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The chemokine receptor CCR9 is required for the T cell-mediated regulation of chronic ileitis in mice

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Abstract

Background & Aims—A balance between effector and regulatory (Treg) T-cell responses is required to maintain intestinal homeostasis. To regulate immunity, T cells migrate to the intestine using a combination of adhesion molecules and chemokine receptors. However, it is not known whether the migration pathways of effector cells and Tregs are distinct or shared. We sought to determine whether interaction between the chemokine CCR9 and its receptor, CCL25, allows effectors or Tregs to localize to chronically inflamed small intestine.

Methods—Using a mouse model that develops Crohn's-like ileitis (TNFΔARE mice) we examined the role of CCL25–CCR9 interactions for effector and Treg traffic using flow cytometry, quantitative reverse transcription PCR, immunohistochemistry, immunoneutralization, and proliferation analyses.

Results—In TNFΔARE mice, expression of CCL25 and the frequency of CCR9-expressing lymphocytes increased during late-stage disease. In the absence of CCR9, TNFΔARE mice developed exacerbated disease, compared with their CCR9-sufficient counterparts, which coincided with a deficiency of $CD4^+/CD25^+/FoxP3^+$ and $CD8^+/CD103^+$ Tregs within the intestinal lamina propria and mesenteric lymph nodes. Furthermore, the $CD8^+/CCR9^+$ subset decreased the proliferation of $CD4^+$ T cells *in vitro*. Administration of a monoclonal antibody against CCR9 to TNFΔARE mice exacerbated ileitis *in vivo*, confirming the regulatory role of $CD8^+/CCR9^+$ cells.

Conclusions—Signaling of the chemokine CCL25 through its receptor CCR9 induces Tregs to migrate to the intestine. These findings raise concerns about the development of reagents to disrupt this pathway for the treatment of patients with Crohn's disease.

Keywords

CD; regulatory T cells; inflammation; immune system

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Introduction

CD and ulcerative colitis result from an imbalance between effector and Treg responses[1]. Although agents that target lymphocyte recirculation have proven therapeutic efficacy in CD (e.g. Natalizumab)[2], none specifically target the traffic of effector T cells, while sparing that of Tregs. Further understanding of the mechanisms that mediate effector and Treg recirculation may result in the development of novel therapeutics with better specificity than those in clinical use.

Lymphocyte recirculation to the intestine is mediated by integrin $\alpha_4\beta_7$, which interacts with Mucosal Addressin Cell Adhesion Molecule-1 (MAdCAM-1)[3]. However, it is not known how T cells recognize specifically the small intestinal microenvironment to maintain the preferential small intestinal localization observed in CD. The expression of CCL25 (TECK) is restricted to the small intestine and thymus, providing a potential molecular mechanism for a trafficking dichotomy into distinct small and large intestinal compartments[4].

CCL25 attracts cells that express its cognate receptor CCR9. This might be of clinical relevance, as patients with small intestinal CD have increased numbers of $CCR9^+$ T cells in blood[5]. CCL25 is additionally induced in the liver of patients with primary sclerosing cholangitis[6]. Thus, it is conceivable that CCL25 serves as a chemoattractant for effector $CCR9^+$ T cells or alternatively, CCL25 may recruit Tregs to help dampen dysregulated inflammation. Consistent with this hypothesis, Papadakis *et al.* recently identified a $CCR9^+$ T cell population that produces IL-10[7].

Preliminary trial data (PROTECT1) suggests that a small molecule inhibitor of CCR9 (CCX282, Traficet-EN, ChemoCentryx) might have a therapeutic effect in CD[8]. However, CCR9 deficiency had no effect on the severity of ileitis in TNFΔARE mice[9], whereas Traficet EN prevented disease onset. Thus, there is disagreement between the existing preclinical and clinical evidence.

The TNF ΔAU-rich element (TNFΔARE) model develops terminal ileitis, reminiscent of human CD in its histological features and the pivotal role played by TNF in its pathogenesis[10]. Thus, we further explored the role of the CCR9/CCL25 axis for the trafficking of T cells in this model, as it is the first chemokine and receptor pair that may account for the lifelong terminal ileal localization shared with the human disease. First, we assessed the expression of CCR9 and CCL25 along the time course of the disease. We then generated CCR9-deficient TNFΔARE mice and assessed the severity of ileitis, noticing increased severity, when compared to CCR9-sufficient TNFΔARE mice. Thus we hypothesized that Tregs might be more dependent on CCL25/CCR9 than effectors for homing into small bowel. To further explore this possibility we assessed the frequency of Tregs in CCR9-sufficient and deficient mice and investigated a potential role for CCR9-expressing $CD8^+$ T cells in the regulation of Crohn's-like ileitis. Finally, the effect of CCR9 immmunoneutralization on disease severity was evaluated.

Results

Increased frequency of CCR9-expressing T cells in lymphoid compartments of TNFΔARE mice

The expression of CCR9 was assessed by flow cytometry in T cells isolated from the spleen, MLN and LP of TNFΔARE mice (solid line) during early (4-), peak (8-)(not shown) and late disease (≥20-weeks-of-age), compared with WT littermates (gray histograms)(Figure 1A). Corresponding isotype antibodies (not shown) and CCR9-deficient lymphocytes (discontinuous histograms) were used as controls. At 4- and at 8-weeks-of-age, we observed

that the percentage of CCR9-expressing cells was not significantly different (not shown). However, at 20-weeks-of-age the absolute counts, calculated by multiplying the percentage positive cells by the cell counts of the organs (see supplementary methods), revealed a significant increase in $CD4^+/CCR9^+$ in TNFΔARE mice spleen ($3\pm0.3\times10^6$ vs. $5\pm0.7\times10^6$, $P=0.03$), MLN ($0.6\pm0.7\times10^6$ vs. $2\pm0.2\times10^6$, $P<0.001$) and LP ($1.4\pm0.1\times10^6$ vs. $3\pm0.3\times10^6$, $P=0.005$) compared with WT mice.

Both the percentage ($P<0.05$) and absolute numbers ($26\pm1\times10^6$ vs. $18\pm2\times10^6$, $P=0.01$) of $CD8^+/CCR9^+$ T cells from 20-week-old TNFΔARE mice were lower in the spleen compared with WT. In contrast, within the MLN both the percentage ($P<0.01$) and absolute counts increased nearly five-fold ($2\pm2\times10^6$ vs. $8.5\pm0.2\times10^6$, $P<0.001$). Within the LP both the percentage ($P<0.05$) and absolute counts ($1\pm0.2\times10^6$ vs. $3\pm0.4\times10^6$, $P=0.02$) of $CD8^+/CCR9^+$ T cells also increased (Figure 1A). Thus there is redistribution of CCR9-expressing $CD8^+$ T cells and accumulation within the ilea and MLN in mice with ileitis.

Ileal expression of CCL25 increased in TNFΔARE mice

To determine whether CCL25 expression was modulated by inflammation, we assessed mRNA transcripts and protein levels in WT and TNFΔARE mice. CCL25 mRNA levels showed a decreasing gradient from proximal to distal small intestine in WT mice[11]. By contrast, expression in ilea from 20-week-old TNFΔARE mice was increased four-fold compared to WT littermates (Figure 1B, $P<0.001$). CCL25 was not significantly different at 4 weeks, regardless of the mRNA source, but was increased 4-fold at 20-weeks-of-age in whole ilea (Figure 1C, $P<0.05$) and 15-fold in enriched epithelial extract (Figure 1D, $P<0.001$). Immunohistochemistry confirmed that CCL25 protein localized predominantly to epithelia and increased at 20- compared with 4-week-old mice. Preincubation of the primary with recombinant CCL25 confirmed the specificity of the signal (Figure 1E, control) and no signal was observed in sections incubated with the secondary without preincubation with the primary (not shown). Thus, expression of CCL25 increased specifically in inflamed terminal ilea and is restricted to intestinal epithelia.

CCR9 deficiency exacerbates chronic murine Ileitis

It has been recently reported that CCR9 deficiency had no effect on the severity of TNF-driven ileitis in TNFΔARE mice[9]. This result was unexpected as CCR9/CCL25 are believed to play a critical role for small intestinal homing[12]. Thus, we generated CCR9-deficient TNFΔARE mice[13]. The full spectrum of disease (early, peak, late) was evaluated in CCR9-sufficient (ΔARE) and CCR9-deficient ($\Delta ARE/CCR9^{-/-}$) mice (Figure 2A). Active indices revealed more severe ileitis at 4-weeks-of-age in $\Delta ARE/CCR9^{-/-}$ compared with ΔARE mice, at 8- and at 20-weeks-of-age. A significant increase in the chronic index was also observed at 8- and at 20-weeks-of-age in $\Delta ARE/CCR9^{-/-}$ mice. Villus distortion was significantly different at 20-weeks-of-age, whereas higher total indices were observed in $\Delta ARE/CCR9^{-/-}$ mice at all time points assessed: 4-(0.7 ± 0.2 vs. 0.2 ± 0.1, $P<0.05$), 8-(18 ± 1 vs. 14 ± 2, $P<0.05$) and 20-weeks-of-age (22 ± 1.3 vs. 16 ± 1.3, $P<0.01$) (Figure 2A). Representative micrographs showed distortion of intestinal architecture and more pronounced transmural infiltrates in $\Delta ARE/CCR9^{-/-}$ mice compared with TNFΔARE mice (Figure 2B). Thus, CCR9 deficiency exacerbates TNF-mediated ileitis.

Decreased $CD4^+/CD25^+/FoxP3^+$ Tregs in the MLN and LP of $\Delta ARE/CCR9^{-/-}$ mice

To examine whether Tregs rely on CCR9/CCL25 interactions to traffic to ileum and MLN in ileitis we assessed the frequency of $CD4^+/CD25^+/FoxP3^+$ Tregs in $\Delta ARE/CCR9^{-/-}$ mice compared with WT and ΔARE mice (Figure 3). The percentage of Tregs increased in the spleen of 20-week-old ΔARE mice compared with WT littermates but not in 8-week-old mice. Within this compartment, there was no difference in the percentage of Tregs in CCR9-

sufficient or -deficient TNFΔARE mice at 8- and 20-weeks-of age. Yet in the MLN, Tregs increased at 8 weeks in ΔARE mice compared with WT (10±0.2 vs. 14±0.7, $P<0.01$) and decreased back to the level of WT mice in ΔARE/CCR9$^{-/-}$ mice (12±0.1, $P<0.05$ vs. ΔARE). Also at 20-weeks-of-age Tregs increased in ΔARE MLN compared to controls decreasing in frequency in ΔARE/CCR9$^{-/-}$ mice. This was also the case in the LP, where Tregs increased at 8 weeks in ΔARE mice compared with WT and decreased in ΔARE/CCR9$^{-/-}$ mice and at 20-weeks (Figure 3A). Representative contour plots highlight the relative deficiency of Tregs in ΔARE/CCR9$^{-/-}$ mice (Figure 3B). Normalization by cell count confirmed these differences (not shown). Thus, Tregs accumulate in the inflamed MLN and LP of TNFΔARE mice compared with WT controls whereas this is not observed in CCR9-deficient animals, despite their more severe inflammation.

Increased CD8$^+$/CD44$^+$ effectors and decreased CD8$^+$/CD103$^+$ T cells during peak and chronic disease in CCR9-deficient TNFΔARE mice

We and others have previously described the effector functions of CD8$^+$/CD44$^+$ T cells in TNFΔARE mice and shown that CD44 deficiency attenuates ileitis[14, 15]. Conversely CD8$^+$/CD103$^+$ T cells inhibit the proliferation of CD4$^+$ T cells *in vitro* and reduced their ability to induce ileitis *in vivo*[16]. Thus an imbalance of these populations may lead to exacerbation of IBD. To determine the effect of inflammation and CCR9 deficiency on the distribution of these subsets, we examined their frequency in WT, ΔARE and ΔARE/CCR9$^{-/-}$ mice. We observed increased effector CD8$^+$/CD44$^+$ T lymphocytes in the spleen of ΔARE compared with WT and a further increase in ΔARE/CCR9$^{-/-}$ at 8- and 20-weeks-of-age. Also within the LP there were significant increases in CD8$^+$/CD44$^+$ T cells between ΔARE and ΔARE/CCR9$^{-/-}$ mice at both 8- and 20-weeks-of-age (Figure 4A, C).

Conversely, CD8$^+$/CD103$^+$ T cells decreased in ΔARE/CCR9$^{-/-}$ mice spleen compared with ΔARE at 8-(49±3 vs. 51±3, N.S. vs. 30±6, $P<0.05$) and at 20-weeks-of-age. In the MLN there was an increase in CD8$^+$/CD103$^+$ T cells between WT and ΔARE, but no change in ΔARE/CCR9$^{-/-}$ at 8 weeks (59±2 vs. 69±3, $P<0.05$ vs. 69±2, N.S.), whereas at 20-weeks-of-age the CD8$^+$/CD103$^+$ subset increased in ΔARE compared to WT mice and decreased in ΔARE/CCR9$^{-/-}$ mice. Within the LP the CD8$^+$/CD103$^+$ T cells increased between WT and ΔARE mice and decreased in ΔARE/CCR9$^{-/-}$ at 8-(40±1 vs. 63±4, $P<0.05$ vs. 47±2, $P<0.05$) and 20-weeks-of-age (Figure 4B). Representative contour plots demonstrate that CCR9 deficiency results in an imbalance between effector and regulatory subsets in ΔARE/CCR9$^{-/-}$ mice (Figure 4C).

Preferential CCR9 expression by CD4$^+$/CD25$^+$/FoxP3$^+$ and CD8$^+$/CD103$^+$ Treg cells

As Tregs failed to accumulate within the LP of CCR9-deficient mice relative to CCR9-sufficient mice (Figures 3, 4), we examined whether such impairment may be due to their reliance on CCR9 to home to small intestine. We compared the surface expression of CCR9 in regulatory subsets to those of cells not expressing Treg markers in 20-week-old TNFΔARE mice. The percentage of CCR9-expressing cells was higher in CD4$^+$/CD25$^+$/FoxP3$^+$ Treg cells from spleen (red histogram) compared with their CD4$^+$/CD25^{neg}/FoxP3^{neg} T cell counterparts (blue histogram). Expression within MLN was also higher and the LP yielded similar results (Figure 5A). Furthermore, within CD8$^+$/CD103$^+$ T cells (red histogram) the percentage of CCR9-expressing cells was higher compared with CD8$^+$/CD44$^+$ T cells (blue histogram) in spleen, MLN and in LP (Figure 5B). Cells isolated from CCR9-deficient mice (discontinuous histograms) served as controls. Together this data show that CCR9 is preferentially expressed by Tregs in TNFΔARE mice and explains the deficiency of CD4$^+$/FoxP3$^+$ and CD8$^+$/CD103$^+$ T cells in the ileal LP of ΔARE/CCR9$^{-/-}$ mice.

CD8$^+$/CCR9$^+$ T cells from TNFΔARE mice decreased proliferation of CD4$^+$ T cells in a dose-dependent manner

We have previously shown that the CD8$^+$/CD103$^+$ subset inhibit CD4$^+$ T cell proliferation *in vitro* and induction of ileitis *in vivo*[16]. Here we additionally show that fifty to eighty percent of these cells co-express CCR9 (Figure 5). To further assess a potential regulatory role of the CD8$^+$/CCR9$^+$ subset, we investigated whether the CD8$^+$/CCR9$^+$ T cells isolated from TNFΔARE mice (Figure 6A), could affect the proliferation of CD4$^+$ T cells after anti-CD3/CD28 stimulation. We observed strong proliferation of CD4$^+$ T cells when cultured alone. Addition of a 10:1 CD4$^+$:CD8$^+$/CCR9$^+$ ratio significantly reduced proliferation of CD4$^+$ T cells between 27 and 61% in cell divisions 1 and 4. A 5:1 ratio reduced proliferation from 40 to 74% and a 1:1 ratio maximally inhibited proliferation between 56 and 90% (Figure 6B, C and supplementary table 1). A 1:1 ratio of CD8$^+$/CCR9$^+$ T cells was required to reduce the proliferation of CD4$^+$ T cells if isolated from WT mice, suggesting that the pro-inflammatory environment present in TNFΔARE mice enhances the anti-proliferative capacity of this T cell subset, as we have shown previously for CD4$^+$ T cells from TNFΔARE mice (supplementary Figure 1)[16]. Thus, CD8$^+$/CCR9$^+$ T cells (particularly those isolated from TNFΔARE mice) exert a potent anti-proliferative effect on CD4$^+$ T cells.

Anti-CCR9 mAb exacerbates ileitis in TNFΔARE mice and depletes CD8$^+$/CD103$^+$Tregs

To evaluate the effect of an anti-CCR9 mAb on the severity of ileitis, we administered mAb CW1.2 or its equivalent IgG2a isotype to 4-week-old TNFΔARE mice. Terminal ilea were harvested and the severity of ileitis was assessed, as described[17]. Treatment with anti-CCR9 mAb significantly worsened active, chronic, villus and total indices (3±1 vs. 9±0.4, $P<0.001$) compared with isotype-treated controls (Figure 7A). Flow cytometry analyses showed no significant change in the frequency of CD4$^+$/CD25$^+$/FoxP3$^+$ Tregs, whereas within the CD8$^+$, the CD44$^+$ subset increased while the CD103$^+$ T cells decreased in mice treated with anti-CCR9 mAb compared with isotype-treated mice ($P<0.05$ vs. isotype for all organs)(Figure 7B). Thus, targeting the CCR9/CCL25 pathway alters the ratio of effector and regulatory CD8$^+$ T cells and exacerbates ileitis.

Discussion

Imprinting of T cells with a small intestinal homing address code (i.e. CCR9) is mediated by retinoic acid (RA)[18]. RA in the presence of TGF-β additionally determines the induction of FoxP3[19], while TGF-β also promotes the expression of CD103[20]. Yet although the induction of these regulatory molecules shares common pathways, little is known about the role of the CCL25/CCR9 axis for Treg trafficking and regulation of immune responses in the small intestine. Here we show high concordant expression of CCR9, FoxP3, and CD103 in Tregs, suggesting that their induction might be coupled within inductive compartments. As CCL25 expression is restricted to small intestine, we evaluated its role in the TNFΔARE model that develops inflammation in the distal small intestine. We show that CCR9$^+$ T cells undergo late inflammation-driven expansion. Furthermore, during late disease, the amount of CCR9 mRNA transcripts and CCL25 protein levels increased in TNFΔARE mice ilea, yet CCL25 protein expression remained localized to the ileal epithelium. Evaluation of disease severity in CCR9-deficient mice revealed more severe disease than that of CCR9-sufficient counterparts. Thus, we investigated whether a preferential dependence by Tregs on the CCR9/CCL25 axis for homing into the chronically-inflamed ileum may account for the accelerated disease. We noticed a deficiency of CD4$^+$/CD25$^+$/FoxP3$^+$ and CD8$^+$/CD103$^+$ Tregs within the LP and MLN of CCR9-deficient mice. In addition, Tregs preferentially expressed CCR9 on their surface than their non-regulatory counterparts and the CD8$^+$/CCR9$^+$ subset decreased the proliferation of CD4$^+$ T cells *in vitro*. Moreover, anti-CCR9 antibodies exacerbated disease and altered the pro- to anti-inflammatory CD8$^+$/

$CD44^+$:$CD8^+$/$CD103^+$ T cell ratio, confirming a predominant role for CCR9/CCL25 for the regulation of ileitis.

CCR9-expressing cells are increased in peripheral blood of patients with small bowel CD[5], thus inflammatory signals might modulate their frequency. The expression of CCR9 is known to be induced by RA[18], while a high antigen dose is suppressive[21]. It is not known whether inflammatory signals (e.g. cytokines) might alter CCR9 expression. Our data suggests that during the late disease of TNFΔARE mice either CCR9 is induced (predominantly in $CD8^+$ T cells) or survival/retention signals result in increases in the number of CCR9-expressing cells within the MLN and LP. Furthermore it appears that CCR9 might be upregulated as the $CD8^+$ T cells migrate out of the LP into the MLN and the periphery. Thus, CCR9 expression appears to be a dynamic process, with cells being able to upregulate and downregulate surface expression perhaps after ligand binding, resulting in internalization, as shown with other chemokine receptors (e.g. CCR7). This may account for the lower mean fluorescence intensities observed in the LP[22]. As we utilized CCR9-deficient lymphocytes as controls and two different mAb clones, our studies convincingly report the true expression of this molecule within the different subsets and lymphoid compartments.

As it has been shown for other chemokines, CCL25 is likely transcytosed across endothelium to be presented at the apical surface[23] where it induces adhesion of CCR9-expressing cells[24]. Although CCL25 has been considered a homeostatic chemokine[25] more recent data have led to the suggestion that its expression may be inducible in intestinal inflammation[26, 27]. CCL25 is additionally expressed aberrantly in the liver of patients with primary sclerosing cholangitis[6]. Yet the specific inflammatory signals that induce its expression in intestine or liver remain to be defined. Ericsson and colleagues exposed small intestinal cell lines to TNF and IFN-γ and mice to LPS *in vivo* and although they observed increased expression of other chemokines, CCL25 remained unchanged[28]. Although our data might suggest that TNF (overproduced systemically in TNFΔARE mice), might be responsible, we observed localized induction within the affected terminal ileum rather than generalized small bowel expression, suggesting that perhaps a combination of mediators within the effector organ triggers expression.

Few studies have looked at the role of CCL25/CCR9 pathway in models of IBD and the limited available literature appears discordant. We have previously evaluated its role in the SAMP1/YitFc model, which spontaneously develops Crohn's-like ileitis and found that the therapeutic efficacy of CCR9 immunoblockade was limited to early disease[27]. However, other work has shown that CCR9 deficiency had no effect on the severity of ileitis in TNFΔARE mice[9], whereas a small molecule inhibitor of CCR9 prevented disease onset[29]. By contrast our studies in TNFΔARE mice showed that CCR9 deficiency exacerbates ileitis. Several differences may account for the discrepancy. First, we evaluated the entire course of the disease from 4- to 20-weeks-of-age, whereas the prior study evaluated disease between 8- and 14-weeks-of-age. Second, while the prior study evaluated the histological severity of disease in cohorts of 3-6 mice, our experimental cohorts included a minimum of 17 mice[9]. Finally, in our studies all mouse strains were on C57BL6/J background and the genetic background affects the severity of IBD in animal models[30].

Tregs, defined by their expression of the transcription factor forkhead box P3 (FoxP3)[31] and production of IL-10 accumulate in intestine of patients with IBD[32]. Expansion of $CD4^+$/$FoxP3^+$ T cells has also been reported in animal models[33]. The molecular basis of their recruitment to the inflamed small intestine has yet to be elucidated. There is evidence to support that $CD4^+$/$CD25^+$/$FoxP3^+$ cells from the small intestinal LP may express more CCR9 on their surface than similar cells from the spleen[34] and a subset of $CD4^+$/$CCR9^+$

cells possesses a Treg cytokine profile[7]. We hypothesized that Treg deficiency in CCR9 deficient mice, due to an increased Treg dependence on CCR9 to traffic to the ileum resulted in exacerbated inflammation. In fact Treg frequency in CCR9-deficient mice was similar to that of controls and lower than in TNFΔARE mice, despite their worse inflammation, supporting a reliance on CCR9/CCL25 for Treg traffic to the inflamed small intestine.

$CD8^+$ T cell subsets in TNFΔARE mice have either an effector or a regulatory function[14, 15,16]. The latter are characterized by expression of CD103 [16] which is present murine and human $CD8^+$ T cells that home to mucosal surfaces. $CD4^+$ T cells that express CD103 exert a regulatory role in colitis[35]. We have previously described that the $CD8^+$/$CD103^+$ subset inhibited the proliferation of $CD4^+$ cells *in vitro* and exerted a regulatory role during TNF-mediated ileitis[16]. Here we observe an imbalance between these subsets in CCR9-deficient TNFΔARE mice, which exhibited increased proinflammatory $CD8^+$/$CD44^+$ T cells while displaying decreased pro-regulatory $CD8^+$/$CD103^+$. This imbalance has implications on the course and severity of disease due to the large disparity between effector and regulatory subsets in CCR9-deficient TNFΔARE mice.

$CD45Rb^{high}$ T cells induce colitis in RAG-deficient or SCID mice[30], whereas co-transfer of Tregs prevented and cured IBD[33], highlighting the critical role of Tregs for maintenance of homeostasis. Lymphocyte recruitment to the small intestine requires local expression of CCL25 that interacts with CCR9 on the T cell surface. However, little is known about the specific homing determinants of Tregs. We show that in TNFΔARE mice, $CD4^+$/$CD25^+$/$FoxP3^+$ and $CD8^+$/$CD103^+$ Tregs show preferential expression of CCR9 compared with their non-regulatory counterparts. The potential dependency on this pathway by Tregs for recirculation to terminal ileum may contribute to the observed exacerbation of disease resulting from genetic ablation of CCR9.

We confirmed that CCR9-expressing $CD8^+$ T cells inhibited the proliferation of $CD4^+$ T cells in a dose-dependent manner. To further define whether this was also true *in vivo* we tested the effect on an anti-CCR9 mAb on disease severity. Antibody treatment significantly worsened all histological parameters and significantly reduced the percentage of the $CD8^+$/$CD103^+$ Tregs in ilea and MLN and increased the effector $CD8^+$/$CD44^+$ subset, confirming a critical role for CCR9-expressing cells for the regulation of ileitis.

Recent trials examined the efficacy of Traficet EN, CCX282-B, which targets the CCL25/CCR9 axis in patients with CD[8]. The preliminary results appear promising both for induction and maintenance of remission (http://www.drugs.com/clinical_trials/chemocentryx-reports-traficet-maintains-remission-crohn-s-digestive-week-ddw-2010-conference-9318.html, http://www.medicalnewstoday.com/articles/172216.php). In addition, preclinical data on the preventive effect of Traficet EN has been published[36]. As the chemokine is not expressed in normal or inflamed colon, it is interesting that the above studies did not focused on patient with ileitis but also included colonic and ileocolonic CD. Our observations of exacerbated disease and decreased Tregs after treatment with an anti-CCR9 mAb or genetic ablation of CCR9 supports the hypothesis that CCR9 plays a major role in the trafficking of Tregs to the chronically inflamed small intestine. Thus, the CCR9/CCL25 axis appears to be more important for the regulation of immune responses than for its induction in this TNF-mediated ileitic model.

Materials and Methods

Mice

The B6.129S-Tnf^{tm2Gkl}/Jarn strain was generated as previously described[14], backcrossed to C57BL6/J for over 30 generations and kept under specific pathogen-free conditions. Experimental animals were either heterozygous for the ΔARE mutation or homozygous wild-type (WT), which served non-inflamed controls. CCR9-deficient TNFΔARE mice were generated from CCR9-deficient mice[13] by mating TNFΔARE males to CCR9-/- females on C57BL6/J background for 2 or more generations. CCR9 deficiency was confirmed by genotyping the progeny and by flow cytometry on isolated T cells. Fecal samples were negative for Helicobacter, protozoa and helminthes. All animals were handled according to procedures approved by the institutional committee for animal use.

Tissue collection and histological analyses

The distal ilea (10 cm) were harvested and histological assessment of ileal inflammation was performed by a single pathologist (PJ) in a blinded fashion, using a semi-quantitative scoring system, as described[17].

Real-Time RT-PCR

CCL25 transcript quantification was performed using TaqMan assays (Mm00436443_m1, Applied Biosystems, Foster City, CA) using an ABI PRISM 7300 Sequence Detection System. Each reaction was performed in duplicate. Thermocycling conditions were as per manufacturer's instructions (TaqMan Reagents, product insert). The expression of 18s (Hs99999901-s1, Applied Biosystems) was measured as an endogenous control. The ratio of messenger RNA expression was calculated by the ΔΔCt method (user bulletin no. 2, Applied Biosystems).

Immunohistochemistry

Terminal ilea were snap frozen, sectioned (5 μm), fixed with acetone and incubated with goat polyclonal anti-CCL25 (AF-481, R&D Systems, Minneapolis, MN) or goat IgG, with or without recombinant mouse CCL25 (R&D Systems). Rabbit anti-goat horseradish peroxidase–labeled antibody was used as secondary (Vector Laboratories, Burlingame, CA). Normal rabbit sera was used to reduce nonspecific binding (Sigma Chemical Co, St Louis, MO).

Lymphocyte isolation

Splenocytes, MLN, and lamina propria mononuclear cells were isolated as previously described[27].

Flow cytometry and intracellular staining

Cells were incubated with fluorescent rat or hamster anti-mouse antibodies with the following specificities CD4 (GK1.5, RM4-5), CD8 (53-6.7), CD19(6D5), CD25 (PC61), CD44 (IM7), CD62L (MEL-14), CD103 (2E7), (BD Biosciences, San Jose CA, USA), FoxP3 (FJK-16s)(e-Biosciences, San Diego CA), and CCR9 (242503) (R&D Systems) or CW-1.2 (e-Biosciences). FoxP3 staining was performed as per manufacturer's instructions (e-Biosciences). Additional controls included cells isolated from CCR9-deficient mice while positive controls were collected from WT thymi. Cells were washed and fixed with 2% paraformaldehyde and seven/eight color analyses were performed using the BD FACSCanto II (BD Biosciences). Cell sorting was performed using the Moflo XDP (Beckman Coulter, Inc, Fullerton, CA). Further analyses were performed using FLOWJo software (Tree Star Inc, Ashland, OR).

In vitro T cell proliferation assay

Splenic and MLN $CD4^+$ and $CD8^+/CCR9^+$ T cells were isolated from TNFΔARE mice, $CD19^+$ cells were depleted using anti-mouse CD19 microbeads (Miltenyi Biotec, Auburn, CA). The flow-through was stained with anti-mouse CD4, anti-mouse CD8 and anti-mouse CCR9. $CD4^+$ and $CD8^+/CCR9^+$ subsets were separated using a Moflo XDP fluorescence-activated cell sorter (Beckman Coulter). $CD4^+$ T cells were stained with CFSE (0.5 μM) and 1×10^6 cells/well were stimulated with anti-CD3/CD28 Dynabeads (Invitrogen, Carlsbad, CA) and cultured for 5 days alone or with $CD8^+/CCR9^+$ T-cells (≥97% pure). Cells were stained with anti-CD4 and analyzed by flow cytometry. Cell proliferation was quantified using the cell proliferation analysis function of FlowJo software (Tree Star Inc.).

Treatment study

TNFΔARE mice received three intraperitoneal doses (200μg) of anti-CCR9 mAb(clone CW 1.2) or isotype every other day for three days. Organs were collected 24 h after the last injection.

Statistics

Statistical analyses were performed using the two-tailed Student's T test. Data were expressed as mean ± standard error of the mean (S.E.M.). Statistical significance was set at $P<0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

IBD	inflammatory bowel disease
CD	Crohn's disease
Treg	regulatory T cell
MLN	mesenteric lymph node
WT	wild type
LP	lamina propria
RA	retinoic acid
mAb	monoclonal antibody

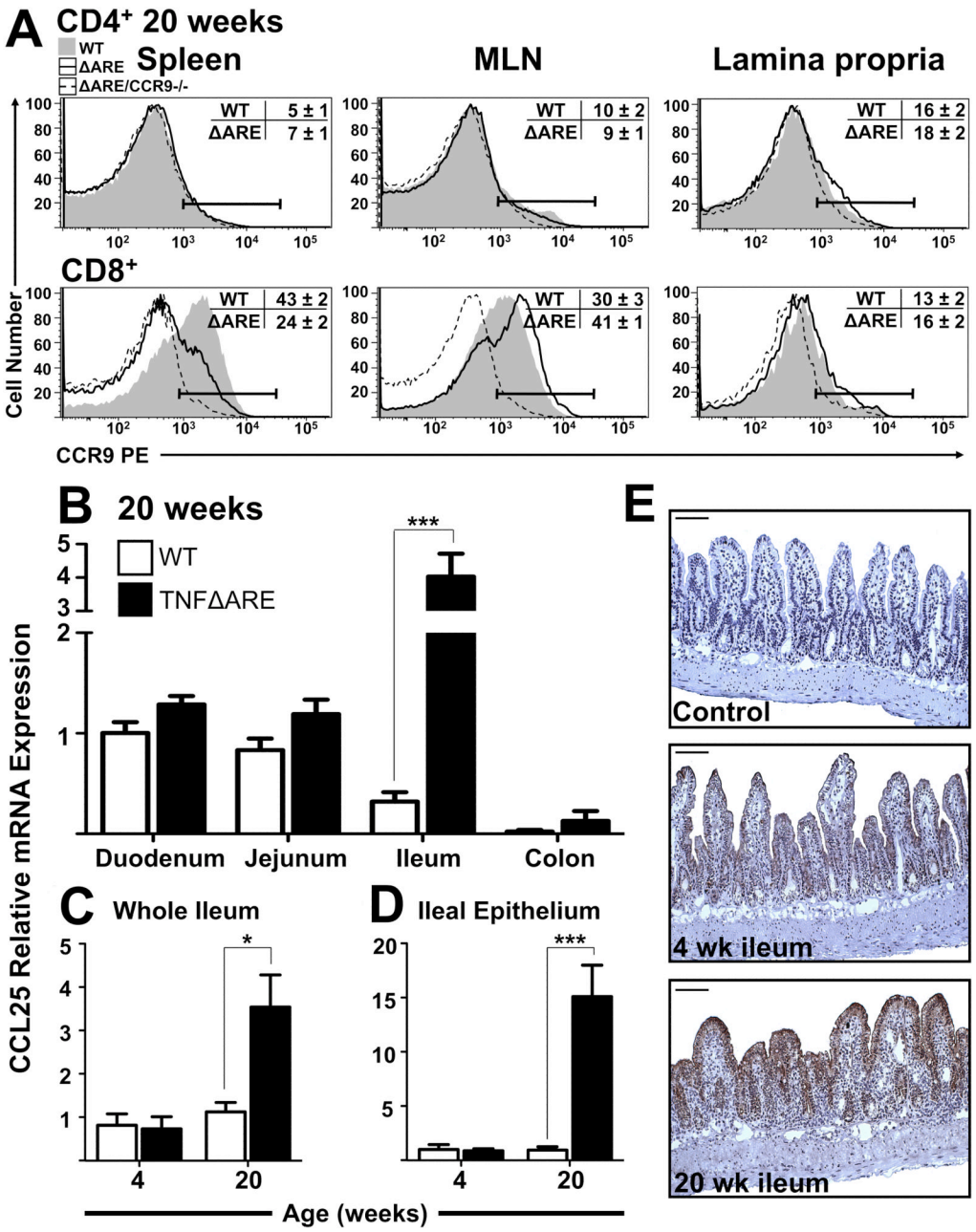

Figure 1.

CCR9 and CCL25 expression in TNFΔARE mice and WT controls. (A) CCR9 expression was analyzed by flow cytometry using CCR9-deficient lymphocytes (dashed line) and isotype-matched antibody (MFI<10^1, not shown) as controls. Cells were gated on FSC, SSC, CD4 or CD8 (Mean ± S.E.M. percent positive and representative histograms obtained from 4 or more independent experiments, *n*=3-4 mice/time point and strain). (B, C, D) CCL25 transcripts were quantified by real-time RT PCR (Mean ± S.E.M. fold differences *n*=5-7 mice/intestinal segment/strain/time point, **P*<0.05, ****P*<0.001). (E) Immunohistochemistry showed CCL25 protein expression. Preincubation with recombinant CCL25 was used as control (Representative micrographs from 3 experiments, 10× magnification, bars=100μm).

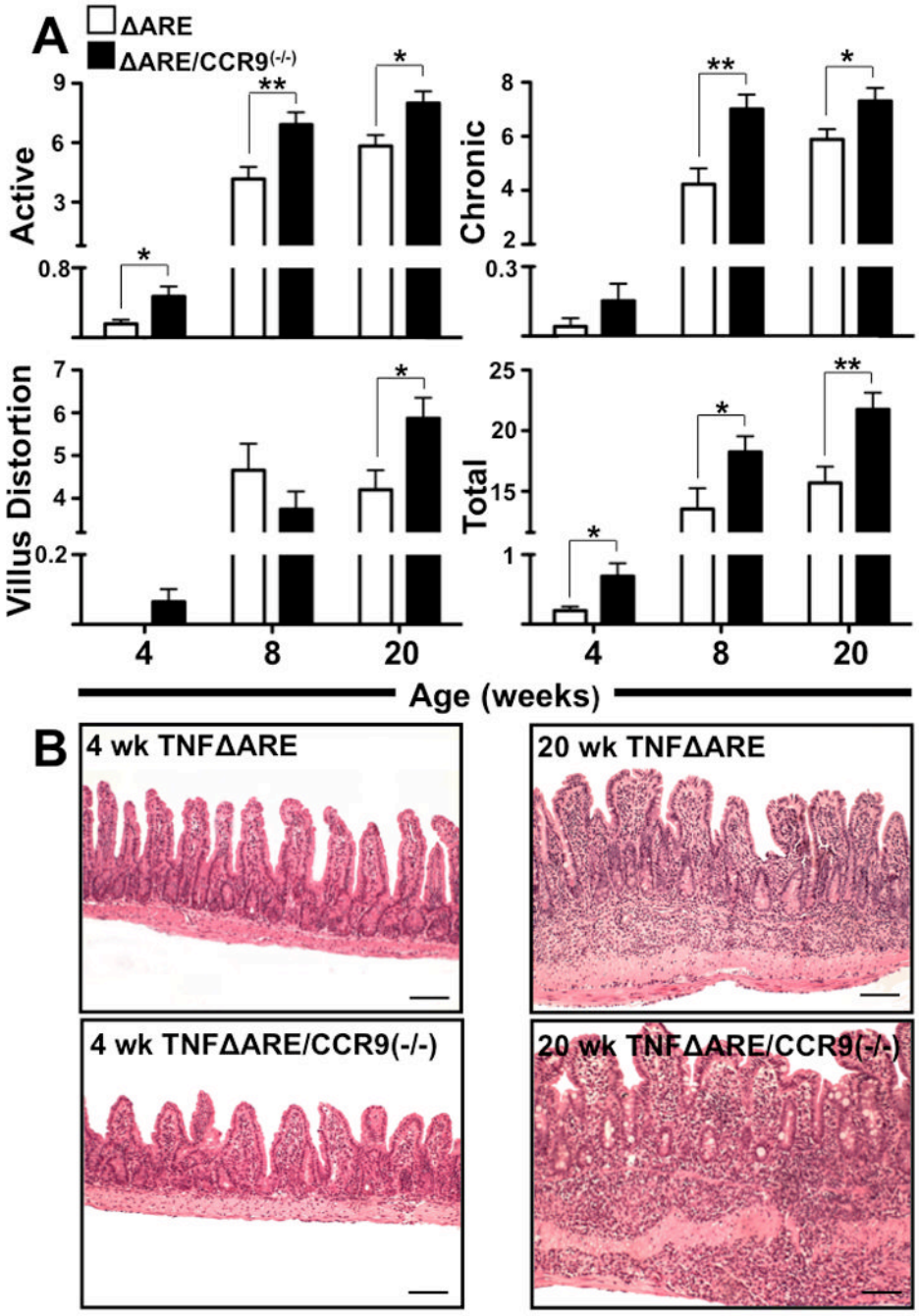

Figure 2.

CCR9 deficiency exacerbated Ileitis. (A) Disease severity in TNFΔARE$^{+/-}$/CCR9$^{+/+}$ (ΔARE) and TNFΔARE$^{+/-}$/CCR9$^{-/-}$(ΔARE/CCR9$^{-/-}$) mice was assessed at indicated ages (Mean ± S.E.M, *n*=17-20 mice/strain/time point, **P*<0.05, ***P*<0.01). (B) Representative micrographs, hematoxylin and eosin, 10× magnification, bars=100μm.

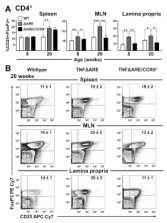

Figure 3.

$CD4^+/CD25^+/FoxP3^+$ Tregs decreased in MLN and LP during peak and late disease in CCR9-deficient TNFΔARE mice. Lymphocytes isolated from indicated compartments of WT, ΔARE and ΔARE/$CCR9^{-/-}$ mice were incubated with indicated antibodies and analyzed by flow cytometry, gating on FSC, SSC and CD4. (A) (Mean ± S.E.M. from 6 independent experiments, *n*=3-4 mice/time point/strain run in duplicate, **P*<0.05, ***P*<0.01, ****P*<0.001). (B)Representative contour plots at 20-weeks-of-age.

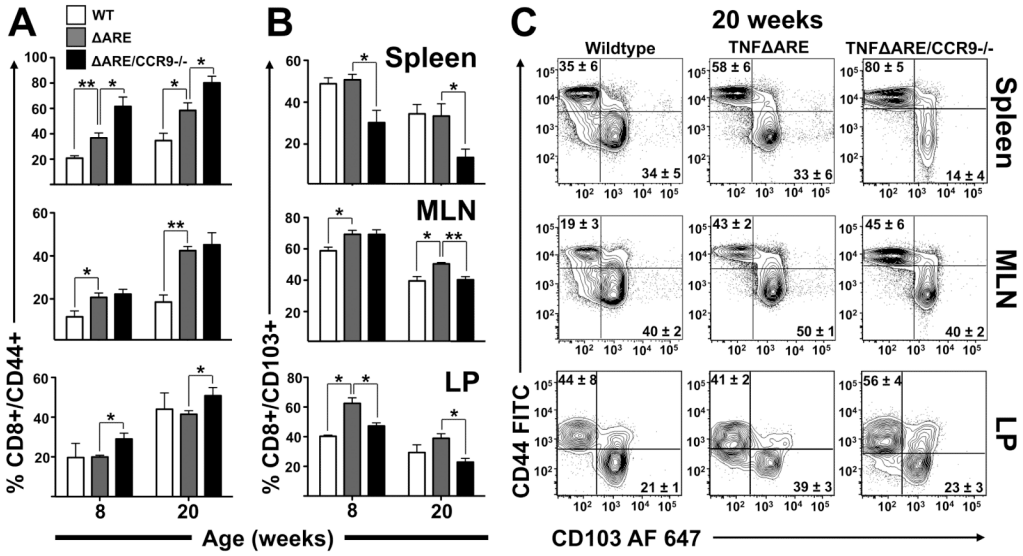

Figure 4.

Imbalance of $CD8^+/CD44^+$ and $CD8^+/CD103^+$ T cells in CCR9-deficient TNFΔARE mice. Lymphocytes from WT, ΔARE and $\Delta ARE/CCR9^{-/-}$ mice were incubated with indicated antibodies and analyzed by flow cytometry after gating on FSC SSC and CD8. (A, B) Percentage of $CD8^+/CD44^+$ and $CD8^+/CD103^+$ T cells in lymphoid compartments at indicated ages (Mean ± S.E.M. from 6 independent experiments, $*P<0.05$, $**P<0.01$). (C)Representative contour plots from $CD8^+$ T cells at 20-weeks-of-age.

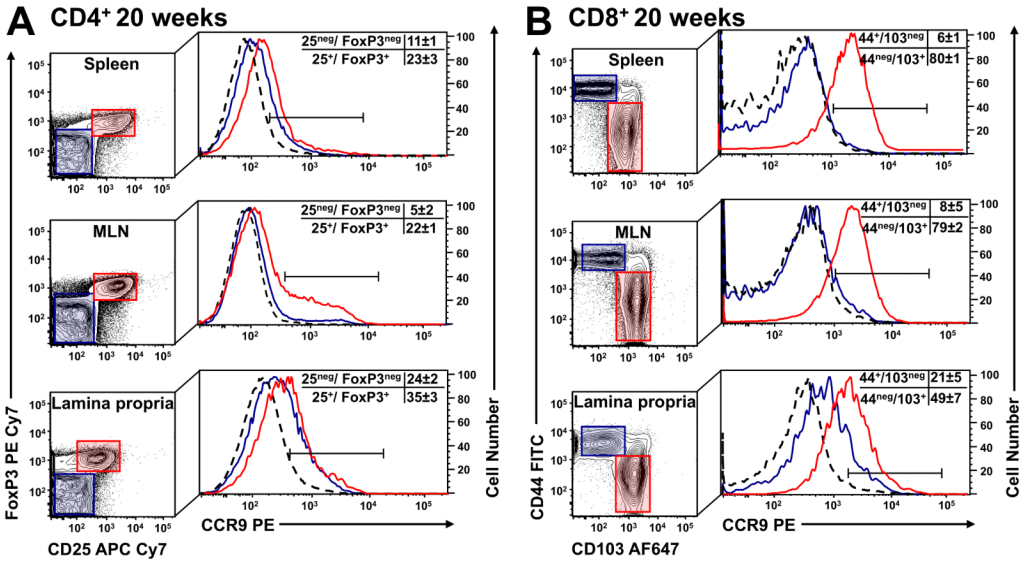

Figure 5.

CCR9 is preferentially expressed by Tregs. (A, B) CCR9 expression was assessed in $CD4^+/CD25^+/FoxP3^+$ (red histograms) and in $CD8^+/CD103^+$ (red histograms) compared to that of $CD4^+/CD25^{neg}/FoxP3^{neg}$ and $CD8^+/CD44^+$ (blue histograms) using cells isolated from CCR9-deficient mice (discontinuous histograms) as controls (Mean ± S.E.M. from 4 experiments, *n*=3 mice/experiment). Representative contour plots from TNFΔARE mice and similarly derived histograms from CCR9-deficient mice gated on FSC, SSC, CD4 and CD8.

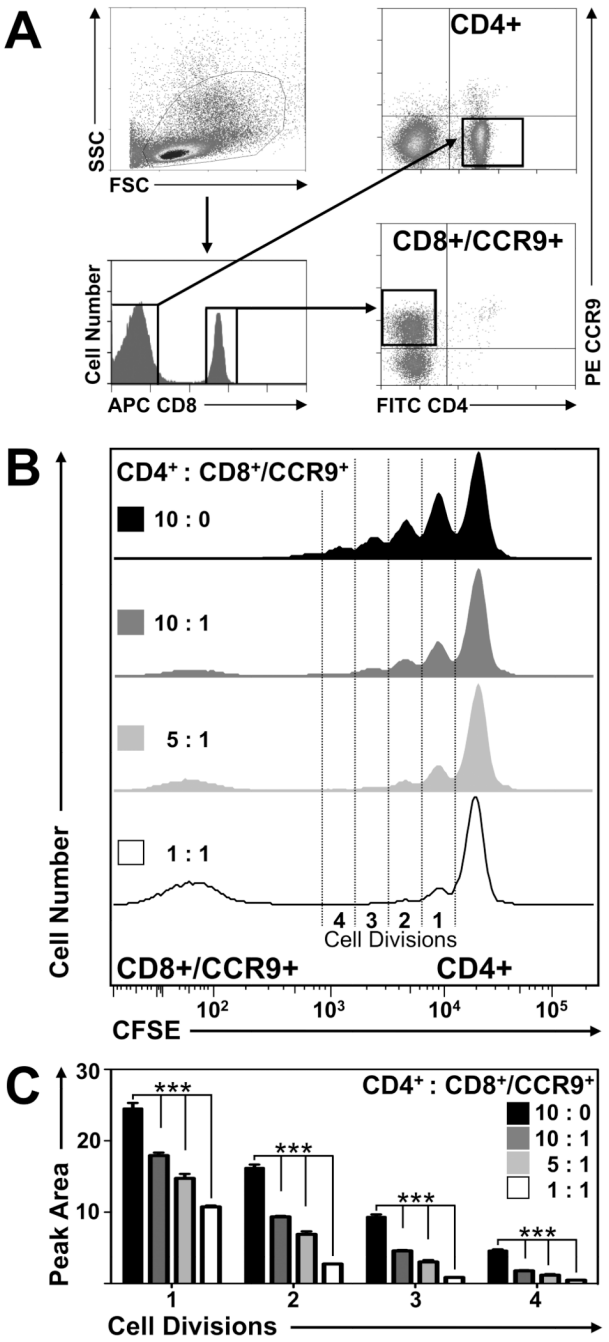

Figure 6.

$CD8^+/CCR9^+$ T cells decreased proliferation of $CD4^+$ T cells. (A) $CD4^+$ and $CD8^+/CCR9^+$ lymphocytes were isolated by flow cytometric sorting as illustrated. (B) CFSE-labeled $CD4^+$ T cells were cultured with or without increasing ratios of $CD8^+/CCR9^+$ T cells. Proliferation was assessed by flow cytometry. (C)Quantification of areas under the curve was performed using FlowJo cell proliferation analysis software (Mean ± S.E.M. from 2 independent experiments run in triplicate, ***P<0.001).

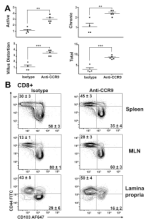

Figure 7.

Anti-CCR9 mAb exacerbated ileitis and reduced $CD8^+/CD103^+$ Tregs. (A) TNFΔARE mice received isotype or anti-CCR9 mAb. The severity of ileitis was assessed as described[17] (Mean ± S.E.M., n=5 mice/group, $^{**}P<0.01$, $^{***}P<0.001$). (B) Lymphocytes from isotype- and anti-CCR9-treated mice were incubated with indicated antibodies and analyzed by flow cytometry. Cells were gated on FSC, SSC and CD8 (Mean ± S.E.M., $P<0.05$ vs. isotype).