF development.

CCL20 has several properties suggesting it is a candidate for participating in ILF development. CCL20 is

Figure 5. CCR6-deficient B lymphocytes have impaired localization to intestinal lymphoid structures. Splenocytes from CCR6^{-/-} (IgH^a allotype) mice and wild-type congenic (IgH^a allotype) mice were transferred together (a–c) or transferred separately (d–f) into B lymphocyte-deficient (JH^{-/-}) recipients as described in Materials and Methods. Transferred cellular populations contained equivalent numbers of B lymphocytes. Mice were evaluated 7 days later for the presence of wild-type or CCR6^{-/-} B lymphocytes by immunohistochemistry with anti-IgM^a (wild type) and anti-IgM^b (CCR6^{-/-}) staining when cotransferred (a–c) or anti-B220 staining when transferred separately (d–f). Wild-type B lymphocytes displayed a threefold greater ability to localize to the PP as determined by the numbers of IgM^{hi} cells present in the PP using allotype specific staining (a–c). There were also a significant number of IgM^{lo} IgM^{a+} (wild type) cells present in the PP, but these could not be accurately quantified due to the intensity of staining (b). CCR6^{-/-} B lymphocytes also displayed a significant, but less pronounced, defect in localizing to CD11c⁺ clusters as determined by anti-B220 staining (d–f). Data in d are displayed as the mean $\pm$ SD. Data were generated from two independent experiments with a total of four mice in each of the transfer groups. * $P < 0.05$.

produced at mucosal surfaces, and specifically produced by the FAE under normal circumstances.¹⁷ Similar to ILF formation, CCL20 expression is induced by inflammatory stimuli, and intestinal CCL20 expression is elevated in inflammatory conditions associated with the formation of lymphoid structures.^{17,27–29} In addition, CCL20 functions to recruit dendritic cells and B lymphocytes; these cell types make up a large proportion of the ILF cellular population.⁴ CCR6, the only identified receptor for CCL20, plays an important role in mucosal immune

responses as evidenced by diminished production of antigen-specific mucosal IgA following oral immunization of CCR6-deficient mice.¹⁰ The specific defects resulting in this phenotype are still being elucidated. Initial studies attributed this defect to the absence of CD11b⁺ dendritic cells in the PP subepithelial dome in CCR6-deficient mice.¹³ However, subsequent studies demonstrated that this dendritic cell population was present but reduced in CCR6-deficient mice.¹⁴ More recent investigations revealed that CCR6 expression by dendritic cells in the subepithelial dome is required to initiate T-cell-dependent responses to an oral pathogen.¹² Despite these observations, the role of CCL20 and CCR6 in the development of ILFs is largely uninvestigated with the exception of one report of normal CP development in CCR6^{-/-} mice.³⁰

Here, we demonstrate that CCL20 expression is increased in ILFs and that CCR6 is highly expressed by B lymphocytes within ILFs. We observed that CCR6-deficient mice have a defect in ILF development; however, the formation of CPs and the recruitment of a substantial population of dendritic cells into these CPs are not altered in these animals. This defect in ILF development correlated with a decreased population of B lymphocytes in the diffuse lamina propria, which contains the CPs and ILFs. Accordingly, this defect was most pronounced in the distal small intestine, the region in which we see the majority of ILFs in wild-type mice. These observations indicate that the requirement for CCR6 in ILF development coincides with the influx of B lymphocytes into the CPs and/or ILFs. Using bone marrow chimeric mice, we confirmed the requirement for CCR6 expression by B lymphocytes in ILF development. These findings were further supported by adoptive transfer studies demonstrating that CCR6^{-/-} B lymphocytes had a reduced ability to localize to PPs and CPs. This demonstrates a specific requirement for CCR6 expression by B lymphocytes in localizing to the intestine and is consistent with a block in ILF development at a stage in which B lymphocytes localize to these structures in CCR6-deficient mice. Although not specifically addressed in the studies presented here, our findings do not preclude, but could suggest, that other CCR6-sufficient cell types play a role in ILF development. We observed a small number of iILFs in recipients of a combination of JH^{-/-} and CCR6^{-/-} bone marrow, suggesting that other pathways can partially compensate for the loss of CCR6 expression by B lymphocytes. In combination with this observation, the absence of ILFs in the recipients of CCR6^{-/-} bone marrow suggests that a CCR6-sufficient non-B lymphocyte plays a role in ILF development. In addition to these observations, we noted a trend toward decreased numbers of large CD11c⁺ clusters in the CCR6^{-/-} mice, suggesting other potential defects in ILF development.

PPs from CCR6^{-/-} mice revealed parallel, but less dramatic, findings. CCR6^{-/-} mice have a normal number of PPs but with diminished size and decreased number of follicles.¹³ We observed that as the CCR6^{-/-} mice are crossed onto the C57BL/6 background, this phenotype has become more pronounced, such that on average PPs in the CCR6^{-/-} mice have less than two follicles. In

our current scoring system, these abnormal PPs are counted as mLFs, thus artificially increasing the number of mLFs in the CCR6^{-/-} mice. Therefore, it is likely that the CCR6^{-/-} mice on the C57BL/6 background are more deficient in mLFs than suggested by the data presented here. We also observed that the PPs in the CCR6^{-/-} mice had small follicular domes, reduced total cellularity, and a reduced proportion of B lymphocytes. The reduced PP total cellular population is consistent with previous reports on the phenotype of the CCR6^{-/-} mice^{11,13}; however, the decreased percentage of PP B lymphocytes and the smaller PP follicular dome in the CCR6^{-/-} mice has not been previously noted. The differences we observed in the PP B-lymphocyte populations were small but statistically significant. Evaluation of older animals revealed an increase in the PP B-lymphocyte populations in both wild-type and CCR6^{-/-} mice; however, CCR6^{-/-} mice maintained a statistically significant decrease in the proportion of PP cells that were B lymphocytes when compared with age-matched wild-type mice (data not shown). This indicates that the small, but statistically significant, difference we observed is maintained with aging. The decreased size of the follicle dome associated with a decreased population of PP B lymphocytes in the PPs of the CCR6^{-/-} mice is supported by a prior study documenting a role for B lymphocytes in facilitating the development of the FAE³¹ as well as a recent study demonstrating a reduced number of M cells in the FAE of CCR6^{-/-} mice.¹¹ The reduced surface area of the FAE and decreased numbers of M cells could provide another explanation for the diminished capacity of the CCR6^{-/-} mice to mount mucosal immune responses to oral antigens.¹⁰ We observed no differences in the proportion of the PP cellular population that were dendritic cells (MH-CII⁺, CD11c⁺) when comparing wild-type and CCR6^{-/-} mice. Our findings are consistent with a recent study using similar methods that found a modest increase in the population of PP cells that are CD11c⁺ in the CCR6^{-/-} mice.¹¹ In contrast, previous reports demonstrated an absent or diminished CD11b⁺ dendritic cell population in the PP subepithelial dome of CCR6^{-/-} mice.^{10,13} Our study did not evaluate dendritic cell subpopulations or the positioning of these subpopulations within the PP. When our findings are evaluated in the context of the significantly decreased total PP cellular population in the CCR6^{-/-} mice, it becomes apparent that PP dendritic cell numbers are decreased in CCR6^{-/-} mice and, therefore, are consistent with a diminished PP CD11b⁺ DC subpopulation as well as potential defects in positioning of these subpopulations within the PP in the absence of CCR6. Overall, our observations indicate that CCR6 deficiency affects the B-lymphocyte population in both forms of organized intestinal lymphoid structures and do not preclude deficiencies in other cellular populations in either structure.

The findings presented here are consistent with previous observations regarding CCR6 expression and function in B lymphocytes. CCR6 expression is acquired by B-2 B lymphocytes as they mature and exit the bone marrow and is largely restricted to mature, follicular (B-2) B lymphocytes and to a lesser extent a small subset of

germinal center and marginal zone B lymphocytes.³² In addition CCR6 expression on follicular B lymphocytes is down-regulated by B-cell receptor ligation.³³ Our observation that ILF B lymphocytes are largely CCR6⁺ is consistent with these findings, as ILF B lymphocytes are exclusively B-2 B lymphocytes and do not have a requirement for antigen activation for their localization to ILFs.^{1,2,25} We observed a hierarchy of CCR6 expression with ILF B lymphocytes having greater CCR6 expression when compared with PP B lymphocytes, and PP B lymphocytes have greater CCR6 expression than the splenic B lymphocytes. This observation is also consistent with previous studies of CCR6 expression by B lymphocytes, as the PPs and the spleen have more developed germinal centers that contain a population of CCR6-negative B lymphocytes, and the spleen also contains a population of CCR6-negative marginal zone B lymphocytes.³² Although we have consistently observed this hierarchy of CCR6 expression on ILF and PP B lymphocytes, the chemotaxis of freshly isolated ILF or PP B lymphocytes in response to CCL20 has been equivalent to or less than that seen in splenic B lymphocytes (data not shown). Possible explanations for this apparent inconsistency include differences in the local environment of the PPs and ILFs that would desensitize B lymphocytes to CCR6-dependent stimuli, including elevated local expression of CCL20, as we have demonstrated here. In the context of these previous observations regarding the restricted patterns of CCR6 expression on B lymphocytes and our observations documenting that ILFs contain polyclonal follicular B-2 B lymphocytes that can be antigen-naïve,^{25,34} we interpret the findings of this study to indicate that CCL20 and CCR6 play a role in recruiting antigen naïve (follicular) B-2 B lymphocytes to the sites of ILF formation.

Two LT-dependent steps have been identified in ILF development, differing in the cellular source of LT. Non-B, non-T lymphocytes can deliver the early signals necessary for the formation of iILFs, these events may be mediated by the lineage c-kit⁺ cells within CPs.⁸ The transition of immature ILFs into mature ILFs requires LT-sufficient B lymphocytes, and these LT-sufficient B lymphocytes can be antigen-naïve.^{2,25} Chemokines play a critical role in the development of organized lymphoid structures, with CXCL13 and its receptor CXCR5 playing an essential role in the development of B-cell follicles in the spleen, lymph nodes, and PPs.³⁵ CXCL13 not only recruits CXCR5⁺ B lymphocytes in this process, but it also induces the expression of LT by antigen-naïve B lymphocytes, thus inducing the production of additional CXCL13 by LTβR-expressing stromal cells and driving the development of a follicle containing antigen-naïve B lymphocytes.³⁵ Given that CCR6 deficiency affected ILF development to a greater degree than PP development, we questioned whether CCL20 might be playing a similar role to CXCL13 in ILF formation. In support of this role, CCL20 is produced following LTβR ligation in epithelial cells.³⁶ Despite these associations, we did not observe the induction of LT expression in B lymphocytes following stimulation with CCL20 (data not shown). An alternative role for CCR6 in ILF development is suggested by prior

studies noting that CCR6⁺ B lymphocytes are also CXCR5⁺³²; therefore these two chemokines may act synergistically, with the recruitment of CCR6⁺ B lymphocytes facilitating the formation of the CXCL13-driven positive feedback loop driving the development of ILFs in response to CCL20 inducing inflammatory stimuli.

PPs and ILFs are distinct members of gastrointestinal-associated lymphoid tissues. Studies to date indicate that these structures can function in similar manners and initiate noninflammatory adaptive immune responses to luminal antigens.^{3,4,34} A primary distinction between these structures is the way in which they are formed, with the formation of PPs being developmentally driven and the development of ILFs occurring after birth and being influenced by luminal stimuli.^{1,2,26} We observed that CCR6 deficiency affects B-lymphocyte populations in intestinal lymphoid structures and resulted in hypoplastic PPs and largely absent ILFs. CCL20 is considered an inflammatory chemokine, and it is reasonable to assume that CCR6 deficiency alters ILF development, which is more affected by inflammatory stimuli, to a greater degree than PP development, which is largely developmentally driven. This assumption is supported by our findings of unchanged numbers of PPs and a more than 10-fold decrease in the numbers of ILFs in the absence of CCR6. Our observations indicate that CCR6 expression by B lymphocytes is essential for the formation of ILFs and suggests that CCL20 production by CPs in response to inflammatory stimuli may be an event facilitating the recruitment of B lymphocytes and the subsequent transition into ILFs.

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