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Chemokine receptor CCR7 regulates the intestinal $T_H1/T_H17/T_{reg}$ balance during Crohn's-like murine ileitis

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ABSTRACT

The regulation of T cell and DC retention and lymphatic egress within and from the intestine is critical for intestinal immunosurveillance; however, the cellular processes that orchestrate this balance during IBD remain poorly defined. With the use of a mouse model of TNF-driven Crohn's-like ileitis ($TNF^{\Delta ARE}$), we examined the role of CCR7 in the control of intestinal T cell and DC retention/egress during experimental CD. We observed that the frequency of CCR7-expressing T_H1/T_H17 effector lymphocytes increased during active disease in $TNF^{\Delta ARE}$ mice and that $\Delta ARE/CCR7^{-/-}$ mice developed exacerbated ileitis and multiorgan inflammation, with a marked polarization and ileal retention of T_H1 effector $CD4^+$ T cells. Furthermore, adoptive transfer of $\Delta ARE/CCR7^{-/-}$ effector $CD4^+$ into lymphopenic hosts resulted in ileo-colitis, whereas those transferred with $\Delta ARE/CCR7^{+/+}$ $CD4^+$ T cells developed ileitis. $\Delta ARE/CCR7^{-/-}$ mice had an acellular draining MLN, decreased $CD103^+$ DC, and decreased expression of RALDH enzymes and of $CD4^+CD25^+FoxP3^+$ T_{regs}. Lastly, a mAb against CCR7 exacerbated ileitis in $TNF^{\Delta ARE}$ mice, phenocopying the effects of congenital CCR7 deficiency. Our data underscore a critical role for the lymphoid chemokine receptor CCR7 in orchestrating immune cell traffic and T_H1 versus T_H17 bias during chronic murine ileitis. *J. Leukoc. Biol.* 97: 1011–1022; 2015.

Introduction

Intestinal immune homeostasis is reliant on maintaining tolerance to food and bacterial antigens while remaining poised to respond to intestinal pathogens. Most lymphocytes within the GALT constantly traffic from blood into tissues, where they may become resident or recirculate through the mesenteric lymphatics back to the circulation. This process allows for the dynamic cellular encounters of antigen-presenting DCs and naïve T cells that shape protective adaptive immunity. Whereas the recirculation of T cells is critical for immunosurveillance, a clear role for T_H1/T_H17 $CD4^+$ T_{EM} subsets in the LP has been implicated in driving the pathogenesis of CD [1–3]. The understanding of the control of cellular recruitment, tissue retention, and egress is critical for the development of novel, therapeutic modalities that target traffic in IBD. However to date, whereas we have been able to therapeutically harness some of the molecules that determine intestinal recruitment (i.e., integrins $\alpha4$ and $\alpha4\beta7$), those that control intestinal retention and lymphatic egress of gut-tropic T cell subsets are less defined.

The chemokine receptor CCR7 was originally believed to be expressed only by activated DCs and naïve T cells, limiting the role of this receptor for homing to lymph nodes. Later, seminal observations demonstrated that CCR7 was re-expressed by activated T cells, allowing their egress from tissues to lymph nodes via afferent lymphatics [4–6]. The expression of CCR7 ligands, CCL19 and CCL21, is tightly regulated within lymphoid organs, acting as chemotactic and retentive signals [7, 8]. However, increased ectopic expression of CCL19 and CCL21 has been reported in the inflamed intestine of patients with CD and in preclinical models of chronic ileitis [9, 10], yet the role of their cognate receptor CCR7 in regulating T_{EM} recirculation during conditions of chronic inflammation is less understood.

Abbreviations: ΔARE = Δ adenylate-uridylate-rich element, $\Delta ARE/CCR7^{+/+}$ = CCR7-sufficient TNF Δ adenylate-uridylate-rich element, $\Delta ARE/CCR7^{-/-}$ = CCR7-deficient TNF Δ adenylate-uridylate-rich element, CD = Crohn's disease, CD62L = cluster of differentiation 62 ligand, DC = dendritic cell, FoxP3 = forkhead box P3, GALT = gut associated lymphoid tissue IBD = inflammatory bowel disease, LP = lamina propria, MLN = mesenteric lymph node, RA = retinoic acid, RALDH =

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The online version of this paper, found at www.jleukbio.org, includes supplemental information.

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Here, we use the TNF$^{\Delta ARE}$ mouse model of chronic Crohn's-like ileitis [11], which develops terminal ileitis reminiscent of human CD, to assess the contribution of the CCR7 chemokine axis to disease pathogenesis. We investigated the expression of CCR7 on cytokine-producing effector T cell subsets infiltrating the ileum and MLN during disease onset. Furthermore, we generated a ΔARE/CCR7$^{-/-}$ substrain and assessed the effects of CCR7 deficiency on disease severity, T cell phenotype, and cytokine production. After the identification of an exacerbation of disease in ΔARE/CCR7$^{-/-}$ mice compared with CCR7-sufficient counterparts, we further defined a role for CCR7 in the intestinal retention of CD4$^+$ T$_{EM}$ by use of T cell adoptive transfer and immunoneutralization studies.

MATERIALS AND METHODS

Mice

The B6.129S-Tnf^{tm2Gkl}/Jarn (TNF$^{\Delta ARE}$) strain was described previously. These mice overproduce TNF and develop CD-like ileitis and arthritis [9, 11]. CCR7-deficient [B6.129P$_2$(C)-Ccr7^{tm1Rfor}/J] and RAG1$^{-/-}$ mice (B6.129S7-*Rag1*^{tm1Mom}/J) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). To generate a CCR7-deficient substrain, TNF$^{\Delta ARE}$ mice were backcrossed to B6.129P$_2$(C)-Ccr7^{tm1Rfor}/J. Experimental mice used were heterozygous for the ΔARE mutation, homozygous for CCR7 deficiency, or CCR7 sufficient. Mice were kept under specific pathogen-free conditions, and fecal samples were negative for *Helicobacter* species, protozoa, and helminthes. Animal procedures were approved by the Institutional Animal Care and Use Committees of the University of Colorado Denver and University of California, San Diego.

Tissue fixation, paraffin embedding, and histologic scoring

Terminal ilea were excised, opened longitudinally, and washed with cold PBS, followed by fixation with 10% buffered formalin. Tissue was subsequently paraffin embedded, cut into 5 μm sections, and stained with H&E. Histologic assessment of ileitis was performed in a blinded fashion by an intestinal pathologist, as described [12].

RNA extraction, cDNA synthesis, and real-time PCR

Total RNA was isolated from homogenized ilea or MLN by use of the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). RNA (500 ng) was reverse transcribed into cDNA with a high-capacity cDNA archive kit (Applied Biosystems, Foster City, CA, USA). Real-time RT-PCR was performed by use of TaqMan gene expression assays (Applied Biosystems) containing forward and reverse primers and a FAM-labeled Minor Groove Binder (MGB) TaqMan probes. PCR assays for IFN-γ (Mm00801778_m1), TNF (Mm00443258_m1), IL-1β (Mm00434228_m1), IL-6 (Mm00446191_m1), IL-12p35 (Mm00434165_m1), IL-12p40 (Mm_00434174_m1), IL-23 (Mm00518984_m1), IL-17a (Mm00439618_m1), IL-17f (Mm00521423_m1), TGF-β (Mm00441727_g1), IL-4 (Mm0045259_m1), IL-10 (Mm01288386_m1), RALDH1 (Mm00657317_m1), RALDH2 (Mm00501306_m1), and RALDH3 (Mm00474049_m1) were performed by use of TaqMan Universal PCR Master Mix with 18s as an endogenous control. Relative gene expression was calculated by use of the $\Delta\Delta$ comparative threshold method with ABI Relative Quantitation (RQ) software (Applied Biosystems).

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retinaldehyde dehydrogenase, RORγt = retinoic acid-related orphan receptor γt, Tbet = T-box transcription factor TB×21 T$_{CM}$ = central memory T cell, T$_{EM}$ = effector memory T cell, T$_{Naive}$ = naïve T cell, T$_{reg}$ = regulatory T cell, WT = wild-type

Leukocyte isolation

Splenocytes, MLN, and ileal LP mononuclear cells were isolated, as described previously [13, 14].

Flow cytometry

Cells from indicated compartments were incubated with fluorescently labeled anti-mouse antibodies against: CD4 (GK1.5) and CD19 (6D5; BioLegend, San Diego, CA, USA); CD8 (Ly-2), CCR7 (4B12), CD62L (MEL-14), CD44 (IM7), MHCII (M5/114.15.2), CD103 (2E7), CD11b (M1/70), CD11c (N418), IFN-γ (XMG1.2), IL-17a (FFA21), CD25 (PC61.5), FoxP3 (FJK-16s), Ki67 (SolA15), RORγt (B2D), $\alpha4\beta7$ (DATK-32), and CCR9 (CW-1.2; eBioscience, San Diego, CA, USA); Tbet (OX-40; BD Biosciences, San Jose, CA, USA); or corresponding isotype controls. Intracellular staining was performed by use of the FoxP3 staining kit (eBioscience), according to the manufacturer's instructions. Intracellular cytokine staining was performed following 4 h stimulation with PMA (50 ng/ml), ionomycin (1 μg/ml), and brefeldin and monensin. Analysis was performed by use of a BD FACSCanto II (BD Biosciences). FACS was performed by use of a BD FACSAria III (BD Biosciences). Further analyses were performed by use of FlowJo software (Tree Star, Ashland, OR, USA).

Adoptive transfer studies

CD4$^+$ T$_{EM}$ (CD44^{high}CD62L^{neg}) from the spleen of TNF$^{\Delta ARE}$/CCR7$^{+/+}$ and TNF$^{\Delta ARE}$/CCR7$^{-/-}$ mice were incubated with fluorescently labeled antibodies, as above, and separated by use of the FACSAria system (BD Biosciences). CD4$^+$ effector fractions (≥98% pure; 1×10^6) were suspended in 200 μL saline and injected i.p. into 8-wk-old RAG1$^{-/-}$ recipients. Ilea and colons were harvested 8 wk post-transfer, and the severity of inflammation was assessed as described [12, 13].

CCR7 immunoblockade

Eight-week-old TNF$^{\Delta ARE}$ mice received 4 i.p. doses (500 μg) of anti-CCR7 mAb (Clone 4B12; R&D Systems, Minneapolis, MN, USA) or rat isotype every 4 days for 2 wk. Organs were collected 24 h after the final injection.

Statistical analysis

Statistical analyses were performed by use of ANOVA or a 2-tailed Student's *t*-test. Data were expressed as mean ± SEM. Statistical significance was set at $P < 0.05$.

RESULTS

T$_H$1/T$_H$17 CD4$^+$ T$_{EM}$ expressing CCR7 are increased in MLN and ilea of TNF$^{\Delta ARE}$ mice with ileitis

The onset of ileitis in TNF$^{\Delta ARE}$ mice is characterized by marked leukocyte infiltration of the ileum and MLN, composed predominantly of CD4$^+$ T cells (**Fig. 1A** and **D**), most of which show an activated effector phenotype (CD44$^+$). Furthermore, we phenotyped effector CD4$^+$ from WT and TNF$^{\Delta ARE}$ mice and assessed their cytokine profile by flow cytometry. Compared with their WT counterparts, effector CD4$^+$ from TNF$^{\Delta ARE}$ displayed enhanced inflammatory cytokine production in the ileum and draining MLN during disease onset at 8 wk of age (Fig. 1B and E).

To begin to understand the role of CCR7 in the traffic of effector CD4$^+$ T cells to and from intestine during ileitis, we assessed the mRNA expression of CCR7 in effector CD4$^+$ T cells from the ileum and MLN. CCR7 mRNA was increased significantly in sorted effector CD4$^+$ (CD44$^+$/CD62L^{neg}) at 8 wk of age (Fig. 1C and F). Thus, expression of CCR7 is not restricted

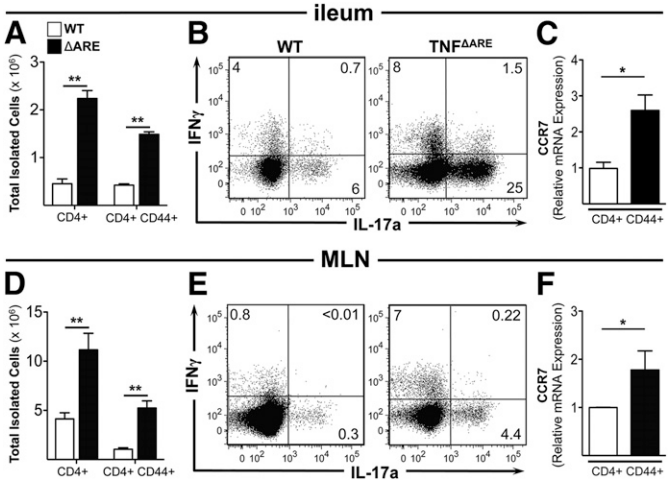

Figure 1. The onset of ileitis in TNF$^{\Delta ARE}$ mice is characterized by increased T_H1/T_H17 CD4$^+$ T cells expressing CCR7. (A and D) Cellular composition of WT and TNF$^{\Delta ARE}$ (ΔARE) ileal infiltrate and MLN, with correlation of CD4$^+$ and CD4$^+$ CD44$^+$ (T_{EM}; CD44^{high} CD62L^{neg}) lymphocytes by flow cytometry. (B and E) Flow cytometry analysis of CD4$^+$ T cells from ilea and MLN of WT and TNF$^{\Delta ARE}$ mice assessing the relative expression of IFN-γ and IL-17a. Gating was performed on live, CD45$^+$MHCII^{neg} CD4$^+$ T cells from indicated compartments at 8–10 wk of age. (C and F) Relative mRNA expression of CCR7 from sorted CD4$^+$CD44$^+$ from MLN and ileum of WT and TNF$^{\Delta ARE}$ mice. Data expressed as mean ± SEM; $*P < 0.05$; $**P < 0.01$ versus age-matched WT mice from 2 independent experiments (n = 5 mice/strain; A and D, ANOVA; C and F, t-test).

to naïve T cells, and IFN-γ- and IL-17a-producing CD4$^+$ effector cells accumulate within the ilea and MLN of TNF$^{\Delta ARE}$ mice during active disease.

CCR7 deficiency exacerbates ileitis in TNF$^{\Delta ARE}$ mice

Then, we generated a CCR7-deficient substrain of TNF$^{\Delta ARE}$ mice and found that ΔARE/CCR7$^{-/-}$ displayed a further increase in leukocytic infiltrate compared with its ΔARE/CCR7$^{+/+}$ counterparts (**Fig. 2A** and **B**). In addition, we assessed the continuum of ileitis progression in ΔARE/CCR7$^{+/+}$ and ΔARE/CCR7$^{-/-}$ mice (Fig. 2C and D). Active indices revealed exacerbated ileitis at 4 wk of age (early disease) in ΔARE/CCR7$^{-/-}$ compared with its ΔARE/CCR7$^{+/+}$ counterparts and at 10 wk of age. A significant increase in the chronic index was also observed at 4, 10, and 20 wk of age in ΔARE/CCR7$^{-/-}$ mice (Fig. 2C and D). The increased leukocyte numbers in the ilea of ΔARE/CCR7$^{-/-}$ mice were accompanied by a marked increase in villus distortion at all time-points assessed (Figure 2C and D). Thus, congenital CCR7 deficiency exacerbated ileitis by enhancing recruitment or by disrupting effector T cell egress from the intestine.

CD4$^+$ T_{EM} are retained within the ileal LP and depleted in the MLN of ΔARE/CCR7$^{-/-}$ mice

We assessed the composition of the cellular infiltrate within the ilea and MLN of TNF$^{\Delta ARE}$/CCR7$^{-/-}$ and TNF$^{\Delta ARE}$/CCR7$^{+/+}$ mice. Flow cytometry revealed significant retention of CD4$^+$ (**Fig. 3A**) and CD8$^+$ (Supplemental Fig. 1A) T cells in the ilea of ΔARE/CCR7$^{-/-}$. Analysis of the draining MLN displayed a converse paucity of cellular infiltrate in ΔARE/CCR7$^{-/-}$ compared with ΔARE/CCR7$^{+/+}$ counterparts (Fig. 3B). Flow cytometric subanalysis revealed that ileal retention and decreased MLN cell counts were not unique to CD4$^+$ but also observed for CD8$^+$ T cells (Supplemental Fig. 1). At 10 wk of age, ΔARE/CCR7$^{-/-}$ mice displayed a greater accumulation of effector CD4$^+$ (T_{EM}) cells in the ileum compared with TNF$^{\Delta ARE}$ littermates (Fig. 3A and B). The frequency of T_{CM} (CD44$^+$/CD62L$^+$) and T_{Naive} (CC44$^-$CD62L$^+$) CD4$^+$ subsets in the ilea appeared unchanged (Fig. 3A). Whereas an increase retention of CD4$^+$ T_{EM} was observed in the inflamed ilea of ΔARE/CCR7$^{-/-}$ mice, the MLN displayed a significant reduction in the percentage and absolute numbers of all T cell subsets analyzed (Fig. 3A and B). Thus, ileitis in ΔARE/CCR7$^{-/-}$ mice is characterized by a greater accumulation/retention of CD4$^+$ T_{EM} cells within intestine and a loss of T_{EM}, T_{CM}, and T_{Naive} trafficking to the draining MLN during chronic ileitis.

CCR7 deficiency results in an altered ratio of T_H1 versus T_H17 CD4$^+$ T cells in ilea of TNF$^{\Delta ARE}$ mice

To understand further the specific CD4$^+$ effector subsets that are retained within the ileum of ΔARE/CCR7$^{-/-}$mice, we performed a series of flow cytometry experiments on cells isolated from the ileum and MLN. CCR7 deficiency resulted in a marked increase in the frequency of CD4$^+$IFN-γ$^+$ in the ileum and MLN compared with its ΔARE/CCR7$^{+/+}$ counterparts (**Fig. 4A** and **C**). Once cytokine percentages are adjusted for the dramatic cellularity differences associated with CCR7 deficiency, the ileum and MLN of CCR7-deficient mice are predominantly populated by IFN-γ$^+$ CD4$^+$ T cells (Fig. 4A and C). There is still an increase in IL-17a$^+$ CD4$^+$ T cells in the ileum compared with CCR7-sufficient counterparts; however, the magnitude of their development is reduced compared with IFN-γ$^+$. An interesting observation is that the MLN displays a reduction in IL-17a compared with ΔARE/CCR7$^{+/+}$ mice (Fig. 4B and D).

Consistent with the increased retention of pathogenic effector cells within the LP of CCR7-deficient animals was the ileal mRNA expression of several cytokines increased in CCR7-deficient animals (Fig. 4E, I–VI). Of note, CCR7 deficiency results in a loss of ileal IL-17a mRNA (Fig. 4E, VII); however, other T_H17-related cytokines—IL-23 and TGF-β—displayed a modest increase compared with TNF$^{\Delta ARE}$ mice (Fig. 4E, I–XII]. Collectively, these data provide evidence for the generation and retention of T_H1 effector CD4$^+$ T cells in the terminal ileum of ΔARE/CCR7$^{-/-}$ mice.

ΔARE/CCR7$^{-/-}$ ileitis results in changes in DC subsets, decreased expression of RALDHs, and an imbalance in CD4$^+$ FoxP3$^+$ CD25$^+$ T_{regs}

Previously, we have demonstrated a role for CD103$^+$ regulatory DC in the control of TNF$^{\Delta ARE}$ ileitis [15, 16]. As DCs use CCR7 to migrate to lymph nodes and educate naïve CD4$^+$ T cells [7, 10, 17], we assessed the potential contribution of CCR7 on DC migration by flow cytometry from ΔARE/CCR7$^{+/+}$ and

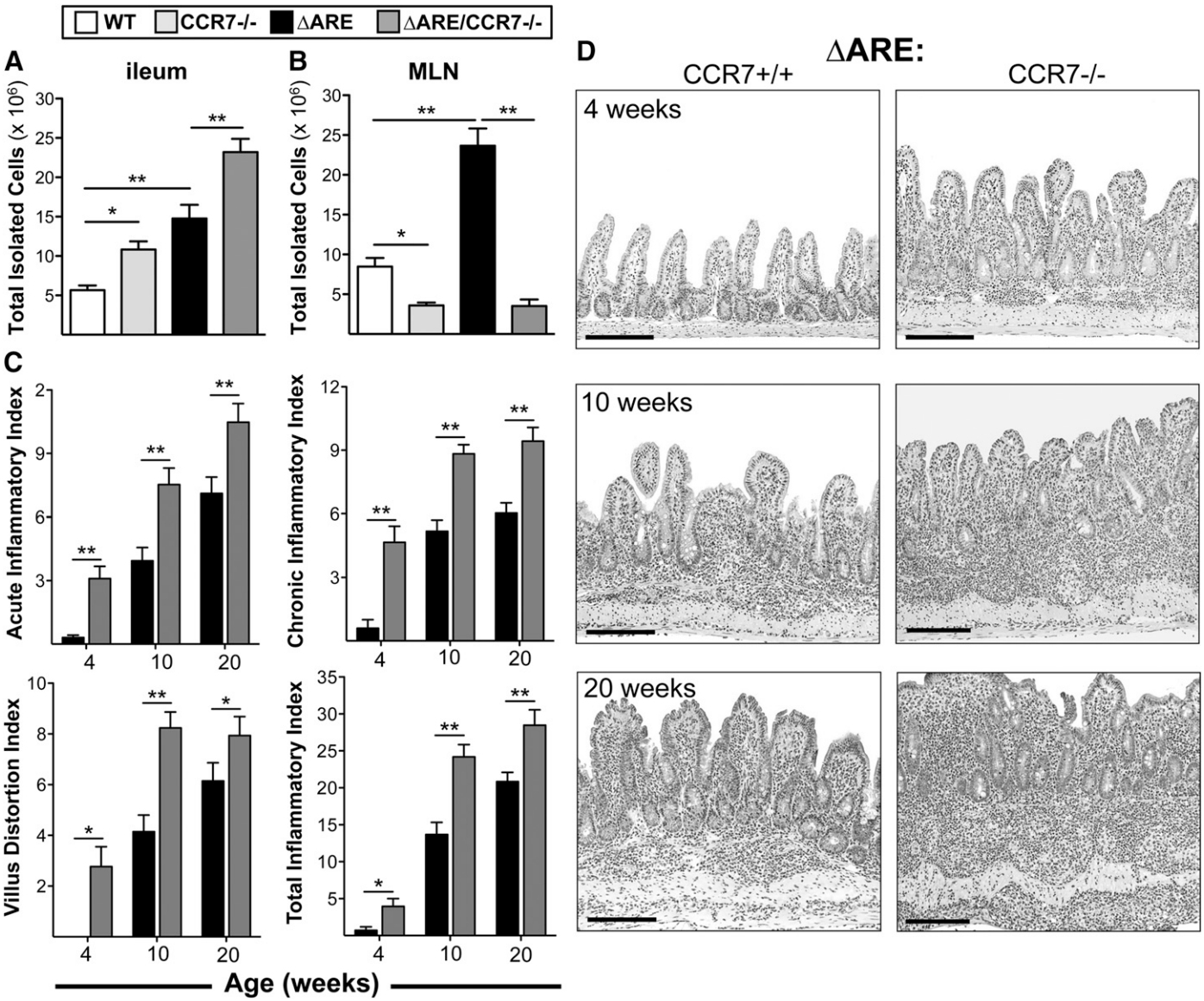

Figure 2. CCR7 deficiency exacerbates ileitis in TNF$^{\Delta ARE}$ mice. (A and B) Cellular composition of WT, CCR7$^{-/-}$, TNF$^{\Delta ARE}$/CCR7$^{+/+}$ (ΔARE) versus TNF$^{\Delta ARE}$/CCR7$^{-/-}$ (ΔARE/CCR7$^{-/-}$) ileal infiltrate and MLN. (C) Histologic assessment of TNF$^{\Delta ARE}$/CCR7$^{+/+}$ and TNF$^{\Delta ARE}$/CCR7$^{-/-}$ mice ilea at indicated ages, according to acute and chronic leukocytic infiltrates, villus distortion, and a combinatorial total inflammatory index. (D) Representative micrographs of ileum H&E from TNF$^{\Delta ARE}$/CCR7$^{+/+}$ and TNF$^{\Delta ARE}$/CCR7$^{-/-}$ mice between 4 and 20 wk of age. Data expressed as mean ± SEM; $*P < 0.05$; $**P < 0.01$ versus age-matched WT mice (n = 10–18 mice/strain, ANOVA). Original scale bars, 100 μm.

ΔARE/CCR7$^{-/-}$mice during peak disease. CCR7 deficiency resulted in a significant loss of CD103^{+}CD11b^{-}CD11c^{+}MHCII^{high} DC in the ilea (**Fig. 5A**). mRNA expression of RALDH1, RALDH2, RALDH3 was reduced compared with its WT counterparts (Fig. 5B), but within the ileum, there were no significant differences between ΔARE/CCR7$^{+/+}$ and ΔARE/CCR7$^{-/-}$mice. In addition, there was a loss of CD103^{+}CD11b^{-} DCs in the draining MLN (Fig. 5D) with a concomitant increase in CD103^{-}CD11b^{+} (Fig. 5D). Lastly, there was a marked decrease in the expression of the RALDH enzymes RALDH2 and RALDH3 in ΔARE/CCR7$^{-/-}$ mice compared with their ΔARE/CCR7$^{+/+}$ counterparts (Fig. 5E). Collectively, these data highlight a critical role for CCR7 during regulatory CD103^{+} DC migration and resultant loss of the RA-producing RALDH enzymes in ΔARE/CCR7$^{-/-}$ mice.

To investigate further the downstream effect of regulatory DC deficiency on T_{reg} numbers in ΔARE/CCR7$^{-/-}$ mice, we assessed the frequency of T_{reg} in the ilea and MLN. During active ileitis at 10 wk of age, ΔARE/CCR7$^{-/-}$ mice had increased CD4^{+}CD25^{+}FoxP3^{+} T_{regs} in the LP compared with TNF$^{\Delta ARE}$ littermates (Fig. 5C). Conversely, the acellular MLN of ΔARE/CCR7$^{-/-}$mice displayed a loss of CD4^{+}CD25^{+}FoxP3^{+} T_{reg} compared with its TNF$^{\Delta ARE}$ counterparts (Fig. 5F). Thus, although CCR7 deficiency does not result in a failure

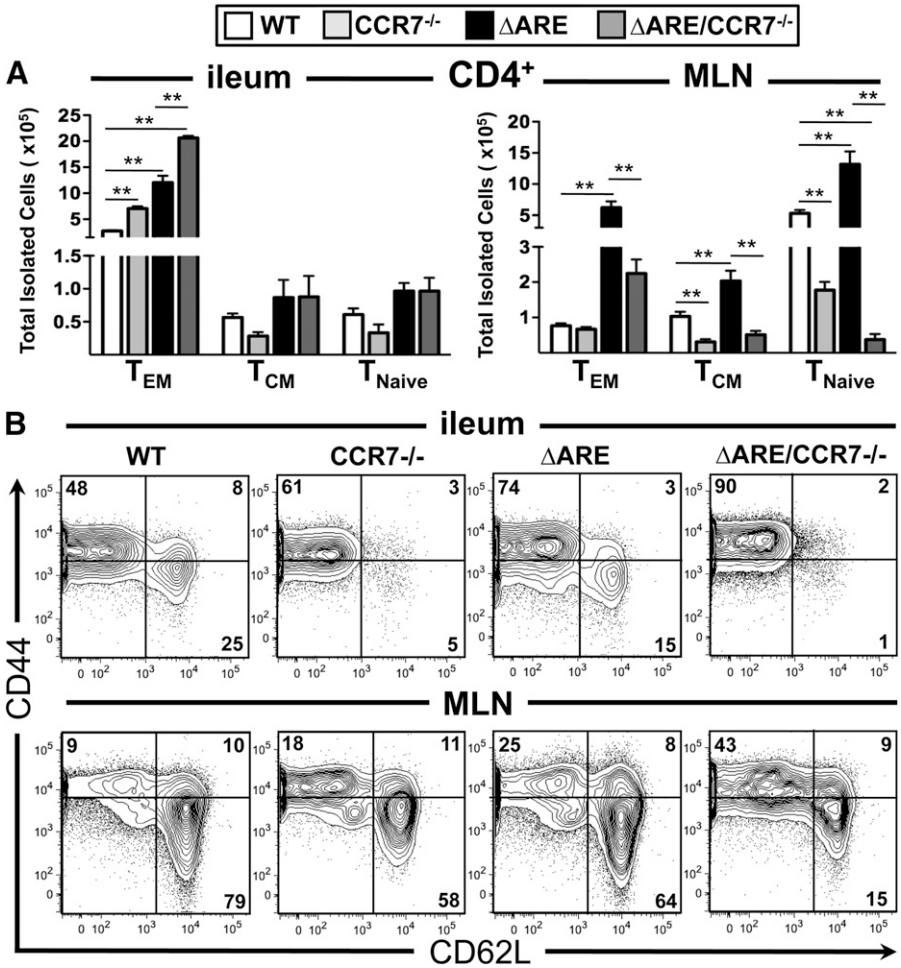

Figure 3. CCR7 deficiency increased ileal retention of CD4$^+$ T_{EM} and inhibits egress to draining MLN. (A) Flow cytometric analyses characterizing the absolute cell numbers of T_{EM}, T_{CM}, and T_{Naive} CD4$^+$ T cells in the ilea and MLN of WT, CCR7$^{-/-}$, ΔARE$^{+/+}$ and ΔARE$^{-/-}$ mice. (B) Representative flow cytometry contour plots depicting frequency of T_{EM}, T_{CM}, and T_{Naive} CD4$^+$ T cells in the ilea and MLN of ΔARE/CCR7$^{+/+}$ or ΔARE/CCR7$^{-/-}$ and WT mice. Data expressed as mean ± SEM; $^{**}P < 0.01$ versus its indicated counterpart from 3 independent experiments (n = 6 mice/strain, ANOVA).

to induce FoxP3$^+$ T cells, it appears to impair their ability to migrate to the MLN.

Anti-CCR7 mAb augments a T_H1 cytokine profile, reduces CD103$^+$ DC subsets, and exacerbates ileitis in TNF$^{\Delta ARE}$ mice

To assess the effects of CCR7 immunoblockade, we administered a neutralizing anti-CCR7 mAb or corresponding isotype control to 8-wk-old TNF$^{\Delta ARE}$ mice. We have shown previously that similar to the observation in genetic CCR7 deficiency, CCR7 mAb immunoblockade exacerbated TNF$^{\Delta ARE}$ ileitis significantly, as indicated by increased infiltration of the terminal ileum and a paucity of leukocytes within the MLN [9]. Furthermore, anti-CCR7 mAb exacerbated all histologic indices significantly compared with vehicle controls (**Fig. 6A**). When compared with its TNF$^{\Delta ARE}$ counterparts, CCR7 deficiency augments an ileal T_H1 cytokine profile (Fig. 6B) but did not alter the expression of ileal IL-17a, IL-17f, or IL-23 (Fig. 6B).

As with congenital CCR7 deficiency, CCR7 blockade resulted in a significant loss of CD103$^+$CD11b$^-$CD11c$^+$MHCII^{high} DC in the ilea compared with its WT counterparts (Fig. 6C and D). Thus, antibody blockade of CCR7 in TNF$^{\Delta ARE}$ ileitis inhibited lymphocyte egress, promoted a T_H1 cytokine profile, decreased RA-producing DCs, and exacerbated intestinal inflammation.

CCR7-deficient CD4$^+$ T_{EM} induce ileo-colitis in RAG1$^{-/-}$ mice

We have reported previously that CD4$^+$ T_{EM} drive ileitis in TNF$^{\Delta ARE}$ mice [13]. As such, we assessed whether CCR7 deficiency altered the capacity of effector CD4$^+$ from TNF$^{\Delta ARE}$ mice to transfer ileitis adoptively into lymphopenic recipients. To address this, effector CD4$^+$ were isolated from spleens of 4- to 6-wk-old ΔARE/CCR7$^{+/+}$ and ΔARE/CCR7$^{-/-}$ mice and adoptively transferred separately into RAG1$^{-/-}$ recipients. Of note, ΔARE/CCR7$^{+/+}$ and ΔARE/CCR7$^{-/-}$ CD4$^+$ CD44$^+$ T cells expressed comparable pretransfer levels of transcription factors, such as FoxP3, Tbet, and RORγt. This was also evident for the intestinal homing markers $\alpha4\beta7$ and CCR9, in addition to cytokines IFN-γ, TNF, and IL-17a (Supplemental Fig. 2). Eight weeks post-transfer, CCR7-deficient effector CD4$^+$ T cells were retained in the ileum and colon, with a paucity of adoptively transferred T cells in the draining MLN (**Fig. 7B**). Surprisingly, flow cytometry assessment of Ki67$^+$ in proliferating CD4$^+$ T cells in the ilea and colon revealed no appreciable difference in the relative percent of Ki67$^+$ from either genotype (ΔARE/CCR7$^{+/+}$ vs. ΔARE/CCR7$^{-/-}$; ileum, P = 0.480; colon, P = 0.864; Fig. 7C and D). This was also evident for the transcription factors Tbet (P = 0.444) and RORγt (P = 0.112; Fig. 4E). Of note, however, ΔARE/CCR7$^{-/-}$ CD4$^+$ T cells developed a predominant T_H1

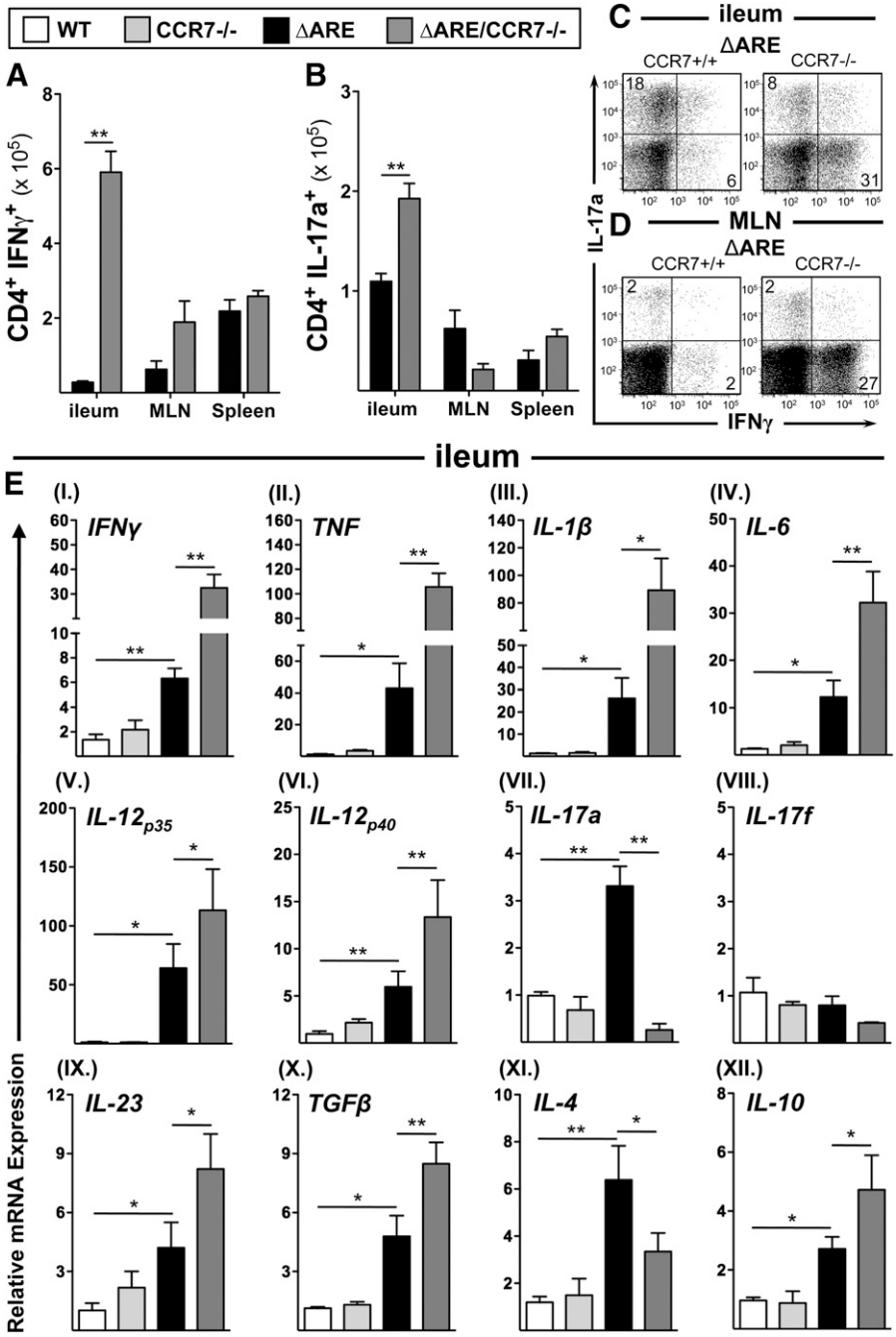

Figure 4. Altered T_H1/T_H17 $CD4^+$ ratio in ΔARE/$CCR7^{-/-}$ mice. (A–D) Absolute numbers and representative flow cytometry plots of IFN-γ^+ versus IL-$17a^+$ from ileum and MLN $CD4^+$ T cells. Gating was performed on live, $CD45^+$ $MHCII^{neg}$ $CD4^+$ T cells at 8–10 wk of age. Data expressed as mean ± SEM; $^{**}P < 0.01$ versus age-matched indicated counterparts from 2 independent experiments (*t*-test). (E, I–XII) Cytokine mRNA analysis was performed by real-Time PCR on ileal tissues from CCR7-sufficient or -deficient WT and ΔARE/$CCR7^{+/+}$ or ΔARE/$CCR7^{-/-}$ mice at 10 wk of age. Data expressed as mean ± SEM; $^{*}P < 0.05$; $^{**}P < 0.01$ versus indicated counterparts from 2 independent experiments ($n = 6$ mice/strain, ANOVA).

profile with a loss of T_H17, similar to effects with congenital CCR7 deficiency (Fig. 7F–I). In addition, mice adoptively transferred with CCR7-deficient effector $CD4^+$ displayed significantly exacerbated histologic parameters of ileitis and colitis compared with CCR7-sufficient controls (Fig. 7J and K). Of note, whereas effector $CD4^+$ from ΔARE mice adoptively transferred ileitis, CCR7-deficient $CD4^+$ cells induced a dysregulated ileocolitis in $RAG1^{-/-}$ mice (Fig. 7J and K). Collectively, these data underscore a critical and previously unappreciated role for CCR7 in the regulation of small intestinal T cell development, intestinal retention, and lymphatic recirculation.

CCR7 deficiency in $TNF^{\Delta ARE}$ mice induces multiorgan inflammation and associated pathology

Whereas pathology is restricted to the terminal ileum in $TNF^{\Delta ARE}/CCR7^{+/+}$ during active disease at 8 wk of age, we sought to assess if the exacerbated inflammation observed in $TNF^{\Delta ARE}/CCR7^{-/-}$ mice allowed for a loss of peripheral tolerance and the development of extraintestinal manifestations. Indeed a multiorgan inflammation and panenteritis were also observed by 8 wk of age in the ΔARE/$CCR7^{-/-}$mice (**Fig. 8A**). With the exception of solid organs, such as the brain and kidney, CCR7 deficiency resulted in a marked inflammatory infiltrate in all

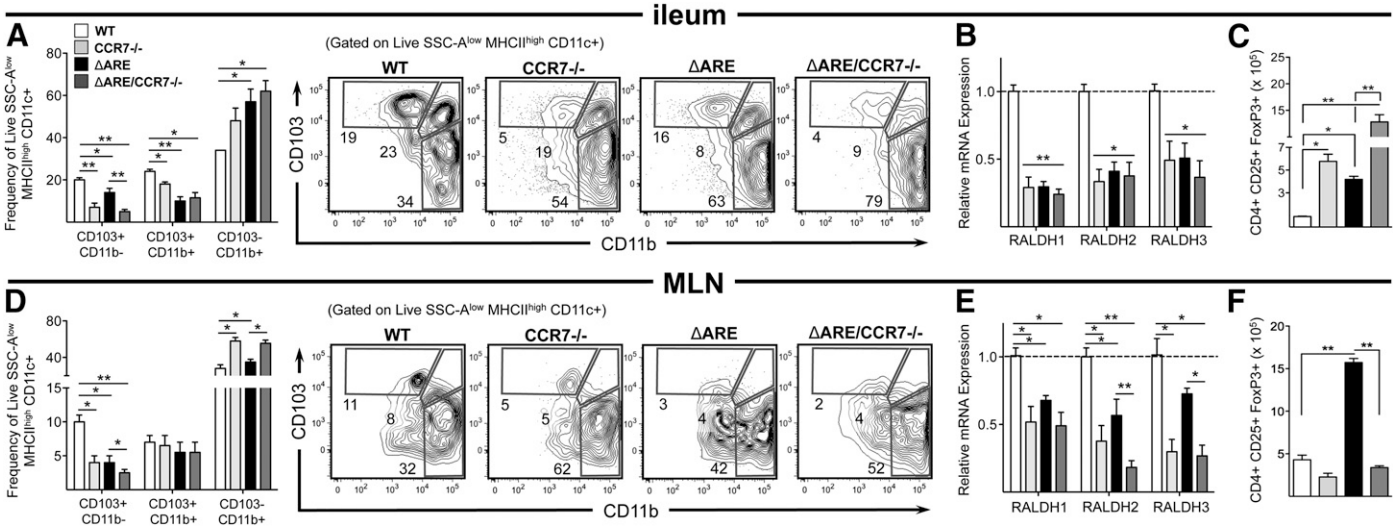

Figure 5. Changes in CD103$^+$ versus CD11b$^+$ DC subsets, loss of RALDH enzymes, and altered T_{reg} profile in ΔARE/CCR7$^{-/-}$ mice. (A) Frequency of ileal CD103$^+$ and CD11b$^+$ DC population and representative flow cytometry plots of ileal CD103$^+$ and CD11b$^+$ DC. SSC-A, Side-scatter-area. (B) Relative mRNA expression analysis of ileal RALDHs (RALDH1, RALDH2, RALDH3) from indicated genotypes. (C) Absolute numbers of CD4$^+$CD25$^+$FoxP3$^+$ T_{regs} from the ileum of CCR7-sufficient or -deficient WT and ΔARE/CCR7$^{+/+}$ or ΔARE/CCR7$^{-/-}$ mice. (D) Frequency of MLN CD103$^+$ and CD11b$^+$ DC population. (E) Relative mRNA expression analysis of RALDHs (RALDH1, RALDH2, RALDH3) in the MLN from indicated genotypes. (F) Absolute numbers of CD4$^+$CD25$^+$FoxP3$^+$ T_{regs} from the MLN of CCR7-sufficient or -deficient WT and ΔARE/CCR7$^{+/+}$ or ΔARE/CCR7$^{-/-}$ mice. Experiments were performed from indicated compartments and genotypes at 8–10 wk of age. Data expressed as mean ± SEM from 3 independent experiments; $^*P < 0.05$; $^{**}P < 0.01$ (n = 4–6 mice/strain, ANOVA).

mucosal sites and a panenteritis of the gastrointestinal tract (Fig. 8A and B). The extent of multiorgan inflammation is also evident from macroscopic assessment at necropsy, where ΔARE/CCR7$^{-/-}$mice display a clear inability to thrive by 8 wk of age (Fig. 8C). Thus, these data are consistent with an absolute requirement for CCR7 in cellular egress out of intestinal tissues and highlight its critical role in controlling intestinal homeostasis.

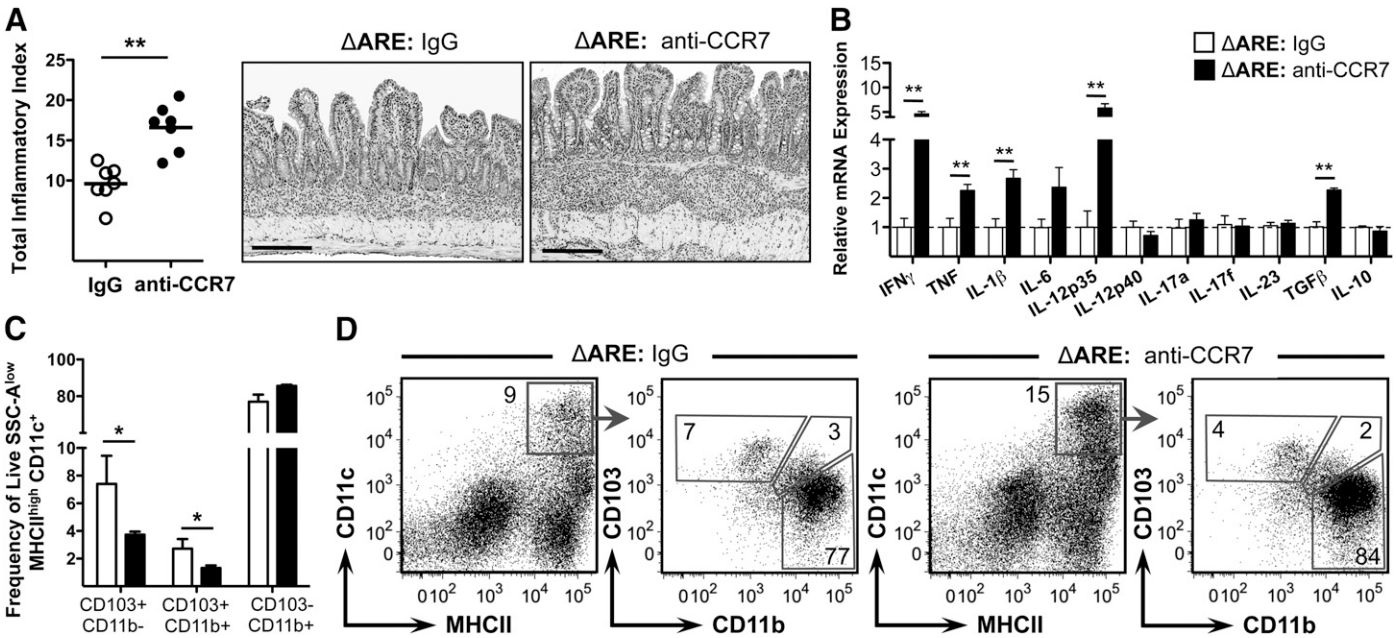

Figure 6. Antibody blockade of CCR7 exacerbates ileitis in TNF$^{\Delta ARE}$ mice by inhibiting lymphatic egress and promoting retention of effector/memory CD4$^+$ T cells. Eight-wk-old TNF$^{\Delta ARE}$ mice received 4 injections (i.p.) of anti-CCR7 (4B12; 500 μg) or IgG2a vehicle every 4 days for 2 wk. (A) Histology indices were assessed for ileal tissue post-treatment with representative micrographs. (B) Cytokine mRNA analysis was performed by real-time PCR on ileal tissues of isotype (IgG2a)- and anti-CCR7-treated mice. (C) Frequency of CD103$^+$ and CD11b$^+$ DC subsets from ilea post-treatment. Data expressed as mean ± SEM; $^*P < 0.05$; $^{**}P < 0.01$ versus IgG2a from 2 independent experiments (n = 6–7 mice/treatment). (A and B) t-test versus IgG2a; (C) ANOVA). Original scale bars, 100 μm.

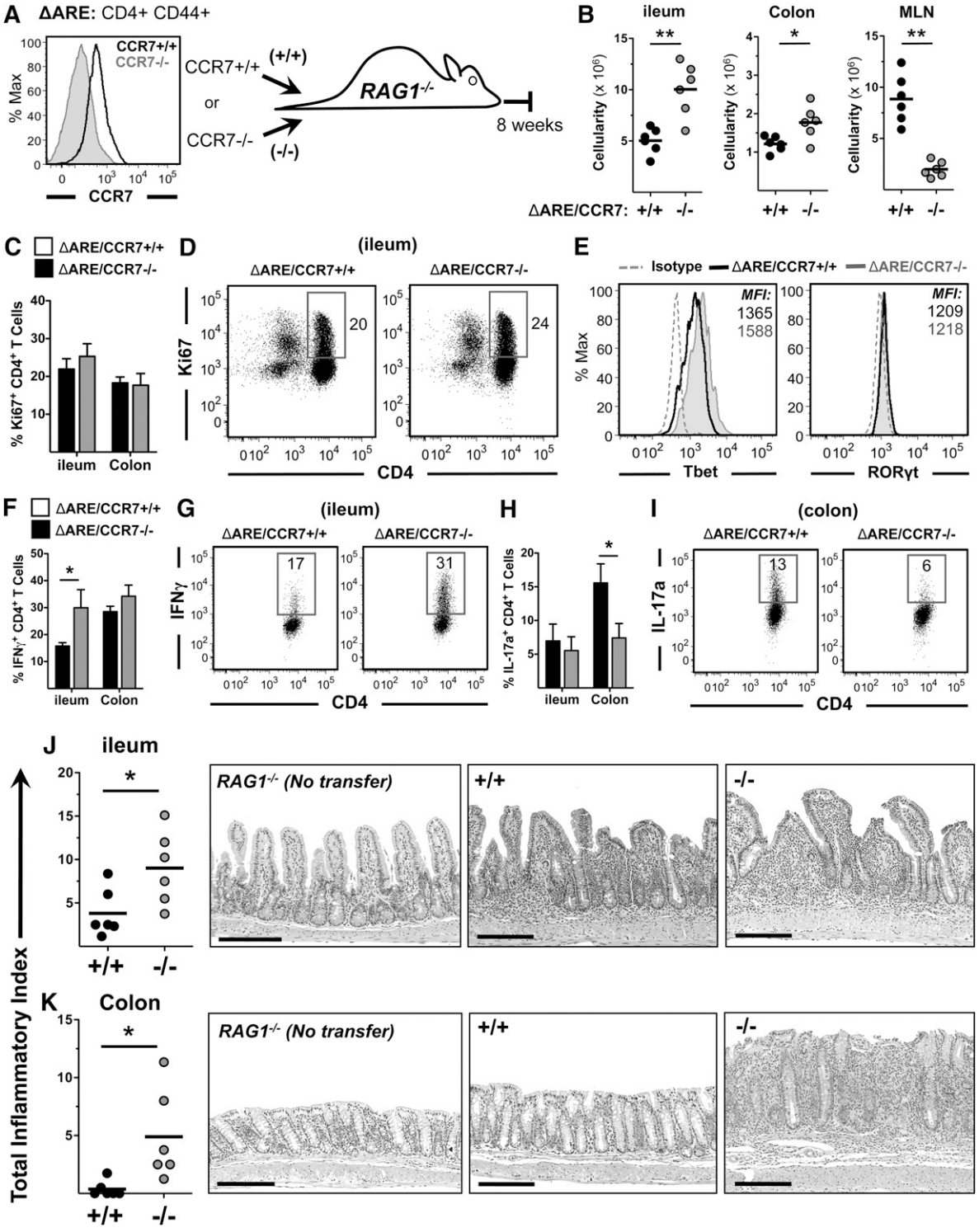

Figure 7. CCR7-deficient CD4$^+$ effector T cells from TNF$^{\Delta ARE}$ mice drive a dysregulated ileo-colitis following adoptive transfer into RAG1$^{-/-}$ mice. CD4$^+$ T_{EM} (CD44^{high} CD62L^{neg}) from the spleen of 4- to 6-wk-old ΔARE/CCR7$^{+/+}$ and ΔARE/CCR7$^{-/-}$ mice were isolated by FACS and adoptively transferred (1×10^6; i.p.) into RAG1$^{-/-}$ recipients. (A) Representative assessment of CCR7 on adoptively transferred CD4$^+$ T cells by flow cytometry. (B) Cell counts were assessed 8 wk post-transfer in ilea, colon, and MLN. (C and D) Percentage of Ki67$^+$ in proliferating CD4$^+$ T cells in the ilea and colon and representative flow cytometry plots from the ileum. (E) Representative flow cytometry histograms of ileal Tbet$^+$ and RORγt$^+$ CD4$^+$. MFI, Mean fluorescence intensity. (F and G) Percentage of ileal IFN-γ$^+$ CD4$^+$ T cells and representative flow cytometry plots. (H and I) Percentage of colonic IL-17a$^+$ CD4$^+$ and representative flow cytometry plots. (J and K) Total inflammatory indices from adoptively transferred RAG1$^{-/-}$ recipient ilea and colons. Data expressed as mean ± SEM; *$P < 0.05$; **$P < 0.01$ versus ΔARE/CCR7$^{+/+}$ from 2 independent experiments (n = 6–7 mice/treatment). (B, J, and K, t-test; F and H, ANOVA). Original scale bars, 100 μm.

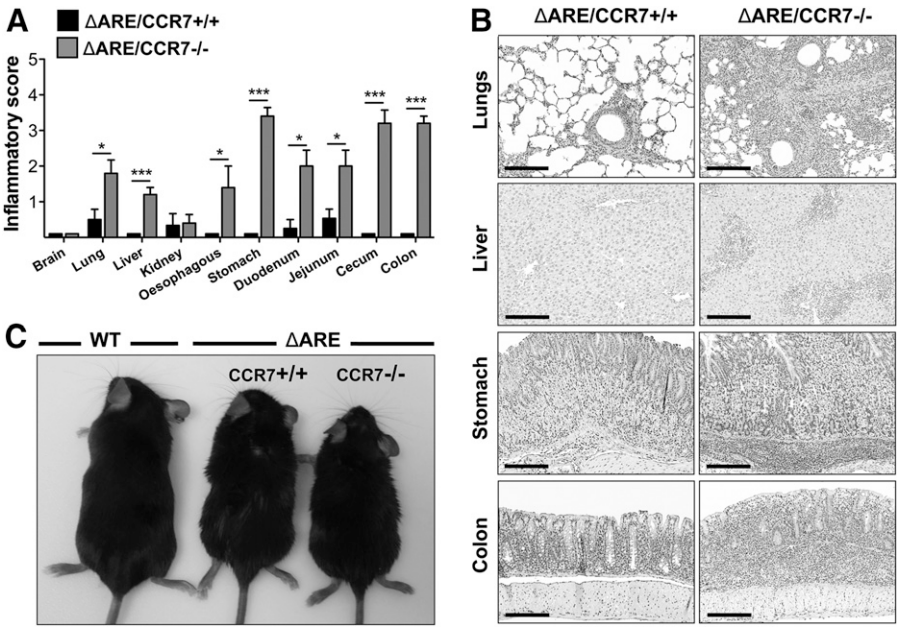

Figure 8. ΔARE/CCR7$^{-/-}$ mice induce multiorgan inflammation and associated pathology. (A) Histologic assessment and pathologic evaluation of ΔARE/CCR7$^{+/+}$ and ΔARE/CCR7$^{-/-}$ mice at 10 wk of age. (B) Representative micrographs of tissue H&E from ΔARE/CCR7$^{+/+}$ and ΔARE/CCR7$^{-/-}$ mice. (C) Representative macroscopic assessment at necropsy of ΔARE/CCR7$^{-/-}$ mice displays a clear inability to thrive by 8 wk of age. Data expressed as mean ± SEM; $*P < 0.05$; $***P < 0.001$ versus age-matched ΔARE/CCR7$^{+/+}$ counterparts (n = 3–5 mice/strain, t-test). Original scale bars, 100 μm.

DISCUSSION

With the recent U.S. Food and Drug Administration-approval of Vedolizumab, the targeting of leukocyte traffic to the intestine has become the next frontier for the treatment of IBD [18, 19]. Natalizumab and Vedolizumab target one of many potentially "drugable" steps within the lymphocyte adhesion cascade; thus, we may envision that additional molecular targets will be identified within this pathway. It is likely that in IBD, there is not only excessive recruitment of proinflammatory T_{EM} to the intestine but also, disproportionate retention within the LP that perpetuates the chronic inflammatory process. Whereas dysregulated recruitment of effector T_H1/T_H17 CD4$^+$ has been implicated in driving disease in CD [20, 21], the specific determinants that control its retention within the intestine and egress through lymphatics remain poorly understood. In this regard, CCR7 serves as a master rheostat for T cell responses, as it guides mature antigen-presenting DCs and naïve T cells to and within lymphoid organs [22]. However, to date, a role for CCR7 in controlling the T cell retention/egress under intestinal homeostasis and inflammation remains poorly defined.

A central dogma had been that homing of cytokine-producing effector CD4$^+$ T cells to nonlymphoid tissues and their subsequent egress was independent of CCR7, a molecule expressed predominantly by naïve T cells to traffic to secondary lymphoid organs [23, 24]. More recent data have challenged that theory and identified a clear ability of cytokine-producing CD4$^+$ T cells to use CCR7 for lymphatic egress and recirculation [4, 5, 25]. We have demonstrated previously that CD4$^+$ T_{EM} subsets are crucial for driving the chronic phase of ileitis in the TNF$^{\Delta ARE}$ model, as evidenced by adoptive-transfer studies in lymphopenic mice [13, 26]; additionally, our data have indicated that LP CD8$^+$ T cell subsets play a redundant role in the pathogenesis of ileitis (contrary to previously published reports [27]) but are critical for the T cell-mediated immunoregulation of disease [26]. Whereas CD8$^+$ T cell and CD19$^+$ B cell subsets can express CCR7 to varying degrees [9] and are dysregulated by the loss of CCR7 in TNF$^{\Delta ARE}$ (Supplemental Fig. 1), based on our data that show maximal retention of CD4$^+$ T_{EM} subsets and our previous work implicating this subset in disease pathogenesis [13], we focused here on understanding the regulation of CD4$^+$ T_{EM} by CCR7.

In an attempt to discriminate CD4$^+$ T_{EM} ileal retention and lack of lymphatic egress from dysregulated $T_H1/T_H17/T_{reg}$ polarization with CCR7 deficiency, we performed a series of adoptive-transfer experiments with CCR7-deficient effector T cells into lymphopenic RAG1$^{-/-}$ mice. Whereas CD4$^+$CD44$^+$ T_{EM} from TNF$^{\Delta ARE}$ adoptively transferred disease, ΔARE/CCR7$^{-/-}$ T_{EM} displayed exacerbated ileitis in addition to dysregulated colitis. Consistent with our data with congenital CCR7 deficiency, transfer of CCR7-deficient CD4$^+$ T_{EM} promoted a marked generation and retention of T_H1 within the ileum and impaired egress to the MLN. This raises the question as to why there is a predominant T_H1 profile in ΔARE/CCR7$^{-/-}$ and a paucity of T_H17. Recent work, assessing the anatomic locations responsible for the homeostatic proliferation of gut-homing $\alpha4\beta7^+$ CD4$^+$ T cells, has shown that fast-dividing, gut-homing CD4$^+$ T cells were composed predominantly by an IL-17a$^+$-producing cohort in the MLN and ileum, whereas splenic-derived, fast-dividing CD44$^+$ CD4$^+$ T cells were mainly composed of IFN-γ^+ [28]. Whereas this is not surprising, given the recent identification of the small intestine as the major site for T_H17 development [29, 30], it would suggest that splenic (or "peripherally")-derived IFN-γ^+ CD4$^+$ T cells override this anatomic divide when CCR7-mediated trafficking to the MLN is lost. This was implicated further when mice devoid of a MLN fail to develop a T_H17 response, but IFN-γ^+ CD4$^+$ T cells are unaffected [28]. Indeed, a T_H1 profile has been observed in CCR7-deficient mice with gastritis after 1 yr [31]. However, based on our data that show no measurable difference in the levels of Ki67$^+$ proliferation in gut-homing, CCR7-deficient CD4$^+$ T cells and previous data showing no appreciable loss of colitogenic

$CD4^+$ T cells in mice deficient in spleen and MLN [32, 33], altered proliferation/differentiation of T_H1 may not fully explain our ΔARE/$CCR7^{-/-}$ phenotype. Indeed, increased T_H1-mediated chemotaxis may augment this pathology, based on our data showing increased "T_H1" chemokines in ΔARE/$CCR7^{-/-}$ mice during peak disease. Whereas not assessed in this study, increased survival signals for T_{EM}, such as IL-2, IL-7, and IL-15, may also predominate the ΔARE/$CCR7^{-/-}$ ileum.

One other possible reason for the dysregulated retention/egress of T_H1 $CD4^+$ in ΔARE/$CCR7^{-/-}$ ileitis may be mediated by effects on DCs, which use CCR7 to home to lymph nodes and educate naïve $CD4^+$ T cells [7, 10, 17]. Indeed, recent clinical data have reported altered cytokine profiles and T cell-generating capacities of MLN DCs from patients with IBD [3]. A subset of intestinal DC [defined by their expression of CCR7 and integrin αE (CD103)] produces RA and in the presence of TGF-β, promotes the induction of T_{regs} [34]. This $CD103^+$ DC subset expresses high levels of RALDH2, an enzyme critical to metabolizing retinaldehyde to RA [34]. We have demonstrated previously that expansion of $CD103^+$ regulatory DCs by Flt3L or exogenous supplementation of RA attenuates ileitis in $TNF^{\Delta ARE}$ mice [15, 16]. As such, it is worth noting that the $TNF^{\Delta ARE}$ mouse model (as with human CD) is highly responsive to imbalances in regulatory DC and T_{reg} populations. In addition to the generation and retention of T_H1 $CD4^+$ T cells and heightened ileitis in ΔARE/$CCR7^{-/-}$mice, we observed reduced numbers of $CD103^+$ regulatory DCs in the ilea and MLN during active ileitis. As $CD103^+$ DC drives the generation of T_{reg} in GALT [34, 35], it is very plausible that the observed reduction in RALDH2 and RALDH3 enzymes, in addition to $CD25^+$ $FoxP3^+$ T_{regs} in the MLN of ΔARE/$CCR7^{-/-}$, compounds their dysregulated inflammation. However, one point to note is that whereas $CCR7^+$ $CD103^+$ DCs are indeed critical for generating T_{regs} and $CCR9^+$ $\alpha4\beta7^+$ gut-homing T cells, they do not affect the relative expression of IFN-γ^+ production from antigen-specific $CD8^+$ T cells. This is evidenced by data showing that OVA-specific OT-I $CD8^+$ T cells show similar proliferation and generation of IFN-γ^+ effectors regardless of whether they are stimulated with $CD103^-$ or $CD103^+$ DCs [36]. Currently, whereas commensal segmented filamentous bacteria have been implicated in inducing gut-homing T_H17 [28, 30, 37], the factors controlling intestinal-specific T_H1 are less well defined.

The predominant T_H1 profile in ΔARE/$CCR7^{-/-}$ mice seems somewhat discrepant from previous reports of T cell profiles in other organs. Specifically, CCR7-deficient $CD4^+$ T cells secrete IL-4 preferentially and potentiate an allergic asthma with elevated IL-4, IL-13, and IgE [17, 38]. Conversely, CCR7 signaling via CCL21 on T cells drives a T_H1-favorable response via increased IFN-γ [39]. In addition, CCL19 and CCL21 induce the T_H1-polarizing cytokine IL-12 from DCs [40]. These data point toward a context-, tissue-, and stimuli-dependent role for CCR7 in controlling T_H responses. It is worth noting that a microbial dysbiosis has been identified in a $TNF^{\Delta ARE}$ model of CD [41]. Antibiotic-induced dysbiosis also results in a marked translocation of noninvasive commensal bacteria to the draining MLN with an increased preference for the production of IFN-γ [42]. Thus, it is plausible that the dysregulated, T_H1-driven inflammation in ΔARE/$CCR7^{-/-}$ mice is mediated, in part, by a lack of $CD103^+$ DC/T_{reg}-mediated tolerance in the draining MLN and/or a heightened bacterial influx.

A striking phenotype of the ΔARE/$CCR7^{-/-}$ mice is a widespread multiorgan inflammation and panenteritis, which these mice develop after weaning. Whereas $TNF^{\Delta ARE}$ mice display pathology restricted to the ileum, CCR7 deficiency renders T cells unable to recirculate, "log jamming" within effector sites and driving dysregulated multiorgan inflammation. Of note, multiorgan inflammation has been reported in older, CCR7-deficient mice [31, 43]; however, our data emphasize the nonredundant role that lymphocyte egress/retention signals have on the early events leading to induction of chronic intestinal inflammation. These data also underscore the critical role played by CCR7 in orchestrating peripheral tolerance and self-reactivity [31, 44, 45]. Whereas $FoxP3^+$ $CD4^+$ T_{regs} are increased in the ilea of ΔARE/$CCR7^{-/-}$ mice, they are devoid in the MLN compared with their ΔARE/$CCR7^{+/+}$ counterparts. Thus, it is also plausible that dysregulated generation of $CD4^+$ T_{EM} in the MLN and defective control of those T cells by T_{regs} may explain the augmented ileitis observed in ΔARE/$CCR7^{-/-}$ mice. T_{regs} play a crucial role in the homeostatic proliferation of gut-homing T cells, in addition to suppressing the development of colitogenic T cells in an OX40/OX40 ligand-dependent manner [28, 46]. Indeed, aberrant lung inflammation in CCR7-deficient mice was also abrogated following adoptive transfer of WT T_{regs} [47]. Furthermore, as cytokines produced by $CD4^+$ effector T cells also regulate diverse functions within peripheral lymph nodes, such as Ig class-switching and B cell help, regulation of T cell differentiation, and DC cross-talk, the paucity of $CD4^+$ T_{EM} in the MLN of CCR7-deficient mice may further exacerbate the lack of immunoregulation during ileitis in $TNF^{\Delta ARE}$ mice via multiple mechanisms.

Recent data have suggested that during the course of an inflammatory insult in the skin, CCR7 plays a crucial role during the acute phase but is redundant in controlling lymphocyte egress during chronic inflammation [48]. A redundant role for CCR7 was also observed during induction of autoimmune encephalitis and allergic asthma [49, 50]. In discordance with these observations, our data point toward a critical role for CCR7 in regulating the balance between T_{EM} retention versus lymphatic egress and highlight the importance of homeostatic lymphatic function in the intestinal mucosa. This may also underscore the differences observed between inducible injury models of inflammation (e.g., Dextran sodium sulfate) and the chronic inflammation that drives ileitis in $TNF^{\Delta ARE}$ mice and human IBD. However, a question remains regarding the exclusive requirement of CCR7 for the homing of T cells to lymph nodes. Both our current data and previous literature [17, 48] indicate that T cells are not completely absent from the lymph nodes of CCR7-deficient mice, suggesting compensatory or overlapping machinery used by T cells to home to draining lymph nodes under homeostatic and inflammatory conditions. Whereas alternative chemokine or selectin receptor-ligand systems may be responsible (e.g., CXCR4-CXCL12, CXCR3-CXCL9/CXCL10, and L-selectin-peripheral node addressin), this remaining population of T cells may also be representative of lymph node follicular T_H. Furthermore, whereas T cells have the

capacity to alter their chemokine receptor profile to migrate into peripheral tissues [51, 52], the molecular machinery and specific stimuli, which induce CCR7 expression, remain to be determined.

Whereas the therapeutic blockade of CCR7 for the prevention of aberrant T cell response to self-antigen in peripheral lymph nodes may have held interest for autoimmune diseases, such as type 1 diabetes [44, 53], or the prevention of lymphatic metastasis in some cancers [54], our data stress a critical role for this chemokine receptor in orchestrating T cell and DC recirculation during murine ileitis and argue against CCR7 blockade as a target for the treatment of IBD.

AUTHORSHIP

E.N.M., M.V., J.C.M., and C.B.C. acquired data and performed data analysis. P.J., G.Y.N., and F.R.B. performed data analysis. E.N.M. and J.R.-N. conceived of and designed the research, drafted the article, and had final approval of the manuscript.

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DISCLOSURES

E.N.M., M.V., J.C.M., C.B.C., P.J., and J.R.-N. disclose no conflicts of interest. For the duration of these studies, F.R.B. and G.Y.N. were employees of Amgen; F.R.B. is currently an employee at the Pfizer Center for Therapeutic Innovation, and G.Y.N. is currently an employee at Zymeworks.

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KEY WORDS:
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