Functional Specializations of Intestinal Dendritic Cell and Macrophage Subsets That Control Th17 and Regulatory T Cell Responses Are Dependent on the T Cell/APC Ratio, Source of Mouse Strain, and Regional Localization

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Functional Specializations of Intestinal Dendritic Cell and Macrophage Subsets That Control Th17 and Regulatory T Cell Responses Are Dependent on the T Cell/APC Ratio, Source of Mouse Strain, and Regional Localization

Timothy L. Denning,*†‡§ Brian A. Norris,*† Oscar Medina-Contreras,§ Santhakumar Manicassamy,*† Duke Geem,§ Rajat Madan,¶ Christopher L. Karp,¶ and Bali Pulendran*†‡§

Although several subsets of intestinal APCs have been described, there has been no systematic evaluation of their phenotypes, functions, and regional localization to date. In this article, we used 10-color flow cytometry to define the major APC subsets in the small and large intestine lamina propria. Lamina propria APCs could be subdivided into CD11c+CD11b−, CD11c−CD11b+ subsets. CD11c+CD11b− cells were largely CD103+ F4/80− dendritic cells (DCs), whereas the CD11c−CD11b+ subset comprised CD11c+CD11b+CD103− F4/80+ DCs and CD11c−CD11b+CD103− F4/80− macrophage-like cells. The majority of CD11c+CD11b+ subsets were CD103− F4/80+ macrophages. Although macrophages were more efficient at inducing Foxp3+ regulatory T (Treg) cells than DCs, at higher T cell/APC ratios, all of the DC subsets efficiently induced Foxp3+ Treg cells. In contrast, only CD11c+CD11b+CD103+ DCs efficiently induced Th17 cells. Consistent with this, the regional distribution of CD11c+CD11b+CD103+ DCs correlated with that of Th17 cells, with duodenum > jejunum > ileum > colon. Conversely, CD11c−CD11b+CD103− DCs, macrophages, and Foxp3+ Treg cells were most abundant in the colon and scarce in the duodenum. Importantly, however, the ability of DC and macrophage subsets to induce Foxp3+ Treg cells versus Th17 cells was strikingly dependent on the source of the mouse strain. Thus, DCs from C57BL/6 mice from Charles River Laboratories (that have segmented filamentous bacteria, which induce robust levels of Th17 cells in situ) were more efficient at inducing Th17 cells and less efficient at inducing Foxp3+ Treg cells than DCs from B6 mice in situ. The functional specializations of APC subsets in the intestine are dependent on the T cell/APC ratio, regional localization, and source of the mouse strain. The Journal of Immunology, 2011, 187: 000–000.

Sensing of microbes by innate immune cells is critical for the establishment of effective adaptive immune responses and host defense (1). APCs, including dendritic cells (DCs) and macrophages, are endowed with the ability to detect microbial components via several evolutionarily conserved receptor/signaling pathways, including TLRs, nod-like receptors, and C-type lectin receptors among others (2–4). The integration of microbial-derived signals via these pathways and local microenvironmental cues ultimately dictates the balance between ensuing tolerogenic and proinflammatory responses (5, 6). Additionally, specific APC subsets, expressing various levels of cell surface markers such as CD11c, CD11b, F4/80, CD8α, and CD4, differentially respond to microbial encounter (7, 8).

DCs and macrophages are situated in various lymphoid and nonlymphoid tissues, and under homeostatic conditions they encounter the majority of microbial Ags at mucosal surfaces (9). In particular, DCs and macrophages in the intestinal Peyers patches (PPs) and lamina propria (LP) are situated just beneath a single layer of epithelial cells that separate them from a vast commensal microflora. The major portals of entry for many microbes are the PPs of the small intestine (10). Within the PPs exist several subsets of DCs that differentially regulate immune responses dependent upon whether they are CD11b−, CD8α−, or CD11b+CD8α− (11). CD11b+ PP DCs can be stimulated to secrete IL-10 and induce the differentiation of T regulatory cells 1 and may promote IgA B cell responses (12). Alternatively, CD8α+ and CD11b−CD8α−PP DCs can produce IL-12 and generate Th1-like cells (13). In the LP, CD8α+ and CD11b+ DCs have been identified, and the functions of these subsets have yet to be clearly defined. CD11b+ LP DCs appear to express tight junction proteins and can extend dendrites that interdigitate between neighboring intestinal epithelial cells to sample luminal microbes (14). The extension of dendrites is controlled by the expression of CX3CR1 by DCs, and such CX3CR1-dependent processes may control intestinal bacterial clearance (15).
Additionally, CD11b+ LP DCs can promote the induction of Th17 cell differentiation either constitutively (16) or after stimulation by bacterial flagellin in a TLR5-dependent manner (17). Intestinal DCs also have been reported to specifically induce the gut-homing molecules a4β7 and CCR9 on responding T and B cells (18–20) and Foxp3+ regulatory T (Treg) cells from naive CD4+ T cells in vitro in a retinoic acid (RA)-dependent fashion (21–23), and recent work from our laboratory has demonstrated a potent role for β-catenin signaling in programming such DCs to a tolerogenic state (24). The ability of LP DCs to induce Foxp3+ Treg cell conversion appears closely linked with the commensal microbiota, because such conversion can be inhibited by bacterial CpG and TLR9-deficient mice have increases in Foxp3+ Treg cells in the LP (25). The role of CD80+ LP DCs is less clear but also may be to induce differentiation of Treg cells (26).

In addition to DCs, a major macrophage network exists in the intestinal LP. LP macrophages are avidly phagocytic and hypo-responsive to various inflammatory stimuli, spontaneously secrete IL-10, and can efficiently promote Foxp3+ Treg cell conversion in vitro in the presence of exogenous TGFB-β (16, 27–31). Steady-state LP macrophages also may dampen immune responses during homeostasis and intestinal inflammation or injury (16, 29, 32–34). For example, depletion of intestinal macrophages using clodronate liposomes led to exacerbated dextran sodium sulfate (DSS)-induced experimental colitis (35). Alternatively, certain macrophage subsets may contribute to the pathogenesis of intestinal inflammation in mice and humans (29, 36). Recently, TREM-1+ and CD14+ LP macrophages were shown to be associated with the pathogenesis of human inflammatory bowel disease (37, 38). Thus, steady-state LP macrophages may play important roles in maintaining tolerance, whereas inflammatory macrophages may contribute to the pathogenesis of intestinal inflammation (36).

Thus, unique APC subsets in different regions of the intestine may contribute to maintaining the balance between tolerogenic and proinflammatory immune responses. In this article, we have defined major DC and macrophage subsets in the small and large intestine LP and demonstrate that the functional specializations of LP DC and macrophage subsets in the intestine are dependent on several parameters, including the T cell/APC ratio, regional localization, and source of the mouse strain. Thus, F4/80+ macrophage subsets are the major IL-10 producers in the LP and concomitantly express TGFB-β and RA-generating enzymes and promote differentiation of inducible Foxp3+ Treg cells. Furthermore, all of the DC subsets could induce Foxp3+ Treg cells at particular T cell/APC ratios. Alternatively, CD11b+CD103+ LP DCs in C57BL/6 mice from Charles River Laboratories uniquely express IL-6 and TGFB-β mRNA as well as RA-generating enzymes and efficiently induce differentiation of Th17 cells. CD11b+CD103+ LP DCs are enriched in the duodenum and rare in the colon during the steady state and accumulate in the colonic LP along with Th17 cells during intestinal inflammation. Strikingly, CD11c+CD11b+ LP DCs from C57BL/6 mice purchased from Charles River Laboratories [that have segmented filamentous bacteria (SFB), which induce robust levels of Th17 cells in situ (39, 40)] were more efficient at inducing Th17 cells and less efficient at inducing Foxp3+ Treg cells than their counterparts from C57BL/6 mice from The Jackson Laboratory. Therefore, these results demonstrate that the functions of intestinal APC subsets are dependent on several parameters, including the T cell/APC ratio, source of the mouse strain, and regional localization.

Materials and Methods

**Mice**

C57BL/6 male mice 6–12 wk of age obtained from Charles River Laboratory were used in all of the studies. In specific experiments, mice obtained from The Jackson Laboratory also were used. OT-II (Rag1−/−) mice, originally obtained from The Jackson Laboratory, were bred on site. IL-10−/−IRE-EGFP reporter (Vert-X) mice (41) were created by insertion of a floxed neomycin–IRE-EGFP cassette between the endogenous stop site and the polyadenosine site of IL-10. The neomycin resistance marker was excised by breeding the mice with Zp3-Cre mice, and successful Cre-mediated deletion was confirmed by Southern blot analysis (41). Mice were maintained under specific pathogen-free conditions in the Emory Vaccine Center or Emory Whitehead Biomedical Research Facility. All of the animal protocols were reviewed and approved by the Institute Animal Care and Use Committee of Emory University.

**Isolation of LP cells**

Briefly, small and large intestines were removed and carefully cleaned of their mesentery, PPs were excised (small intestine), and intestines were opened longitudinally and washed of fecal contents. Intestines then were cut into 0.5-cm pieces, transferred into 50-ml conical tubes, and shaken at 250 rpm for 20 min at 37˚C in HBSS medium (Life Technologies) supplemented with 5% FBS (Mediatech) containing 2 mM EDTA. This process was repeated two additional times. The cell suspensions were passed through a strainer, and the remaining intestinal tissue was washed and then minced, transferred into a 50-ml conical tube, and shaken for 20 min at 37˚C in HBSS + 5% FBS containing type VIII collagenase at 1.5 mg/ml (CollagenTech). The cell suspension was collected, passed through a strainer, and pelleted by centrifugation at 1200 rpm. CD11b+ or CD11c+ cells were enriched by positive selection with CD11b or CD11c microbeads (Miltenyi Biotec), respectively. For FACS experiments, CD11b+CD103+ enriched cells were stained with PE-conjugated anti-CD103 (clone M290; BD Pharmingen), PerCP-conjugated anti-CD45 (clone 30-F11; BD Pharmingen), PE-Cy7 conjugated anti-F4/80 (clone BM1) and anti-CD11c (clone H3B6; BioXCell), 7AAD750-conjugated anti-IAα (clone M5/114), and Pacific blue conjugated anti-CD11b (clone M1/70) (all from eBioscience unless otherwise noted). Stained cells were sorted to purified indicated populations on a FACSAria at the Emory Vaccine Center Flow Cytometry Core Facility.

**Flow cytometry**

Isolated splenocytes or small intestine LP cells were resuspended in PBS containing 5% FBS. After incubation for 15 min at 4˚C with the blocking Ab 2.4G2 anti-FcγRIII/B7, the cells were stained at 4˚C for 30 min with labeled Abs. Samples then were washed two times in PBS containing 5% FBS. The samples were analyzed immediately at this point, or they were fixed in PBS containing 2% paraformaldehyde and stored at 4˚C. Abs used for analysis were from eBioscience unless otherwise noted: FITC-conjugated anti-mouse B220, PE-conjugated anti-mouse CD103 (BD Pharmingen), PE-Texas red-conjugated anti-mouse CD8α (Caltag Labatories), PerCP-conjugated anti-mouse CD45 (BD Pharmingen), PE-Cy7-conjugated anti-mouse F4/80, allophycocyanin-conjugated anti-mouse CD11c, Alexa Fluor 700-conjugated anti-mouse IAα, allophycocyanin-Alexa Fluor 750-conjugated anti-mouse Gr-1, Pacific blue-conjugated anti-mouse CD11b, and Alexa Fluor 430 (live/dead stain; Invitrogen). Intracellular IL-17 or Foxp3 staining was performed using PE-conjugated anti-mouse IL-17 or Foxp3 (eBioscience). Aldehyde dehydrogenase (ALDH) activity was determined using ALDEFLUOR staining kit (Stemcell Technologies) according to the manufacturer’s protocol. In brief, cells were suspended at a concentration of 10^6 cells/ml in ALDEFLUOR assay buffer containing activated ALDEFLUOR substrate (final concentration of 1.5 μM) with or without the ALDH inhibitor diethylamino-benzaldehyde (DEAB) (final concentration of 15 μM) and incubated at 37˚C for 30 min. Flow cytometric analysis was performed on a BD Biosciences FACSCalibur or LSR II flow cytometer at the Emory Vaccine Center.

**Imaging**

To analyze cell morphology, sorted LP APCs were spun onto glass slides using a CytoSpin centrifuge, allowed to air dry for 1 h, fixed in 100% methanol, and then stained using Wright–Giemsa stain.

**Real-time PCR**

Total RNA was isolated from FACS-sorted LP APCs or enriched, splenic DCs (CD11c+ cells) or macrophages (CD11b+ cells) using the Quagen RNeasy Mini Kit according to the manufacturer’s protocol with on-column DNA digestion using the Qiagen RNeasy Mini Kit protocol. cDNA was generated using the Superscript First-Strand Synthesis System for RT-PCR and oligo(dT) primers (Invitrogen) according to the manufacturer’s protocol. cDNA was used as a template for quantitative real-time PCR using
SYBR Green Master Mix (Bio-Rad). PCR and analysis was performed using a MyiQ iCycler (Bio-Rad). Gene expression was calculated relative to that of gapdh.

Stimulation of lymphocytes

In vitro FACS-sorted LP APCs (1 x 10^5 unless otherwise noted) were cocultured with naive CFSE-labeled or unlabeled CD4+CD62L− OT-II T cells (1 x 10^5) and OVA (ISQVHAAHAEINEAGR; 10 µg/ml) in 200 µl RPMI 1640 complete medium in 96-well round-bottom plates. For CFSE experiments, cells were harvested after 90 h. As a positive control for CFSE culture stimulation oCD3/28-coated beads (Dynal) were used at a ratio of one bead per cell. For restimulation, cells were harvested after 90 h of primary culture before being restimulated with PMA (50 ng/ml) and ionomycin (500 ng/ml) for 6 h (intracellular cytokine detection) in the presence of GolgiPlug (BD Pharmingen). Small/large intestine LP lymphocytes were stimulated with PMA and ionomycin for 6 h in the presence of GolgiPlug. For FoxP3+ Treg cell induction assays 1 ng/ml human rTGFB-β (PeproTech) was added to the cultures.

Cytokine detection

For intracellular cytokine analysis, cells were stimulated with PMA and ionomycin for 6 h in the presence of GolgiPlug and subsequently incubated with the blocking Ab 2.4G2 anti-Fc and CytoPEP-conjugated anti-CD4 and allophycocyanin-conjugated anti-mouse TCR-β (BD Pharmingen). Cells were permeabilized by using Cytofix/Cytoperm (BD Pharmingen) and stained by using fluorochrome-conjugated Abs according to the manufacturer’s protocol. IL-10 ELISAs (eBioscience) were performed according to the manufacturer’s protocol.

Suppression assay

Naive OT-II CD4+ T cells (1 x 10^5) were cultured with CD11c− LP macrophages (1 x 10^5) in the presence of 1 ng/ml human rTGFB-β for 90 h, then harvested, washed, and added at a ratio of 1:1 to cultures of CFSE-labeled naive polyclonal CD4+CD45.1+ splenic T cells stimulated with a polyclonal anti-CD4 and allophycocyanin-conjugated anti-mouse TCR-β (BD Pharmingen). Cells were harvested after 48 h and analyzed for CFSE dilution after gating on CD4+CD45.1+ cells.

Induction and assessment of chronic colitis

Chronic colitis was induced by multiple-cycle administration of DSS in drinking water (42, 43). Mice received either regular drinking water (control) or 3% (w/v) DSS in drinking water (m.w. 40,000–50,000) (MP Biomedicals) on days 1–5, 8–12, 15–19, and 22–26, and mice were sacrificed on day 29. Histology was used to evaluate inflammation, and colonic shortening was analyzed. This model of colitis is referred to as “chronic,” because it involved four cycles of DSS treatment over the course of nearly 1 mo and is distinct from the commonly used “acute” model of DSS-induced colitis where mice are typically treated for 5–7 d.

In order to visualize the cell morphology of each of these populations, Giemsa staining was performed on FACS-sorted cells. CD11b dull−CD11c+ cells, which were largely CD103+CD11c+ population, displayed classical DC morphology with numerous dendrites extending from the cell surface and few detectable phagocytic vacuoles (Fig. 1C). Conversely, the CD11b dull−CD11c− population also contained variable levels of CD11b dull−CD11c− cells and was generally included in the analyses. LP CD103+CD11c+ population was almost entirely CD11b dull−CD11c+ (79.2 ± 2%). CD11c−F4/80+ LP macrophages and CD11b+CD11c dull− population were almost entirely CD11b+CD11c dull− (9.2 ± 2%). In the CD11c−F4/80− CD11c dull− population, the most abundant population was identified in patients with chronic colitis. CD11c dull− population was characterized by abundant phagocytic vacuoles and a relatively low level of dendrites. As previously reported by our group, CD11b+CD11c dull− cells also displayed typical macrophage morphology (16). These results collectively demonstrate that CD11b+ cells in the small intestine LP are defined largely by the expression of CD11b with variable expression of CD103 (hereafter referred to as CD11b+CD103− and CD11b+CD103+ LP DCs for simplicity), whereas IAα+ macrophages are defined by the expression of CD11b and F4/80 with variable expression of CD11c (hereafter referred to as CD11c−F4/80− and CD11c+F4/80+ LP macrophages). Additionally, we calculated absolute cell numbers for each of these populations. As shown in Fig. 1D, both LP DC subsets as well as CD11c−F4/80− LP macrophages were present in similar numbers, whereas CD11c+F4/80− LP macrophages were ~10-fold more abundant (p < 0.05). Of note, our strategy of precising on CD45+IAα+ cells for these analyses intentionally did not include what may be considered side scatter−IAα− macrophages (44) or eosinophils (17).

Results

Characterization of intestine LP DC and macrophage subsets

A clear definition of intestine LP DCs and macrophages has been plagued by a lack of specific markers that clearly delineate each population and subsets existing within. Therefore, we performed 10-color flow cytometry on isolated small intestine LP cells combining the use of 9 markers (CD8α, CD11b, CD11c, CD45, CD103, IAα, F4/80, Ly-6C/G, and B220) and a vital dye. After gating on live, CD45+IAα+ cells, three major cell populations expressing either CD11b and/or CD11c were identified (Fig. 1A). Two populations of CD11c+ cells were distinguished by differential expression of CD11b, with the CD11b−CD11c+ cells (4.6 ± 2.2%) ~2.5-fold more abundant than the CD11b dull−CD11c+ subset (1.6 ± 0.2%). This difference was statistically significant (p < 0.05). Notably, CD11b dull−CD11c+ cells were a discrete subset, whereas CD11b+CD11c+ cells displayed variable levels of CD11c that formed a continuum. The gate for CD11b+CD11c− cells, with regard to CD11c intensity, was identified based upon the same level of CD11c expression as that for the clearly defined CD11b dull−CD11c− subset. The other abundant population of cells identified was CD11b+CD11c dull− cells (22.7 ± 1.4%), which were 5.4-fold more abundant than the CD11b+CD11c+ subset and 13.8-fold more abundant than the CD11b dull−CD11c+ subset. These differences were statistically significant (p < 0.05). All of the above-mentioned populations and subsets did not express B220 or Ly-6C/G (data not shown). Additionally, cells that did not stain for either CD11b or CD11c included B cells as identified by B220 or CD19 staining and other undefined cells (data not shown).

To further characterize each of the three major populations expressing either CD11b or/and CD11c, they were analyzed for expression of CD103 and F4/80. The large majority (78.9 ± 2.2%) of CD11b dull−CD11c+ LP cells expressed high levels of CD103 and were negative for F4/80 (Fig. 1B). Interestingly, CD11b+CD11c+ cells included approximately equal frequencies of CD103+F4/80− and CD103+F4/80+ subsets (39 ± 2.5% and 42.7 ± 1.3%, respectively). The most abundant CD11b+CD11c dull− population was almost entirely CD103−F4/80+ (79.2 ± 2%). In order to visualize the cell morphology of each of these populations, Giemsa staining was performed on FACS-sorted cells. CD11b dull−CD11c+ cells, which were largely CD103+F4/80−, displayed classical DC morphology with numerous dendrites extending from the cell surface and few detectable phagocytic vacuoles (Fig. 1C). Similarly, among the CD11b+CD11c+ population the CD103+F4/80− subset was characterized by classical DC morphology, whereas the CD103−F4/80+ subset displayed typical macrophage morphology characterized by abundant phagocytic vacuoles and a relative lack of dendrites. As previously reported by our group, CD11b+CD11c dull− cells also displayed typical macrophage morphology (16). These results collectively demonstrate that IAα+ DCs in the small intestine LP are defined largely by the expression of CD103 with variable expression of CD11b (hereafter referred to as CD11b+CD103− and CD11b+CD103+ LP DCs for simplicity), whereas IAα+ macrophages are defined by the expression of CD11b and F4/80 with variable expression of CD11c (hereafter referred to as CD11c−F4/80− and CD11c+F4/80+ LP macrophages). Additionally, we calculated absolute cell numbers for each of these populations. As shown in Fig. 1D, both LP DC subsets as well as CD11c−F4/80− LP macrophages were present in similar numbers, whereas CD11c+F4/80− LP macrophages were ~10-fold more abundant (p < 0.05). Of note, our strategy of precising on CD45+IAα+ cells for these analyses intentionally did not include what may be considered side scatter−IAα− macrophages (44) or eosinophils (17).

Because LP DCs and macrophages have been reported to generate FoxP3+ Treg cells via the production of RA and IL-10 in the presence of TGF-β1, we analyzed the expression of the RA-generating enzymes retinaldehyde dehydrogenase 1 and 2 (aldh1a1 and aldh1a2), il10, and il10r1 by quantitative real-time PCR. For comparative purposes, splenic DCs (CD11c+ cells) and macrophages (CD11b+ cells) were included in the analyses. LP DCs and macrophages further displayed a clear difference in expression of RA-generating enzymes. CD11c+F4/80+ LP macrophages expressed high levels of aldha1, D11c+F4/80+ LP macrophages and CD11b+CD103− LP DCs expressed intermediate levels, and CD11b+CD103+ LP DCs expressed very low levels that were comparable to those of splenic DCs and macrophages (Fig. 2A). Alternatively, CD11b+CD103− and CD11b+CD103+ LP DCs expressed high levels of aldha2 mRNA, and both LP
macrophage populations displayed significantly lower levels of this RA-generating enzyme \( (p < 0.05) \). Thus, although both LP DCs (22) and macrophages (16) are capable of generating RA and inducing T\(_{reg}\) cells in a RA-dependent fashion, they may differentially employ \( \text{aldh1a1} \) and \( \text{aldh1a2} \) for this purpose. To assess whether such differences correlated with differences in ALDH activity, CD11b\(^+\)CD103\(^-\) and CD11b\(^-\)CD103\(^+\) LP DC subsets and CD11c\(^-\)F4/80\(^+\) and CD11c\(^+\)F4/80\(^+\) LP macrophage subsets were incubated with the fluorescent ALDH substrate ALDEFLUOR, and ALDEFLUOR expression was assessed by flow cytometry. CD11b\(^+\)CD103\(^-\) and CD11b\(^-\)CD103\(^+\) LP DC subsets and CD11c\(^-\)F4/80\(^+\) and CD11c\(^+\)F4/80\(^+\) LP macrophage subsets exhibited significantly \((p < 0.05)\) higher ALDEFLUOR expression than CD11c\(^-\)F4/80\(^+\) and CD11c\(^+\)F4/80\(^+\) LP macrophage subsets in the small intestine, as measured by mean fluorescence intensity (MFI) for ALDEFLUOR expression. The change in MFI \((\Delta \text{MFI})\) was calculated by subtracting the MFI for DEAB-treated samples from the MFI for the corresponding non–DEAB-treated samples \((\text{Fig. 2B})\). In all of the conditions, ALDH activity was blocked by the ALDH inhibitor DEAB. Interestingly, lower ALDH activity was measured in the large intestine, especially for CD11b\(^+\)CD103\(^-\) and CD11b\(^-\)CD103\(^+\) LP DC subsets. Although ALDH expression was significantly lower among LP macrophage subsets when compared with that in LP DC subsets in the small intestine, all of the subsets possessed some specific ALDH expression. Given that LP macrophages 1) express both \( \text{aldh1a1} \) and \( \text{aldh1a2} \) mRNA (albeit at significantly lower levels than LP DCs) and 2) are noticeably positive using the ALDEFLUOR assay (albeit at significantly lower levels than LP DCs) and 3) that the addition of LE540 to block RA signaling reduces the ability of LP macrophages to induce Foxp3\(^+\) T\(_{reg}\) cells in vitro [similar to LP DCs (16)], this strongly suggests that these cells indeed do possess the ability to generate RA.

Although all of the populations of LP DCs and macrophages expressed \( \text{tgfb}1 \), both macrophage subsets expressed significantly more mRNA than either DC subset \((\text{Fig. 2C})\). We next probed the expression of \( \text{il6} \) mRNA among LP DCs and macrophages, because it has been shown to inhibit Foxp3\(^+\) T\(_{reg}\) cell differentiation and can combine with TGF-\( \beta \) to generate IL-17–producing...
T cells. Interestingly, CD11b+CD103+ LP DCs expressed significantly higher levels of \( \text{il6} \) mRNA than the other APC subsets (Fig. 2C); however, this cytokine was undetectable by ELISA and intracellular cytokine staining, suggesting low level protein expression. mRNAs for IL-12p35, IL-12p40, and IL-23p19 were not detectable in any of the groups that we analyzed (data not shown). Interestingly, CD11c\(^2\)F4/80+ and CD11c+F4/80+ LP macrophages both expressed significantly higher levels of \( \text{il10} \) mRNA than CD11b\(^{-}\)CD103\(^+\) and CD11b\(^{-}\)CD103\(^+\) LP DCs (\( p < 0.05 \)). The statistically (\( p < 0.05 \)) different expression of \( \text{il10} \) was confirmed at the protein level using ELISAs (Fig. 2D) and Vert-X mice (Fig. 2E). IL-10–GFP was detected clearly in CD11c\(^2\)F4/80+ LP macrophages and to a lesser extent in CD11c+F4/80+ LP macrophages but was undetectable in CD11b\(^{+}\)CD103\(^{+}\) and CD11b\(^{-}\)CD103\(^{+}\) LP DCs (Fig. 2E, 2F). Collectively, these data provide evidence that LP macrophages are unique in their expression of high levels of \( \text{il10} \), \( \text{tgfb1} \), and \( \text{aldh1a1} \), although both LP DC populations express high levels of \( \text{aldh1a2} \) and CD11b+CD103+ LP DCs specifically express high levels of \( \text{il6} \). These data for each LP DC and macrophage subset are summarized in Table I.

### CD11b\(^{+}\)CD103\(^{+}\) LP DCs induce Th17 cell differentiation

In order to address the differential roles of LP DC and macrophage populations in inducing the differentiation of Th17 or Foxp3-expressing Treg cells in vitro, we setup an Ag presentation assay as described previously (16) by coculturing freshly isolated LP DCs or macrophages with naive OT-II CD4\(^{+}\)CD62L\(^{+}\) T cells and OVA in the absence (Th17 assay) or presence (Treg assay) of

### Table I. Summary of intestinal APC subsets in C57BL/6 mice from Charles River Laboratories: phenotype and cytokine/RA expression

<table>
<thead>
<tr>
<th>Major Subsets of CD45(^{+})IAb(^{+}) LP Cells</th>
<th>Phenotype</th>
<th>Cytokine/RA Expression</th>
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<tr>
<td><strong>CD11c(^{+})CD11b(^{-})</strong></td>
<td>CD103(^{+})F4/80 ( \text{Dendrites or Vacuoles} )</td>
<td>( \text{il6} ) ( \text{il10} ) ( \text{tgfb1} ) ( \text{aldh1a1} ) ( \text{aldh1a2} )</td>
</tr>
<tr>
<td><strong>CD11c(^{+})CD11b(^{+})</strong></td>
<td>CD103(^{+})F4/80 ( \text{Dendrites} )</td>
<td>++</td>
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<tr>
<td><strong>CD11c(^{+})CD11b(^{+})</strong></td>
<td>CD103(^{+})F4/80 ( \text{Dendrites} )</td>
<td>++</td>
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<tr>
<td><strong>CD11c(^{+})CD11b(^{-})</strong></td>
<td>CD103(^{-})F4/80 ( \text{Vacuoles} )</td>
<td>++</td>
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<tr>
<td><strong>CD11c(^{+})CD11b(^{+})</strong></td>
<td>CD103(^{-})F4/80 ( \text{Vacuoles} )</td>
<td>++</td>
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</tbody>
</table>

FIGURE 2. Expression of immunoregulatory cytokines and RA-generating enzymes by LP DCs and macrophages (Mf). A, RNA was isolated from FACS-sorted CD11b\(^{-}\) LP DCs, CD11b\(^{+}\) LP DCs, CD11c\(^{+}\) LP M\( \phi \), CD11c\(^{-}\) LP M\( \phi \), or splenic (SPL) DCs or M\( \phi \), and expression of \( \text{aldh1a1} \) and \( \text{aldh1a2} \) was analyzed by quantitative real-time PCR. Values are expressed relative to that of \( \text{gapdh} \). Bars indicate means ± SEM from one of two independent experiments. B, ALDH activity on FACS-sorted CD11b\(^{-}\) LP DCs, CD11b\(^{+}\) LP DCs, CD11c\(^{+}\) LP M\( \phi \), and CD11c\(^{-}\) LP M\( \phi \) without (filled gray) or with (open black) the addition of the DEAB inhibitor. MFI data from two independent experiments are summarized as means ± SEM. ALDEFLUOR ΔMFI was calculated by subtracting the MFI for DEAB-treated samples from the MFI for the corresponding non-DEAB-treated samples. C, RNA was isolated from FACS-sorted CD11b\(^{-}\) LP DCs, CD11b\(^{+}\) LP DCs, CD11c\(^{+}\) LP M\( \phi \), CD11c\(^{-}\) LP M\( \phi \), or splenic (SPL) DCs or M\( \phi \), and expression of \( \text{tgfb1} \), \( \text{il6} \), and \( \text{il10} \) was analyzed by quantitative real-time PCR. Values are expressed relative to that of \( \text{gapdh} \). Bars indicate means ± SEM from one of two independent experiments. D, FACS-sorted CD11b\(^{-}\) LP DCs, CD11b\(^{+}\) LP DCs, CD11c\(^{+}\) LP M\( \phi \), and CD11c\(^{-}\) LP M\( \phi \) were cultured for 24 h, and supernatant was analyzed for IL-10 using ELISA. Bars indicate means ± SEM from one of two independent experiments. E, Cells were isolated from the total small intestine of Vert-X mice and analyzed by flow cytometry. Histograms are pre-gated on live cells and CD45\(^{+}\) and I-Ab\(^{+}\) and then into the indicated subsets based on CD11b, CD11c, CD103, and F4/80 expression as defined in Fig. 1. F, Data from two independent experiments are summarized as means ± SEM. *\( p < 0.05 \).
exogenous TGF-β1. After 90 h of coculture, both CD11b−CD103+ and CD11b+CD103+ LP DCs as well as CD11c−F4/80+ and CD11c+F4/80+ LP macrophages induced similar levels of CFSE dilution that were of the same magnitude as when αCD3/28 beads were used to stimulate OT-II T cells (Fig. 3A). Interestingly, although LP macrophage subsets induced similar levels of CFSE dilution as LP DC subsets, LP macrophages induced less T cell accumulation in culture (∼3-fold less than that of LP DC subsets) and less T cell clustering and IL-2 production (data not shown).

We next observed that among the major LP DC and macrophage populations CD11b+CD103+ LP DCs preferentially induced significant IL-17–expressing T cell differentiation (6.1%, p < 0.05) as detected by intracellular cytokine staining (Fig. 3B). These data are summarized in Fig. 3B (right panel), which displays means ± SEM from three independent experiments. Importantly, this ability of CD11b+CD103+ LP DCs to induce IL-17–expressing T cells was highly dependent on IL-6 production (Fig. 3C; p < 0.05), because the addition of neutralizing Ab to IL-6 but not isotype control Ab (rat IgG) significantly reduced IL-17–expressing T cells.

Foxp3+ Treg cell differentiation by LP DCs and macrophages is ratio dependent

With the same Ag presentation assay described above but in the presence of exogenous TGF-β1, various LP DC and macrophage populations were probed for their abilities to promote the induction of Foxp3 in differentiating, FACS-sorted naïve Foxp3−CD4+CD62L+ cells. As shown in Fig. 4A, CD11c−F4/80+ and CD11c+F4/80+ LP macrophages but not CD11b−CD103+ or CD11b+CD103+ LP DCs can induce Foxp3-expressing Treg cell differentiation at an T cell/APC cell ratio of 1:1. Because it has been reported that CD103+ and CD103− LP DCs can induce Foxp3-expressing Treg cell differentiation at higher T cell/APC ratios (22), we performed a titration of LP DCs or macrophages while keeping the number of OT-II T cells constant to increase the T cell/APC ratio. Changes in the T cell/APC ratio from 1:1 up to 40:1 did not dramatically alter the high frequency of Foxp3+ Treg cells induced by either CD11c−F4/80+ or CD11c+F4/80+ LP macrophages. Alternatively, LP DCs, which were poor at generating Foxp3+ Treg cells at a ratio of 1:1, were capable of inducing Foxp3 in responding T cells at T cell/APC ratios of 5:1 and higher. These reproducible data confirm previous observations that LP DCs and macrophages differentially induce Foxp3-expressing Treg cells at high T cell/APC ratios in the induction of Foxp3+ Treg cells by LP DCs in vitro. As has been reported for LP DCs (21, 22), Foxp3+ Treg cells induced by CD11c−F4/80+ LP macrophages were capable of suppressing naïve CD4+ responder cell proliferation as analyzed by CFSE dilution (Fig. 4B). The majority of naïve CD4+CD45.1+ responder cells proliferated four to five times in the absence of Foxp3+ Treg cells, although the addition of CD11c−F4/80+ LP macrophage-induced putative Treg cells (∼55% Foxp3+) prevented most
responder cells from dividing at all (66.6% suppression in experiment 1 and 71% suppression in experiment 2; Fig. 4).

**Ability of APC subsets to induce Foxp3+ Treg cells versus Th17 cells is dependent on the source of C57BL/6 mice**

Recent work has demonstrated striking differences in the microbiota and intestinal Th17 cells in genetically identical strains of mice from different vendors (39). Thus, C57BL/6 mice from Charles River Laboratories have SFB that induce high levels of intestinal Th17 cells in contrast to the same strain of mice from The Jackson Laboratory (40). We thus determined whether the intestinal DC subsets in C57BL/6 mice from the different vendors had intrinsic differences in their capacities to induce Th17 versus Foxp3+ Treg cells. Both CD11c+CD11b− and CD11c+CD11b+ LP cells isolated from The Jackson Laboratory mice were more efficient at inducing Foxp3+ Treg cells (Fig. 5A), and the preferential induction of Foxp3+ Treg cells by CD11c+CD11b+ LP cells was significant (p < 0.05). Alternatively, both CD11c+CD11b− and CD11c+CD11b+ LP cells from Charles River Laboratories mice were significantly (p < 0.05) more efficient at inducing Th17 cells than those from The Jackson Laboratory mice (Fig. 5B), and the CD11c+CD11b+ LP cells from Charles River Laboratories mice were more efficient than CD11c+CD11b− LP cells from Charles Laboratories mice at inducing Th17 cells, thus confirming previous results (Fig. 3B). In light of these data using CD11c+CD11b+ LP cells from Charles River Laboratories mice, which contain a mixture of CD103+ LP DCs and F4/80+ macrophages, we further confirmed the preferential and significant (p < 0.05) Th17 cell-inducing capacity of the more specifically defined CD11c+CD11b+CD103+ LP DC subset that was sorted from Charles River Laboratories mice (Fig. 5C). Importantly, differences in the capacities of Charles River Laboratories and The Jackson Laboratory LP cells to induce Th17 versus Foxp3+ Treg cells were not due to differences in the abundances of DC and/or macrophages subsets, because phenotypic characterization of these subsets revealed no significant changes (Fig. 5D). These data reveal that the source of the mouse strain is a critical determinant of intestinal APC function, most likely due to differences in the microbiota.

**Region-specific localization of LP DCs and macrophages**

To further probe the relationship between specific LP DC populations and Th17 and Treg cells in situ, we performed a detailed analysis of the duodenum, jejunum, ileum (defined as equal...
thirds by length), and colon (not including cecum). After live, CD45+IAb+ cells were gated, the frequencies and absolute cell numbers of CD11b+CD103+ and CD11b2CD103+ LP DCs and CD11c+F4/80+ and CD11c2F4/80+ LP macrophages were determined. The frequencies of CD11b2CD11c+ LP DCs (R1) were significantly (p < 0.05) enriched in the colon, whereas the frequencies of CD11b+CD11c+ cells (R2) were significantly (p < 0.05) enriched in the duodenum, as compared with those of other regions. Further, the frequencies of CD11b+CD11c2 cells (R3) were least abundant in the duodenum (p < 0.05) and showed similar accumulation in the jejunum, ileum, and colon (Fig. 6A). Among CD11b+CD11c+ LP cells, the duodenum also displayed the highest frequencies of CD11c+F4/80+ LP macrophages within the CD11b+CD11c+ population increased from 26.1 ± 2.5% in the duodenum to 37.3 ± 5.6% in the jejunum and 38.5 ± 3.9% in the ileum and nearly 46.8 ± 4.8% in the colon. The duodenum contained the lowest frequencies of CD11c+F4/80+ LP macrophages within the CD11b+CD11c+ population (p < 0.05) compared with those of all of the other regions, whereas the colon contained the highest (p < 0.05). This increase in frequency was not directly consistent with changes in absolute cell number due to decreased total cellularity in equal length pieces of intestine obtained from descending intestinal regions.

Another important means of comparing relative changes in the two major LP DC subsets along the intestinal tract is to pregate on live, CD45+IAb+CD11c+CD103+ DCs and then to analyze expression of CD11b to discern the relative abundances of CD11b+CD103- and CD11b2CD103- LP DC subsets. In doing so, a clear gradient from high levels of CD11b+CD103- LP DCs in the duodenum (69.7 ± 2.4%) to intermediate levels in the jejunum (54.9 ± 3.1%) and ileum (47.3 ± 3.2%) to low levels in the colon (26.1 ± 1.3%) was observed (Fig. 6C), which is consistent with that observed with the previous gating strategy (Fig. 6A). These data are summarized in Fig. 6C, which displays means ± SEM from all of the experiments. All of the regions are significantly

**FIGURE 5.** Th17 and Foxp3+ Treg cell differentiation by LP DCs and macrophages is dependent on source of the mouse strain. Intracellular staining and flow cytometry to assess Th17 cell and Foxp3 expression by naive OT-II CD4+ T cells cocultured with FACS-sorted CD11b+CD11c+ or CD11b+CD11c- LP cells and OVA in the absence (Th17) or presence (Foxp3) of TGF-β for 4 d. A and B, Intestinal lamina propria lymphocytes were isolated directly stained for Foxp3 without restimulation (A) or restimulated in vitro with PMA and ionomycin for 5 h and stained for IL-17 (B). All of the panels are pregated on TCRβ+CD4+ cells. Numbers next to the boxes indicate the percentages of positive cells in each plot. Data are representative of two independent experiments with the means ± SEM plotted in the lower panel bar graphs. C, Summary of flow cytometry data of the intracellular production of IL-17 by FACS-sorted CD11b+CD103+ LP DCs from Charles River Laboratories (CRL) or The Jackson Laboratory (JAX) mice cocultured for 4 d with OT-II T cells and OVA and then restimulated for 6 h with PMA and ionomycin. Data from two independent experiments are summarized as means ± SEM. D, Characterization of intestinal LP DC and macrophage subsets in CRL and JAX mice as defined by gating strategy in Fig. 1A. *p < 0.05.
different from each other (p < 0.05) except for the jejunum and ileum. These data demonstrate that when descending the intestinal tract CD11b^+CD103+ LP DCs decrease in abundance, whereas CD11b^2CD103+ DCs increase in abundance. The region-specific localization and T cell differentiation data for each LP DC and macrophage subset are summarized in Table II.

**Intestinal localization of Th17 and Foxp3^+ Treg cells**

We next evaluated the presence of IL-17–producing CD4^+ T cells and Foxp3-expressing T reg cells in the three regions of the small intestine and large intestine. Remarkably, the presence of IL-17–producing CD4^+ T cells was highly enriched in the upper small intestine, with 25.5 ± 1.8% of CD4^+ T cells isolated from the duodenum expressing IL-17 upon ex vivo restimulation with PMA and ionomycin. CD4^+ T cells isolated from the jejunum and ileum expressed 16.6 ± 0.9% and 12.3 ± 0.8% IL-17–producing CD4^+ T cells, respectively, and colonic CD4^+ T cells displayed the lowest frequency of IL-17^+ T cells at 8.3 ± 1.1% (Fig. 7A). All of the regions were significantly different from each other (p < 0.05) in both frequency and absolute cell number, thus highlighting a clear and statistically significant gradient for IL-17–producing CD4^+ T cells along the intestinal tract (Fig. 7B). Of note, the majority of IL-17–expressing CD4^+ T cells did not coexpress IFN-γ, and the majority of IFN-γ–expressing CD4^+ T cells were detected in the colon.

In contrast to the decreased representation of Th17 cells in descending regions of the intestinal tract, an opposite trend was observed for Foxp3-expressing Treg cells in the LP. The presence of Foxp3-expressing Treg cells was the lowest in the upper small intestine at 15.7 ± 0.9% CD4^+ T cells. CD4^+ T cells isolated from the jejunum and ileum expressed 17.3 ± 0.9% and 16.3 ± 1.8% CD4Foxp3^+ T cells, respectively, and colonic CD4^+ T cells displayed the highest frequency of CD4^+Foxp3^+ T cells at 33.7 ± 2%, which was ~2-fold higher than the corresponding frequencies in small intestine regions (Fig. 7A). The enrichment of CD4^+ Foxp3^+ T cells was highest in the lower small intestine (jejenum) and colonic regions.

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**Table II. Summary of intestinal APC subsets in C57BL/6 mice from Charles River Laboratories: regional localization and CD4^+ T cell differentiation capacity**

<table>
<thead>
<tr>
<th>Major Subsets of CD45^+IAb^+ LP Cells</th>
<th>Phenotype</th>
<th>Regional Localization</th>
<th>CD4^+ T Cell Differentiation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD103 F4/80</td>
<td>Duo</td>
<td>Jej</td>
</tr>
<tr>
<td>CD11c^+CD11b^-</td>
<td>CD103^F4/80^-</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>CD11c^+CD11b^x</td>
<td>CD103^F4/80^x</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>CD11c^-CD11b^x</td>
<td>CD103^F4/80^x</td>
<td>++</td>
<td>+++</td>
</tr>
</tbody>
</table>
Foxp3+ T cells in the colon was statistically significant ($p < 0.05$) compared with all of the other regions (which were not statistically different from one another) (Fig. 7B). The opposite trends of Th17 and Foxp3+ Treg cell representation along the length of the intestine, in both frequency and absolute cell number (Fig. 7B), suggest that the milieu of the upper small intestine favors Th17 cell differentiation and that the large intestine microenvironment is conducive to Foxp3+ Treg cell differentiation. These gradients for Th17 and Foxp3+ Treg cells appear to be lost during DSS-induced intestinal inflammation, with no statistically significant difference in either Th17 cells or Foxp3+ Treg cells observed in the duodenum, jejunum, and ileum (Fig. 7C). Of note, the frequencies of IL-17–producing CD4+ T cells were higher in the inflamed small intestine compared with those in the normal small intestine.

During the analysis of the intestinal region-specific presence of DC and macrophage populations and Th17 and Foxp3+ Treg cells, several strong correlations were noted. In particular, the trend of Th17 cells in specific regions of the intestine correlated with the abundance of the CD11b+CD103+ LP DC subset ($r = 0.92$; Fig. 7D). Alternatively, the abundance of Foxp3+ Treg cells correlated with the abundance of the CD11b+CD11c+ LP DC subset ($r = 0.76$) and also with CD11c+ F4/80+ LP macrophages ($r = 0.67$) and to a lesser extent with CD11c+ F4/80+ LP macrophages ($r = 0.63$; Fig. 6C).

**Accumulation of CD11b+CD103+ LP DCs and Th17 cells during colitis**

Having noted a paucity of IL-17–producing CD4+ T cells in the colon, we next examined if these cells were increased during colonic inflammation and if any such increases correlated with the accumulation of CD11b+CD11c+ LP DCs. Thus, we induced chronic colitis in mice using four (1 wk each) cycles of DSS...

**FIGURE 7.** Intestinal localization of Th17 and Foxp3+ Treg cells. A, Intestinal lamina propria lymphocytes were isolated and directly stained for FoxP3 (upper panels) or restimulated in vitro with PMA and ionomycin for 5 h and stained for IL-17 and IFN-γ (lower panels). All of the panels are pregated on TCRβ+CD4+ cells. B, Inverse correlation between the presence of Foxp3+ T cells and IL-17–producing T cells in regions of the intestine in both frequency (upper panel) and absolute cell number (lower panel). FACS data frequencies or absolute cell numbers from three independent experiments are summarized as means ± SEM and plotted in the bar graphs. C, Isolated intestinal lamina propria lymphocytes from the indicated regions of the small intestines of DSS-treated mice were restimulated in vitro with PMA and ionomycin for 5 h and stained for IL-17 and IFN-γ. Cells are pregated on TCRβ+CD4+ cells. FACS data from three independent experiments are summarized as means ± SEM and plotted in the bar graph. D, Positive correlation between the presence of IL-17–producing T cells and CD11b+CD103+ LP DCs in regions of the intestine. Positive correlation between Foxp3+ T cells and CD11b+CD103+ LP DCs, CD11c+F4/80+ LP Mφ, and CD11c+ F4/80+ LP Mφ. *$p < 0.05$. **$p < 0.05$. C, colon; D, duodenum; I, ileum; J, jejunum.
administration in the drinking water. Colonic shortening (data not shown) and prominent mononuclear cell infiltration (Fig. 8A) were observed, and this treatment led to the development of chronic colitis associated with Th1 and Th17 responses (42, 43). When LP cells were isolated and restimulated, CD4+ T cells from colitic DSS-treated mice expressed high levels of IL-17 (29%) compared with those of control mice (7.6%). Data from all of the mice analyzed were averaged and summarized in Fig. 8B (right panel), and statistically significant differences are indicated. Unlike the pattern observed in control mice, the majority of the IL-17–producing cells in the colon of the DSS-treated group of mice coexpressed IFN-γ (Fig. 8B). Associated with the ∼5-fold increase in IL-17–producing CD4+ T cells in the LP of DSS-treated mice (Fig. 8B), a specific and statistically significant (p < 0.05) accumulation in the frequencies of CD45+IAb+ cells, CD11b+CD11c− cells (Fig. 8C), and CD11b+CD103+ LP DCs (Fig. 8D) was noted in comparison with those of cells isolated from control mice. The increased frequency of CD11b+CD103+ LP DCs in DSS-treated mice compared with that in control mice was reflected especially in the absolute number of cells (Fig. 8E; p < 0.05), because the increased cellularity in the inflamed colon led to increases in total cell counts among all of the LP DC and macrophage subsets analyzed (p < 0.05). Collectively, these data suggest that the abundance of CD11b+CD103+ LP DCs positively influences the differentiation of Th17 cells both in the upper small intestine of normal mice and in the large intestine during colitis; however, it deserves mention that CD11b+CD103+ LP DCs in the inflamed intestine did not correlate as strongly with Th17 cells as they did in healthy intestine.

Discussion

In this article, we have shown that the functional specializations of DC and macrophage subsets in the intestine are a complex function of several variables, including the regional localization of the

**FIGURE 8.** Accumulation of CD11b+CD103+ LP DCs and Th17 cells during colitis. Mice were given DSS in their drinking water for 5 d and then changed to regular water for 2 d for a total of four cycles. A, H&E staining was performed on tissue sections from colons of control or DSS-treated mice. Original magnification ×250. B, Isolated intestinal lamina propria lymphocytes were restimulated in vitro with PMA and ionomycin for 5 h and stained for IL-17 and IFN-γ. Cells are pregated on TCRβ+CD4+ cells. FACS data from three independent experiments are summarized as means ± SEM and plotted in the bar graph. C, Cells were pregated on live cells, then gated on CD45+ and IAb+ (upper panels), CD11b and CD11c (lower panels). FACS data from three independent experiments are summarized as means ± SEM and plotted in the bar graph. D, LP DCs defined as CD11c+ and CD103+ cells were analyzed subsequently for CD11b expression. FACS data from three independent experiments are summarized as means ± SEM and plotted in the bar graph. E, Absolute cell numbers for major LP DCs and LP macrophage subsets isolated from control or DSS-treated mice represent means ± SEM. *p < 0.05.
subsets, the T cell/APC ratio, and source of the mouse strain. In order to systematically characterize the DC and macrophage subsets in the small and large intestine LP, we established a 10-color FACS panel to phenotypically identify five major subsets of APCs: CD11c<sup>e</sup>CD11b<sup>i</sup>CD103<sup>f</sup>F4/80<sup>−</sup> and CD11c<sup>i</sup>CD11b<sup>e</sup>CD103<sup>f</sup>F4/80<sup>−</sup> LP DCs, CD11c<sup>e</sup>CD11b<sup>i</sup>CD103<sup>f</sup>F4/80<sup>−</sup> and CD11c<sup>i</sup>CD11b<sup>e</sup>CD103<sup>f</sup>F4/80<sup>−</sup> LP macrophages, and CD11c<sup>e</sup>CD11b<sup>i</sup>LP macrophages that may include B cells and other non-DC, non-macrophage APCs as well as several rare subsets including CD11c<sup>i</sup>CD11b<sup>e</sup>CD103<sup>f</sup>F4/80<sup>−</sup>, CD11c<sup>i</sup>CD11b<sup>e</sup>CD103<sup>f</sup>F4/80<sup>−</sup>, CD11c<sup>e</sup>CD11b<sup>e</sup>CD103<sup>f</sup>F4/80<sup>−</sup>, and CD11c<sup>i</sup>CD11b<sup>e</sup>CD103<sup>f</sup>F4/80<sup>−</sup> APCs.

Intestinal F4/80<sup>−</sup> macrophage subsets, regardless of CD11c expression, were the major IL-10 producers in the LP and promoted the differentiation of inducible Foxp3<sup>e</sup> T<sub>reg</sub> cells but not Th17 cells. Furthermore, all of the DC subsets could promote Foxp3<sup>e</sup> T<sub>reg</sub> cells, albeit less efficiently than the macrophages, and particularly at higher T cell/APC ratios. The CD11b<sup>e</sup>CD103<sup>i</sup> LP DCs from C57BL/6 mice from Charles River Laboratories efficiently differentiated the Th17 cells. However, such DCs from C57BL/6 mice from The Jackson Laboratory were not efficient inducers of Th17 cells, and in fact all of the DC subsets from this strain were much more potent inducers of Foxp3<sup>e</sup> T<sub>reg</sub> cells.

The CD103<sup>i</sup> LP DC subsets displayed striking region-specific localization along the intestine, with the presence of CD11b<sup>e</sup> CD103<sup>i</sup> LP DCs strongly correlating with the abundance of Th17 cells in the healthy, upper small intestine and the inflamed large intestine. Our results emphasize the complexities of the intestinal LP DC and macrophage network in terms of phenotypic characterization, regional localization, and unique functional properties and suggest that these cells may be targets for modulating T<sub>reg</sub> and Th17 cell responses in the intestine during health and disease.

Recently, several studies have analyzed LP DCs and macrophages based on the expression of cell surface Ags that are well appreciated to demarcate each cell population in other tissues (17, 18, 21, 22, 45–47). We and others have relied upon CD11c as the bona fide marker of DCs and CD11b expression (in the absence of CD11c) as a useful, albeit not specific, marker for LP macrophages. In this article, it is demonstrated that CD11b and CD11c each have limitations for identifying CD45<sup>i</sup>I<sub>A<sub>2</sub></sub>LP DCs and macrophages when used in the absence of CD103 and F4/80 costaining. It has been argued that DCs and macrophages (of the LP and elsewhere) are not different cell types or lineages but rather a continuum of cells of the mononuclear phagocyte system (48). Although our data do not specifically address this issue, we operationally define LP DCs as cells that are CD45<sup>i</sup>I<sub>A<sub>2</sub></sub>CD11c<sup>i</sup>CD103<sup>e</sup>CD11b<sup>i</sup>F4/80<sup>−</sup> or CD45<sup>i</sup>I<sub>A<sub>2</sub></sub>CD11c<sup>i</sup>CD11b<sup>i</sup>F4/80<sup>−</sup>CD103<sup>e</sup>, protrude many dendrites, possess few phagocytic vacuoles, and induce robust CD4<sup>e</sup> T cell proliferation in vitro. In contrast, we operationally define LP macrophages as cells that are CD45<sup>i</sup>I<sub>A<sub>2</sub></sub>CD11c<sup>e</sup>CD103<sup>f</sup>F4/80<sup>−</sup> or CD45<sup>i</sup>I<sub>A<sub>2</sub></sub>CD11c<sup>e</sup>CD103<sup>f</sup>F4/80<sup>−</sup>, protrude few dendrites, possess many phagocytic vacuoles, and induce CFSE dilution of responding CD4<sup>e</sup> T cell proliferation in vitro that is equivalent to that induced by LP DCs, albeit with less T cell clustering and overall expansion. Importantly, several recent reports demonstrated that CXCR3<sup>+</sup> and CD103<sup>+</sup> LP DCs are of distinct origins and perform separate roles, with the CD103<sup>+</sup> subset performing classical DC functions (49–51).

The functional capacity of LP DCs and macrophages in modulating CD4<sup>e</sup> T cell differentiation has gained significant attention recently. Several groups reported that LP (22, 25, 52) or mesenteric lymph node (MLN) (21, 23, 53, 54) DCs express RA-generating enzymes and can induce gut-homing molecules and the in vitro differentiation of Foxp3<sup>e</sup> T<sub>reg</sub> cells via RA. Furthermore, we demonstrated a potent role for LP macrophages at inducing Foxp3<sup>e</sup> T<sub>reg</sub> cells via the RA pathway (16). In this article, we reveal several previously unappreciated complexities in determining the functions of intestinal APC subsets. Our data clearly show that the ability of intestinal DC and macrophage subsets to induce Foxp3<sup>e</sup> T<sub>reg</sub> versus Th17 cells is dependent on several factors, including: 1) the ratio of T cells to APCs, 2) the regional localization of the intestine, and 3) the source of the mouse strain. With regard to the latter, our results indicate that DC subsets from C57BL/6 mice from The Jackson Laboratory are much more efficient at inducing Foxp3<sup>e</sup> T<sub>reg</sub> cells and less efficient at inducing Th17 cells than those from the same strain from Charles River Laboratories. This is consistent with recent work from our laboratory in which we had observed robust induction of Foxp3<sup>e</sup> T<sub>reg</sub> cells and weak induction of Th17 cells by intestinal DCs obtained from The Jackson Laboratory mice (24). Recently, it has been established that specific bacterial species, especially SFB, may preferentially drive Th17 cell differentiation in the intestinal LP (39, 40, 55) and that SFB are absent in The Jackson Laboratory mice. Therefore, the relative absence of Th17 cells in the LP of The Jackson Laboratory mice may be due to the lack of CD11b<sup>e</sup>CD103<sup>i</sup> LP DC stimulation by SFB or other Th17 cell-inducing factors. Thus, the differences observed between LP DC subsets in Charles River Laboratory mice might be less striking in The Jackson Laboratory mice and potentially other strains of mice lacking Th17 cell-inducing bacteria/factors.

With regard to the T cell/APC ratio, when LP DCs were reduced in number relative to CD4<sup>e</sup> T cells, such conditions permitted the differentiation of Foxp3<sup>e</sup> T<sub>reg</sub> cells, although not as efficiently as CD11c<sup>i</sup> and CD11c<sup>e</sup>F4/80<sup>−</sup> LP macrophages that induced Foxp3<sup>e</sup> T<sub>reg</sub> cells at all of the ratios tested. The exact factor(s) that account for this ratio-dependent induction of Foxp3<sup>e</sup> T<sub>reg</sub> cells by LP DCs are not clear, but these data suggest that at ratios near 1:1 there are DC-expressed cell surface molecules and/or cytokines that are capable of preventing Foxp3 induction in responding CD4<sup>e</sup> T cells. IL-6 is one candidate cytokine secreted by CD11b<sup>e</sup> CD103<sup>i</sup> LP DCs that may inhibit Foxp3 induction (56) at higher concentrations and lose such an effect as it is diluted away with increasing T cell/DC ratios. However, we did not observe robust IL-6 mRNA expression by CD11b<sup>e</sup>CD103<sup>i</sup> LP DCs, suggesting that other factors may play a role in this process as well. Another important difference between our culture conditions and those reported previously (25) is that those studies supplemented cultures with exogenous IL-2, which is a known inducer of Foxp3<sup>e</sup> T<sub>reg</sub> cells (57) and an inhibitor of Th17 cell differentiation (58). It remains to be determined how well each of these in vitro culture conditions mimics actual T cell/DC ratios and local concentrations of TGF-β and IL-2 in the intestinal milieu. Additionally, although all of the LP DC and macrophage subsets can promote Foxp3<sup*e</sup> T<sub>reg</sub> cell differentiation under certain conditions in vitro, which of these populations/subsets are capable of inducing Foxp3<sup*e</sup> T<sub>reg</sub> cells in the LP in vivo remains unresolved. Beyond de novo induction of Foxp3 in responding T cells by LP APC subsets, it has been demonstrated recently that IL-10–producing myeloid cells in the intestinal LP are crucial for maintaining Foxp3 expression and suppressor function in T<sub>reg</sub> cells in the CD45RB<sup>i</sup> model of colitis (59). Therefore, LP DCs and macrophages may play a dual role in the induction and maintenance of Foxp3 in CD4<sup>e</sup> T cells.

We have demonstrated in this article that CD11b<sup>e</sup>CD103<sup>i</sup> LP DCs from C57BL/6 mice from Charles River Laboratories expressed i<sub>B</sub> and t<sub>gfb1</sub> as well as RA-generating enzymes and efficiently induced the differentiation of Th17 cells in vitro. These data are consistent with our original report (16) that CD11b<sup>e</sup>
CD11c⁺ LP DCs preferentially induced Th17 cells and further define the specific role for the CD103⁺ LP DC subsets in this process. Without methods to specifically deplete CD11b⁺CD103⁺ LP DCs, however, the ability of this DC subset to induce Th17 cell responses in vivo remains to be demonstrated formally. Additional signals that condition CD11b⁺CD103⁺ LP DCs to efficiently generate Th17 cells are beginning to be defined and may involve sensing of bacterial components such as flagellin via TLR5. Uematsu et al. (17) recently reported that CD11b⁺CD11c⁺ LP DCs expressed TLR5 and induced Th17 and Th1 cells when stimulated with flagellin. Interestingly, we did not detect robust levels of lrr5 mRNA in CD11b⁺CD103⁺ LP DCs, and our analysis of TLR5-deficient mice revealed no detectable differences in Th17 cells in the intestinal LP (data not shown). Additionally, it is appreciated now that constitutive Th17 cell development in the LP (60) is independent of the MyD88 and Trif pathways yet highly dependent on the bacterial microbiota (46). Thus, it is possible that TLR5 detection of bacterial flagellin by CD11b⁺CD103⁺ LP DCs may play a role in exacerbating Th17 cell responses during intestinal inflammation but not in the homeostasis of natural Th17 cells resident in the normal intestine.

Although in vitro modeling of intestinal LP DC and macrophage functions is important, we also complimented our in vitro observations with characterization of the regional distribution of these cells and Th17 and Foxp3⁺ Treg cells ex vivo. Although the entire small and large intestines drain to the MLNs, region-specific differences were noted in DCs, macrophages, and CD4⁺ T cells in the LP. In particular, CD11b⁺CD103⁺ LP DCs followed a gradient where they were enriched in the duodenum and rare in the large intestine, a pattern that mimicked that of Th17 cells. Alternatively, CD11b⁺CD103⁺ LP DCs as well as CD11c⁺ and CD11c⁺/F4/80⁺ LP macrophages followed an opposite gradient and were enriched in the large intestine where Foxp3⁺ Treg cells were abundant. These data suggest that unique factors in the upper small intestine favor the differentiation and/or retention of Th17-inducing LP DCs, whereas the large intestinal milieu preferentially supports the differentiation and/or retention of Foxp3⁺ Treg cell-inducing LP DCs and macrophages. Because Th17 cells may mediate antibacterial activities, it is possible that these cells are enriched in the relatively low bacterial load of the upper small intestine to quickly screen for potentially pathogenic bacteria that emigrate from the stomach. The vast number of commensal bacteria in the large intestine may induce an immunoregulatory developmental program that conditions DCs and/or macrophages to generate Foxp3⁺ Treg cells that restrain immunoreactivity toward the microbiota. Another potential explanation for the region-specific differences observed is that some “LP” DCs and/or macrophages may reside in gut-associated lymphoid tissue such as isolated lymphoid follicles. These structures are more abundant in the descending gastrointestinal tract (61) and could explain the abundance of CD11b⁻ LP DCs found in these regions. Alternatively, some of these “LP” DCs and/or macrophages may reside within submucosal regions (49).

The important issue of where steady-state LPT cells differentiate in vivo remains incompletely understood. If differentiation were to take place primarily in the MLN as a result of LP DCs draining, then one would expect to find increased frequencies of Th17 cells and Foxp3⁺ Treg cells in this locale, which is not observed (data not shown). Thus, an alternative possibility is that, in the steady state, Th17 and Foxp3⁺ Treg cell differentiation does not efficiently take place in the MLN but rather in the LP of the intestine after migration of naive T cells to this site (62). Another possibility is that T cells may initiate differentiation in the MLN and upregulate homing receptors that will enhance migration to specific regions of the intestine. In support of this, CCR6 has been demonstrated to be an important homing receptor involved in the recruitment and/or retention of Th17 cells during inflammation (63) and in PPs (64) in normal mice. CCR6 does not appear to significantly affect the presence of Th17 cells in the normal small intestine LP, however, so the enrichment of Th17 cells that we have observed in the upper small intestine is not easily explainable by differential homing due to CCR6. It is possible that in the steady state Th17 and Foxp3⁺ Treg cells differentiate in the LP under the influence of region-specific DC and macrophage subsets presenting Ag and that during intestinal inflammation Th17 and Foxp3⁺ Treg cells and other effector T cells undergo more complete differentiation in the MLN due to increased LP DC trafficking to the MLN (65–68).

When we induced chronic colitis in mice, an interesting change in the composition of large intestine LP DCs and Th17 cells was noted. Not only did Th17 cells significantly increase in the diseased tissue but CD11b⁺CD103⁺ LP DCs also did so. Thus, altered frequencies and functions of large intestine LP DCs during colitis may contribute to inflammation (69). Additionally, DSS-induced colitis altered the normal decreasing gradient of Th17 cells along the length of the small intestine and promoted high levels of Th17 cells in the duodenum, jejunum, and ileum. Thus, increases in Th17 cells and/or CD11b⁺CD103⁺ LP DCs and loss of the typical gradient of these cells may be involved in intestinal inflammation. These data are intriguing in light of the recent description of increased IL-17 levels in the colonic mucosa of patients with Crohn’s disease and ulcerative colitis and in mouse models of colitis (70). Whether IL-17 plays a protective (71, 72) or pathogenic (73) role in intestinal inflammation remains a complex issue, because it is secreted by several cell types and exhibits antimicrobial activity (74). IL-23 is a key cytokine of the IL-12 family that can be secreted by DCs (75) and induce IL-17 secretion by responding CD4⁺ T lymphocytes. IL-23 is now documented as playing a critical role in the pathogenesis of several autoimmune diseases (76), and il23r has been shown recently to be an inflammatory bowel disease gene in adult (77) and pediatric (78) Crohn’s disease patients. Thus, LP DCs also may be a source of IL-23 that drives Th17 cells during intestinal inflammation.

In addition to the increased frequency and cell number of CD11b⁺CD103⁺ LP DCs in the inflamed colon, the other APC subsets also were increased numerically due to increased cellularity in inflamed tissue. Thus, it cannot be excluded that cells other than CD11b⁺CD103⁺ LP DCs contribute to Th17 cell differentiation during intestinal inflammation. Additionally, intestinal macrophages are well appreciated to contribute to the pathogenesis of intestinal inflammation (37, 79, 80). Therefore, although intestinal macrophages appear to play an anti-inflammatory role in the steady state, they can mediate proinflammatory functions during colitis. Whether such diverse functions are performed by different subsets of macrophages or rather the plasticity of individual cells is unclear; however, recent data suggest that a unique subset of TLR2⁺CCR2⁺CX3CR1⁺Ly-6C⁺Gr-1⁺ macrophages secretes TNF-α and promotes intestinal inflammation (81). Another recent report suggested that E-cadherin⁺ DCs are inflammatory APCs that may contribute to the pathogenesis of T cell-mediated colitis (82). Further studies should help to clarify whether these inflammatory E-cadherin⁺ cells are DCs or macrophages.

Overall, our data demonstrate that LP DCs and macrophages play fundamentally important, yet distinct, roles in directing CD4⁺ T cell differentiation. Importantly, we show that the functional specializations of DC and macrophage subsets are dependent on the T cell/APC ratio, their regional localization, and source of the mouse strain from which they were isolated. Further clarifying the
functional development and digestive aspects of these and other LP DC subsets along the length of the gastrointestinal tract during health and disease will likely contribute to a better understanding of mucosal immune regulation, which may direct efforts aimed at improving mucosal vaccination regimens and therapeutic intervention for Crohn’s disease and ulcerative colitis.

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References


