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SAMP1/YitFc Mice Develop Ileitis via Loss of CCL21 and Defects in Dendritic Cell Migration

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Abstract

Background & Aims—The lymphatic chemokine CCL21 is required for dendritic cell (DC) migration from tissues to lymph nodes, which helps establish tolerance to foreign yet harmless antigens. We demonstrate that CCL21 is almost completely absent from SAMP1/YitFc (SAMP) mice, which spontaneously develop chronic ileitis that resembles Crohn's disease, and that DC migration is severely impaired in these mice compared with AKR mice (controls). Toll-like receptor (TLR) agonists like the TLR7 agonist R848 induce DC maturation and mobilization.

Methods—We collected intestinal and other tissues and mesenteric lymph nodes (MLN) from SAMP mice. Expression of CCL21 was measured by quantitative PCR and immunofluorescence analyses; spontaneous and induced migration of DCs were assessed by flow cytometry. We analyzed production of retinoic acid by DCs and their ability to induce development of T-regulatory (Treg) cells. Mice were fed R848 to determine its effects on migration of DCs and development of ileitis in SAMP mice.

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Author Contributions:
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Results—SAMP mice expressed almost no CCL21 in any tissue tested. Their $CD11b^{+}CD103^{+}$ DCs were defective in migration from the ileal lamina propria to the MLN. DCs from SAMP mice also had a greatly reduced ability to produce retinoic acid and induce development of Treg cells, compared with control mice. Young SAMP mice had reduced CCL21 expression and decreased DC migration before developing ileitis. Administration of R848 to these mice increased migration of DC to the MLN and development of Treg cells there, and reduced the severity of ileitis.

Conclusions—Loss of CCL21 signaling and DC migration is required for development of ileitis in SAMP mice. Reagents such as R848, which activate DC migration to the MLN, might be developed as treatments for patients with Crohn's disease.

Keywords

immune regulation; oral tolerance; CD; small intestine

Introduction

Crohn's disease is thought to be caused by an abnormal immune response to commensal bacteria in a genetically susceptible host[1]. Although the adaptive immune system plays an important role, defects in the macrophage and dendritic cell (DC) compartment have been reported, and a possible pathogenic role is supported by clinical data[2]. Migratory DCs are crucial in establishing tolerance towards foreign yet harmless antigens[3]. Crohn's patients are unable to establish tolerance to orally delivered experimental antigens[4], consistent with a migratory DC defect.

SAMP1/YitFc (SAMP) mice develop spontaneous chronic ileitis that resembles Crohn's disease. This mouse strain was derived from AKR mice[5], which do not develop intestinal inflammation. The most severe inflammatory changes in SAMP mice appear in the terminal ileum. Inflammation is transmural and discontinuous, with presence of normal areas of gut mucosa alternating with inflamed regions[6] and shares many properties with Crohn's disease including alterations in epithelial morphology, granulomas, crypt hyperplasia, infiltration of both acute and chronic inflammatory cells, spontaneous skin lesions and in some instances perirectal fistulas[6, 7].

Both Th1 and Th2 adaptive immune responses are detectable during the course of the disease[8]. Ileitis in SAMP mice improves upon administration of corticosteroids[9] or TNF blockade[6], treatments that are also effective in Crohn's patients[1].

Several genetic defects have been described but the ultimate cause of ileitis in SAMP mice is unknown[5, 10]. Bone marrow transfer experiments have shown that the primary disease-causing defect resides in the non-hematopoietic compartment of SAMP mice[11]. Increased permeability of intestinal epithelial cells has been demonstrated in SAMP mice[11, 12] along with defects in Treg function[13] and abnormal NOD2 responses[14].

Several subsets of DCs are constitutively found in the lamina propria (LP) of the terminal ileum. LP DCs are $CD64^{-}MHCII^{+}CX3CR1^{-}CD11c^{+}CD103^{+}$ or $CD103^{-}$ cells that can be further subdivided based on the expression of CD11b[15]. Migratory DCs (mDCs) that include

MHCII$^+$CD11c$^+$CD103$^+$CD11b$^+$ cells have been implicated in mounting immune responses towards pathogens. Migration of DCs from the LP to the MLN is also crucial in establishing oral tolerance to harmless food and commensal antigens[16]. Tolerogenic properties of CD103$^+$ DCs depend on the production of TGF-β and retinoic acid (RA). RA is involved in B cell isotype switching to IgA, in Treg generation in lymph nodes[16–19] and in imprinting Tregs with gut-homing receptors[20, 21].

Once mDCs acquire antigens and maturation signals in the LP, they migrate to MLN[22–25]. This requires the expression of the chemokine receptor CCR7 on DCs and its ligand CCL21 in the afferent lymphatics[16, 26]. CCL21 has a highly charged C-terminus that facilitates binding to glycosaminoglycans displayed on the surface of lymphatic endothelial cells[27]. Mice express at least two isoforms of CCL21[28]: CCL21-Ser which is expressed in lymphatic organs, including lymph nodes and spleen and CCL21-Leu which is expressed by lymphatic endothelial cells in peripheral organs[29]. In the terminal lymphatic vessels of mouse skin, CCL21 is organized in discrete deposits that form portals through which DC crawl into the lymphatic system[30]. Away from the lymphatic vessels CCL21 is arranged in a gradient that directs mDCs to terminal lymphatics[31]. CCL21 is produced by lymphatic endothelial cells, high endothelial venules and fibroblastic reticular cells in lymph nodes[32].

In preliminary experiments, we saw that CD11c$^+$MHCII^{hi}CD103$^+$CD11b$^+$ DCs were missing from SAMP MLN. We therefore hypothesized that a potential defect in DC migration from LP to MLN may contribute to ileitis in SAMP mice by impairing tolerogenic responses. We discovered that CCL21 is absent from SAMP lymphatic vessels. Overcoming the DC migration defect significantly diminished ileitis in SAMP mice, suggesting that defective CCL21 expression is pathogenic. Manipulating DC trafficking may open new avenues for treatment of Crohn's disease.

Results

Reduced numbers of mDCs in SAMP MLN

In the MLN, DCs account for less than one percent of all cells. To study DCs, we performed flow cytometry analysis of MLN and gated out B cells (by CD19), αβ T cells (by TCRβ) and granulocytes (by side scatter, Figure 1A). CD11c$^+$ DCs in the MLN form two distinct populations[33], migratory MHCII^{hi} and resident MHCII^{int} DCs (Figure 1B). MHCII^{hi} cell populations can be further subdivided by expression of CD11b and CD103 ([22], Figure 1C). We found the MHCII^{hi} population reduced in SAMP mice (Figure 1, B and D), with the most severe reduction by more than 75% in CD11b$^+$CD103$^+$ mDCs (Figure 1E). CD103$^+$CD11b$^-$ cells were reduced by about half, and CD103$^-$ cells were reduced by about 30% (Figure 1E). Reduction in the frequency of DCs was not due to a mere expansion of other leukocyte subsets, as total numbers of CD103$^+$CD11b$^+$ cells was reduced (Figure 5B). We conclude that mDCs are diminished in MLN of SAMP mice.

Defective egress of CD11b$^+$CD103$^+$ DCs from the SAMP terminal ileum

The relative decrease of CD11b$^+$CD103$^+$ cells in the MLN could result from a developmental or a migration defect. In the former case, CD11b$^+$CD103$^+$ DCs would be

expected to also be reduced in the small intestinal LP, whereas in the latter case, these cells would accumulate in the LP. We analyzed DCs in the LP of young (5–7 week old) SAMP and AKR control mice (Figure 2) by gating on live $CD11c^+CD103^+$ cells (Figure 2A). In SAMP mice, there was a significant accumulation of $CD11b^+CD103^+$ DCs in the LP (Figure 2, B and C), suggesting that these mDCs were retained in the LP of SAMP mice. To facilitate immunostaining, we took advantage of the fact that CD103 pairs with β_7 to form $\alpha_E\beta_7$ integrin (Figure 2D). Immunofluorescence analysis of the LP of the small intestine showed accumulation of $CD11b^+\beta7$ $integrin^+$ DCs in SAMP mice (Figure 2E). The collecting lymphatic vessels labeled with Lyve1 (Lymphatic Vessel Endothelial Receptor 1) were much wider in adult SAMP mice (Figure 2E) but had normal diameter in 10 days old mice (data not shown).

Lack of CCR7 chemokine ligands in SAMP mice

DC egress to the MLN from the LP is CCR7-dependent[24, 27]. Flow cytometry analysis of MLN confirmed that $CD11c^+MHCII^{high}$ DCs are greatly reduced in MLN of CCR7-deficient mice (Supplementary Figure 1). The defect was mostly due to a sharp reduction in $MHCII^{hi}$ DCs and an almost complete absence of $MHCII^{hi}$ $CD11b^+CD103^+$ DCs. Since the migration defect affected the same cell type that failed to accumulate in the MLN of SAMP mice, we hypothesized that CCR7 expression in SAMP mice may be defective. However, we did not find any difference in CCR7 levels between SAMP and AKR DCs (Supplementary Figure 3B). Next, we tested whether the defect was in the expression of the CCR7 ligands, CCL19 and CCL21, in peripheral and lymphatic organs. In the small intestine of neonatal (before ileitis onset) and terminal ileum of adult SAMP mice, CCL21 mRNA expression was sharply reduced (Figure 3A) and CCL19 expression was reduced 4–8 fold when compared to AKR mice (Figure 3B). To test whether the chemokine expression defect was limited to the ileum, we measured CCL21 and CCL19 mRNA in skin, mesenteric and peripheral lymph nodes and found that expression of both chemokines was also defective there, suggesting that both CCL21 isoforms are depleted in SAMP mice (Figure 3A,B and data not shown). Abnormal expression of CCL19 and CCL21 mRNA was also seen in colonic biopsies from ulcerative colitis and Crohn's disease patients (Supplementary Figure 4), corroborating previous reports[41,43]. Confocal immunofluorescence staining of intestinal whole mounts showed abundant CCL21 immunoreactivity (green) along initial lymphatic vessels in intestinal villi (labeled with Lyve1 in magenta, Figure 3C) of AKR control mice. In the lymph nodes of AKR mice CCL21 was concentrated in $Lyve1^-$ cells as well as present in the form of diffuse pool. In both analyzed tissues, CCL21 was only sporadically detectable by microscopy in SAMP mice (Figure 3C,D). CCR7 ligands also play an important role in homing of naïve T cells to secondary lymphoid organs[27], and adoptively transferred T cells homed less efficiently to lymph nodes of SAMP mice (Supplementary Figure 2 and Supplementary Video 1). Taken together, these findings suggest that defective DC homing from the LP to the MLN of SAMP mice is due to defective expression of CCL21 in lymphatics or MLN.

Defective functions of SAMP MLN DCs

Induced regulatory CD4 T cells (iTreg), which express the transcription factor Foxp3[34] are found in intestinal tissue and contribute to controlling inflammation[35]. In intestinal tissues,

iTregs are induced by so-called tolerogenic DCs, which express TGFβ and RALDH activity[36]. Physiologically, RALDH enzymes convert retinal to RA, which is known to promote iTreg differentiation[35, 37]. RALDH activity can be monitored in single cells by the fluorogenic substrate Aldefluor[38]. More than 60% of MHCII^{high} MLN DCs isolated from control AKR mice express Aldefluor activity, contrasting with less than 30% of MHCII^{high} SAMP MLN DCs (Figure 4A). Also, the amount of enzymatic activity per cell as detected by median fluorescence intensity (MFI) was much lower in SAMP than in AKR MHCII^{hi} DCs (Figure 4B). To test whether this defective RALDH expression resulted in a reduced ability to induce iTregs *in vitro*, we incubated naïve splenic AKR CD4^{+}CD45Rb^{high}CD25^{-} T cells with CD11c^{+} DCs isolated from MLNs of AKR or SAMP mice in the presence of anti-CD3 and TGFβ. While AKR DCs induced 18±2% Foxp3-expressing iTregs, SAMP DCs induced only 11±0.4% (Figure 4C, D).

Next, we tested how defective DC migration and diminished DC RALDH expression correlated with disease activity (ileitis). The terminal ileum of AKR and SAMP mice was harvested at 4–5 (before ileitis onset), 6–7 or 10–11 weeks of age, fixed, embedded and analyzed by scoring H&E stained slides as described[9]. The number of CD11c^{+}MHCII^{high} DCs in the MLN decreased over time, but the reduction was significantly more pronounced in SAMP than in AKR mice (Figure 5A). The absolute number of MLN CD103^{+}CD11b^{+} DCs also decreased more in SAMP than in AKR mice, reaching statistical significance at 6–7 weeks (Figure 5B). This DC defect coincided with the appearance of inflammation as determined by lymphocyte and neutrophil infiltration (Figure 5C and D).

TLR7 agonist R848 can overcome DC recruitment defect in SAMP mice

Attempts to directly correct the defective CCL21 expression in lymphatics of SAMP mice were not successful (data not shown). Since Toll-like receptor (TLR) agonists are known inducers of DC maturation and mobilize DCs *in vivo*[39], we tested if AKR and SAMP DC can be mobilized from LP to MLN, by the TLR7 agonist R848[23, 25]. Eighteen hours after oral gavage, R848 augmented the percentage of MHCII^{high} MLN DCs in AKR mice 3–4 fold (Figure 6A), due to an increase in CD103^{+}CD11b^{-} and CD103^{+}CD11b^{+}, but not the CD103^{-}CD11b^{+} subsets (Figure 6B and D). In SAMP mice, the frequency of DCs was sharply lower than in AKR mice, but the R848 gavage induced a similar 3–4 fold increase (Figure 6A–D). R848 treatment restored all MHCII^{high} subsets to levels similar to untreated AKR mice (Figure 6C, D). RALDH activity in CD11c^{+}MHCII^{hi} cells was also increased after R848 treatment (Figure 6E).

Encouraged by these results, we tested the clinical effect of 7-day treatment with R848 (0.5 μg/g, by oral gavage daily) on ileitis in 30 weeks old SAMP mice (4–6 mice/group). As a positive control we treated a group of SAMP mice with dexamethasone (Dex), which is known to reduce inflammation in SAMP mice[9]. Treatment with R848 resulted in an improvement in villus architecture, decreased infiltration of inflammatory cells and reduction in thickening of the gut wall (Figure 7A and B). Interestingly, R848 treatment was as effective as the positive control dexamethasone, bringing the inflammatory score down by more than half (Figure 7B). Unlike corticosteroid treatment, R848 induced a significant

increase in the MHCII^{hi} DCs (Figure 7C, D) and a small but statistically significant increase in CD4^{+}Foxp3^{+} cells in the MLN (Figure 7E).

Taken together, our data show that SAMP mice have a defect in trafficking of DCs from LP to MLN due to sharply reduced CCL21 expression. The remaining MLN DCs isolated from SAMP mice are defective in RALDH activity and iTregs induction, suggesting that the migration defect affects tolerogenic DCs. Since the defect in trafficking of tolerogenic DCs precedes disease onset, this suggests that DCs play a major role in the SAMP model of ileitis. Consistent with this, promoting LP DCs migration into the MLN by administration of an oral TLR7 agonist dramatically reduced disease scores.

Discussion

Our data show that homeostatic DC trafficking is dramatically disturbed in the small intestine of SAMP mice. The loss of mDCs in MLN results from aberrant migration of MHCII^{hi}CD103^{+}CD11b^{+} DCs from the LP to the MLN due to defective CCL21 expression in lymphatics. Because mDCs are missing in the MLN of SAMP mice, they cannot effectively induce Tregs. Increasing DC trafficking by treatment with the orally available TLR7 ligand R848 improved DC migration, RA production and enhanced induction of Foxp3^{+} cells. This data suggests that therapies aimed at improved DC trafficking might be useful in patients with Crohn's disease.

The inflamed mucosa in Crohn's patients and SAMP mice harbors increased numbers of DCs[40, 41]. Many of these cells express CD83 and CCR7, suggesting that they are mature DCs[41]. Normally, these cells would be expected to migrate to the MLN, but in Crohn's patients they appear to be retained in the LP. This is similar to the phenotype we found in the SAMP model. In patients, increased retention of CCR7^{+} DCs in the inflamed LP is thought to be mediated by excessive production of CCL19 by DCs and CCL21 by fibroblastic reticular cells and lymphatic endothelial cells[41]. This can be reproduced in animal tissues, where providing exogenous CCL21 also resulted in DCs stranded in the parenchyma[31]. Transgenic overexpression of CCL21 under the H-2K^{b} promoter caused a decrease in DC migration to the draining lymph node in a *Leishmania major* infection model[42]. In healthy tissue CCL21 forms an orderly gradient which guides DCs into the terminal lymphatics[31]. Overexpression masks this gradient and prevents mature DCs from migrating toward lymphatics and leaving the inflamed LP. Dysregulation of CCL21 and CCL19 production in the ileum and MLN was also found in another mouse model of chronic ileitis (TNFΔARE)[43]. Aberrant expression of CCR7 ligands impairs leukocyte trafficking leading to accumulation of T cells in inflamed gut[43]. The same group previously identified an impaired balance between CD103^{+} and CD103^{-} dendritic cells in the MLN of TNFΔARE[44]. In the SAMP model, the CCL21 gradient is absent, which has the same functional consequence. Taken together, our and previously published data strongly suggest that chemokine defects are tightly linked to two relevant murine models of chronic small intestinal inflammation. The accumulation of DCs in the LP of SAMP mice is remarkably similar to that found in Crohn's patients and emphasizes the relevance of this mouse model. Our data suggest that the DC migration defect depends on the reduced expression of CCL21, as this chemokine is sufficient in guiding DC trafficking in CCL19 knockout mice[26].

Histopathological evaluations of intestinal material obtained from Crohn's patients before the wide-spread use of disease-modifying drugs showed dilation of lymphatic terminals, submucosal edema, lymphocytic thrombi within lymphatics, and aggregates of lymphocytes often containing granulomas[45, 46]. Here we report dilated lymphatics and edema in the SAMP model, underlining the critical involvement of the lymphatic system in both Crohn's disease and SAMP ileitis.

Gut CD103$^+$ mDCs have the unique potential to drive conversion of naïve CD4 T cells into iTregs in a process that depends on TGF-β and is potentiated by RA[34]. Our results show that defects in CD103$^+$ migratory SAMP DCs and significant loss of RA-producing DCs in the MLN translates into less efficient conversion of naïve CD4 T cells into Foxp3$^+$ Tregs. Recent studies showed that though mutually redundant[47, 48], CD103$^+$CD11b$^-$ and CD103$^+$CD11b$^+$ cells are jointly required for Treg homeostasis[49]. Data from our *in vivo* rescue experiments demonstrate that R848-induced mobilization of intestinal DCs in SAMP mice increased the frequency of Foxp3$^+$ cells in the MLN. Extrathymically generated iTregs are critically involved in controlling mucosal inflammation[35]. A recent study suggested that dysfunctional Tregs may play an important role in SAMP ileitis[13]. In addition, we found that SAMP DCs were less efficient at inducing iTregs.

Loss of CD103$^+$CD11b$^+$ mediated by targeted DC-specific deletion of Notch2[47, 50] or the transcription factor IRF4[51] does not predisposes mice to spontaneous ileitis, highlighting the redundancy of regulatory mechanisms. Similarly, Crohn's requires a combination of multiple genetic and environmental factors[1]. It is likely that SAMP mice combine several defects that culminate in the ileitis phenotype. Disrupted mucosal barrier integrity in SAMP mice[11] might be particularly important as a mechanism which allows for increased translocation of food and bacterial antigens. This in conjunction with deficits in tolerogenic DCs and aberrant naïve T cells homing might promote ileitis. Because in SAMP mice both CCL21 and CCL19 are defective from birth, it is very likely that their absence impacts the development of mucosal immunity. Our finding that DC mobilization improves ileitis suggests that manipulation of dendritic cell trafficking might provide novel therapeutic avenues for patients affected by Crohn's disease.

Materials and Methods

Mice and treatments

SAMP1/YitFc mice (SAMP), a substrain of the SAMP1/Yit line developed at Yakult Central Institute for Microbiological Research (Tokyo, Japan), B6.129P2(C)-*Ccr7*^{tm1Rfor}/J (CCR7-deficient), C57BL/6-*Itgb7*^{tm1Cgn}/J (integrin β_7-deficient), C57BL/6J and AKR/J mice were obtained from established colonies at the La Jolla Institute for Allergy and Immunology (La Jolla, CA). Mice were treated with 0.5 mg/kg of R848 (Invivogen, San Diego, CA) by oral gavage. Control mice received sterile water. A group of mice received 100 μg dexamethasone (Butler Schein, Dublin, OH) intraperitoneally every second day. All animals were housed in a specific pathogen–free facility, and all experiments were approved by the institutional committee for animal use

Sample preparation

Mice were euthanized by CO_2 inhalation at times required by the experimental design. Human colonic material was harvested from patients undergoing flexible sigmoidoscopy or colonoscopy for diagnostic or surveillance purposes. All studies were approved by the Internal Review Board of Case Medical Center. Detailed methods for immunohistochemical and immunofluorescence analysis, quantitative polymerase chain reaction, cell isolation, flow cytometry and cell sorting, cell cultures and short term homing are available in Supplementary Methods.

Statistical analysis

Results are expressed as mean ± SEM unless indicated otherwise. Data were analyzed using GraphPad Prism 6 (GraphPad Software, La Jolla, CA). Differences between individual groups were assessed using two-sided unpaired Student t test. Multiple groups were compared with control group using a one-way analysis of variance followed by Dunnett's multiple comparisons test. $P \leq 0.05$ was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

DC	dendritic cell
SAMP	SAMP1/YitFc
Tregs	regulatory T cells
MLN	mesenteric lymph nodes
LP	lamina propria
mDC	migratory DC
RA	retinoic acid
MFI	median fluorescence intensity
iTreg	induced regulatory T cells
TLR	Toll-like receptor

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Author names in bold designate shared co-first authorship.

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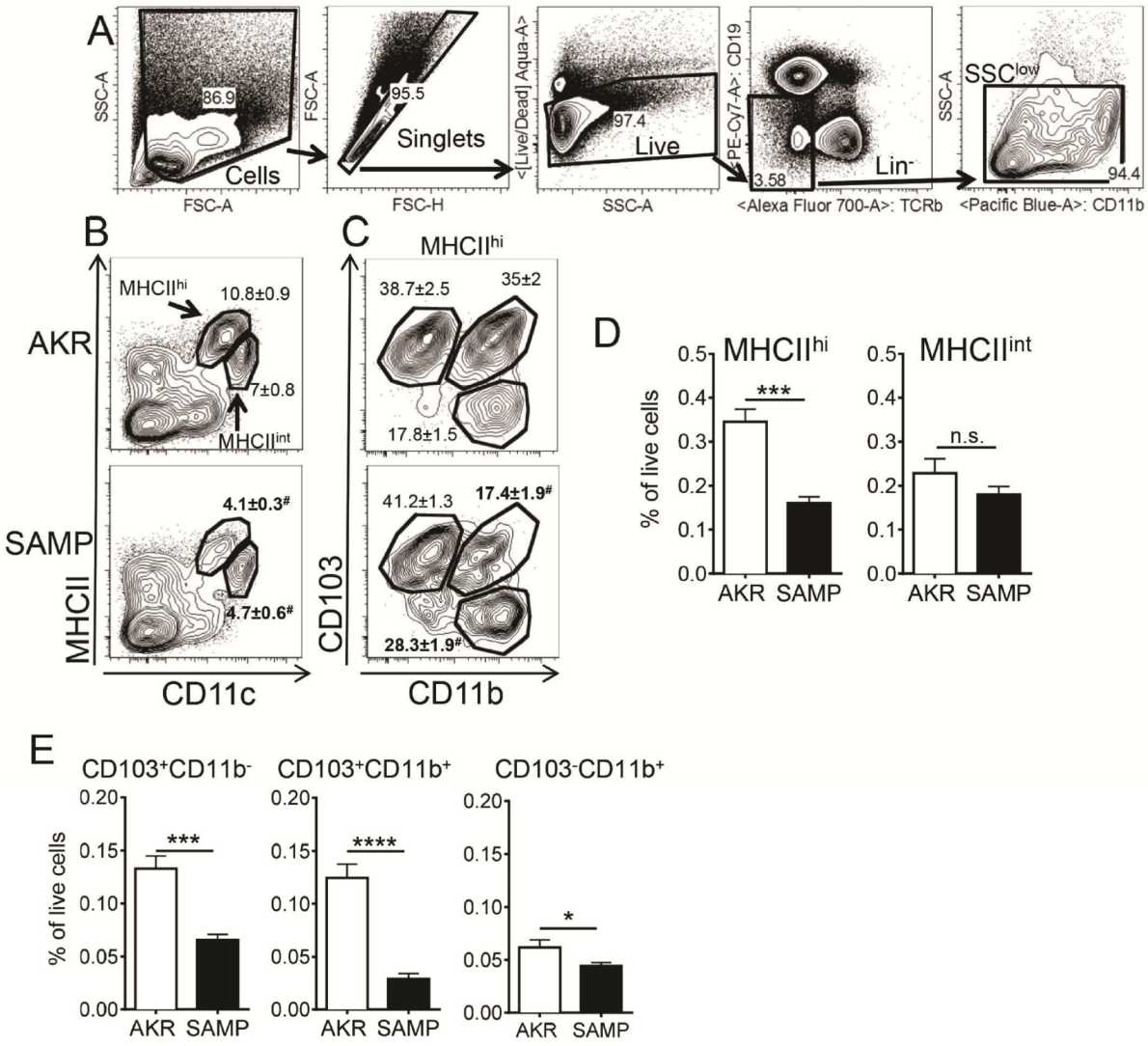

Figure 1. Defective MLN DC in SAMP mice

(A) Single cell suspensions were prepared by incubating minced tissues in collagenase and DNAse I. Sequential gates were set to exclude debris, doublets, dead cells, cells of B and T cell lineage and granulocytes (SSC^{hi}). (B) Representative flow cytometry analysis of DC in AKR and SAMP MLN from 10–20 weeks old mice. Two populations of $CD11c^{+}MHCII^{hi}$ and $CD11c^{hi}MHCII^{int}$ MLN DCs were detected. (C) $MHCII^{hi}$ cells contained 3 major subpopulations: $CD103^{+}CD11b^{+}$, $CD103^{+}CD11b^{-}$ and $CD103^{-}CD11b^{-}$ cells. Mean of percentages of parent gate ± SEM are indicated on the plots, # denotes statistically significant differences between AKR and SAMP mice. (D) The fraction of $MHCII^{hi}$ and $MHCII^{int}$ cells and (E) subpopulations of $MHCII^{hi}CD11c^{+}$ cells was calculated as % of all live cells. Results are the mean ± SEM of 3 experiments with n=5 in AKR and n=6 in SAMP groups. n.s – not significant, **** $p<0.0001$, *** $p<0.001$, ** $p<0.01$, * $p<0.05$ by two-sided Student's t test

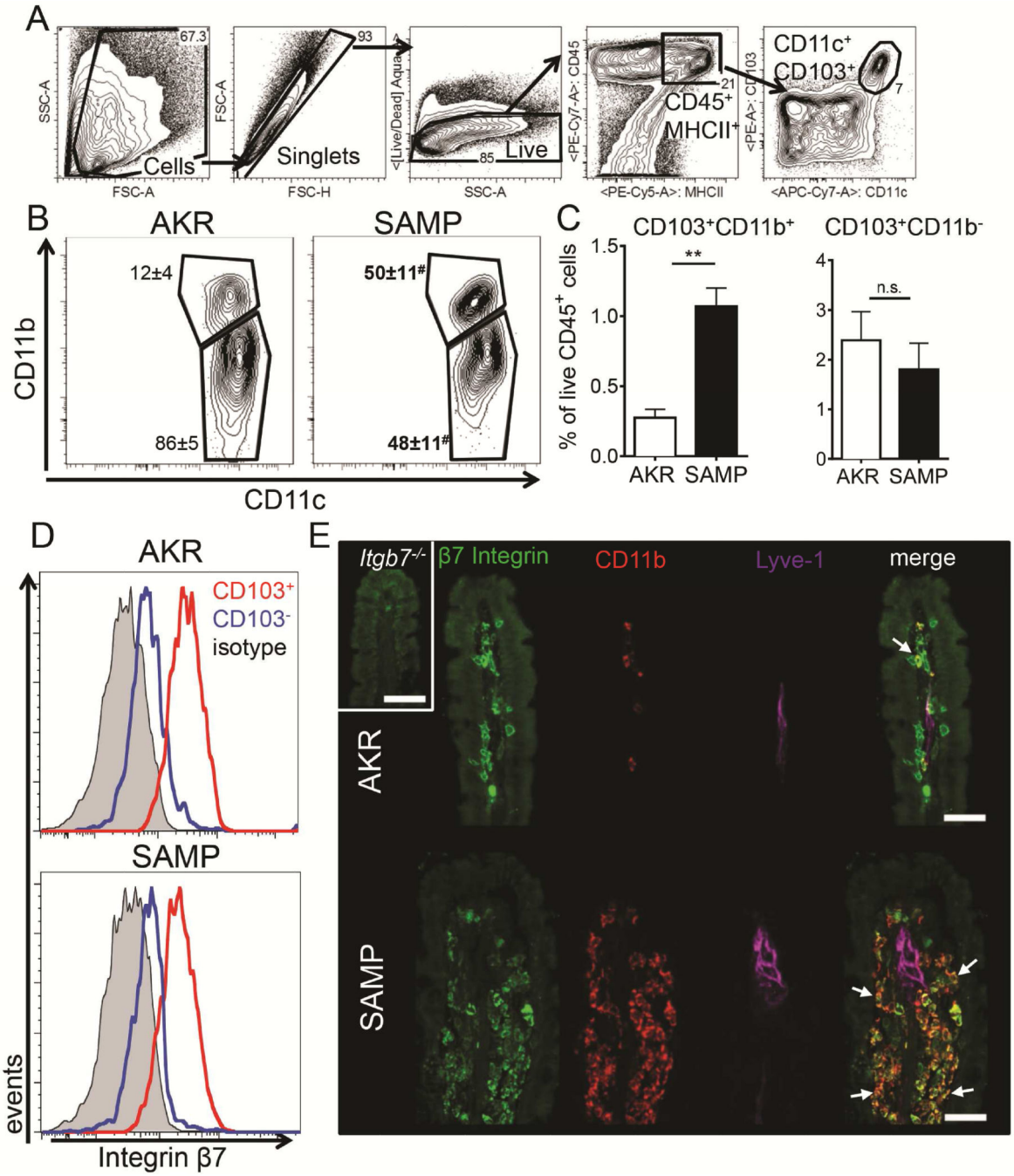

Figure 2. Accumulation of CD103$^+$CD11b$^+$ mDC in the terminal ileum LP of SAMP mice

LP cells were prepared from 5–7 weeks old AKR and SAMP mice by digestion and gated (A) on live single CD45$^+$MHCII$^+$CD103$^+$CD11c$^+$ cells. (B) Representative flow cytometry plots of CD103$^+$CD11c$^+$CD11b$^+$ and CD11b$^-$ population in AKR and SAMP LP. Percentages of parent gate (mean ± SEM) are indicated on the plots, # denotes significant differences between AKR and SAMP mice. (C) Frequency of gated populations among all live leukocytes. Results are the mean ± SEM of 2 experiments with n=4 for AKR and n=3 for SAMP groups. (D) Expression of β_7 integrin on CD103$^+$ (*red*) and CD103$^-$ (*blue*)

MHCII^{high} MLN DCs. Isotype control (*grey*) was done on MHCII^{high} cells. (E) Sections of terminal ileum from 20 weeks old AKR and SAMP mice were stained with antibodies to β_7 integrin (*green*), CD11b (*red*), and Lyve1 (Lymphatic Vessel Endothelial Receptor 1, *magenta*). Shown is maximum intensity projection of a 6 μm confocal stack. $\beta7^+CD11b^+$ cells (*arrows*) are enriched in SAMP LP. Control staining (*insert*) was done on tissue from β_7-deficient mouse. Representative of 2 experiments with at least 2 mice per group. Bar = 20 μm. ** $p<0.01$, $^{\#}$ $p<0.05$

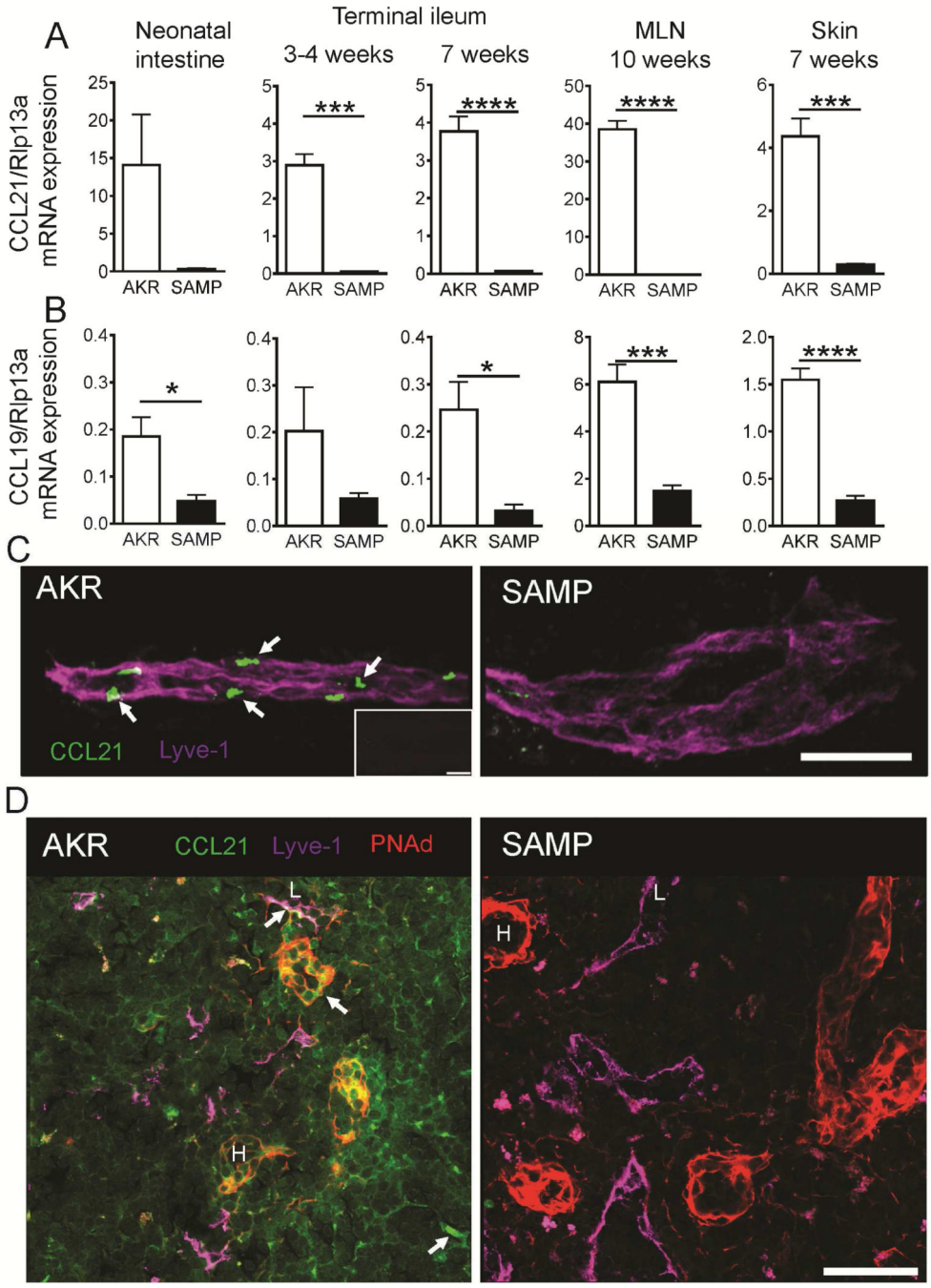

Figure 3. Reduced CCL21 expression in SAMP mice

Quantitative RT-PCR for CCL21 (A) and CCL19 (B) in neonatal intestine, terminal ileum, mesenteric lymph nodes and skin of AKR and SAMP mice; mean±SEM for n=3–5 mice/group. Ribosomal protein L13A (Rpl13a) mRNA was used as a reference. (C) Whole mount staining of terminal ileum with antibodies to CCL21 (*green*) and Lyve1 (*magenta*). Confocal immunofluorescence demonstrates abundant presence of CCL21 protein (*arrows*) in Lyve1^{+} lymphatic vessel of AKR, while weak expression of CCL21 can be seen only sporadically in SAMP mice; Maximum intensity projection of 15 × 1 μm Z stacks. Bar = 100 μm

Representative of 3 experiments with total n=4 mice/strain. Isotype control is shown in the *insert*. Bar = 30 μm. (D) Sections of MLN form AKR and SAMP mice were stained with antibodies to CCL21 (*green*), peripheral node addressin (*red)* and Lyve-1 (*magenta*). Diffuse and concentrated (arrows) pools of CCL21 in lymphatic vessels *(L)* and high endothelial venules *(H)* are absent in SAMP lymph nodes. Bar = 50 μm. Representative of 2 experiments.

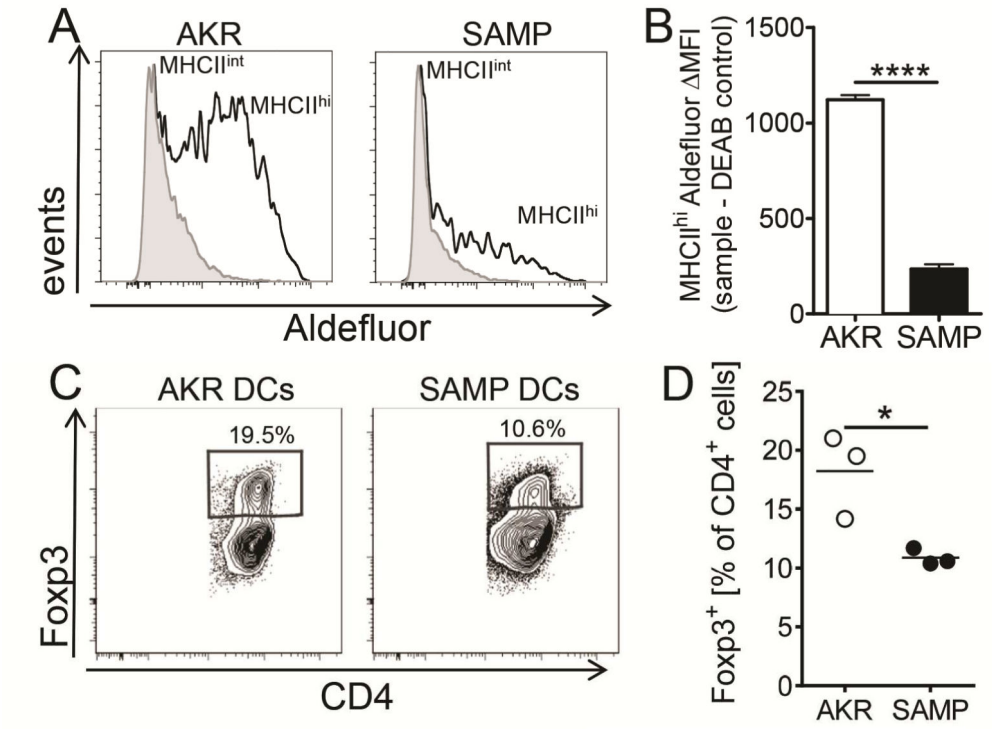

Figure 4. SAMP DCs are defective in generating Foxp3$^+$ cells in vitro

Capacity of SAMP and AKR MLN DCs to produce RA was measured by RALDH activity in Aldefluor assay. (A) Representative histograms of MLN MHCII^{int} (gray) and MHCII^{hi} (open) cells from AKR and SAMP mice (B) Aldefluor ΔMFI signal was calculated by subtracting median fluorescence intensity of MLN MHCII^{hi} cells treated with RALDH inhibitor DEAB from corresponding non-treated samples. Mean ± SEM from 3 experiments and n=4 mice for each group. (C–D) Naïve AKR CD4$^+$CD45Rb^{hi}CD25$^-$ T cells were incubated with magnetically enriched and FACS sorted CD11c+ DC from AKR or SAMP MLN for 5 days in the presence of 2 μg/ml anti-CD3ε and 5 ng/ml TGFβ1. (C) Representative flow cytometry plots of Foxp3 expression on cultured CD4$^+$ cells. (D) Data from 2 experiments are presented and mean is indicated. **** $p<0.0001$, * $p<0.05$ by two-sided Student's t test

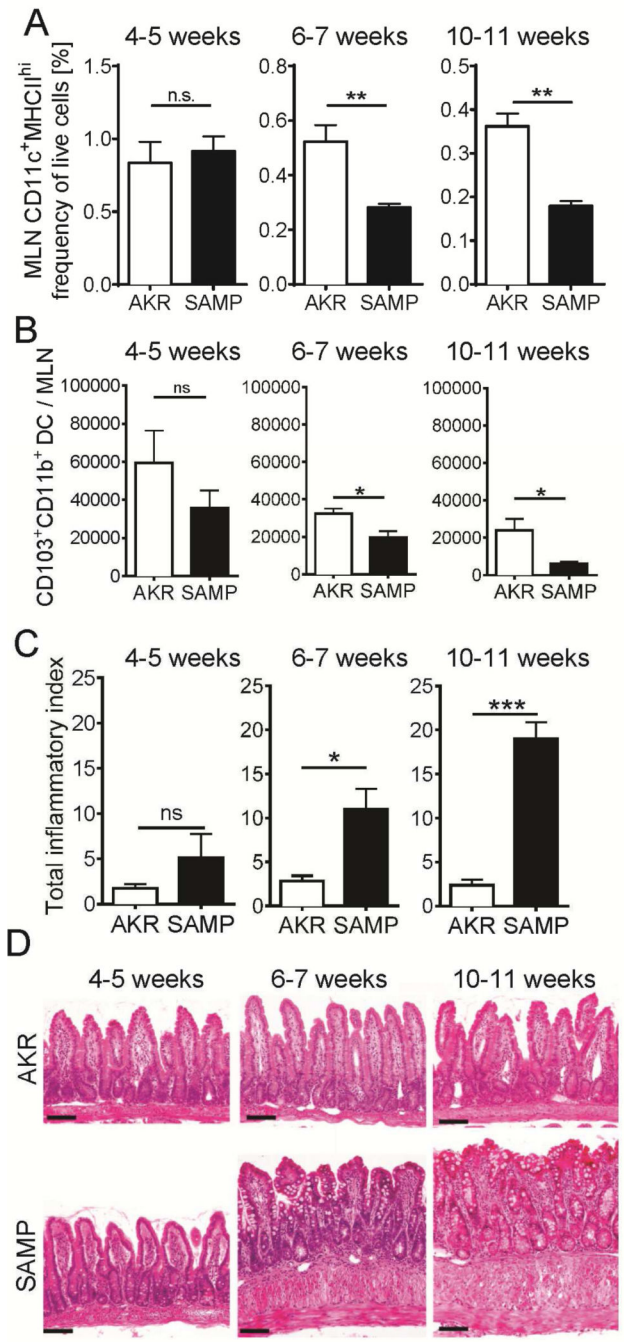

Figure 5. Loss of MHCII^{hi} DC in MLN of SAMP mice is concomitant with the onset of clinical disease

(A–B) MLN MHCII^{hi} DCs and ileitis (C) were analyzed in 4–5, 6–7 and 10–11 weeks old mice (mean ± SEM, n≥3). (B) Diminished numbers of CD11c^{+}MHCII^{hi}CD103^{+}CD11b^{+} cells in the MLNs of >6 weeks old SAMP mice. (D) Sections of terminal ileum were stained with H&E. Bar = 100 μm. n.s – not significant, *** p<0.001, ** p<0.01, * p<0.05 by two-sided Student's t test

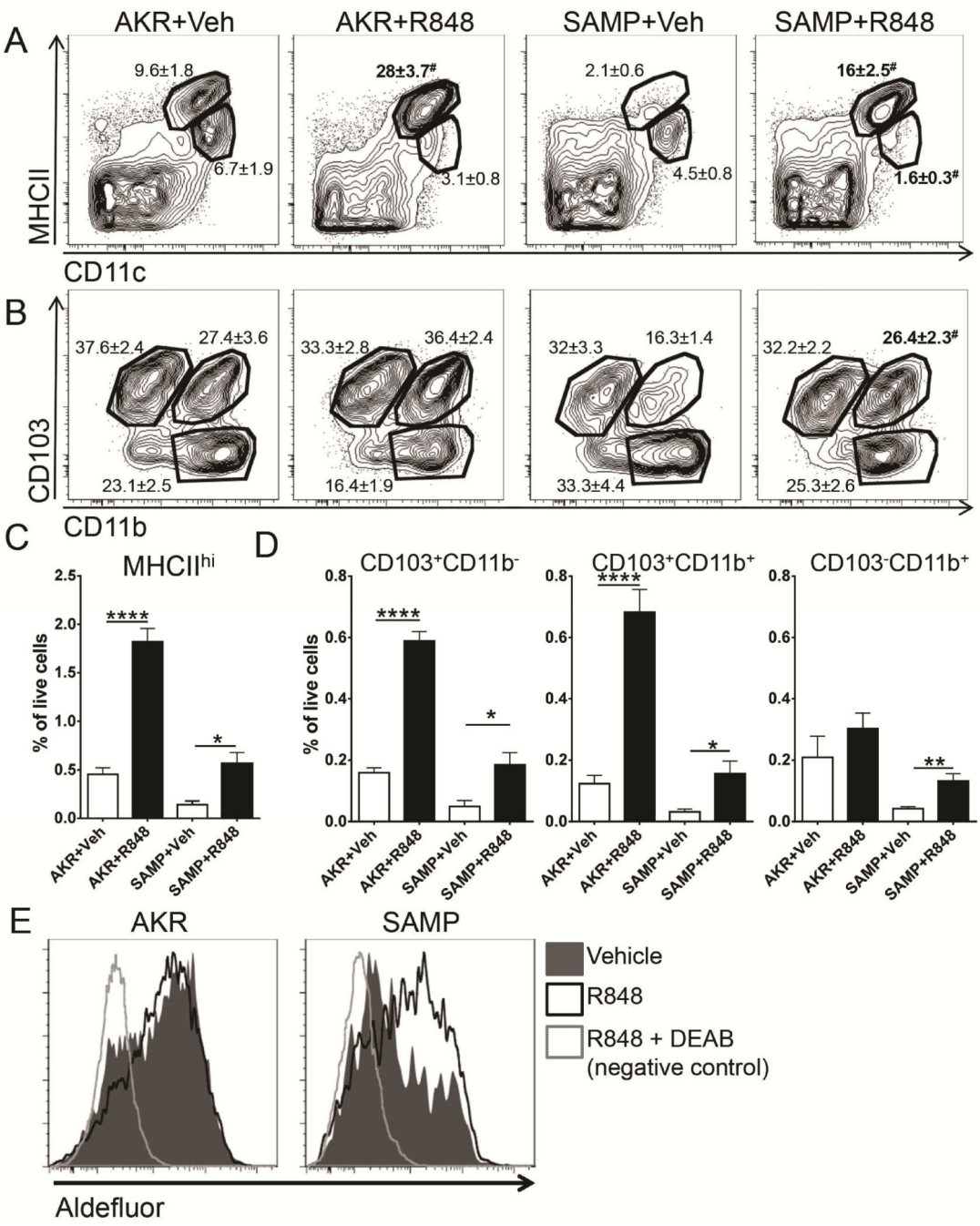

Figure 6. TLR7 agonist R848 can overcome DC recruitment defect in SAMP mice

(A) Migration of $CD11c^{+}MHCII^{hi}$ DC into MLN was measured 18h after gavage of R848 (0.5 μg/g). (B) Treatment induced migration of $CD103^{+}$ $CD11b^{+}$ and $CD11b^{-}$ cells in the AKR mice. Percentages of parent gate ± SEM are indicated on the plots, # denotes significant differences between vehicle and R848 treated mice of the same strain. (C) Frequency of migratory cells at baseline (vehicle treated mice) and after TLR induction (R848) was lower in MLN of SAMP mice. n=5–6 in each group. (E) Activity of RA-synthesizing enzyme in $CD11c^{+}MHCII^{hi}$ MLN cells following TLR7 ligand treatment was

measured by flow cytometry. Filled histograms show vehicle-treated mice, black open histograms depict R848-treated mice. Control sample treated with RALDH inhibitor DEAB is shown as gray open histograms. Data are representative of 3 experiments. **** $p<0.0001$, ** $p<0.01$, * $p<0.05$, # $p<0.05$ by two-sided Student's t test

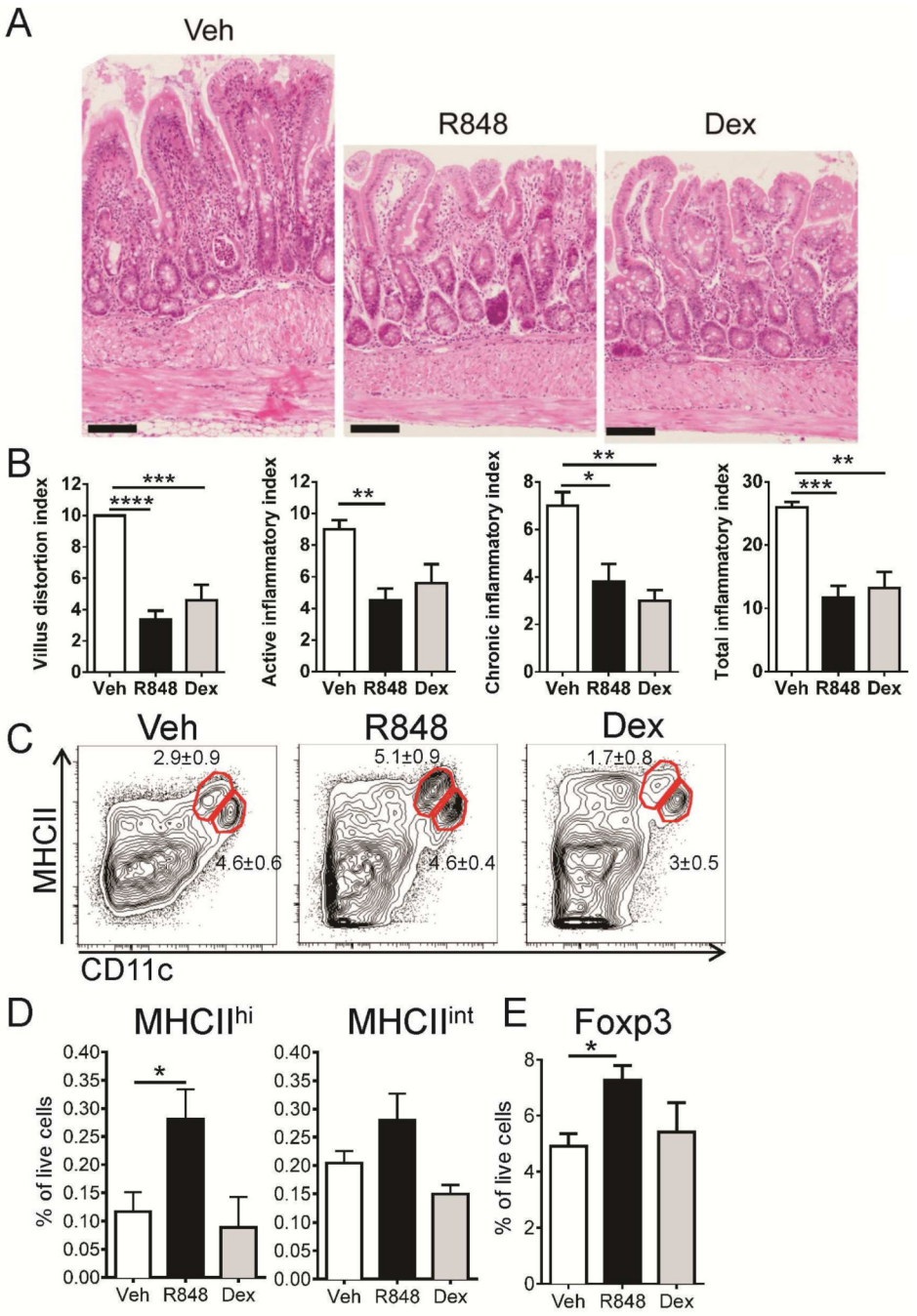

Figure 7. Treatment with R848 reduces inflammation in the terminal ileum

(A–B) Effect of treatment with R848 on ileitis. Outcomes of a 7 day course of R848 treatment (0.5 μg/g body mass, by oral gavage) on >30 weeks old mice. Control animals received either vehicle (H_2O) by daily gavage or dexamethasone (Dex) by IP injection (100 μg every second day). (A) Ileal swiss-rolls were stained with H&E and (B) total inflammatory score was assessed. n=4–8 per group. (C–F) R848 induces mDCs and MLN Foxp3$^+$ cells. (C) Representative flow cytometry plots and (D) frequency of DC subsets were analyzed among all live cells recovered from the lymph nodes. (E) Cells were gated to

include $CD4^+$ $TCR\beta^+$ T cells and expression of Foxp3 was determined by intracellular staining. Shown is percentage of $CD4^+Foxp3^+$ cells among all live MLN cells. Data from 2 experiments are presented as mean ± SEM, n=7–12 per group. **** $p<0.0001$, *** $p<0.001$, ** $p<0.01$, * $p<0.05$ by one-way analysis of variance followed by Dunnett's multiple comparisons test