

Research Article

CCR9 signaling in dendritic cells drives the differentiation of Foxp3⁺ Tregs and suppresses the allergic IgE response in the gut

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The chemokine receptor CCR9 and its only known ligand CCL25 play an important role in gut inflammation and autoimmune colitis. The function of CCR9-CCL25 in the migration of immune cells is well characterized. However, its role in the immune cell differentiation is mostly not known. Using dextran sodium sulfate (DSS)-induced gut inflammation model, we showed that CCR9⁺ dendritic cells (DCs) specifically CD11b[−]CD103⁺ DCs were significantly increased in the gut-associated lymphoid tissues (GALT) compared to control mice. These CCR9⁺ DCs express lower MHC II and CD86 molecules and had regulatory surface markers (FasL and latency-associated peptide, LAP) in the GALT. In the presence of CCL25, CCR9⁺ DCs promoted *in vitro* differentiation of Foxp3⁺ regulatory CD4⁺ T cells (Tregs). CCL25-induced differentiation of Tregs was due to intrinsic signaling in the DCs but not through CD4⁺ T cells, which was driven by the production of thymic stromal lymphopoietin (TSLP) and not IL-10. Furthermore, adoptive transfer of CCR9⁺ DCs in C57BL/6 mice promoted Tregs but reduced the Th17 cells in the GALT, and also suppressed the OVA-specific gut-allergic response. Our results suggest CCR9⁺ DCs have a regulatory function and may provide a new cellular therapeutic strategy to control gut inflammation and allergic immune reaction.

Keywords: CCL25 · CCR9 · dendritic cells · Foxp3 · mucosal tolerance



Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

Chemokine (C-C motif) receptor 9 (CCR9) is expressed on IgA-producing B cells, CD8⁺ T cell, CD4⁺ T cells, $\gamma\delta$ T cells, and DCs, and drives the migration to its only known chemokine ligand CCL25, which is constitutively expressed on small intestinal epithelial cells and thymic epithelial cells in mice and humans

[1–4]. Intestinal epithelial cells during gut inflammation or in the inflammatory bowel disease (IBD) produce several folds higher CCL25 and drive the recruitment of CCR9⁺ immune cells. Due to its gut tropism activity, CCR9 and CCL25 are suggested as a potential therapeutic target [1, 5, 6]. A phase 2 study using a CCR9 antagonist (vercirnon or GSK1605786A, also known as CCX282-B) showed clinical remission in moderate to severe Crohn's disease (CD) [7], but subsequent phase 3 study failed to improve clinical response and remission [6]. Moreover, CCR9 antagonist treatment showed a dose-dependent adverse reaction in almost all the patients [6, 8, 9]. The data from animal models also showed

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that dextran sodium sulfate (DSS)-induced colitis in CCR9^{-/-} or CCL25^{-/-} mice or adoptive transfer of wild-type naïve CD4⁺ T cells in RAG1^{-/-}CCL25^{-/-} mice have exacerbated colitis [10, 11]. CCR9^{-/-} animals also show the development of myelin oligodendrocyte glycoprotein peptide (MOG)-induced severe EAE [4]. It has been reported that increased colonic expression of CCL25 reduces the DSS-induced severity of the colitis in mice [11]. CCR9 is expressed on both effector and regulatory CD4⁺ T cells and help to traffic into the small intestine. The antibody against CCL25/CCR9 ameliorates the early, but not the chronic phase of murine colitis [12]. Interestingly, the adoptive transfer of CCR9^{-/-} naïve CD4⁺ T cells in lymphopenic host lead to trafficking of these cells in the colon and cause colitis, and co-transfer of CCR9^{-/-} Tregs inhibit colitis [12]. Together, these results suggest that CCR9 expression of effector/memory T cells or Tregs is not required for the trafficking to the colon and its function. It has been reported that the adoptive transfer of wild-type naïve CD4⁺ T cells into RAG2^{-/-}CCR9^{-/-} mice leads to the development of colitis, and this was due to altered localization and function of plasmacytoid DCs (pDCs) within the small intestine [13]. However, in another study, DSS-treated CCR9^{-/-} mice or colitis-induced by adoptive transfer of naïve CD4⁺ T cells in RAG1^{-/-}CCL25^{-/-} recipients showed no change in the frequency of pDCs [10]. Most of the previous study shows that blockade of CCR9/CCL25 interaction exacerbated colitis [10, 12] and was independent of CCR9 expression on T cells, suggesting the importance of CCR9 on innate immune cells.

A possible role for CCR9 on the conventional DC has not yet elucidated. The conventional DCs (cDCs) play a critical role in gut inflammation and autoimmunity in both humans and mice [14]. cDCs are known to drive the effector immune response, but some subsets of cDCs also orchestrate the protective immune response and tolerance in the gut [15]. DCs play a crucial role in maintaining the subtle balance between immunity and tolerance in the intestine [16]. Based on CD11b and CD103 expression, cDCs are categorized into four subsets: CD103⁺CD11b⁻, CD103⁻CD11b⁺, CD103⁺CD11b⁺, and CD103⁻CD11b⁻ in the small intestine and colon [17]. DCs migrate from the peripheral lymph nodes to the mLN to prime naïve T cells and promote the differentiation of regulatory or effector T cells [18]. DCs phenotype is largely based on their activation status after antigen encounter in the mucosal compartment, which elicits different signaling pathways that skew immune response toward activation or tolerance. In the present study, we hypothesized that intrinsic CCR9 signaling in DCs might promote the distribution and differentiation of Tregs in the gut mucosa and help in maintaining mucosal tolerance.

In the present study, we explore the role of CCR9 in the migration of conventional DCs and CCR9-CCL25 interaction in modulating the Th17 and Treg response. We used the DSS-induced colitis model [19] and studied the phenotype of CCR9⁺ DCs in a homeostatic and inflammatory condition. Further, we investigated the functional role of CCR9⁺ DCs in the presence of CCL25 in the differentiation of regulatory CD4⁺ T cells (Treg) or Th17 cells. Our results showed that CCR9⁺ DCs preferentially CD11b⁻CD103⁺

DCs recruited to the gut and gut-associated lymphoid tissues during gut inflammation. CCR9⁺ DCs induces Treg differentiation in the presence of CCL25. Further, we demonstrate that thymic stromal lymphopoietin (TSLP) produced by CCR9⁺ DCs but not IL-10 promotes the differentiation of Treg. CCR9⁺ DCs in the GALT show an immature and regulatory phenotype and suppressed the cholera toxin and OVA-induced gut allergic response in mice.

Results

Gut inflammation alters the frequency of various subsets of DCs in the GALTs

Based on the development, phenotype, and function, different types of DCs have been characterized during gut inflammation and autoimmunity [17, 20]. To test the changes in the various subsets of DCs in the GALTs during gut inflammation, we induced inflammatory colitis in C57BL/6 mice by giving 2% DSS in the drinking water. Our results showed that compared to control mice, DSS-treated mice showed >25% weight-loss in 10 days (Fig. 1A). Our data showed that the absolute number and frequency of DCs were increased in the Peyer's patch (PP) and lamina propria (LP) in DSS-treated mice as compared to control (untreated) mice (Fig. 1B; Supporting Information Fig. 1A). The gut-homing DCs expressing CD103⁺ and CD11b⁺ are known to play an important role in the inflammation and mounting an effector immune response [17, 21, 22]. Our results showed that DSS-treated mice had increased frequency of CD11b⁻CD103⁺ DCs in the mesenteric lymph nodes (mLN) and PP as compared to the control mice (Fig. 1C; Supporting Information Fig. 1B). In PP, CD11b⁺CD103⁺ DCs and CD11b⁺CD103⁻ DCs showed increased frequency in the DSS-treated mice as compared to control group (Fig. 1C). These results suggest that various subsets of DCs have variable homing potential and maybe having a different function in the GALTs to control the inflammation.

DCs show higher CCR9 expression during gut inflammation

The continuous migration of APCs to and from LP and PP to mLN plays a crucial role in mounting an effective immune response and maintaining the gut homeostasis and tolerance [1]. Chemokine receptor CCR9 expression controls the migration of these immune cells into the gut and contributes to the gut inflammation [1, 23, 24]. We tested the expression of CCR9 on various subsets of DCs in the DSS-induced gut inflammation (Supporting Information Fig. 2A). Our results showed that DSS treatment increased the frequency of total CCR9⁺ DCs in the PP (Fig. 2A). We also analyzed CCR9 expression on various subsets of DCs and showed that CD11b⁺CD103⁺, CD11b⁻CD103⁺, and CD11b⁻CD103⁻ DCs had a significantly higher frequency and MFI of CCR9 in the mLN and PP in DSS-treated mice as compared to

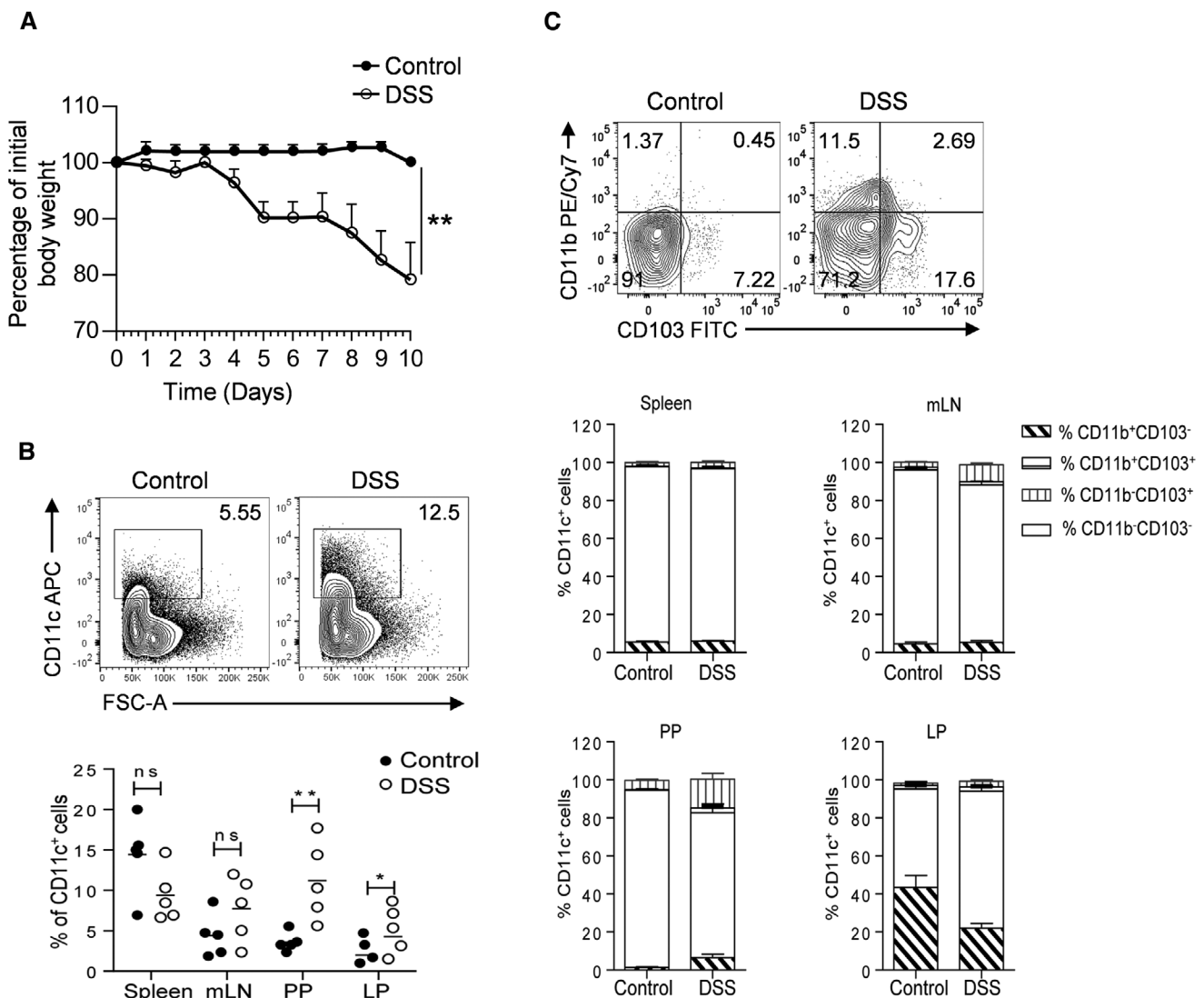


Figure 1. Gut inflammation increases the early recruitment of DCs in the gut-associated lymphoid tissues (GALTs). C57BL/6 mice were given drinking water with or without 2% DSS. (A) Loss of body weight was monitored and plotted as percent weight loss from the initial weight with time. Error bars represent the mean $\pm$ SD. (B) On day 10 of DSS treatment, mice were sacrificed. CD11c⁺ DCs were analyzed after gating on live singlet cells using flow cytometry and plotted (upper panel). Numbers in the contour plot show the percentage of cells in the gated region. The mean percentage of DCs in the spleen, mLN, PP, and LP, were calculated plotted (lower panel). (C) Based on the CD11b and CD103 expression, various subsets of DC were analyzed. The numbers in the contour plots indicate the percentage of cells in the gated region (upper). The mean percentage of various subsets of DCs was analyzed and plotted (lower). Each symbol represents data from individual mice (B). Error bars indicate the mean $\pm$ SEM (B and C). $n = 5$ mice/group. Unpaired Student's t -test was used (A, B, and C). * $p < 0.05$; ** $p < 0.01$; ns, not significant (A and B). Data shown are representative of one of the three independent experiments (A, B, and C).

control mice (Fig. 2B; Supporting Information Fig. 2B), and these staining of CCR9 on each subsets of DCs were specific (Supporting Information Fig. 3). Recruitment and accumulation of CCR9⁺ DCs may be the consequence of transportation of the high number of bacteria in PP during inflammation. It is reported that immature DCs accumulate in the subepithelial dome (SED) and helps in the internalization of nonpathogenic bacteria for the onset of mucosal inflammation [25]. The DSS treatment increased the expression of CCL25 levels in the intestine and colon (Supporting Information Fig. 2C). The immunohistological analysis showed that CCR9⁺ DCs were mostly localized near the villi of small intestine and

colon in DSS-treated mice that were otherwise located at the base in control mice (Fig. 2C; Supporting Information Fig. 2D). These results suggest that gut inflammation not only increased the frequency of CCR9⁺ DCs but also altered the anatomical localization in the gut. CCR6 also plays an important role in the homing of immune cells in the gut [1, 26]. Our analysis of CCR6 expression on CD11c⁺CCR9⁺ DCs showed no alteration of CCR6 with DSS treatment (Supporting Information Fig. 2E). Together, these results suggest that inflammation affects the expression CCR9 in the specific subset of DCs and their localization in the gut.

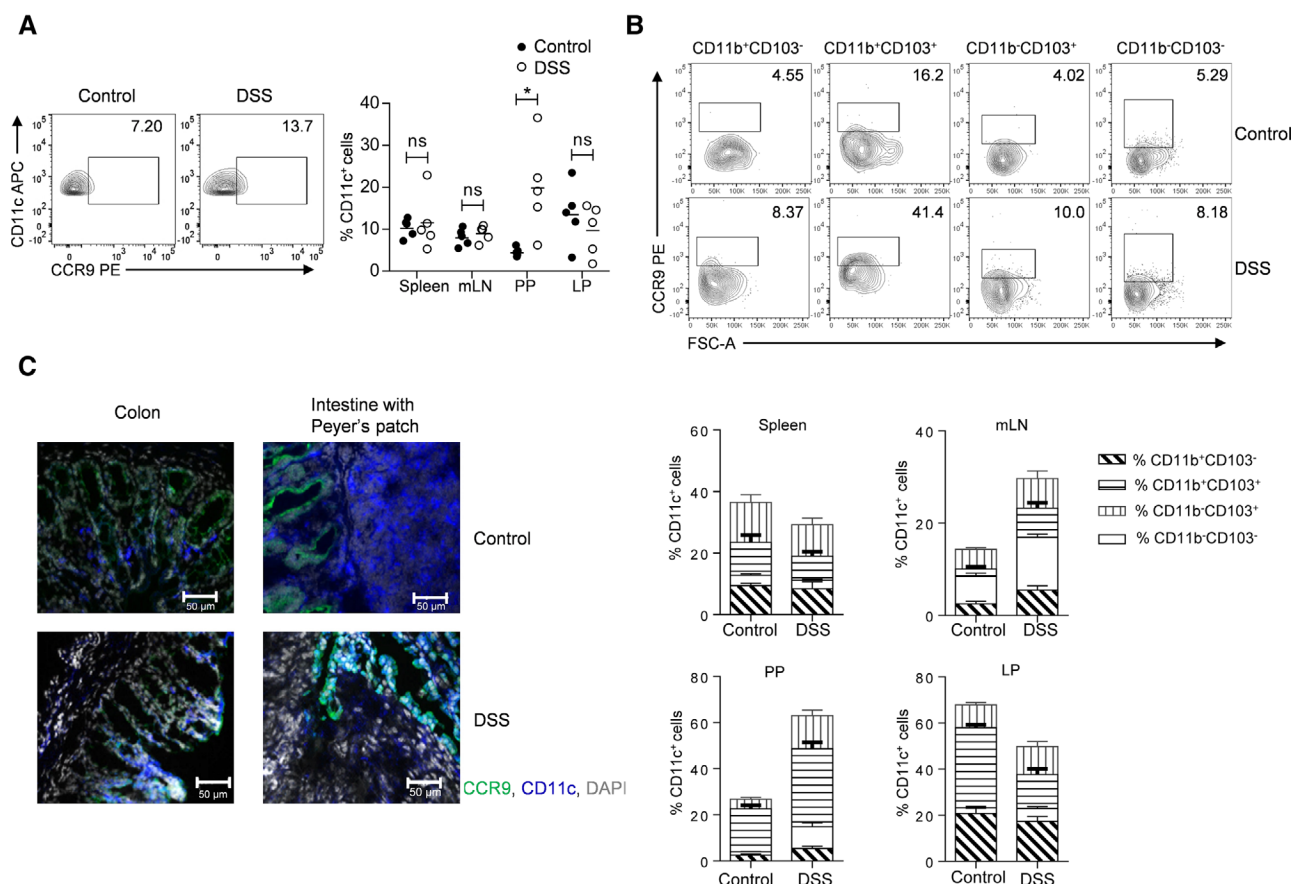


Figure 2. CCR9 expression on various subsets of DCs during gut inflammation. C57BL/6 mice were given drinking water with or without 2% DSS. (A) On day 10, mice were sacrificed, and the expression of CCR9 on CD11c⁺ cells were analyzed by flow cytometry. Cells in the contour plots are gated on CD11c⁺ cells (left panel). The numbers in the contour plot show the percentage of cells in the gated region. The mean percentage of CCR9⁺ DCs in the spleen, mLN, PP, and LP were analyzed and plotted (right panel). Each symbol represents data from individual mice. Error bars indicate the mean $\pm$ SEM. $n = 5$ mice per group. Unpaired Student's t -test was used. * $p < 0.05$ and ns, not significant. (B) CCR9 expression on various subsets of DCs was analyzed after gating on specific subsets of DCs, as shown in Supporting Information Figure 2A and plotted (upper panel). Numbers indicate the percentage of cells in the gated region. The mean percentage of CCR9⁺ on the various subset of DCs was analyzed plotted (lower panel). Error bars indicate the mean $\pm$ SEM. $n = 5$ mice per group. (C) On day 10, CCR9 expression in the colon and intestine with Peyer's patch were analyzed by immunohistological staining. The representative images stained for CCR9 (green), CD11c (blue), and nuclear stain DAPI (grey) are shown. Scale bar 50 μ m. Original magnification is 400 $\times$. Data shown are representative of one of the three independent experiments (A, B, and C).

CCR9⁺ DCs show lower MHC II and CD86 expression but higher LAP and FasL expression

Activation of DCs cause increased surface expression of CD40, CD80, CD86, and MHC II molecules [27]. We tested the expression of these co-stimulatory molecules on CCR9⁺ as well as CCR9⁻ DCs under homeostasis and during gut inflammation. Our results showed that expression of MHC II and CD86 molecules was decreased on CCR9⁺ DCs, whereas CCR9⁻ DCs showed higher expression of these molecules in the gut-associated lymphoid tissues, PP and LP, in DSS-treated animals as compared to control mice (Fig. 3A and B). It has been shown that tolerogenic DCs express FasL and latency-associated peptide (LAP) molecules and inhibit the T cell response to control gut inflammation and helps in maintaining mucosal tolerance [28, 29]. Our results showed that CCR9⁺ DCs had similar levels of FasL expression in the spleens, however, had higher FasL expression in PP dur-

ing gut inflammation as compared to the homeostatic condition in the PP (Fig. 3B). Moreover, CCR9⁻ DCs showed higher FasL expression in the spleen and LP with DSS treatment as compared to control groups (Fig. 3B). Similarly, our data showed increased LAP expression on the CCR9⁺ DCs specifically in the PP in DSS-treated mice as compared to control mice (Fig. 3B), but CCR9⁻ DCs did not show any change in the expression (Fig. 3B). Together, these results suggest that CCR9⁺ DCs show immature phenotype and express anti-inflammatory molecules during gut inflammation.

CCR9⁺ DCs promote Foxp3⁺ Treg and inhibit Th17 cell differentiation

Since our results showed that CCR9⁺ DCs had a tolerogenic phenotype, we investigated the potential function of CCR9⁺ DC in

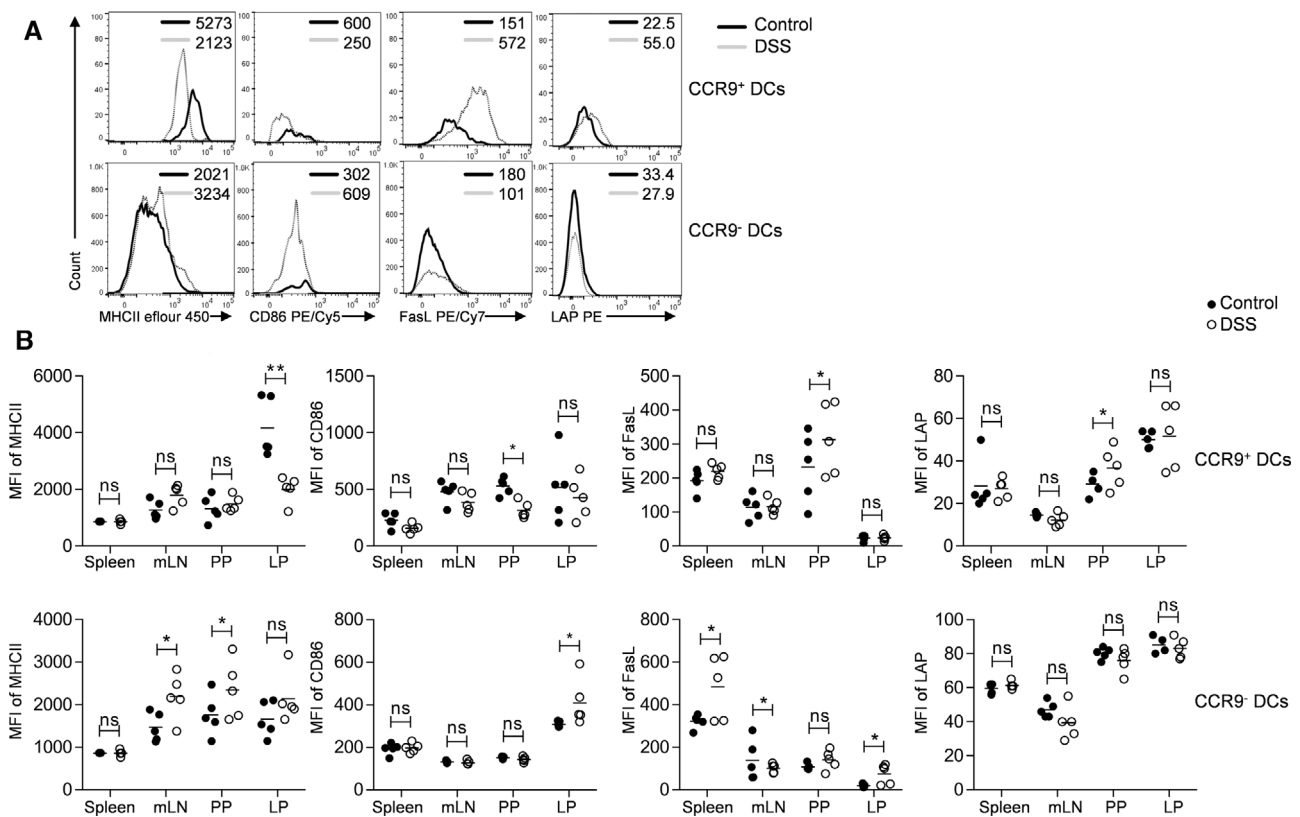


Figure 3. CCR9⁺ DCs show regulatory phenotype during gut inflammation. C57BL/6 mice were given drinking water with or without 2% DSS. On day 10, expression of MHC II, CD86, FasL, and LAP was analyzed on CD11c⁺CCR9⁺ and CD11c⁺CCR9⁻ DCs using flow cytometry. (A) A representative histogram showing overlap between control and DSS-treated mice cells gated on CCR9⁺CD11c⁺ or CCR9⁻CD11c⁺ DCs are depicted. Numbers in the histogram indicate the MFI of indicated markers in the gated region (upper panel). (B) The MFI of MHCII, CD86, FasL, and LAP on DCs in the spleen, mLN, PP, and LP were calculated and plotted (lower panel). Each symbol represents data from the individual mice. Error bars indicate the mean $\pm$ SEM. $n = 5$ mice per group. Unpaired Student's *t*-test was used. * $p < 0.05$; ** $p < 0.01$; ns, not significant. Data shown are representative of one of the three independent experiments.

promoting the differentiation of Foxp3⁺ Treg cells or Th17 cells in culture. To test this, CCR9⁺ DCs were cultured with purified naïve CD4⁺ T cells (CD4⁺CD25⁻CD44⁻ cells) under Treg or Th17 differentiation condition in the presence or absence of CCL25. Results showed that CCL25 promoted the differentiation of naïve CD4⁺ T cells into Foxp3⁺ Tregs (Fig. 4A). However, CCL25 reduced the differentiation of ROR γ t⁺ Th17 cells under Th17 condition (Fig. 4B and C). Also, we found that this increase in FoxP3 expression was CCL25 concentration dependent (data not shown). CCL25 did not have any effect on the differentiation of Th1 and Th2 cells (data not shown). CCL25 did not affect the differentiation of Foxp3⁺ Tregs with CCR9⁻ DCs (Supporting Information Fig. 4). It has been demonstrated that CCR9⁺ thymic DCs are the potent inducer of natural Tregs in the thymus [30, 31]. We also compared the Treg differentiation potential of peripheral CD11c⁺CCR9⁺ with thymic DCs and thymic epithelial cells. Our results showed that gut CCR9⁺ DCs, thymic DCs, and mTEC cells were able to drive the differentiation of DCs, and the addition of CCL25 significantly promoted the Treg differentiation with CCR9⁺ DCs but not with CCR9⁻ DCs (Fig. 4D). Collectively, these results suggest that CCR9⁺ DCs promote Treg but inhibit the differentiation of inflammatory Th17 cells.

To test the effect of CCL25 is only through CCR9 expressed on DCs but not on CD4⁺ T cells, we cultured the naïve CD4⁺ T cells in the presence of plate-bound anti-CD3 ϵ mAb, soluble anti-CD28 mAb and with or without CCL25. In the absence of DCs, CCL25 did not show any effect on the differentiation of Treg cells (Fig. 4E and F). Interestingly, the effect of CCL25 on Th17 differentiation was independent of DCs (Fig. 4E and F). These data suggest that CCL25-CCR9 signaling on DCs affect the Treg and Th17 cell differentiation.

CCL25 induced Treg differentiation is affected by TSLP

Gut DCs are known to produce various molecules such as IL-10, TGF- β , retinoic acid (RA), and TSLP that drive the differentiation of Foxp3⁺ Tregs [32]. To test the increased differentiation of Tregs with CCR9⁺ DCs in the presence of CCL25 was due to the secretion of IL-10 in the culture supernatant, naïve CD4⁺ T cells were cultured in Treg and Th17 differentiation condition for 4 days. Our results showed no change in the IL-10 secretion in culture supernatant in Treg condition, whereas Th17 condition showed reduced IL-10 levels in culture (Fig. 5A). We further confirmed that

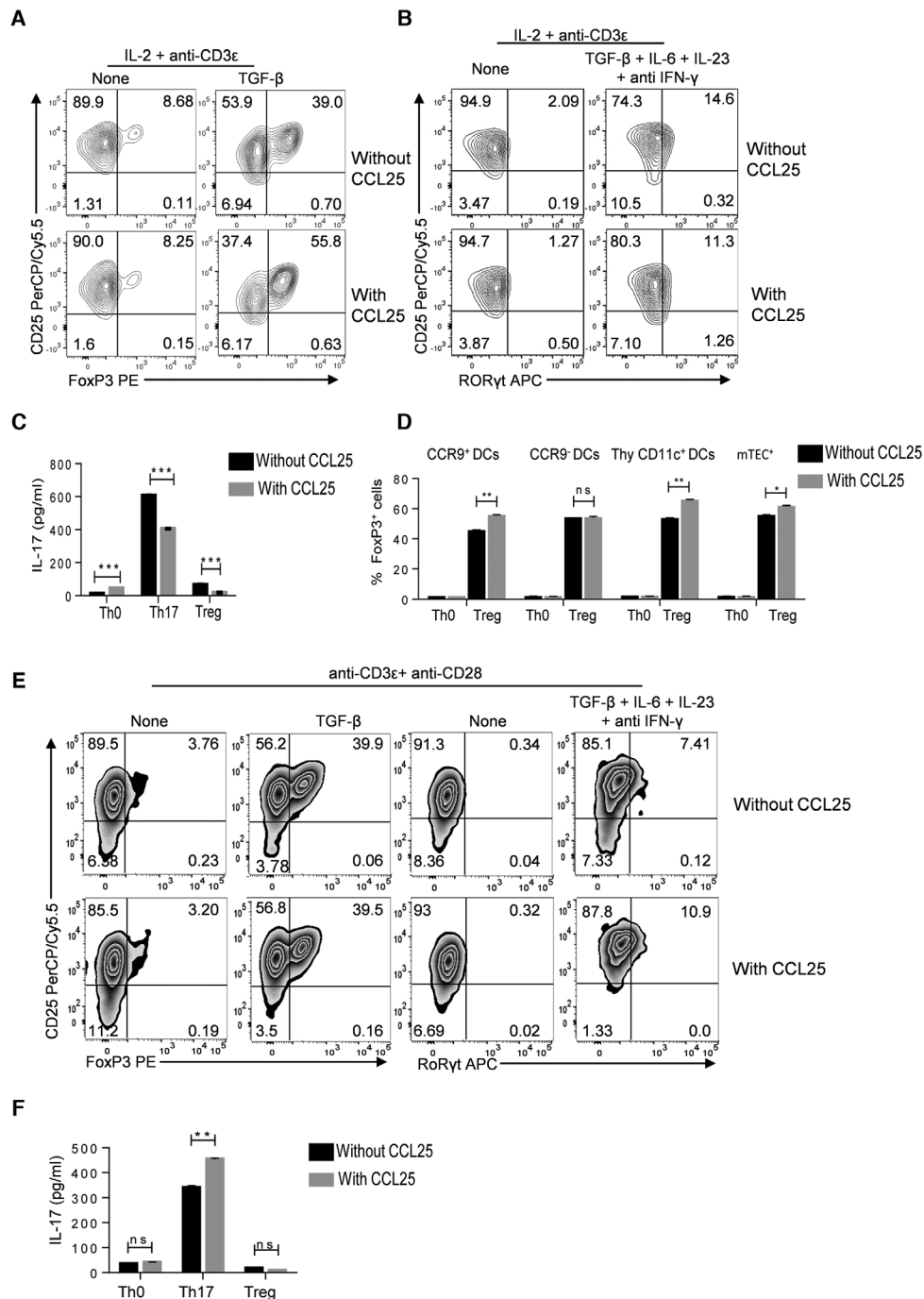


Figure 4. CCR9 $^{+}$ DCs promote Foxp3 $^{+}$ Tregs and inhibit Th17 cell differentiation. Naïve CD4 $^{+}$ T cells (CD4 $^{+}$ CD25 $^{-}$ CD44 $^{-}$ cells) were cultured with purified CCR9 $^{+}$ CD11c $^{+}$ DCs in presence or absence of CCL25 under Treg or Th17 differentiation conditions for 4 days. After 4 days, cells were surface stained for CD4, CD25, and intracellular Foxp3 and ROR γ t and analyzed by flow cytometry. (A) The expression of Foxp3 was analyzed after gating CD4 $^{+}$ cells. (B) The expression of ROR γ t was analyzed after gating on CD4 $^{+}$ cells. The numbers in the contour plots show the percentage of cells in the marked quadrants (A and B). Data shown are representative of one of the three experiments (A and B). (C) After 4 days of culture, the supernatant was collected, and the secretion of IL-17 was measured by ELISA. Error bars indicate the mean $\pm$ SD. Data shown are representative of one of the two independent experiments. Unpaired Student's t-test was used. *** p < 0.001. (D) Naïve CD4 $^{+}$ T cells were cultured with peripheral CD11c $^{+}$ CCR9 $^{+}$ DCs, CD11c $^{+}$ CCR9 $^{-}$ DCs, thymic CD11c $^{+}$ DCs, and thymic medullary epithelial cells (mTEC $^{+}$ cells) in presence of CCL25 under Treg differentiation condition and the percentage of Foxp3 $^{+}$ cells were analyzed by flow cytometry. Error bars indicate the mean $\pm$ SD. Data shown are representative of one of the two independent experiments. Unpaired Student's t-test was used. * p < 0.05, ** p < 0.01, and ns, not significant. (E) Naïve CD4 $^{+}$ T cells were cultured in anti-CD3 ϵ mAb-coated 96-well plates in the presence of soluble anti-CD28 mAb along with Th17 and Treg differentiating condition in the absence of DCs. After 4 days of cultures, Foxp3 and ROR γ t expression were analyzed using flow cytometry. Data shown are representative of one of the three independent experiments. The numbers in the contour plot show the percentage of cells in the marked quadrants. (F) The secretion of IL-17A in culture supernatant from (E) was measured by ELISA and plotted. Error bars indicate mean $\pm$ SD. Data shown are representative of one of the two independent experiments. Unpaired Student's t-test was used. ** p < 0.001; ns, not significant.

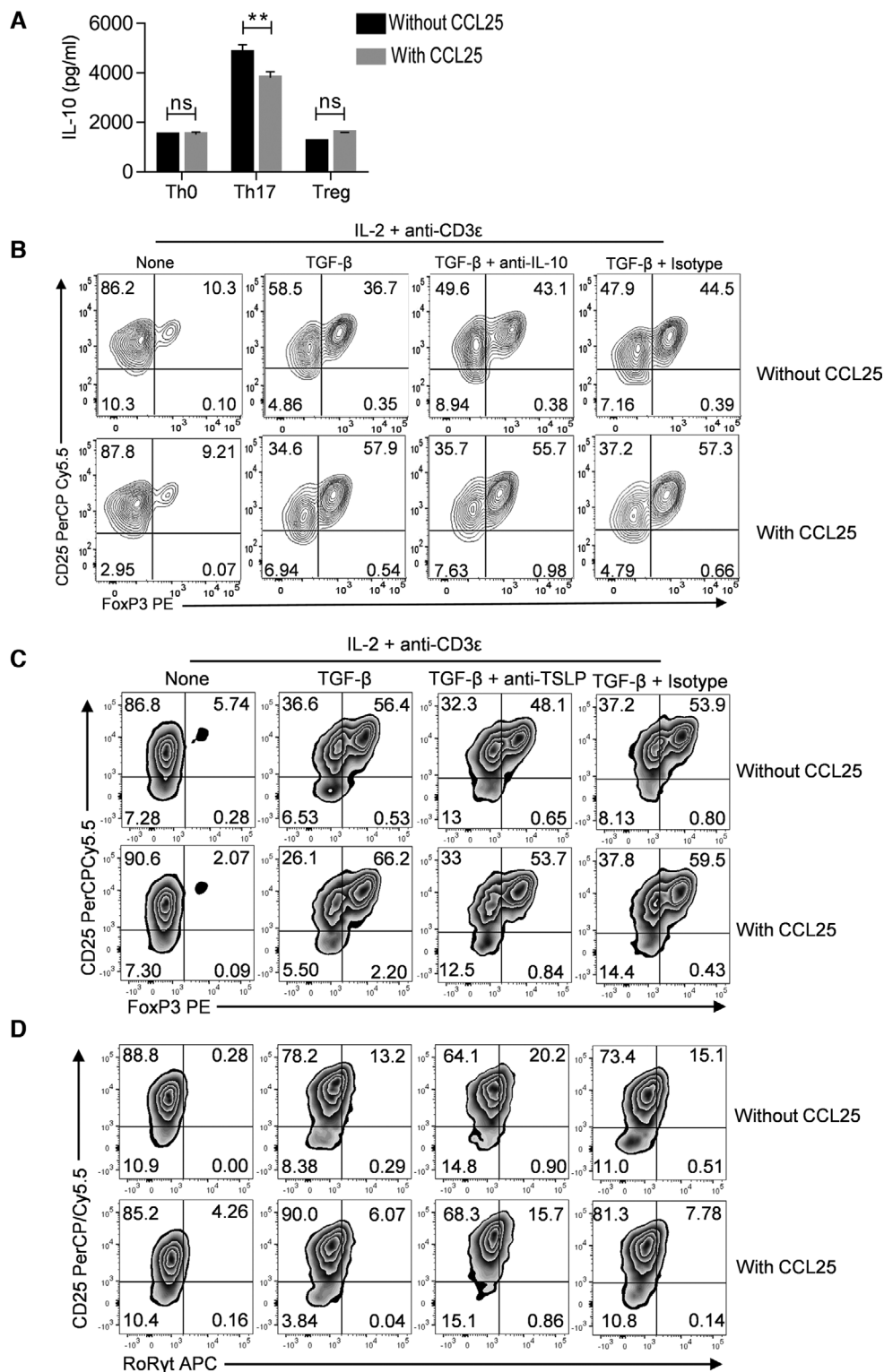


Figure 5. TSLP produced in culture promotes the differentiation of Foxp3⁺ Tregs. Naïve CD4⁺ T cells (CD4⁺CD25⁻CD44⁻ cells) were cultured with purified CCR9⁺CD11c⁺ DCs in presence or absence of CCL25 under Treg or Th17 differentiation condition for 4 days. (A) Culture supernatant was collected, and the secretion of IL-10 was measured by ELISA. Error bars represent mean $\pm$ SD. Data shown are representative of one of the two independent experiments. Unpaired Student's t-test was used. ** $p < 0.01$, ns, not significant. (B) During Treg differentiation culture, IL-10 was neutralized using anti-IL-10 mAb or isotype control mAb was added in culture. Foxp3 expression was monitored by flow cytometry after gating on CD4⁺ T cells. (C and D). During Treg or Th17 cells differentiation culture, TSLP was blocked using anti-TSLP mAb or added only isotype control mAb in culture. The expression of Foxp3 or ROR γ t was monitored after gating on CD4⁺ T cells using flow cytometry. The numbers in the graph represent the percentage of cells in each quadrant (B–D). Data shown are representative of one of the three independent experiments.

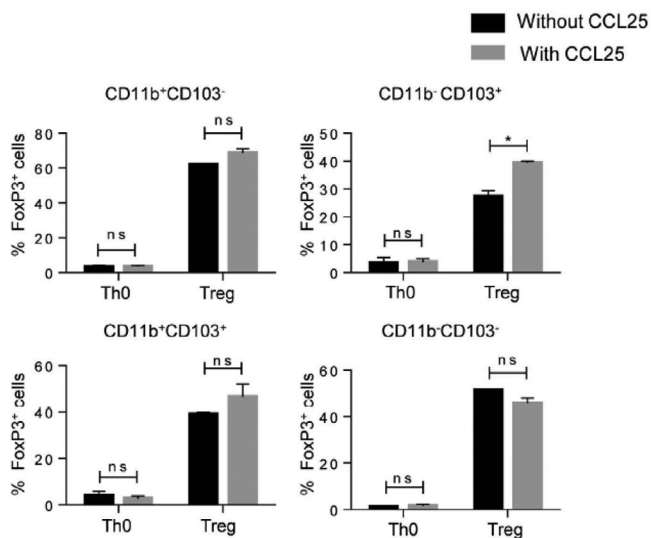


Figure 6. CD11b⁺CD103⁺ DC subset promotes Foxp3 Treg differentiation. Naïve CD4⁺ T cells (CD4⁺CD25⁻CD44⁻ cells) were cultured with purified indicated subsets of DCs in the presence or absence of CCL25 under Th0 or Treg differentiation condition for 4 days. Foxp3 expression in CD4⁺ T cells was monitored using flow cytometry. Data shown are mean of three independent experiments, and error bars represent mean $\pm$ SEM. Unpaired Student's t-test was used. * $p < 0.05$, ns, not significant.

the neutralization of IL-10 with anti-IL-10 mAb in culture did not affect the CCL25 driven differentiation of Tregs (Fig. 5B). Interestingly, blocking of TSLP with anti-TSLP mAb in culture lowered the CCL25 induced differentiation of FoxP3⁺ Tregs as compared to isotype control mAb (Fig. 5C; Supporting Information Fig. 4B). With the blocking of TSLP, CCL25 promoted the Th17 cell differentiation under Treg culture condition (Fig. 5D). It is known that TGF- β with TSLP helps in the maintenance of tolerance; however, under inflammatory conditions, TSLP level decreases and show increased differentiation of Th17 cells [32], this further supports our results on the role of TSLP promotes Tregs in culture.

CD11b⁺CD103⁺ DCs is a potent inducer of Foxp3⁺ Treg with CCL25

Our results showed that various subsets of DCs express CCR9 and migrate into the GALT during gut inflammation (Fig. 2B). We analyzed the Treg induction potential of these DCs in the presence of CCL25. To test this, we cultured various subsets of the DCs with naïve CD4⁺ T cells in the presence or absence of CCL25 and monitored the differentiation of Foxp3⁺ Tregs in the culture. Our results showed that in the presence of TGF- β CD11b⁺CD103⁻ DCs are a potent inducer of Treg, however, among various subsets of DCs, only CD11b⁺CD103⁺ DCs significantly increased the Foxp3⁺ Treg differentiation with CCL25 (Fig. 6). Together these results showed that among all four subsets of DCs analyzed, CD11b⁺CD103⁺ DC is the most potent inducer of Foxp3⁺ Tregs.

CCR9⁺ DCs suppress OVA-specific allergic response

Treg cells play an important role in controlling gut tolerance and allergic response [33, 34]. Since CCR9⁺ DCs induce strong Treg response after exposure to CCL25, we next examined the tolerogenic potential of CCR9⁺ DCs using the animal model of OVA-induced allergy [28]. In this experiment, to break the mucosal tolerance in C57BL/6 mice, animals were orally given OVA and cholera toxin (Ctx) for three consecutive days. The control mice were given orally either PBS or OVA alone. On day 7, the subcutaneous injection of OVA in a CFA was given and then purified CCR9⁺ DCs or CCR9⁻ DCs isolated from naïve C57BL/6 mice were adoptively transferred. After 7 days of adoptive transfer of DCs, animals were sacrificed, and the differentiation of Foxp3⁺ Treg and ROR γ t⁺ Th17 cells and IgE response was monitored. Our results showed that the group that received the adoptive transfer of CCR9⁺ DCs had a significantly higher frequency of Foxp3⁺ Tregs in the spleen and PP (Fig. 7A). Interesting, analysis of LP of mice received the adoptive transfer of CCR9⁻ DCs had a significantly higher frequency of ROR γ t⁺ Th17 cells compared to other groups (Fig. 7B). These results were also consistent with our in vitro data, where CCR9⁺ and CCR9⁻ DCs had a differential effect on the regulation of Foxp3⁺ Tregs and Th17 cells.

In our OVA allergy model, we further investigated the effect CCR9⁺ DCs on IgE⁺ B cells in the spleen and GALTs. Our results showed that the adoptive transfer of CCR9⁺ DCs significantly lowered the frequency of IgE⁺ B cells in the spleen, mLN, PP, and LP as compared to other groups, including CCR9⁻ DC group (Fig. 7C; Supporting Information Fig. 5). Further, we investigated the OVA-specific IgE levels in the serum, and our results showed that mice that received CCR9⁺ DCs had marginally lower OVA-specific serum IgE levels compared to other groups (Fig. 7D). As expected, the OVA-specific IgE level in serum was not as significant as we found in the mucosal compartment. However, CCR9⁺ DCs mice able to abrogate IgE response also in the systemic compartment. Together these results suggest that CCR9⁺ DCs control the gut mucosa-associated differentiation of Tregs and Th17 cells and also contribute to controlling the gastrointestinal allergic response.

Discussion

DCs are innate immune cells and have been implicated in inducing tolerance as well as inflammatory response in the gut [35]. The heterogeneity and migratory properties of DCs make them unique cell types of the immune system to maintain immune tolerance in the peripheral compartment, including the gut [36]. The intestine is daily exposed to trillions of microbes, and DCs in the gut capture microbial antigens and present to the T cells. For migration to different effector sites, DCs use various chemokine receptor-like CCR6, CCR7, and CCR9 [37]. It has been reported that dendritic cells extend their dendrites through M-cell specific transcellular pores to sample the gut antigens in a controlled manner for induction of protective immunity [38]. However, during IBD,

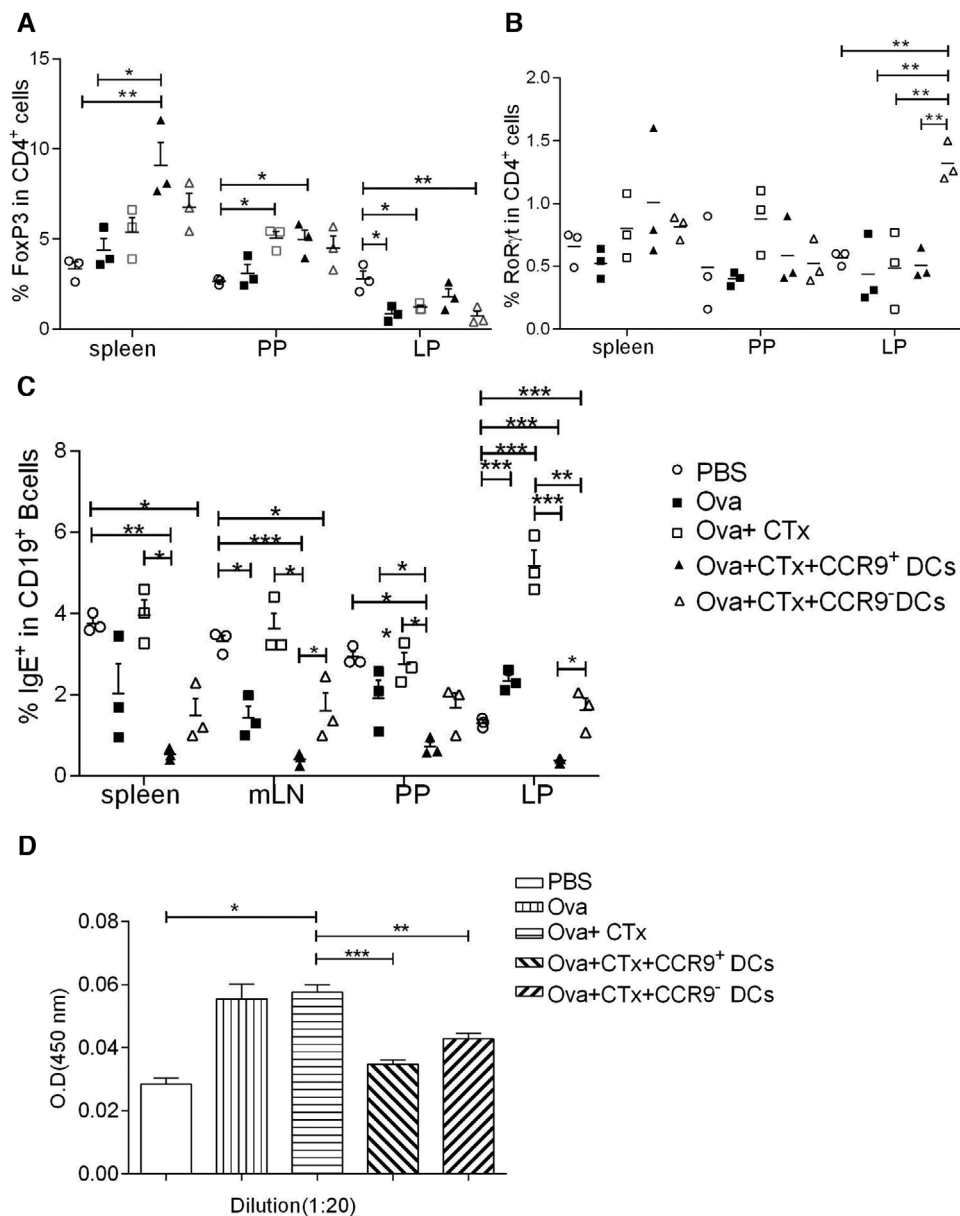


Figure 7. CCR9⁺ DCs promote Treg differentiation and suppress allergic IgE responses in GALT. C57BL/6 mice were orally given PBS, OVA, or OVA plus cholera toxin (OVA plus CTx) for 3 constitutive days. On day 7, OVA plus CTx group was divided into three groups, and adoptively transferred purified CD11c⁺CCR9⁺ DCs, CD11c⁺CCR9⁻ DCs, or sham transferred. These mice were also given a subcutaneous injection of OVA in CFA at the time of DC injection. After 7 days, animals were sacrificed, and Treg, Th17, IgE⁺ B cells, and serum IgE response were monitored. (A) Foxp3⁺ cells were analyzed by flow cytometry after gating on CD4⁺ T cells in spleen and GALTs and plotted. (B) Analysis of RORγt⁺ Th17 cells using flow cytometry after gating on CD4⁺ T cells. (C) IgE⁺ B cells were analyzed by flow cytometry after gating on CD19⁺ B cells. Each symbol represents data from individual mice (A–C). Error bars represent mean ± SEM (A–C). (D) OVA-specific IgE was monitored in the serum by ELISA and plotted. Error bars represent mean ± SEM (D). Data shown are from a single experiment (A–D). *n* = 3 mice per group (A–D). One-way ANOVA with Tukey's multiple comparisons test was used (A–D). **p* < 0.05; ***p* < 0.01; and ****p* < 0.001. Non-significant values are not marked.

immature DCs accumulate in PP, and helps in the internalization of bacteria, which causes the onset of inflammation [25]. Here, we show that chemokine receptor CCR9 expressed on various subsets of DC in the secondary lymphoid organs and GALT. During gut inflammation, CCR9⁺ CD11c⁺ DCs show altered localization in the Peyer's patch and intestine as compared to homeostatic conditions. Since CCR9 is a G protein-coupled receptor (GPCR), besides migration, it also affects several other functions of DCs [39]. We

show that CCR9⁺ DCs show immature phenotype and promote the differentiation of Tregs in vitro as well as in vivo and suppress the gut allergic response, whereas CCR9⁻ DCs expanded inflammatory Th17 cells in the GALT. Immune suppression by CCR9⁺ DCs is mediated by TSLP, implying that CCR9⁺ DCs activate the TSLP pathway for the differentiation of Foxp3⁺ Tregs cells and neutralization of TSLP in culture drives them toward the Th17 pathway.

DSS damages the intestinal epithelial layer and cause acute inflammation, which results in the migration of APCs at the inflamed site [40]. Based on CD11b and CD103 expression, gut DCs are characterized into four groups [17]. Our data show that CD11b⁺CD103⁺, CD11b⁺CD103⁻, and CD11b⁻CD103⁺ infiltrate into the gut during inflammation, and CD11b⁻CD103⁺ DC shows higher capacity to drive Treg differentiation. What is the functional relevance of CCR9 expression by DCs in turns of their migration and tolerogenic function? CCL25 abundantly expressed on intestinal and thymus epithelium and allows the CCR9⁺ immune cell chemotaxis at CCL25 rich sites [41, 42]. We show that CCR9 is expressed on cDCs and preferentially recruits CCR9⁺ DCs in the inflamed gut and GALT, which is in contrast to previous reports that showed that CCR9 was not involved in the migration of cDCs [43]. This observed discrepancy might be due to the overlapping phenotypic marker to distinct DCs population [17]. Previous studies have shown that migratory DCs express different phenotypic markers during maturation and antigen presentation [35, 44, 45]. Surprisingly, we observed that CCR9⁺ DCs have immature phenotype as confirmed by reduced expression of MHC II and CD86, and increased expression of LAP and FasL. These observations postulate our hypothesis that CCR9 activation not only affects migration but also might be associated with regulating cellular functions. Further, how CCR9-CCL25 intrinsic signaling in DCs affects the antigen presentation pathways and the APC function need detailed investigation.

In the present study, we dissected how CCR9 signaling affects the function of DCs or CD4⁺ T cells. Our results suggest that CCR9⁺ DCs present in the mLN and PP induce increased Treg differentiation as compared to CCR9⁻ DCs in the presence of CCL25. We further report that CCR9⁻ DCs modulate the differentiation of the Th17 cells in the presence of CCL25. High expression of CCR9 causes low stimulation of T cell by inhibition of IL-2 production, which govern T cell apoptosis or induce suppressor function in T cell. Interaction of CCR9^{low} DCs with T cell enhances their proliferation and secretion of pro-inflammatory cytokines [46]. In the absence of CCR9 signaling, possibly T cells able to skew immune response toward the inflammatory phenotype. The reason for this is still not clear. The detailed molecular signaling and effect on gene expression in DCs need to be further investigated. The tolerogenic function of DCs and their associated molecules are well studied [47], and DCs mediate their regulatory function by secreting cytokines or by contact-dependent suppression [32]. TGF- β and IL-10 are the most studied molecules that are involved in Treg generation in the gut [32]. Our results showed that Treg induction by CCR9⁺ DCs was not affected by IL-10 and TGF- β in the culture. Apart from these molecules, we also studied TSLP, a molecule known to secreted by DCs, and contribute to the induction of Tregs [29]. TSLP is a major cytokine consecutively expressed by intestinal epithelial cells (ECs) and known to play an important role in the generation of tolerogenic DCs, which in turn promote Treg differentiation and class switching in B cells [48]. TSLP triggers DCs to induce expression of both B-cell activating factor (BAFF) and A proliferation-inducing ligand (APRIL), which in turn promote IgA class-switching in the intestine [49]. TSLP

also inhibits the production of IL-12 by DCs in the intestine. However, in Crohn's disease, the absence of TSLP due to disruption of physiological communication between ECs and DCs failed to control IL-12 production by DCs and promote Th1/Th17-related pathology in the intestine [50].

PP is the most prominent inductive site for IgA⁺ B cell response in the gut-associated lymphoid tissues (GALTs). After entering into PP, B cells upregulate expression of CCR6 and migrate toward (sub-epithelial dome) SED, which expresses its ligand CCL20 and interacts with tolerogenic DCs. Direct interaction of tolerogenic DCs with B cells activates TGF- β production leading to IgA class-switch recombination (CSR) [51]. These plasma cells precursor migrate through the lymph via the thoracic duct into the blood and then into Lamina Propria (LP) by upregulating expression of chemokines specific to intestines such as α 4 β 7, CCR9, and CCR10. Our results showed that DSS treatment increased the frequency of total CCR9⁺ DCs in the PP in an inflammatory condition and also had a tolerogenic phenotype. So, we assumed that CCR9⁺ DCs could interact with B cells in SED and regulate IgA class-switching in PP. Further, these B cell precursor migrates to LP by upregulating CCR9 expression. Nevertheless, we also found that the adoptive transfer of CCR9⁺ DCs significantly lowered the frequency of IgE⁺ B cells in LP. Although our result indicates that CCR9 might have a role in B cell class-switching in allergic conditions, further study needed to reveal the exact role of CCR9⁺ DCs in the intestine during colitis.

In the gut, specifically, CD103⁺ DCs highly express TSLP and promote the differentiation of Foxp3⁺ Treg, while limiting the differentiation of Th17 through direct interaction with the TSLP receptor on CD4⁺ T cells [52]. In DSS-induced colitis models, TSLP expression decreases with increasing expression of IL-23R, and this may help in the downregulation of Foxp3⁺ Treg [52, 53]. We show that inhibition of TSLP marginally impairs the suppressive capacity of CCR9⁺ DCs in comparison to CCR9⁻ DCs. TSLP is produced by tolerogenic subset CD103⁺ DCs, and its expression is attenuated during colitis. Inflammation does not affect TSLP production in the other subset of DCs. TSLP production by all subsets of DCs in the presence or absence of CCL25 needs detailed investigation. In the intestine, DCs subset has a differential ability to modulate T cell response [54]. CD11b⁺CD103⁺ DCs differentiate T cells either into Treg or Th17 cells based on cytokine milieu in the gut microenvironment [18]. Our results suggest that in the gut, there is a subset of DCs that respond to CCL25 and drive the tolerogenic function. Hence, it is possible that CD11b⁻CD103⁺ DCs with the help of CCR9 migrate into PP and induce T cell response by producing TSLP. We also considered that CD11b⁺CD103⁻ DC is also a potent inducer of Treg in the absence of CCL25. After migration in the gut, CD11b⁺CD103⁻ may lose their CCR9 expression and trapped in mLN induce Th1/Th17 immune response. Alternatively, CCR9 expression itself promotes tolerogenic function in a specific subset of DCs through intrinsic signaling. Our findings suggest an unanticipated function of CCR9 expressing CD103⁺ DCs in controlling intestinal homeostasis.

It has been reported that oral administration of OVA induces gut mucosal tolerance to OVA-antigen in mice by affecting both

cellular and humoral immune responses [28]. CTx is a mucosal adjuvant, and along with OVA, it breaks the OVA-specific gut tolerance by increasing the production of inflammatory cytokines and activating B and T cells in the mucosal compartment [28]. Single parenteral boosting with OVA plus CFA elicits low impact systemic immune response with an increase in IgE in the serum. We showed that the adoptive transfer of CCR9⁺ DCs in this OVA-specific gut mucosal allergic model promotes Foxp3⁺ Tregs in the spleen and GALTs and attenuates IgE⁺ B cells in the GALTs and OVA-specific IgE response in serum. Interestingly, CCR9[−] DCs induce Th17 responses as compared to CCR9⁺ DCs in the GALTs. CCR9 is a G protein-coupled receptor and signaling through CCR9 is known to alter the PI3K/AKT-dependent signaling [55]. CCL20 is another chemokine upregulated in intestinal epithelial cells during gut inflammation and autoimmunity [1]. Unlike CCR9, we have previously shown that CCR6-CCL20 signaling in CD4⁺ T cells inhibit the differentiation of Foxp3⁺ Tregs and inhibit its suppressive function and drive the differentiation of Th17 cells [53]. Together, these findings suggest that gut tropic chemokine–chemokine receptor signaling may have a different function in adaptive and innate immune cells during gut inflammation and autoimmunity. It could be possible that CCR9-CCL25 interaction specifically activates these pathways to promote the secretion of anti-inflammatory molecules. However, the role of CCR9 in DCs needs a detailed investigation. Understanding the intrinsic molecular and cellular signaling in the specific cell types will provide new opportunities to control gut inflammation and autoimmunity.

In conclusion, our results suggest that CCR9⁺ DCs contribute to gut tolerance by inducing Tregs and controls the inflammation and suppress the gut allergic response. These results help in unravelling a new field of research in mucosal immunology. Further, understanding the cellular and molecular mechanism of chemokine-induced changes in the DCs will provide essential insight in designing better strategies to control gut inflammation.

Materials and methods

Mice

Six to eight weeks old wild-type C57BL/6 and B6.Cg Foxp3^{tm2tecl} (Foxp3gfp) mice were used. These mice were procured from the Jackson Laboratory (Maine, USA) and bred in the NCCS experimental animal facility. All experimental animal procedures used in the present study were approved by the Institutional Ethics Committee of Animals Usage (Reference ID: EAF/2016/B-279 and EAF/2015/B-257).

Antibodies and other reagents

FITC-anti-mouse CD11c (N418), APC-anti-mouse CD11c (N418), Biotin-anti-mouse CD11c (N418), FITC-anti-mouse CD4 (GK1.5), Alexa Fluor 488-anti-mouse CD4 (GK1.5), Alexa Fluor 647-anti-mouse CD4 (GK1.5), PE-anti-mouse CD8a (53-6.7), Pacific blue-

anti-mouse CD44 (IM7), APC/Cy7-anti-mouse CD44 (IM7), PE-anti-mouse CCR9 (CW-1.2) FITC-anti-mouse CCR9 (CW-1.2), APC-anti-mouse CCR9 (CW-1.2), PerCP/Cy5.5-anti-mouse CD25 (PC61), PE/Cy7- anti-mouse T-bet (4B10), Pacific blue-anti-mouse CD80 (16-10A1), PE/Cy5-anti-mouse CD86 (GL-1), Biotin-anti-mouse CD317 (927), Biotin-anti-mouse FasL (MFL3), PE-anti-mouse LAP (TW7-16B4), FITC-anti-mouse CD103 (2E7), PE- anti-mouse CD103 (2E7), Pacific blue-anti-mouse B220 (RA3-6B2), APC-Cy7-anti-mouse CD19 (6D5), PerCP/Cy5.5-anti-mouse-IgG1 (RMG1-1), APC-anti-mouse IgG2a (RMG2a-62), IgG2b (RMG2b-1), Alexa Fluor 488-anti-mouse GATA3 (16E10A23), Biotin-anti-mouse CD8a (53-6.7), PE/Cy7-anti-mouse IFN- γ (XMG1.2), Biotin-anti-mouse IL-10 (JES5-16E3), Pacific blue-anti-mouse IL-17A (TC11-18H10.1), PE-anti-mouse GM-CSF (MP1-22E9), PerCP/Cy5.5-anti-mouse CD8a (53-6.7), APC-Cy7-Streptavidin, purified recombinant mouse IFN- γ , purified recombinant mouse IL-2, purified recombinant human TGF- β 1, purified recombinant mouse IL-23, purified recombinant mouse IL-6, purified anti-mouse TSLP (28F12), purified rat IgG2a, κ isotype control (RTK2758) purified mouse IgG2b κ isotype control (MG2b-57), and brefeldin-A solution were purchased from BioLegend (San Diego, CA). eFluor 450-anti-mouse CCR9 (CW-1.2), APC-anti-mouse ROR γ t (AFKJS-9), PE-anti-mouse Foxp3 (NRRF-30), Pacific blue-anti-mouse CD317 (eBio927), eFluor 450-anti-mouse MHC II (AF6-1201), purified anti-mouse IL-10 (JES5-16E3), purified anti-mouse IL-4 (11B11), eFluor 450-anti-mouse IgM (eB121-15F9), FITC-anti-mouse IgE (23G3), PE-anti-mouse IgA (mA-6E1), and APC-eFluor 780 anti-Mouse CD4 (GK1.5) were procured from eBioscience (San Diego, CA). PE/Cy7-anti-mouse CD11b (M1/70) and FITC-anti-mouse CD8a (53-6.7) were from BD Bioscience (San Jose, CA). Purified anti-mouse IFN- γ (XMG1.2) and purified anti-mouse CD3 ϵ (145-2C11) were purchased from BioXcell (West Lebanon, NH). Recombinant mouse CCL25 was from R&D Systems (Minneapolis, MN). Dylight 649-strptavidin was from Jackson ImmunoResearch (West Grove, PA).

Induction of acute colitis

The gut inflammation was induced in wild-type C57BL/6 mice by giving 2% dextran sodium sulfate (DSS; w/v, MW 36–50 kDa; MP Biomedicals, Santa Ana, CA) in drinking water [53]. These mice developed colitis within 10 days of DSS treatment. The development and progression of colitis were monitored daily for weight-loss, loose stool, and bloody diarrhea.

Preparation of the single-cell suspension

The intestine was excised, and feces and PP were removed and chopped into small pieces using fine scissors. Tissues pieces were resuspended in 1 $\times$ Hank's balanced salt solution (HBSS)-containing collagenase type IV (300 U, Sigma), DNase I (0.02 mg/mL, Sigma) and EDTA (5 mM), and incubated at 37°C for 30–90 min in a shaking water bath. Single-cell suspensions were

obtained by passing through a 70 μm pore-size cell strainer (BD Biosciences, San Jose, CA). Following the removal of debris and washing with RPMI 1640 medium, the cell suspensions were used for analysis as per the recommended guidelines [56] using flow cytometry (FACS Canto II, BD Bioscience). The single-cell suspensions of the spleen, mLN, and PP were prepared by mechanical disruption and passed through a 70 μm size pore-containing strainers. RBCs were removed by treating with ACK lysis buffer, washed with RPMI medium, stained, and resuspended in RPMI 1640 medium. Cells were used for flow cytometry sorting or analysis of subsets of DCs and CD4⁺ T cells. The purity of sorted cells was >96%.

Immunohistochemical staining

For immunohistochemical staining, tissues were embedded in OCT freezing medium (Sakura Finetek, Torrance, CA), snap-frozen in liquid nitrogen and stored in -80°C till further use. Tissue sections (7 μm thick) were fixed in chilled acetone for 5 min, air dried, and washed with cold PBS. The tissues were blocked with 10% horse serum (Jackson Immuno Research, West Grove, PA) at room temperature (RT) for 30 min. Sections were washed with PBS and incubated with indicated fluorochrome-conjugated primary Abs (1:200 dilution) for 45 min; followed by four times wash with cold PBS, and further incubated with secondary antibodies (1:1000 dilution) for 30 min. Sections were washed five times with PBS, fixed with 1% paraformaldehyde, and mounted in aqueous mounting medium containing DAPI (Electron Microscopy Sciences, Hatfield, PA) and images were acquired using Leica DMI6000 microscope (Leica Microsystems, Germany).

In vitro culture of CD4⁺ T cells

Naive CD4⁺ T cells (CD4⁺CD25[−]CD44[−]) were purified from C57BL/6 or FoxP3-GFP mice spleen using flow cytometry sorting. For isolation of CD11c⁺CCR9⁺ DCs or CD11c⁺CCR9[−] DCs, single-cell suspension from mLN and PP were incubated with biotinylated antibodies for 30 min, followed by incubation with streptavidin-conjugated magnetic beads (BD Biosciences). Cells were purified according to the manufacture's protocol (BD Biosciences). The enriched DCs were used for purifying CD11c⁺CCR9⁺ DCs or CD11c⁺CCR9[−] DCs using a flow cytometry sorter (FACS Aria III, BD Bioscience). The purity of each cell after sorting is >90%. The purified naïve CD4⁺ T cells (5×10^4 cells/well) and DCs (1×10^4 cells/well) were cultured in 200 μL of complete RPMI media in the presence of IL-2 (20 ng/mL), anti-mouse CD3 ϵ (5 $\mu\text{g}/\text{mL}$) in U-bottom 96-well plates at 37°C in a 5% CO_2 incubator for 4 days. Purified recombinant human TGF- β 1 (10 ng/mL) was added to induce differentiation into Treg lineage. Purified recombinant human TGF- β 1 (10 ng/mL), purified recombinant mouse IL-23 (10 ng/mL), and purified recombinant mouse IL-6 (20 ng/mL) were added in culture to induce the differentiation into Th17 lineage. For the activation of CCR9, CCL25 (100 ng/mL) was added to the culture. Cells were harvested and stained with CD4, CD25,

ROR γ t, and Foxp3, and analyzed using flow cytometry (FACS Canto II, BD Bioscience, CA).

Induction of OVA-specific gut allergic response

To study the fate of CCR9⁺ DCs in an in vivo condition, we used OVA-specific gut allergic model. C57BL/6 mice were given OVA (25 mg/mice) and CTx (5 $\mu\text{g}/\text{mice}$) orally in PBS every day for 3 days. Control mice were given the same dose of OVA or PBS alone. For adoptive transfer experiments, CCR9⁺ or CCR9[−] DCs were sorted from mLN and PP from wild-type C57BL/6 mice. On day 7, mice that received OVA and CTx also received either adoptively transferred of purified CCR9⁺ DCs or CCR9[−] DCs (0.5×10^6 cells/mouse) and subcutaneous injection of OVA (100 $\mu\text{g}/\text{mouse}$) in the CFA. After 7 days, mice were euthanized, and spleen, mLN, PP, and LP were harvested, and the immune response was monitored using flow cytometry. Serum was also collected before and after immunization from all the groups and stored in -80°C for ELISA.

Enzyme-linked immunosorbent assay

After 4 days of cell culture, supernatants were collected, and secretion of IL-10 and IL-17 was determined using an ELISA kit from BioLegend. To determine the antigen-specific antibody responses in the mice serum, we monitored the antigen-specific IgE antibody. Briefly, ELISA plates were coated with OVA solution (10 $\mu\text{g}/\text{mL}$) in carbonate buffer (9.5 pH) by incubating at 4°C for overnight. The next day, plates were washed with Tween-20 (0.05%-containing PBS, and wells were blocked with 2% skimmed milk at 37°C for 2 h. After blocking, plates were washed and incubated with serially diluted serum for 2 h. Plates were washed, and biotin-anti-mouse IgE (1:500 dilution) was added, incubated for 1 h, washed, and subsequently incubated with HRP-conjugated anti-rat IgG antibody (1:1500 dilution), and incubated at 37°C for 1 h, washed four times and TMB substrate (BioLegend) was added, and reaction was stopped with 2.5 M H_2SO_4 and absorbance was measured at 450 and 570 nm in ELISA reader (Multiscan GO, Thermo Scientific).

Statistical analysis

All the analyses were carried out either using unpaired Student's *t*-test or one-way ANOVA with Tukey's multiple comparisons test. The *p*-values < 0.05 were considered significant.

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Author contributions: M.P. performed the experiments, analyzed the data, and wrote the manuscript. P.P., N.H., Shilpi, N.K., and S.A.S. helped in some of the experiments including setting up adoptive transfer experiments. G.L. designed the study, interpreted the data, and wrote the manuscript.

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- Abbreviations:** CCR9: chemokine (C-C motif) receptor 9 · DCs: dendritic cells · DSS: dextran sodium sulfate · Foxp3: forkhead box P3 · GALT: gut-associated lymphoid tissues · LP: Lamina propria · mLN: mesenteric lymph nodes · PP: Peyer's patch · RORγt: RAR-related orphan receptor gamma t · Tregs: regulatory CD4+ T cells · TSLP: thymic stromal lymphopoietin
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**CCR9 signaling in dendritic cells drives the differentiation of Foxp3⁺ Tregs and
suppresses
the allergic IgE response in the gut**

Supplementary Figures and Figure legends

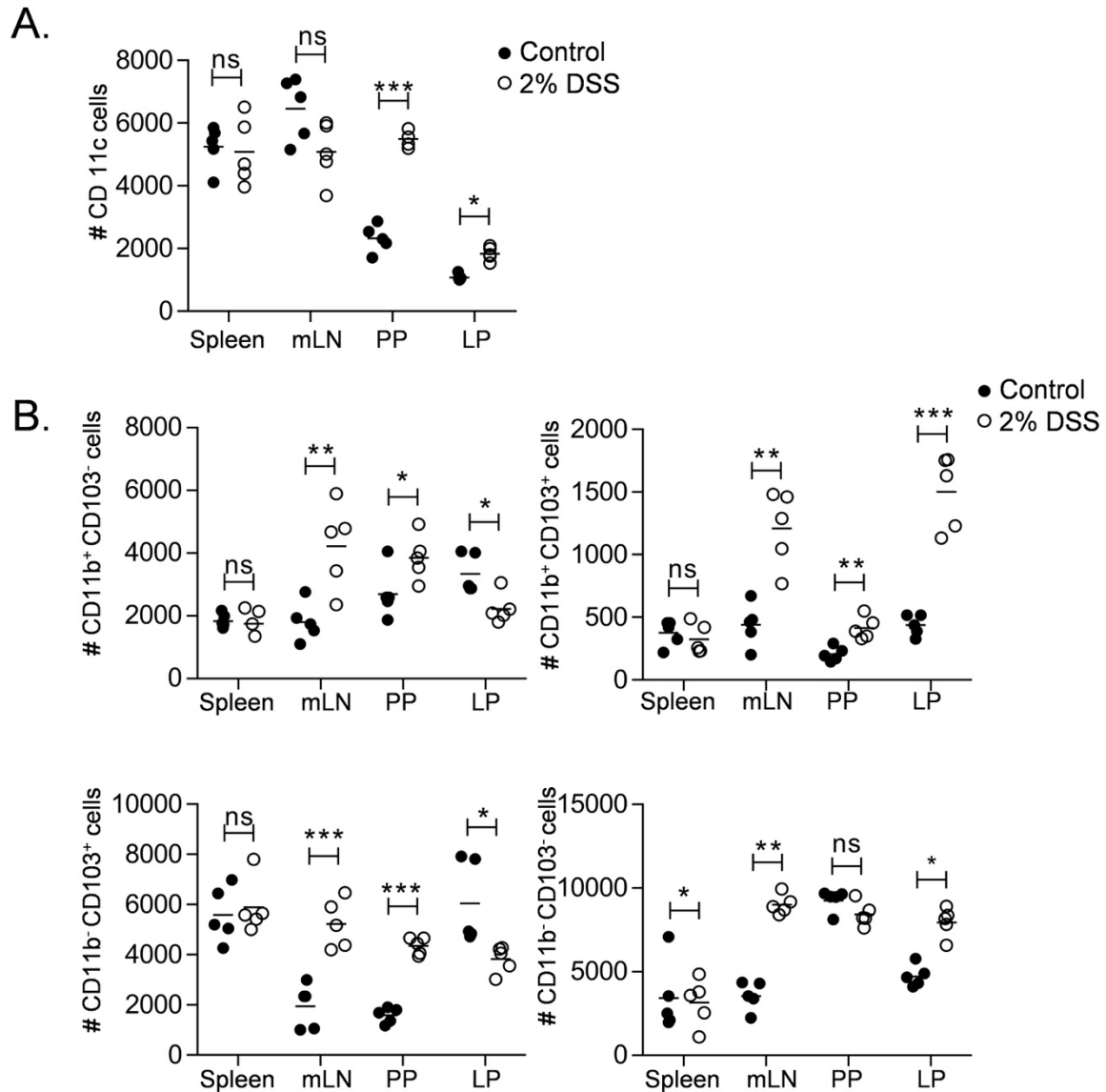


Figure S1

Figure S1: Gut inflammation increases the recruitment of DCs in the secondary lymphoid tissues. C57BL/6 mice were given with or without 2% DSS in drinking water. On day 10, mice were sacrificed, and cells in the secondary lymphoid tissues were analyzed using flow cytometry. **(A)** The absolute number of CD11c⁺ DCs were counted in different lymphoid tissues and plotted. **(B)** The absolute numbers of indicated CD11c⁺ DCs subsets were counted and plotted. Each symbol represents data from individual mice **(A, B)**. Error bars indicate the mean $\pm$ SEM. n = 5 mice/group. *p < 0.05; **P < 0.01; ***P < 0.001; ns, not significant. Data shown are representative of one of the three independent experiments.

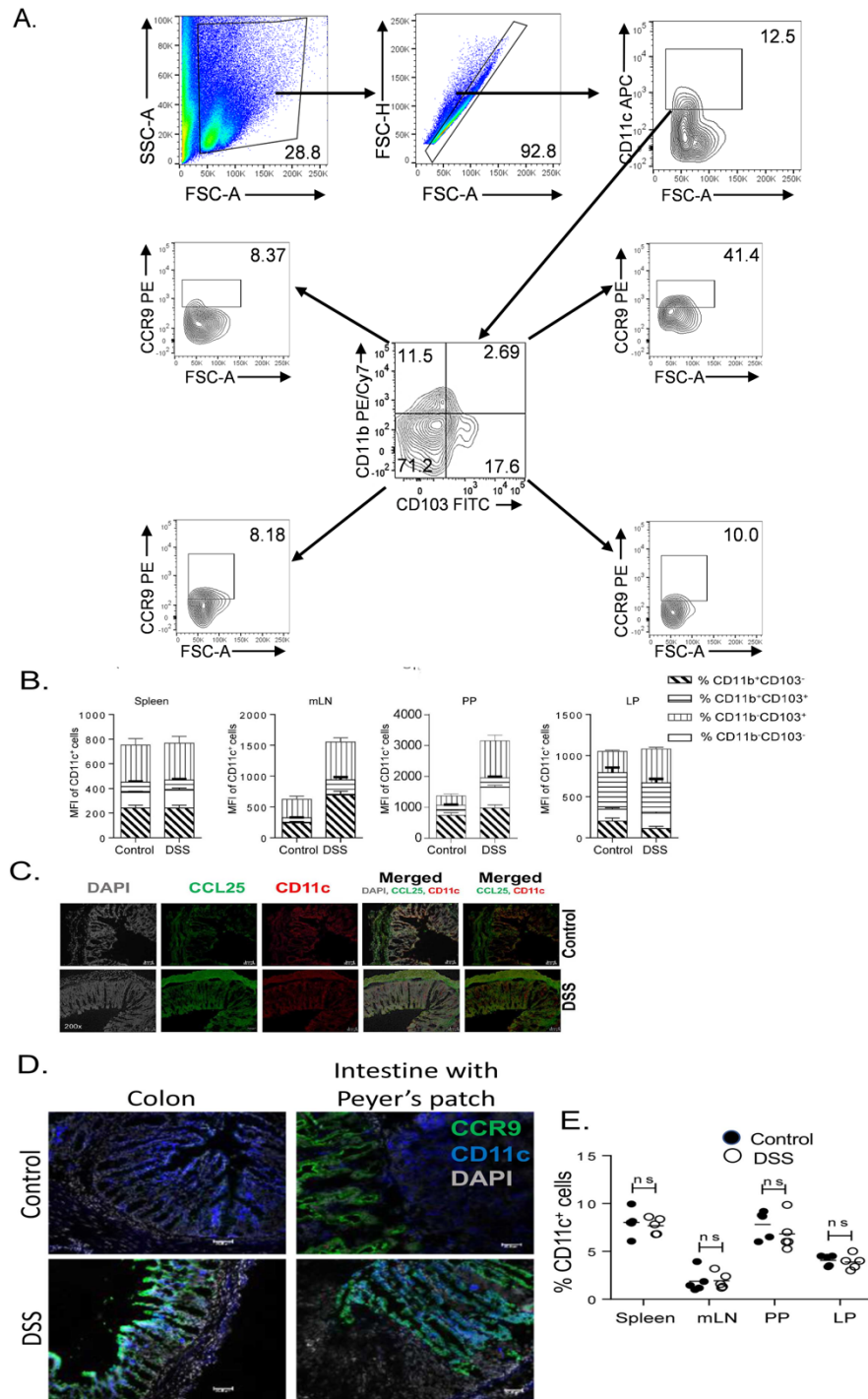


Figure S2

Figure S2: CCR6 and CCR9 expression in DCs during gut inflammation. C57BL/6 mice were given with or without 2% DSS in drinking water. On day 10, mice were sacrificed. CCR9 expression on DCs was analyzed. **(A)** Gating strategy used for monitoring the expression of the chemokine receptor on the various subset of DCs. **(B)** The expression of CCR9 on various

subsets of CD11c⁺ DC was analyzed. The mean fluorescence intensity (MFI) of CCR9 on indicated DC subsets were calculated and plotted. Error bars represent mean $\pm$ SEM. n = 5 mice/group. **(C)** Colon was harvested and analyzed using immunofluorescence staining. The representative images stained with CCL25 (green), CD11c (red), and nuclear stain DAPI (grey) are shown. Original magnification 200X. **(D)** Colon and intestine with Peyer's patch were harvested and analyzed using immunohistological staining. The representative images stained with CCR9 (green), CD11c (blue), and nuclear stain DAPI (grey) are shown. Original magnification 200X. **(E)** The frequency of CCR6⁺ cells gated on CD11c⁺CCR9⁺ cells was measured and plotted. Each symbol represents data from individual mice. n = 5 mice/group. ns, not significant. Data shown are representative of one of the three independent experiments **(B, E)**.

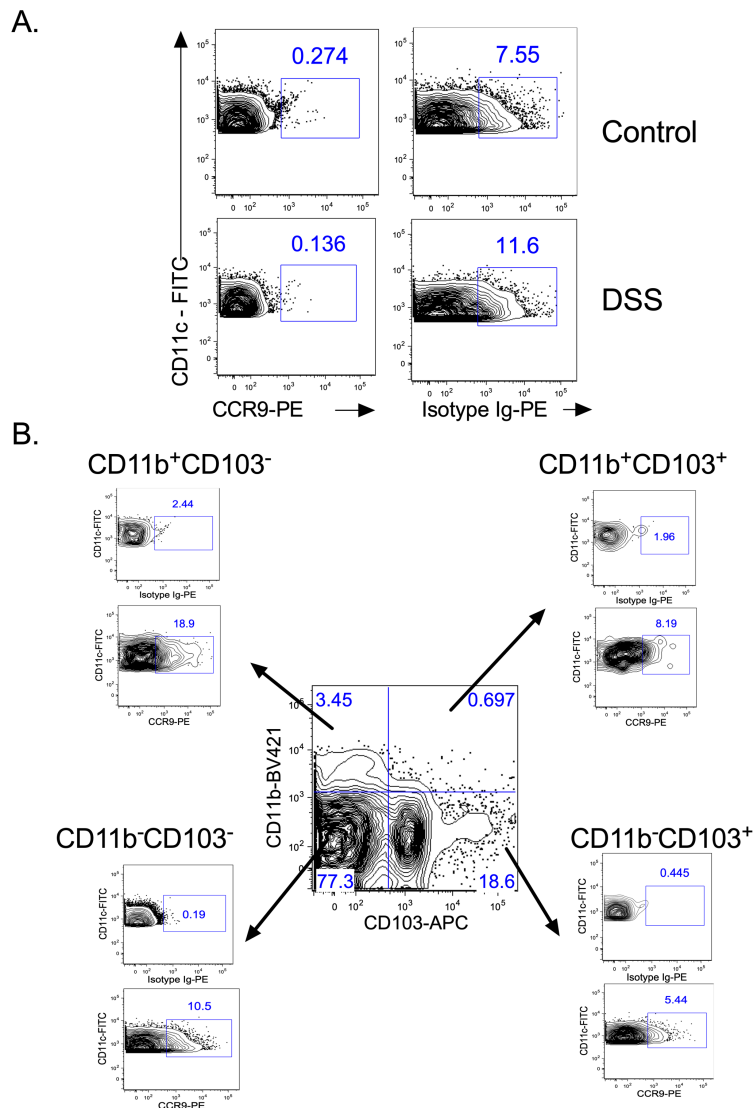
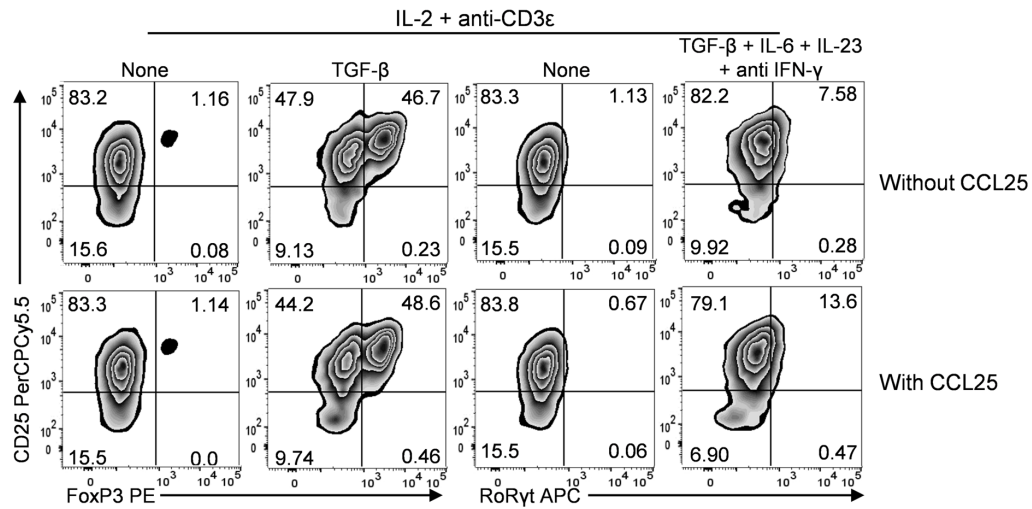


Figure S3

Figure S3: Specific staining of CCR9 on the various subset of DCs. C57BL/6 mice were given with or without 2% DSS in drinking water. On day 10, mice were sacrificed. CCR9 expression on DCs was analyzed in Peyer's patch. **(A)** Cells were first gated on lymphocytes (FSC-A Vs. SSC-A), followed by singlet cells (FSC-A Vs. SSC-A gate). **(A)** Singlet cells were gated on CD11c⁺ cells and plotted. Isotype control Ig staining (left) and CCR9 staining (right) are shown between control and DSS treated mice. **(B)** Gating strategy used for monitoring the expression of the CCR9 on the various subset of DCs. CD11c⁺ cells were gated as in A, and plotted as CD11b and CD103 (center). Isotype antibody (top) and CCR9 antibody (bottom) staining of each subset of DCs are shown.

A.



B.

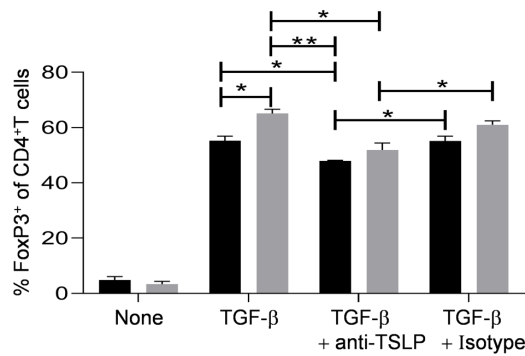


Figure S4: CCR9⁺ DCs do not affect the differentiation of Foxp3⁺ Treg but promotes Th17 cell differentiation. (A) Naïve CD4⁺ T cells (CD4⁺CD25⁺CD44⁺ cells) were cultured with purified CD11c⁺CCR9⁺ DCs in presence or absence of CCL25 under Treg or Th17 differentiation conditions for 4 days. Expression of Foxp3 and RORγt in CD4⁺ T cells were analyzed, and data shown are gated on CD4⁺ T cells. Data shown are representative of one of the two independent experiments. The numbers in the plots show the percentage of cells in the marked quadrants. **(B)** During Treg cells differentiation culture, TSLP was blocked using anti-TSLP mAb or added only isotype control mAb in culture. The expression of Foxp3 was monitored after gating on CD4⁺ T cells using flow cytometry. Data shown are mean of two independent experiments, and error bars represent mean ± SEM. *P < 0.05, **P < 0.01.

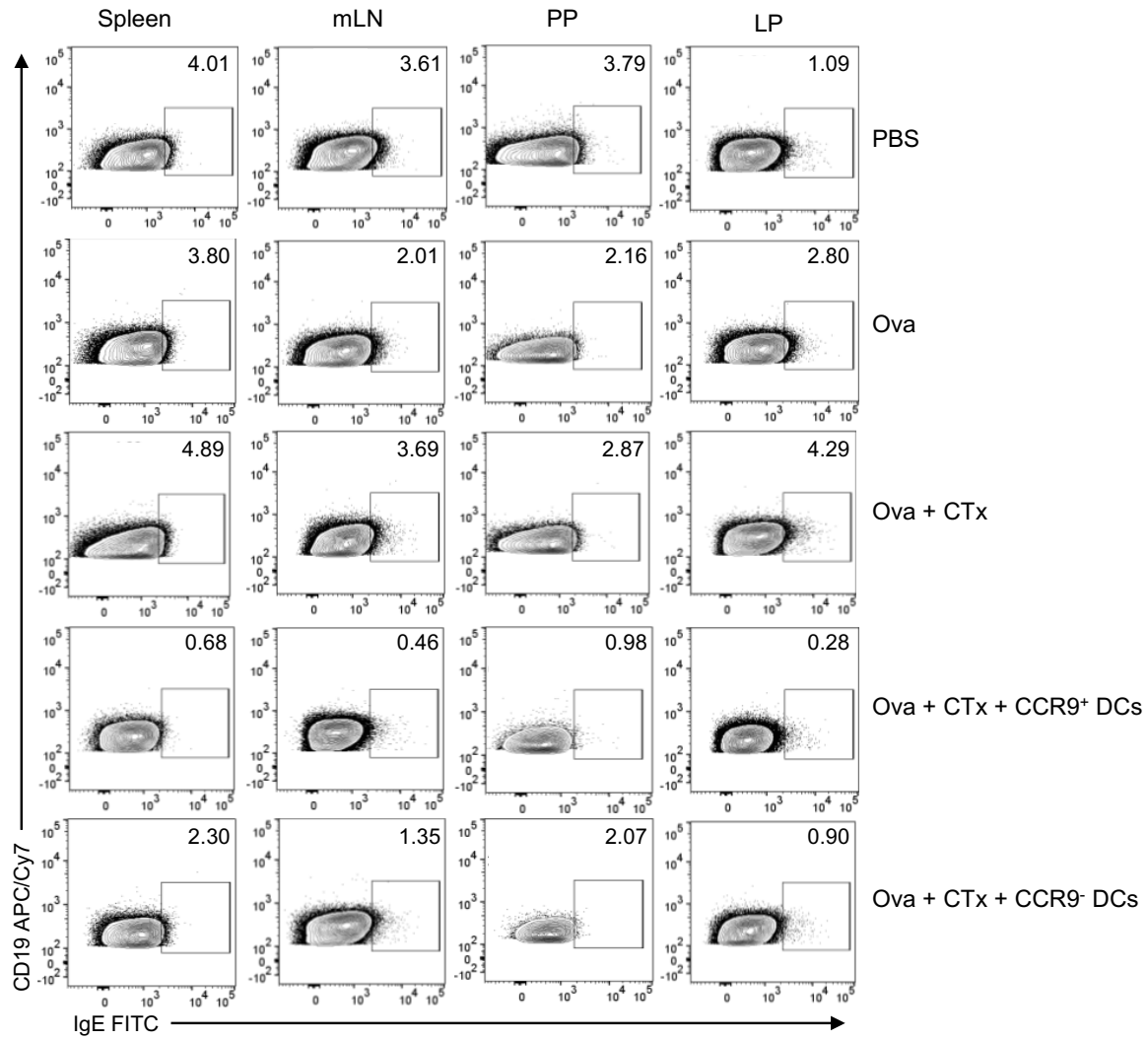


Figure S5: CCR9⁺ DCs suppress CD19⁺ IgE response in the mucosal compartment. C57BL/6 mice were orally given PBS, ova, or ova plus cholera toxin (Ova plus Ctx) for three constitutive days. On day 7, Ova plus CTx group was divided into three groups, and adoptively transferred purified CD11c⁺CCR9⁺ DCs, CD11c⁺CCR9⁻ DCs, or sham transferred. These mice were also given a subcutaneous injection of ova in CFA at the time of DC injection. After seven days, animals were sacrificed, and CD19⁺ IgE cells were monitored. Cells in the contour plots are gated on CD19⁺ B cells. The numbers in the contour plot show the percentage of CD19⁺ IgE cells in the gated region.