CCR4-dependent regulatory T cell function in inflammatory bowel disease

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Inflammatory bowel disease (IBD) is an idiopathic inflammatory disease of the intestine. CD4$^+$ T lymphocytes play an important role in both initiating and regulating intestinal inflammatory immune responses. CD4$^+$CD25$^+$CD45RB^{low} regulatory T (T reg) cells are capable of preventing the development of colitis in a mouse model of IBD. The precise mechanism of T reg cell–mediated prevention of colitis in this model is unclear, and the role of chemokine receptors in the trafficking and function of T reg cells in this model has not been determined. We examined the role of the chemokine receptor CCR4 in in vivo trafficking and suppressive function of T reg cells in a mouse adoptive transfer model of IBD. CCR4-deficient T reg cells failed to accumulate in the mesenteric lymph nodes (MLNs) at early time points (2–5 d) after adoptive transfer, resulting in a failure to suppress the generation of pathogenic T cells and the development of colitis. Moreover, although CCR4-deficent T cells had equivalent in vitro suppressive activity and accumulated in MLNs at later time points (42–56 d), they were unable to suppress colitis. Our study demonstrates that CCR4 plays an important role in T reg cell trafficking in LNs and that this is critical for T reg cell suppressive function in vivo.

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The pathogenesis of human inflammatory bowel disease (IBD) is thought to involve a dysregulated immune response to common luminal bacterial antigens (1). An intact mucosal barrier and regulatory mechanisms normally prevent the gut immune and inflammatory response, which protects the host from pathogenic agents, from proceeding to tissue injury and autoimmunity. Activated T lymphocytes have been shown to play an important role in chronic inflammation of the intestine in both human IBD and in experimental mouse models of colitis.

In a mouse adoptive transfer model of IBD, transfer of naive CD4$^+$ cells into a lymphocyte-depleted mouse induces mucosal inflammation of the colon (2). However, this intestinal inflammation is prevented when CD4$^+$ regulatory T cells are concomitantly transferred with naive CD4$^+$ T cells (2), demonstrating a potent suppressive role for regulatory T cells in intestinal inflammation in vivo. Regulatory T (T reg) cells are a subset of CD4$^+$ T cells capable of inhibiting autoimmune responses. Naturally occurring T reg cells arise spontaneously in vivo, express a unique transcription factor, Foxp3, and control responses to tissue-specific autoantigens that are presented to the immune system via mucosal surfaces (3). Immune tolerance is an important mechanism to prevent an excessive adverse reaction to the diverse luminal foreign antigens and bacterial pathogens in the intestine, and is mediated in part by subsets of CD4$^+$ T cells (T reg cells), which are characterized by the secretion of TGF-β and IL-10. Important questions remain unanswered regarding where this inhibition takes place and the molecular mechanism of T reg cell trafficking in vivo.

Chemokines are a superfamily of chemotactic cytokines that control leukocyte migration via G protein–coupled receptors expressed on target cells (4). The chemokine receptors CCR4 and CCR8 were found to be functionally expressed on human peripheral blood CD4$^+$CD25$^+$ T reg cells (5). In patients with ovarian carcinoma, the host response to the tumor was shown to be inhibited by Foxp3$^+$CCR4$^+$ T reg cells that were recruited into the tumor as a result of the tumor-derived CCR4 ligands, CCL17 and CCL22 (6). In a mouse cardiac allotransplantation model, CCR4 and CCL22 were up-regulated in tolerized allografts, and tolerance induction could not be achieved in

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CCR4-deficient ($CCR4^{-/-}$) recipients, indicating an important role of CCR4 in the generation and/or recruitment of $Foxp3^+$ T reg cells into cardiac allografts (7). Furthermore, CCR5 was recently shown to play a role in the migration of T reg cells into dermal sites of chronic cutaneous *Leishmania major* infection (8), whereas another recent study found that CCR7 was required for T reg cell function in the LN in a mouse model of IBD (9).

In the current study, we examined the role of CCR4 in T reg cell function and trafficking in the mouse adoptive transfer model of IBD. $CCR4^{-/-}$ T reg cells demonstrated delayed accumulation in mesenteric LNs (MLNs) at early time points after adoptive transfer and ineffective accumulation within the MLN at later time points. This impaired the ability of T reg cells to suppress the generation of autoimmune pathogenic effector T cells and the development of colitis in recipient mice.

RESULTS AND DISCUSSION

Chemokine receptor expression in T reg cells

To determine the expression of chemokine receptors in wild type (WT) T reg cells, we isolated $CD4^+CD25^+CD45RB^{low}$ T reg cells and naive $CD4^+CD25^-CD45RB^{high}$ T cells from WT C57BL/6 mice and performed quantitative PCR (QPCR) analysis. QPCR was used because of the limited availability of mAbs to mouse chemokine receptors for FACS analysis. As expected, in naive T cells, CCR7 and CXCR4 were the two chemokine receptors highly expressed, which is consistent with their naive phenotype (Fig. 1 A, shaded bar). In T reg cells, CCR7 and CXCR4 were expressed, but at much lower levels than in naive T cells. Interestingly, there were several additional chemokine receptors expressed in T reg cells, including: CCR1, CCR3, CCR4, CCR6, CCR8, CCR10, CCR11, CXCR3, CX3CR1, and the leukotriene B_4 receptor BLT1 (Fig. 1 A, open bar). This differential chemokine receptor expression between T reg cells and naive T cells implies distinct trafficking and functional characteristics between theses two T cell populations.

In the mouse model of IBD using adoptive transfer of pathogenic $CD4^+CD45RB^{high}$ T cells, 8 wk after adoptive transfer when clinical disease is evident, mRNA levels for CXCR3, CCR4, CCR5, and CCR6 have been found to be increased in the inflamed colon, suggesting that these chemokine receptors may play a role in $CD4^+CD45RB^{high}$ T cell trafficking and function (10). We sought to determine the expression of chemokine receptors on T reg cells capable of inhibiting the development of inflammatory colitis. To do so, 8 wk after cotransfer with naive $CD4^+CD25^-CD45RB^{high}$ T cells into T and B cell–deficient $Rag\text{-}2^{-/-}$ recipient mice, T reg cells were recovered from the spleen, lamina propria of the colon, and peripheral LNs and MLNs of recipient mice by FACS cell sorting, taking advantage of the Thy-1.1 (CD90.1) and Thy-1.2 (CD90.2) congenic markers. Naive pathogenic T cells were from the Thy-1.1 congenic strain, and T reg cells were from the congenic Thy-1.2 strain. Expression of chemokine receptors in recovered T reg cells varied markedly, depending on the anatomical sites, and the levels were much higher compared with T reg cells before adoptive transfer (Fig. 1 B). CCR4, CCR5, CCR6, CCR8, CCR9, CXCR3, and BLT1 were highly up-regulated in T reg cells recovered from MLNs. Of note, these T reg cells expressed TGF-β1, but not IL-10 (Fig. 1 B). These results suggest that the microenvironment in

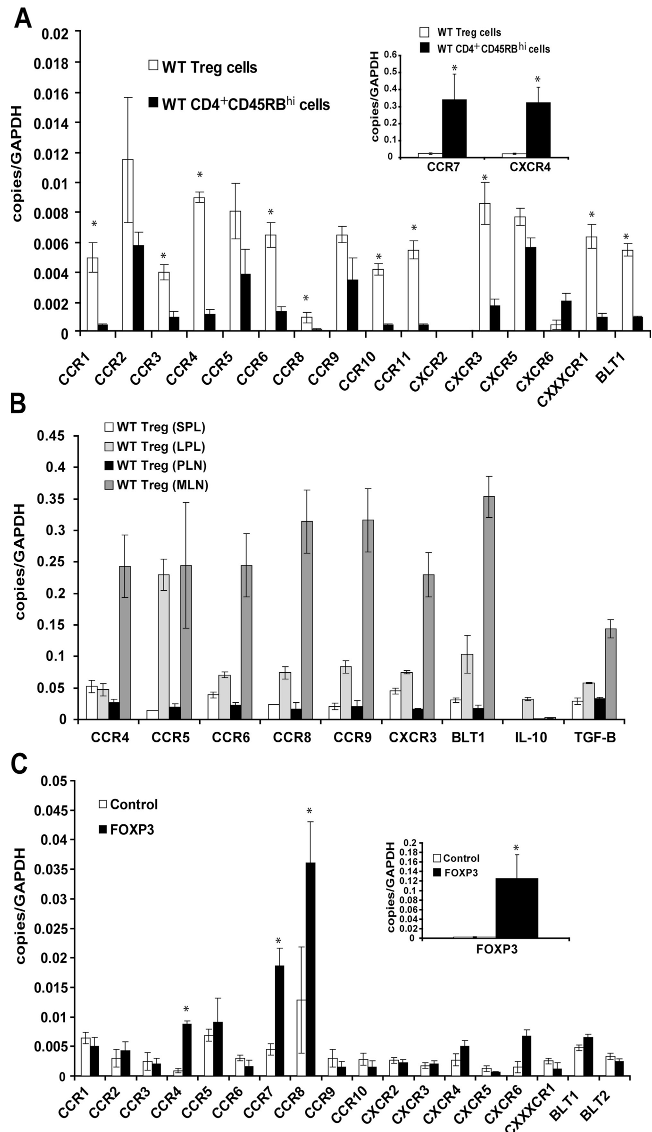

Figure 1. Expression of chemokine receptors in T reg cells. (A) QPCR analysis of chemokine receptor expression in freshly isolated WT $CD4^+CD25^+CD45RB^{lo}$ T reg cells (open bar) and $CD4^+CD25^-CD45RB^{hi}$ naive T cells (shaded bar). (B) Expression of chemokine receptors in WT T reg cells recovered from the spleen (open bar), lamina propria of the colon (light gray bar), peripheral LN (filled bar), and MLN (dark gray bar) 8 wk after cotransfer with pathogenic T cells. (C) Expression of chemokine receptors in virally transduced naive T cells expressing Foxp3 (shaded bar) or control MIGR vector (open bar). *, $P < 0.001$, as determined by a Student's *t* test. Error bars indicate the SD of triplicate measurements. In A and B, T reg or naive T cells were isolated from five different mice, and the data are representative of three similar independent experiments.

the lymphoid organ draining the site of inflammation plays an important role in regulating chemokine receptor expression in T reg cells, hence regulating the trafficking and function of these cells in vivo.

Retroviral gene transfer of Foxp3 has been shown to convert naive T cells into functional T reg cells (11). We introduced Foxp3 into naive $CD4^+CD25^-CD45RB^{high}$ T cells by retroviral transduction, and analyzed expression of chemokine receptors in these virally transduced $Foxp3^+$ T cells. Compared with T cells transduced with vector alone (controls), expression of CCR4, CCR7, and CCR8 was markedly upregulated in cells transduced with $Foxp3^+$ (Fig. 1 C, shaded bars). Interestingly, these chemokine receptors have all previously been implicated in T reg cell trafficking and function, including CCR7, which was shown to control the migration of $CD4^+CD25^+CD69^-$ human T reg cells to tonsilar germinal centers (12). Collectively, these results indicate that a subset of chemokine receptors expressed in T reg cells likely play important roles in T reg cell trafficking and function.

$CCR4^{-/-}$ T reg cells fail to protect development of colitis in vivo

To determine the importance of CCR4 in T reg cell in vivo function, we performed the mouse model of IBD by adoptively transferring WT naive T cells with or without cotransferring WT or $CCR4^{-/-}$ T reg cells into $Rag\text{-}2^{-/-}$ recipient mice. As expected, recipient mice that received WT naive $CD4^+CD25^-CD45RB^{high}$ T cells (pathogenic) alone developed severe colitis 8 wk after cell transfer, with marked edema and thickening of the colon and loss of normal, pellet-shaped stools (Fig. 2 A, first panel, top). There was also a marked mononuclear cellular infiltrate and an alteration of normal colonic mucosal architecture (Fig. 2 A, first panel, bottom). $Rag\text{-}2^{-/-}$ mice that received cotransfer of WT $CD4^+CD25^+CD45RB^{low}$ T reg cells and WT naive T cells were protected from developing colitis with a normal transparent appearance of the colon, normal pellet-shaped stools, and normal mucosal structure (Fig. 2 A, second panel). In marked contrast, $CCR4^{-/-}$ T reg cells failed to prevent the development of colitis after being cotransferred with WT naive T cells, and the severity of colitis was comparable to that in mice that received naive T cell alone (Fig. 2 A, third panel). We also examined the effects of two other chemokine receptors expressed in T reg cells, CCR2 and CCR5, on T reg function in the model. To do so, we coinjected $Rag\text{-}2^{-/-}$ mice with $CCR2^{-/-}$ T reg cells and WT naive T cells and $CCR5^{-/-}$ T reg and WT naive T cells. We found that both groups of recipient mice were protected from developing colitis (Fig. 2 A, fourth and fifth panels), suggesting that in contrast to CCR4, CCR2 and CCR5 are not required for T reg cell function in this model. To quantify the severity of colitis, we compiled the disease activity index (DAI) and

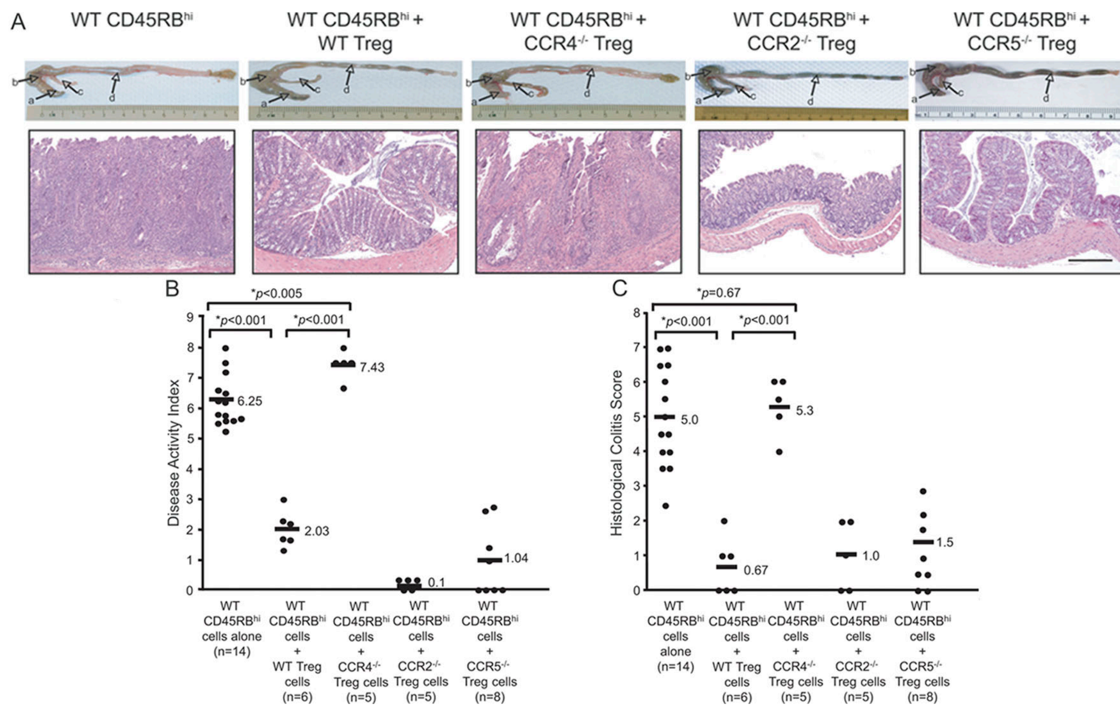

Figure 2. Development of colitis after adoptive cell transfer. (A, top) Gross appearance of colon in representative $Rag\text{-}2^{-/-}$ recipient mice 8 wk after cell transfer. Arrows indicate the distal cecum (a), the proximal cecum (b), the terminal ileum (c), and the colon (d). (bottom) Histological appearance of colon in representative mice. Bar, 250 μm. (B) 8 wk after cell transfer, DAI was compiled from individual mice based on wasting, stool consistency, and gross appearance of the colon. Higher number indicates more severe colitis. (C) Histological colitis score. Sections of colon from individual mice were imbedded in formalin and stained with hematoxylin and eosin. The severity of colitis was scored by our participating pathologist, who was blinded to each experiment. Number of mice/group indicated on figure (*n*) from three independent experiments.

histological colitis score from individual recipient Rag-$2^{-/-}$ mice 8 wk after cell transfer. The DAI was compiled as previously described (13) based on the general appearance of the recipient mice (wasting), stool consistency, and the gross appearance of the colon, with higher scores representing more severe colitis. The histological colitis score was determined from hematoxylin and eosin–stained colon sections scored for the presence of crypt abscesses (0–1), the degree of mucosa thickness (0–3), and the degree of inflammatory infiltrate (0–3). The maximum score of the histological index was 7.

The mean DAI in mice that received WT naive pathogenic T cells alone was 6.25 ($n = 14$), whereas in mice that received cotransfer of WT T reg cells and WT naive pathogenic T cells the mean DAI was only 2.03 ($n = 6$; $P < 0.001$; Fig. 2 B). In contrast, mice that received cotransfer of CCR$4^{-/-}$ T reg cells and WT naive T cells, the mean DAI was 7.43 ($n = 5$), which was significantly higher than mice that received cotransfer of WT T reg cells and naive pathogenic T cells (2.03; $P < 0.001$). The mean DAI in mice that received cotransfer of CCR$2^{-/-}$ T reg cells and WT naive T cells was 0.1 ($n = 5$) and mice that received cotransfer of CCR$5^{-/-}$ T reg cells and WT naive T cells was 1.04 ($n = 8$; Fig. 2 B). Furthermore, transfer of CCR$4^{-/-}$ naive pathogenic T cells induced colitis similar to WT naive T cells, demonstrating that the effect of CCR4 was specific for T reg cell function and not T effector cell function in this model (unpublished data).

The mean histologic colitis score in mice that received WT naive T cells alone was 5 ($n = 14$), whereas the mean colitis score in mice that received cotransfer of WT T reg cells and naive T cells was 0.67 ($n = 6$; $P < 0.001$; Fig. 2 C). In mice that received cotransfer of CCR$4^{-/-}$ T reg cells and WT naive T cells, the mean colitis score was 5.3 ($n = 5$), which is virtually indistinguishable from that in mice that received naive T cells alone (mean colitis score of 5.0; $P = 0.67$, not significant; Fig. 2 C). In contrast, the mean colitis score in mice that received cotransfer of CCR$2^{-/-}$ T reg cells and WT naive T cells was 1.0 ($n = 5$), and the mean colitis score in mice that received cotransfer of CCR$5^{-/-}$ T reg cells and WT naive T cells was 1.5 ($n = 8$), which were not statistically significant from that in mice that received WT T reg cells and WT naive T cells (mean colitis score of 0.67; $n = 5$; $P = 0.56$). These results indicate that the chemokine receptor CCR4 is critical for T reg cell function in vivo.

To further explore the mechanism of CCR4-dependent T reg cell in vivo function, we compared the expression of T reg cell markers and suppressive function of CCR$4^{-/-}$ and WT T reg cells. We found no significant difference in the expression of Foxp3, CTLA4, integrin α^E, GITR, and the cytokines IL-10 and TGF-β between the two groups of T reg cells (Fig. 3 A). We also found no difference in the ability of CCR$4^{-/-}$ and WT T reg cells to inhibit the proliferation of naive T cells in vitro (Fig. 3 B). These results indicate that difference in the suppressive function between CCR$4^{-/-}$ and WT T reg cells is likely a direct result of deficient CCR4 expression.

CCR$4^{-/-}$ T reg cells have an altered pattern of in vivo trafficking

We hypothesized that the specific subset of chemokine receptors expressed by T reg cells will affect their function in vivo by influencing their homing properties. In the aforementioned in vitro T reg cell assay, cells are incubated in wells with a small area, and thus migration is likely less important for localization of T reg with DC and pathogenic T cells than in lymphoid tissues. Therefore, we determined the trafficking patterns of CCR$4^{-/-}$ T reg cells and WT T reg cells at different time points after adoptive transfer into Rag-$2^{-/-}$ mice. We analyzed the distribution of CCR$4^{-/-}$ and WT T reg cells after cotransfer with WT naive T cells into Rag-$2^{-/-}$ recipient mice. CCR$4^{-/-}$ T reg cells and WT T reg cells were recovered from different lymphoid organs at different time points after adoptive transfer into Rag-$2^{-/-}$ mice, and the percentage and absolute numbers of T reg cells in each organ were determined by FACS analysis. The naive pathogenic T cells were from the Thy-1.1 strain, and the CCR$4^{-/-}$ T reg cells and WT T reg cells were from the congenic Thy-1.2 strain. The percentage of CCR$4^{-/-}$ T reg cells of total $CD4^+$ cells and the absolute cell numbers of CCR$4^{-/-}$ T reg cells were significantly less than that of the WT T reg cells in the MLN 2 and 5 d after cell transfer

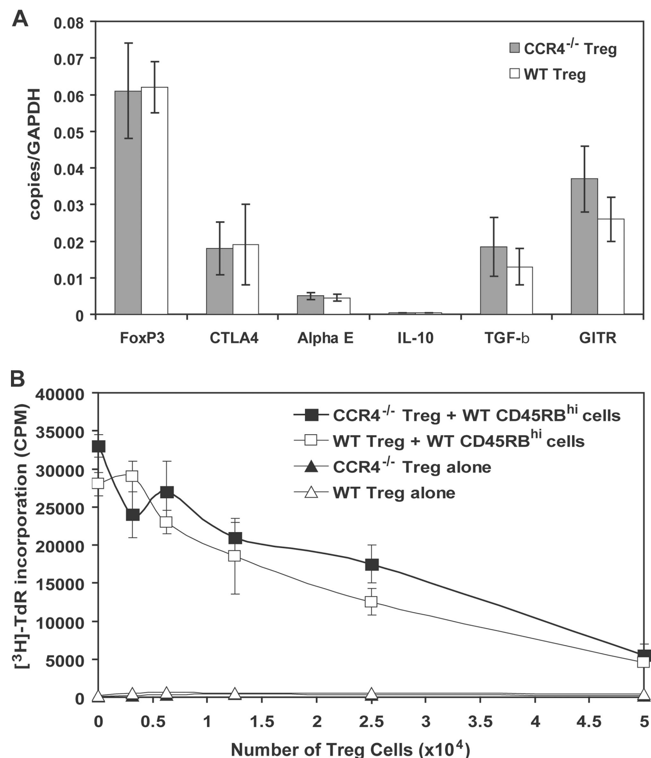

Figure 3. In vitro acitivity of CCR$4^{-/-}$ T reg cells. (A) Expression of T reg cell–related markers and cytokines by QPCR in CCR$4^{-/-}$ T reg cells (shaded bar) and WT T reg cells (open bar) ($n = 3$ mice/group). (B) Inhibition of naive T cell proliferation by CCR$4^{-/-}$ T reg cells (closed square) and WT T reg cells (open square). Closed and open triangles represent CCR$4^{-/-}$ T cells alone and WT T reg cells alone, respectively.

(Fig. 4, A and B). This most likely represents impaired homing and/or retention of CCR4$^{-/-}$ T reg cells in the MLN, as adoptively transferred T reg cells do not begin to divide until 1 wk after transfer (14). However, from day 42 after cell transfer onward, the percentage and absolute number of CCR4$^{-/-}$ T reg cells in the MLN were greater than that of WT T reg cells (Fig. 4, C and D). At these later time points, CCR4$^{-/-}$ T reg cells are likely recruited and/or retained in the MLN by inflammation-induced non-CCR4 chemokines, where their expansion is driven by the profound inflammation that develops in these mice 6 wk after adoptive transfer of CCR4$^{-/-}$ T reg cells, in contrast to the absence of disease in mice that received WT T reg cells. There were no significant difference in the percentage and cell numbers between CCR4$^{-/-}$ T reg cells and WT T reg cells in the colon, peripheral nondraining LNs, and spleen at all time points examined (unpublished data).

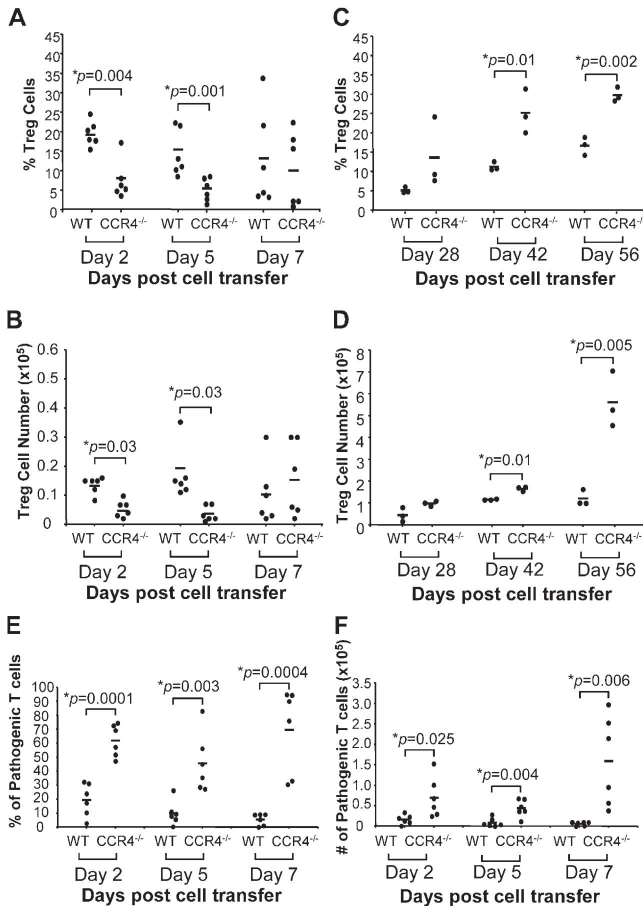

Figure 4. Accumulation of WT and CCR4$^{-/-}$ T reg and pathogenic T cells in the MLN. (A and C) Percentage of T reg cells in total CD4^{+} cells in the MLN after adoptive cell transfer with pathogenic T cells. (B and D) Absolute numbers of T reg cells in the MLN after adoptive cell transfer with pathogenic T cells. (E) Percentage and (F) absolute numbers of WT pathogenic T cells in the MLN after adoptive cell transfer with WT and CCR4$^{-/-}$ T reg cells. The bars represent the means of the indicated number of mice from two independent experiments.

The decrease in CCR4$^{-/-}$ T reg accumulation resulted in an increase in the percentage and numbers of WT pathogenic T cells generated in the MLN at all time points studied compared with mice that received WT T reg cells (Fig. 4, E and F). These data suggest that inhibition of T cell priming in the draining LN is critical for T reg cell–mediated suppression of colitis development, and implies that the MLN is likely the anatomical site where WT T reg cells exert their suppressive effect upon pathogenic T cells. Evidence exists to support the notion that the ability of T reg cells to home to LN is critical for their in vivo function. Although CD62L^{high} and CD62L^{low} T reg cells have similar in vitro suppressive capacity, CD62L^{high}CD4^{+}CD25^{+} T reg cells that home to LNs have a greater in vivo suppressive activity than CD62L^{low} T reg cells that home to the periphery in preventing autoimmunity and graft-versus-host disease (15–17). This concept was further supported by the observation that T reg cells deficient in integrin β_7 expression were unable to traffic to the colon after adoptive cell transfer in the mouse adoptive transfer model of IBD, but still had similar in vivo suppressive effect, as WT T reg cells in preventing development of colitis (18). Because migration of T reg cells into the MLN is not mediated by the integrin β_7, control of the induction phase of inflammation within the MLN was not impaired. Furthermore, in an IBD model similar to the one used here, CCR7 function was recently shown to be required on T reg cells for their ability to suppress colitis; this was also correlated with the ability of T reg cells to enter LNs (9). Collectively, these studies and ours support a critical role for T reg cells in the MLN to suppress colitis in this model.

Intravital microscopy has shown that T reg cells directly interact with antigen-bearing DCs and can down-modulate CD4^{+} T cell immune responses by inhibiting stable interactions between reactive CD4^{+} T cells and DCs during priming in LNs (19, 20). Both reactive CD4^{+} T cells and T reg cells migrate adjacent to CD11c^{+} DCs at the T cell–B cell boundary of the pancreatic LNs in nonobese diabetic mice (19). In the model of IBD used in our study, T reg cells have been found adjacent to clusters of CD11c^{+} cells and pathogenic T cells in the MLN (21). At early time points (2–5 d) after cell transfer, few CCR4-deficient T reg cells accumulate in the MLN. Reduced numbers of CCR4- deficient T reg cells compared with WT T reg cells in the MLN may result from their decreased entry into and/or decreased retention within the LN, which is caused by an impaired ability to migrate within the LN and make effective cell contacts. In support of the notion that CCR4 is important for T reg cell homing within the MLN, we found that the CCR4 ligands CCL17 and CCL22 were highly expressed in the MLNs of both Rag-2$^{-/-}$ mice and WT mice, as compared with the colon (Fig. 5, A and B). They were also highly expressed in the MLN after adoptive transfer of WT naive T cells and WT T reg cells during the entire course of the model (Fig. 5 C), suggesting that this chemokine sytem can regulate T reg cell function throughout the model. Previous studies have demonstrated that CCL22 is expressed by maturing DCs that

have migrated from the periphery and entered the T zone of LNs (22) and can mediate T cell–DC cluster formation, which is mediated by CCR4 expressed on T cells and CCL22 produced by DCs (23). Thus, WT T reg cells may migrate toward DC-produced CCL22, form conjugates with the antigen-presenting cell, and be retained within the LN. In contrast, CCR4-deficient T reg cells may fail to migrate toward DCs and/or form conjugates with antigen-presenting cells, and instead continue their transit through the LN and exit into the lymph. At later time points (42–56 d), we find that T reg cells greatly accumulate in the MLN, perhaps responding to the production of inflammatory chemokines within the LN, as we found that T reg cells recovered from the MLN 8 wk after adoptive transfer expressed several additional chemokine receptors, including CCR5, CCR6, CCR8, CCR9, and CXCR3. However, CCR4-deficient T reg cells are still unable to control disease, perhaps because of an inability to make effective contacts with DCs in vivo. This may explain our observation that even though $CCR4^{-/-}$ T reg cells expanded in the MLN at late time points after transfer and were equally effective at inhibiting T cell proliferation as WT T reg cells in vitro, they were unable to effectively inhibit colitis at these later time points. This interpretation is supported by the observation that WT T reg cells are able to suppress ongoing disease in the model, even when added after colitis has already developed (21). Thus, the absence of CCR4 on T reg cells impairs their early accumulation within the MLN and likely also impairs their migratory behavior and/or positioning once in the LNs, resulting in their functional impairment.

In summary, using the mouse adoptive transfer model of IBD, we demonstrated that CCR4 plays an important role in T reg cell trafficking and function in vivo. Failure of $CCR4^{-/-}$ T reg cells to prevent the development of colitis after cotransfer with pathogenic T cells was the result of an inability of $CCR4^{-/-}$ T reg cells to control the generation of pathogenic T cells resulting from impaired trafficking of $CCR4^{-/-}$ T reg cells in the draining MLNs. Our results provide direct evidence of an important role for CCR4 in T reg cell trafficking and function within the MLNs in vivo in a mouse model of IBD, with implications for human IBD.

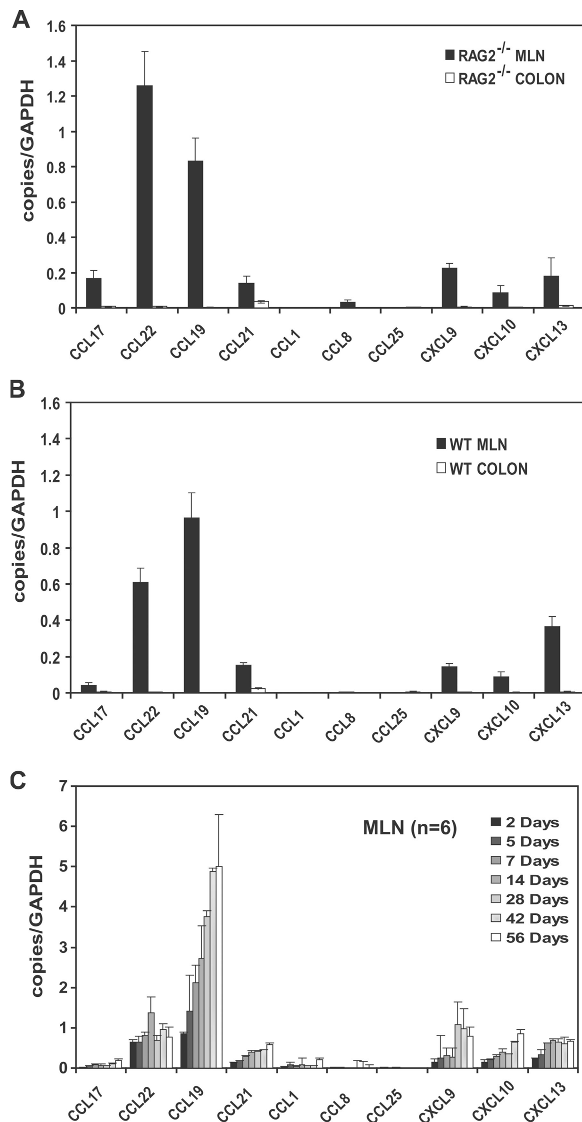

Figure 5. Chemokine expression in MLN and colon. Levels of chemokines in the MLN (closed bars) and the colon (open bars) of the naive $Rag\text{-}2^{-/-}$ mice (A) or WT C57BL/6 mice (B). The bars represent the means of $n = 6$ mice from two independent experiments. (C) Levels of chemokines in the MLN at the indicated time points after adoptive transfer of WT naive T cells and WT T reg cells into $Rag\text{-}2^{-/-}$ mice.

MATERIALS AND METHODS

Mice. WT C57BL/6 mice (Thy-1.2, CD90.2), WT congenic Thy-1.1 mice (CD90.1), and $CCR4^{-/-}$ (24), $CCR2^{-/-}$, and $CCR5^{-/-}$ mice on C57BL/6 background were obtained from The Jackson Laboratory. $Rag\text{-}2^{-/-}$ mice (C57BL/6 genetic background) were purchased from Taconic. All mice were kept in the specific pathogen-free barrier room at our animal facility. Donor mice (C57BL/6, Thy-1.1, and $CCR4^{-/-}$ mice) were used at 6–8 wk of age, and recipient $Rag\text{-}2^{-/-}$ mice were used at 8–10 wk of age. All mice were age and gender matched for every experiment. The experimental protocol was approved by the Subcommittee of Research Animal Care of Massachusetts General Hospital according to Institutional Animal Care and Use Committee guidelines before the start of the study.

mAbs. Anti–mouse CD90.2-PE (Thy-1.2; clone 30-H12), anti–mouse CD90.1-FITC (Thy-1.1; clone OX-7), anti–mouse CD45RB-FITC (clone 16A), anti–mouse CD25-PE (clone PC61), and anti–mouse CD3-APC (clone 145-2C11) were purchased from PharMingen. Anti–mouse CD4-Cy5.5 (clone RM4-5) was purchased from Caltag.

Cell isolation. Naive $CD4^+CD25^-CD45RB^{hi}$ T cells and $CD4^+CD25^+$ $CD45RB^{low}$ T reg cells were isolated from the LNs and spleen of donor mice by FACS cell sorting. Single-cell suspension was stained with a cocktail of directly conjugated mAbs, including anti–CD45RB-FITC, anti–CD25-PE, anti–CD3-APC, and anti–CD4-Cy5.5. The naive T cells and T reg cells were isolated by cell sorting using a MoFlo Cell Sorter (DakoCytomation).

Mouse model of experimental colitis. Immediately after cell sorting, naive T cells (4×10^5) and T reg cells (10^5) in 300 μl of cold Hanks' medium containing 2% FCS (Sigma-Aldrich) and 10 mM Hepes (Life Technologies)

were injected into recipient Rag-$2^{-/-}$ mice through the tail veins. In mice that received naive T cell alone, 4×10^5 naive T cells were injected. Recipient mice were kept in the specific pathogen–free barrier room for 8–10 wk after cell transfer, with close monitoring for weight loss, diarrhea, and general wellbeing.

Assessment of colitis. Recipient mice were weighed weekly to follow colitis-associated wasting, and they were killed 8–10 wk after cell transfer. The clinical DAI was compiled as described by de Jong et al. (13), with higher scores representing more severe colitis. Histological colitis scores were obtained on sections of colons stained with hematoxylin and eosin and reviewed by our participating pathologist, Dr. A. Bhan, who was blinded to each experiment. The sections are scored for the presence of crypt abscesses (0–1), the degree of thickness (0–3), and the degree of inflammatory infiltrate (0–3). The maximum score of histological index is 7.

Isolation of colonic lamina propria lymphocytes from the recipient mice. Isolation of lamina propria lymphocytes was performed according to published protocols (25), with modifications. In brief, the entire colon (excluding the cecum) was removed from the animals and opened longitudinally. 1–2-mm pieces were prepared with surgical scissors and incubated in RPMI containing 70 μg/ml Liberase Blendzyme 3 (Roche), 3 μg/ml DNase I (Sigma-Aldrich), and 1 mM DTT (Sigma-Aldrich) at 37°C for 90 min with constant shaking (200 rpm). The cell suspension was filtered through a 70-μM filter (Falcon). Enterocyte contamination was removed by passing cell suspension through a glass-wool column. The flow-through faction was collected, and it contained a mixture of lamina propria lymphocytes and intraepithelial lymphocytes.

Proliferation assay. Naive $CD4^+CD25^-CD45RB^{hi}$ T cells were isolated from the spleen and LNs of C57BL/6 mice by FACS sorting, and cultured at 5×10^4 cells/well in triplicate in the presence of irradiated APC and 5 μg/ml soluble anti-CD3, in a 96-well flat-bottom tissue culture plate (Falcon). T reg cells were added to each well in varying numbers, with the ratio to naive T cells at 1:8, 1:4, 1:2, and 1:1. After culture for 72 h, 0.5 μCi of tritiated thymidine (NEN) were added to each well for the last 18 h.

QPCR. Total RNA was purified using the RNeasy kit (QIAGEN). After DNase I (Invitrogen) treatment, 2 μg of total RNA was used from reverse transcription reaction (Applied Biosystems). Primers used to examine the expression of chemokine receptors were designed using Primer Express software 1.0 (PE Biosystems). All oligonucleotide primers were synthesized by Invitrogen. The QPCR reactions were perfomed in optical 96-well strips with optical caps (Stratagene) using the MX4000 Multiplex QPCR system (Stratagene). Quantity values generated for gene expression were obtained by comparison of the fluorescence generated by each sample with standard curves of known quantities and were divided by the quantity of total RNA present in each reaction.

Retroviral transduction of Foxp3 in naive T cells. Plasmid DNA containing MigR1 vector and MigR1-Foxp3 were provided by A. Rudensky (University of Washington, Seattle, WA). Both MigR1-Foxp3 and MigR1 plasmids were transfected into the packaging cell line, Plat E, using Fugene (Roche). Naive $CD4^+CD25^-CD45RB^{hi}$ T cells were isolated from C57BL/6 mice and were transduced with Foxp3 or MigR1 vector-containing viral supernatant supplemented with 8 μg/ml polybrene, followed by centrifugation for 90 min at 2,000 rpm and 72 h of incubation at 37°C, as previously described (26, 27). Foxp3-transduced cells were isolated by cell sorting for GFP expression.

Statistical analysis. Student's *t* test (unpaired, two-tailed) was used to calculate significant levels for all measurements. $P < 0.05$ was considered to be statistically significant.

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