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CD134L Expression on Dendritic Cells in the Mesenteric Lymph Nodes Drives Colitis in T Cell-Restored SCID Mice[1]

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CD134L Expression on Dendritic Cells in the Mesenteric Lymph Nodes Drives Colitis in T Cell-Restored SCID Mice[1]

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Transfer of CD45RB^{high} CD4$^+$ T cells to immune-deficient mice in the absence of regulatory T cells leads to a Th1-mediated colitis. In this study, we show that intestinal inflammation is characterized by a 15-fold increase in the number of CD134L$^+$ (OX40L$^+$)-activated DC in the mesenteric lymph nodes (MLNs) compared with BALB/c mice. This was important functionally, as administration of an anti-CD134L mAb inhibited the proliferation of T cells in the MLNs as well as their expression of the gut-homing integrin $\alpha_4\beta_7$. Most importantly, the anti-CD134L mAb completely blocked development of colitis. Surprisingly, CD134L was found to be expressed by a proportion of dendritic cells (DC) in the MLNs of unreconstituted SCID mice, suggesting that CD134L can be induced on DC in the absence of T cell-derived signals. These results indicate that some DC in the MLNs of SCID mice express an activated phenotype and that CD134L expression by these cells is involved in the development of colitis induced by T cell transfer. Accumulation of CD134L$^+$ DC was inhibited by cotransfer of regulatory T cells, suggesting that inhibition of the accumulation of activated DC is one mechanism by which these cells prevent immune pathology. ***The Journal of Immunology,*** **2001, 166: 6972–6981.**

There is now accumulating evidence from a number of model systems that the numbers of T cells in the periphery are under very tight control. It has long been appreciated that transfer of T cells to immune-competent recipients results in limited expansion of the transferred cells, whereas significant expansion, estimated to be in the order of up to 10,000-fold, has been observed after transfer to immune-deficient recipients (1–3). Autoimmune/inflammatory disorders have been shown to develop in rodents with experimentally induced lymphopenia, suggesting that dysregulated peripheral T cell expansion may be involved in the pathogenesis of these diseases (4–6).

Transfer of small numbers of CD45RB^{high} CD4$^+$ T cells to SCID mice led to significant expansion of these cells in the periphery. This was most marked in the intestine, as the majority of mice developed a Th1 cell-mediated chronic colitis (7–11). Intestinal pathology resembled that seen in inflammatory bowel disease (IBD)[5] in humans and was characterized by an extensive lymphocytic infiltrate, comprising CD4$^+$ T cells and macrophages, epithelial cell hyperplasia, ulceration, and depletion of mucin-secreting goblet cells (12). T cell expansion was significantly reduced and intestinal pathology absent when T cells were transferred to SCID mice raised under germ-free conditions, indicating that the indigenous microbiota play an important role in driving these pathogenic responses (13, 14). Importantly, cotransfer of the reciprocal CD45RB^{low} subset inhibited both the dysregulated expansion of CD45RB^{high} progeny and the development of colitis by a mechanism that involved both IL-10 (15) and TGF-β (16). Recently, these regulatory T (Treg) cells have been shown to be contained within the CD25$^+$ CD45RB^{low} CD4$^+$ subset and to be dependent on CTLA4 for their function (17). CD25$^+$ CD4$^+$ cells have also been shown to prevent the development of autoimmune disease induced after T cell depletion, indicating that functionally specialized Treg cells, present in the Ag-experienced pool of normal mice, play an important role in the control of peripheral T cell responses (18–20).

It is now widely accepted that T cell activation involves signals transduced by the TCR complex after recognition of Ag as well as from costimulatory molecules after encounter with their ligands present on APC (21). Members of the TNFR superfamily, including CD27, CD30, 4-1BB (CD137), and OX40, have been shown to mediate costimulatory activity (22). The OX40 molecule (CD134) is expressed transiently on activated CD4$^+$ T cells (23, 24) and on some CD8$^+$ cells. It interacts with OX40L (CD134L) (25, 26), which is a type II membrane protein with amino acid sequence similarity to TNF. CD134L has been reported to be present after activation on B cells (27, 28), dendritic cells (DC) (29), microglia (30), and human endothelium (31). Studies in both the mouse and human indicate that CD134-CD134L interactions are required for optimal B cell responses (27, 32, 33). More recent studies have revealed that these interactions also play a pivotal role in T cell responses. Activation of T cells in the presence of CD134L-expressing cells led to enhanced clonal expansion and secretion of cytokines (24). Similar results have been observed in vivo, as treatment of mice with anti-CD134 mAb in the presence of LPS led to enhanced clonal expansion and development of T cell memory (34). Consistent with these findings, CD134 knockout mice were

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[5] Abbreviations used in this paper: IBD, inflammatory bowel disease; DC, dendritic cell(s); EAE, experimental autoimmune encephalomyelitis; MLN, mesenteric lymph node; Rag, recombinase-activating gene; s, soluble; Treg, regulatory T.

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found to have reduced T cell proliferative responses and cytokine production defects (35, 36). Similarly, CD134L knockout mice exhibited impaired T cell priming and cytokine production in vivo. Furthermore, DC isolated from these mice had impaired costimulatory activity in vitro, indicating that CD134L expression plays an important role in APC function. (37, 38).

Several studies have highlighted the importance of CD134-CD134L interactions in vivo. Thus, $CD134^+$ T cells are found in inflammatory lesions, and blocking CD134-CD134L interactions via administration of a CD134-Ig fusion protein led to amelioration of experimental autoimmune encephalomyelitis (EAE) (30) and of intestinal inflammation (30, 39, 40). Although these studies clearly show that CD134-CD134L interactions play a role in T cell-mediated immune pathology, they do not reveal precisely how. The fact that CD134L is expressed on a variety of activated APC as well as on activated endothelium makes it a possibility that CD134-CD134L interactions are involved in a range of T cell functions, including expansion and survival, differentiation and effector function, as well as migration to sites of inflammation.

Given the important role that CD134-CD134L interactions play in the function of T cells in an intact immune system, it was a possibility that these interactions were involved in T cell-mediated immune pathology induced under conditions of lymphopenia. To test this, we have generated a mAb reactive with murine CD134L. Using this reagent in SCID mice restored with $CD45RB^{high}$ $CD4^+$ T cells, we show that CD134-CD134L interactions are essential for dysregulated T cell expansion and development of colitis, and that it is expression of CD134L by activated DC that drives the pathogenic response.

Materials and Methods

Mice

C.B-17 SCID, 129/SvEv recombinase-activating gene (Rag) 2-deficient ($Rag2^{-/-}$), BALB/cJ, and 129/SvEv mice were bred under specific pathogen-free conditions and kept in microisolators with filtered air in the Biomedical Service Unit at the John Radcliffe Hospital. Mice were used at 8–12 wk of age.

Antibodies

The following mAbs were used for cell purifications: YTS169, anti-mouse CD8; TIB120, anti-mouse MHC class II (American Type Culture Collection (ATCC), Manassas, VA); M1/70, anti-mouse Mac-1 (TIB128; ATCC); RA3-6B2, anti-mouse B220 (41); PE-conjugated anti-mouse CD45RB (clone 16A; PharMingen, San Diego, CA); Cy-Chrome-conjugated anti-mouse CD4 (clone RM4-5; PharMingen). The following Abs were used for flow cytofluorography: TIB 139, anti-mouse H-2b, CD4-PerCP (RM4-5), CD45RB FITC (16A), $\alpha_4\beta_7$ PE (LPAM-7), α_E-bio (M290), CD25-bio (7D4), CD11c PE (HL3), CD40 FITC (HM40-3), CD80 FITC (16-10A1), CD86 FITC (GL1), SAv-APC (all PharMingen), and CD134 FITC (OX86) (Serotec, Oxford, U.K.). Stained cells were run on a FACSort and analyzed using CellQuest Software (Becton Dickinson, San Jose, CA).

OX89, a rat IgG1 anti-OX40L mAb, was produced by standard procedures involving the fusion of NS1 myeloma cells and spleen cells from a PVG rat that had been immunized, s.c., with 20 μg CD4.CD134L (see below) in complete Freund's adjuvant. After 2 wk, the rats received CD4.CD134L (20 μg) in incomplete Freund's adjuvant, and after a further 3-wk interval they received 20 μg of CD4.CD134L i.v. Splenocytes were taken 4 days after the last immunization. Initial screening was on recombinant protein, and activated T cells and specificity were confirmed on fibroblasts transfected with rat OX40L (24). For in vivo use, anti-mouse CD134L (OX89) and the rat anti-mouse IgG1 isotype control (GL113) were purified from hybridoma supernatant by affinity chromatography and shown to contain <1 EU endotoxin per mg of protein.

Blocking studies

Soluble (s)CD4.CD134 protein contains the TNFR-like repeat region of rat CD134 that binds to mouse CD134L plus domains 3 and 4 of rat CD4, and was produced and purified, as described (26). A multimeric form of the protein was generated by binding CD4.CD134 recombinant protein to streptavidin-coated fluorescent Sphero beads (Spherotech, Libertyville, IL) via a biotinylated OX68 Ab, as described previously (42, 43). The cells and Sphero beads were incubated on ice for 40 min and analyzed by flow cytofluorography (42, 43). The chimeric sCD48.CD4 protein contained domains 3 and 4 of CD4 as for CD134 (42).

Cell purification and flow cytofluorography

$CD4^+$ T cell subsets were isolated from spleens, essentially as previously described (9). Briefly, single cell suspensions were depleted of $CD8^+$, MHC class II^+, $Mac\text{-}1^+$, and $B220^+$ cells by negative selection using sheep anti-rat-coated Dynabeads (Dynal, Oslo, Norway). In some cases, $CD4^+$-enriched cells were stained with Cy-Chrome-conjugated anti-CD4 and PE-conjugated anti-CD45RB Abs and $CD4^+$ $CD45RB^{high}$ and $CD45RB^{low}$ $CD4^+$ fractions sorted on a FACSVantage (Becton Dickinson). Alternatively, cells were stained with FITC-conjugated anti-CD45RB, PE-conjugated anti-CD25, and CyChrome-conjugated anti-CD4, and $CD45RB^{high}$ $CD4^+$and $CD25^+$ $CD4^+$ populations were sorted. Cells were >97% pure on reanalysis. In some experiments, $CD4^+$ T cells were purified by positive selection using CD4 Dynabeads and the DETACHaBEAD system (Dynal), followed by MACS separation to yield $CD45RB^{high}$ $CD4^+$ cells. For the MACS separation, $CD4^+$ cells were stained with FITC-conjugated anti-CD45RB (16A; PharMingen), followed by anti-FITC microbeads, according to the manufacturer's instructions, and run over MS^+ mini columns (Miltenyi, Bergisch Gladbach, Germany). Bound cells were eluted from the column and were 95% $CD45RB^{high}$ $CD4^+$.

CFSE labeling of T cells

T cell division in vivo was assessed by flow cytofluorography of CFSE-labeled cells. MACS-sorted $CD45RB^{high}$ $CD4^+$ cells were stained in vitro with the cytoplasmic dye CFSE (Molecular Probes, Leiden, The Netherlands) before reconstitution (44). Briefly, cells were incubated for 7 min at 37°C with 5 μM CFSE. The reaction was quenched by washing in ice-cold DMEM supplemented with 10% FCS. Cell viability was assessed by trypan blue exclusion. CFSE staining gave one sharp peak, as assessed by flow cytofluorography.

T cell reconstitution and Ab treatment

Eight- to 10-wk-old C.B-17 SCID mice were injected i.p. with 4×10^5 sorted $CD45RB^{high}$ $CD4^+$ cells alone or in combination with $CD45RB^{low}$ $CD4^+$ or $CD25^+$ $CD4^+$ Treg cells, as indicated. The 129 $Rag2^{-/-}$ mice were reconstituted with 2.5×10^6 CFSE-labeled MACS-sorted $CD45RB^{high}$ $CD4^+$ cells. mAbs (500 μg) were injected i.p. in PBS the day after reconstitution and twice per week for the duration of the experiment.

Immunohistochemistry

Frozen tissue was sectioned and fixed in 2% formaldehyde in PBS. Endogenous peroxidases were neutralized by incubation with 1% H_2O_2. The primary Abs were used at a concentration of 5 μg/ml (OX89) or as unpurified tissue culture supernatant (N418). The secondary Ab was a biotinylated anti-rat or anti-Armenian hamster. Positive brown staining was developed by the ABC-elite kit, followed by diaminobenzidine substrate (Vector Laboratories, Burlingame, CA). Sections were counterstained with hematoxylin.

Cell purification

For enumeration of CD4 cells in the intestine lamina propria, lymphocytes were isolated from the colon, as described (15). The total number was determined by multiplying the number of leukocytes by the frequency of $CD4^+$ cells. The latter was determined by flow cytofluorography. DC were prepared essentially as in Vremec and Shortman (45). Briefly, mesenteric lymph nodes (MLNs) were cut into pieces and incubated for 25 min under agitation at 37°C in the presence of 1 mg/ml collagenase/dispase (Sigma, St. Louis, MO) and 100 U/ml DNase (Sigma) before 5 min of deaggregation in the presence of 0.1 M EDTA. The tissue was then passed through a 70-μm membrane to generate single cell suspensions.

Clinical and microscopic examination

T cell-restored SCID mice were weighed weekly and sacrificed after 8 wk or when they had lost 20% of their initial weight. A 0.5-cm piece of the distal colon was removed and fixed in formal saline. Paraffin-embedded sections (5 μm) were cut and stained with hematoxylin and eosin and used for microscopic assessment of colitis. Tissues were graded semiquantitatively from 0 to 5 in a blinded fashion. A grade of 0 was given when there were no changes observed. Changes typically associated with other grades are as follows: grade 1, minimal scattered mucosal inflammatory cell infiltrates, with or without minimal epithelial hyperplasia; grade 2, mild scattered to diffuse inflammatory cell infiltrates, sometimes extending into the submucosa and associated with erosions, with minimal to mild epithelial hyperplasia and minimal to mild mucin depletion from goblet cells; grade 3, mild to moderate inflammatory cell infiltrates that were sometimes transmural, often associated with ulceration, with moderate epithelial hyperplasia and mucin depletion; grade 4, marked inflammatory cell infiltrates that were often transmural and associated with ulceration, with marked epithelial hyperplasia and mucin depletion; and grade 5, marked transmural inflammation with severe ulceration and loss of intestinal glands.

Statistics

The Mann-Whitney *U* test was used for comparison of weights, clinical scores, cell numbers, and levels of activation markers. Disease incidence was analyzed by the Fischer exact test.

Results

Generation and molecular characterization of an anti-CD134L mAb

The CD134L mAb (OX89) was produced by the fusion of spleen cells from a rat that had been immunized with a recombinant chimeric protein consisting of domains 3 and 4 of rat CD4 and the extracellular region of mouse CD134L. Hybridomas were screened for their ability to bind to recombinant protein, activated T cells, and cells transfected with recombinant mouse CD134L. As shown in Fig. 1*A*, OX89 bound specifically to CD134L-transfected fibroblast cells. Consistent with previous reports of expression of CD134L, OX89 was also found to bind to B cells and DC activated by LPS and anti-CD40 mAb, but not to anti-CD3-activated T cells (data not shown). Immunohistochemical analysis of spleens from naive BALB/c mice revealed only scattered OX89-positive cells that were located outside the T and B cell areas and had DC-like morphology (data not shown).

To further characterize OX89 mAb, its ability to block CD134-CD134L interactions was examined. As the interaction between CD134L (trimeric) and recombinant extracellular region of CD134 (monomeric) is very weak (26), the latter was made multivalent by coupling the extracellular domain of CD134 to anti-CD4-coated fluorescent beads (43). Flow cytofluorography showed that CD134-coated beads bound to CD134L-expressing fibroblast cells and not the parental line, and that control beads coated with CD48 (its ligand, CD2, is not expressed on mouse fibroblasts) did not bind to either cell line (Fig. 1*B*). Preincubation with the OX89 mAb reduced CD134 bead binding by ~70%. Thus, these data show that OX89 binding is able to partially block multimeric CD134 from binding to trimeric CD134L (Fig. 1*C*). Thus, biological effects of OX89 (see below) could be due to blocking the interaction, although other effects such as signaling through CD134L cannot be ruled out.

OX89 treatment inhibits wasting disease and colitis

To investigate the effects of OX89 on T cell-mediated immune pathology, C.B-17 SCID mice were reconstituted with CD45RB^{high} CD4$^+$ T cells and treated with either OX89 or an isotype control mAb. Weights were followed throughout the experiments. Mice were sacrificed after 8 wk, and the development of colonic inflammation was assessed. As expected, the majority of

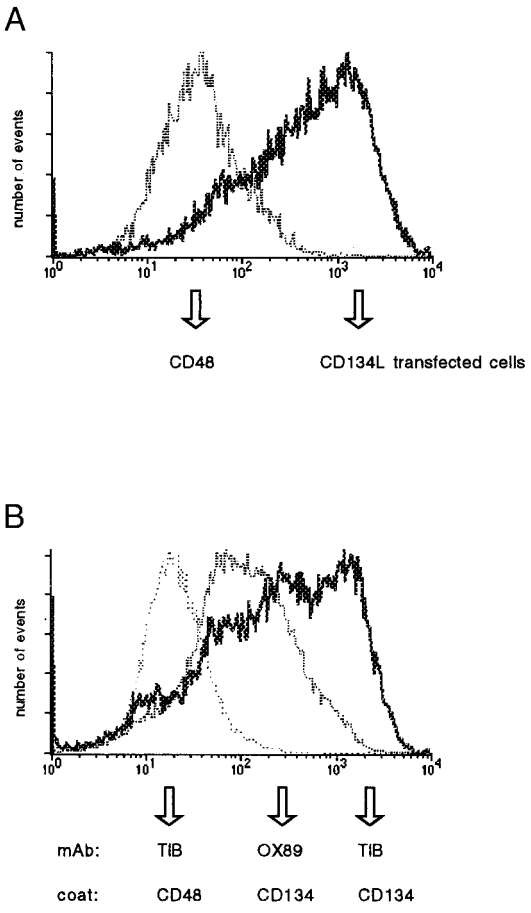


FIGURE 1. Characterization of OX89, an anti-CD134L mAb. *A*, Flow cytofluorography shows labeling with OX89 (solid line) of fibroblast cells transfected with CD134L, but not the irrelevant CD48 mAb (dashed line). *B*, Assay for multimeric binding of CD134 to CD134L-transfected cells by flow cytofluorography. sCD4.CD134, or control protein sCD48.CD4, was coupled to streptavidin-coated beads and mixed with CD134L-transfected cells. OX89 gives partial inhibition of binding of CD134 beads (thin line) compared with blocking with an irrelevant mAb recognizing MHC class I (TIB) (thick line) and the control sCD48.CD4 beads, which gives background fluorescence (dashed line).

control mice developed wasting disease and colitis. In contrast, none of the anti- CD134L-treated mice had significant inflammatory changes in the intestine (Fig. 2*A*) and gained weight throughout the course of the experiment (Table I). As has been previously described, colitis was accompanied by an expansion of CD4$^+$ T cells in the intestine ($341 \times 10^3 \pm 89 \times 10^3$; Fig. 2*B*). In OX89-treated mice, colons had to be pooled to obtain quantifiable numbers of CD4 cells. Pools of two colons yielded $36.8 \times 10^3 \pm 11.8 \times 10^3$ CD4$^+$ cells, meaning that there were ~10- to 20-fold lower numbers of CD4 cells per colon in OX89-treated mice. Taken together, these data demonstrate that administration of OX89 prevents T cell accumulation in the intestine of T cell-restored SCID mice and inhibits the development of Th1-mediated colitis.

To determine whether continual treatment with OX89 was required to prevent development of colitis, in some experiments Ab treatment was discontinued after 2 wk. Under these circumstances, only 2 of 10 treated mice developed colitis compared with 7 of 10 in the control group, suggesting that OX89 treatment early after T cell reconstitution has a long lasting protective effect (Table I).

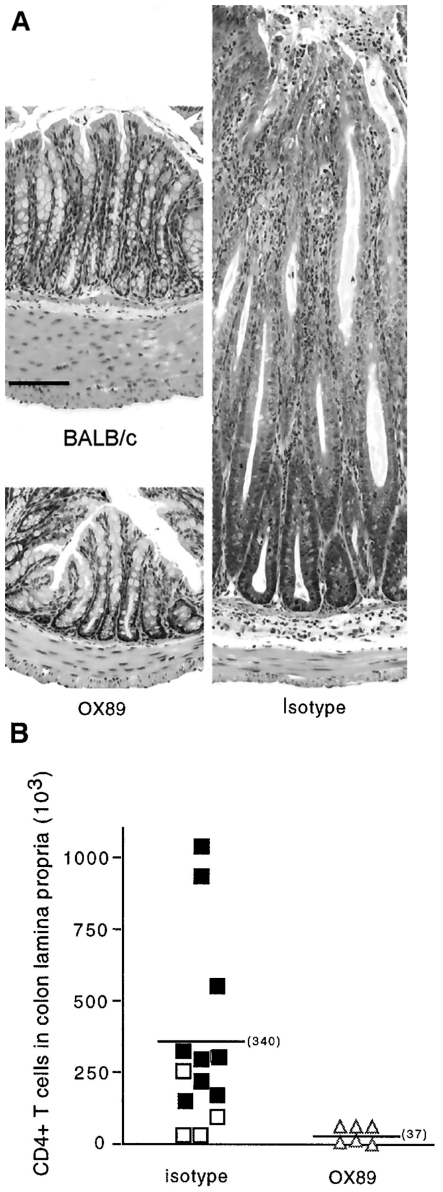


FIGURE 2. OX89 therapy prevents the development of colitis. *A*, Hematoxylin and eosin stainings of representative colons from normal BALB/c and reconstituted C.B-17 SCID mice treated with either isotype or OX89 Ab. Images are at the same magnification. Bar represents 100 μm. *B*, The total number of $CD4^+$ T cells found in the colon correlates with disease. Data represent the number of $CD4^+$ T cells isolated from the colon of individual mice (isotype treated) or pooled from two mice/group (OX89 treated). Black boxes represent mice that developed colitis, and horizontal lines indicate the mean that also appears in parenthesis. Data are pooled from two independent experiments.

OX89 treatment inhibits T cell expansion and homing in T cell-restored immune-deficient mice

Colitis develops in SCID mice after transfer of as few as 10^4 $CD45RB^{high}$ $CD4^+$ cells (9), and is consistently accompanied by significant numbers of $CD4^+$ T cells in the large intestine as well as expansion in peripheral lymphoid organs. This suggests that induction of disease is dependent on initial peripheral expansion of T cells, followed by homing of these cells to the intestinal mucosa. OX89 may be acting at several points in this pathway, including inhibition of peripheral T cell reconstitution in the spleen or MLN or subsequent homing of cells to the intestine. To further investigate this, the number of T cells in different compartments early after T cell reconstitution was analyzed.

Table I. *OX89 treatment prevents wasting disease and colitis in reconstituted SCID mice*[a]

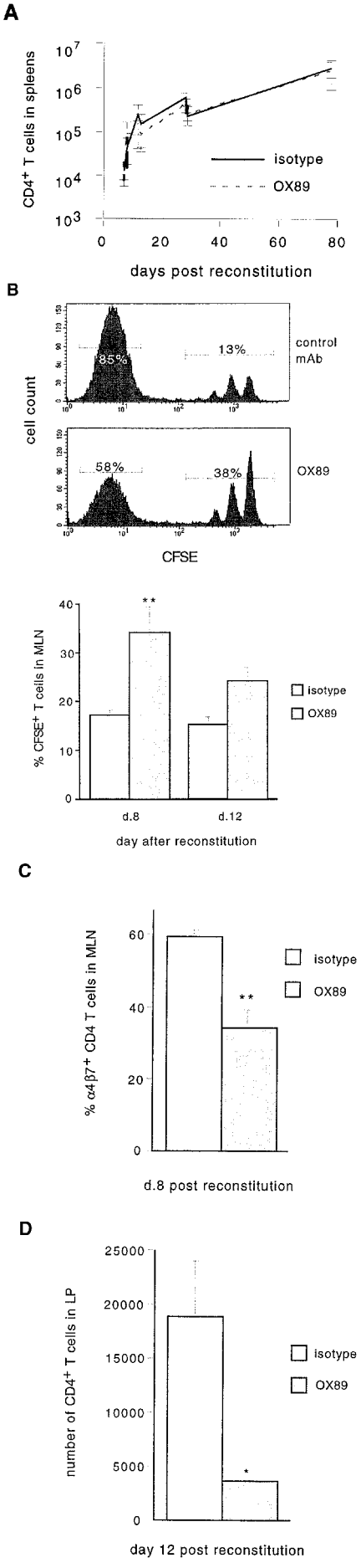
mAb Treatment[b]	Length of Treatment	% Colitis (*n*)[c]	% Weight Change[d]
Isotype	8 wk	69 (13)	91 ± 15
OX89	8 wk	0 (12)**	119 ± 6***
Isotype	First 2 wk	87 (8)	87 ± 10
OX89	First 2 wk	20 (10)**	104 ± 3***

[a] All mice were reconstituted with 4×10^5 $CD45RB^{high}$ $CD4^+$ cells. Experiments were terminated after 8 wk.
[b] Ab (0.5 mg) was given i.p. twice weekly for the given time period.
[c] n = total numbers of mice. **, $p < 0.005$.
[d] % of initial weight (±SD). ***, $p < 0.001$.

Total $CD4^+$ T cell numbers in spleens were determined at various time points after T cell reconstitution in OX89-treated and control mice. As can be seen in Fig. 3*A*, OX89 treatment had no effect on T cell accumulation in the spleen, as T cell numbers were similar in both groups. To assess proliferation, $CD45RB^{high}$ $CD4^+$ cells were labeled with the cytoplasmic dye CFSE and transferred to 129 $Rag2^{-/-}$ mice, which were treated with OX89 or control mAb. Analysis of CFSE expression among T cells in the spleen 8 days after T cell reconstitution showed that the majority had undergone over five divisions, as less than 10% retained detectable levels of CFSE. This was the same in OX89- and control-treated groups (data not shown). These results suggest that T cell accumulation in the spleen is not dependent on CD134-CD134L interactions. However, a more extensive kinetic analysis would be required to provide definitive evidence on this point.

To assess T cell activation and proliferation more directly relevant to bacteria-driven immune responses, we analyzed the effect of OX89 treatment on T cell proliferation in the MLN as well as the expression of activation markers and gut-homing molecules. Eight days after T cell reconstitution, significantly higher number of T cells retained CFSE in OX89-treated mice, suggesting reduced proliferation among T cells in the MLN of these mice (Fig. 3*B*). Consistent with the impaired proliferative response in OX89-treated mice, there was reduced expression of markers of T cell activation among $CD4^+$ cells in the MLN, including CD25 (data not shown).

T cell homing to the gut, including the intestine and its associated lymphoid tissue, involves the interaction of the $\alpha_4\beta_7$ integrin on T cells with its ligand mucosal addressin cell adhesion molecule 1, expressed on the vascular endothelium (46, 47). Analysis of $\alpha_4\beta_7$ expression on $CD4^+$ cells in the MLN revealed an ~2-fold reduction in the frequency of $\alpha_4\beta_7{}^+$ cells present in OX89 compared with isotype-treated mice (Fig. 3*C*). This reduction was specific for this gut-homing molecule, as levels of $\alpha_E\beta_7$, thought to be involved in retention of T cells in the gut (48), were unchanged (data not shown). Consistent with the reduction in the frequency of $\alpha_4\beta_7{}^+$ cells, there was also a striking reduction in the number of $CD4^+$ T cells present in the intestine early after T cell reconstitution in OX89-treated mice ($3.5 \times 10^3 \pm 0.23 \times 10^3$) compared with control mice ($18.8 \times 10^3 \pm 5.2 \times 10^3$; Fig. 3*D*). When lamina propria lymphocytes were analyzed from mice reconstituted with CFSE-labeled cells, the lamina propria lymphocytes were always CFSE negative, indicating that cells here had undergone at least six to eight divisions, most likely before their migration to the intestine. These results suggest that OX89 inhibits intestinal inflammation in part as a result of its ability to inhibit Ag-driven T cell activation and expansion in the MLN at early



time points after T cell reconstitution. These experiments do not rule out the possibility that OX89 also inhibits T cell expansion in the spleen or the survival or effector function of T cells present in the intestine.

Colitis is characterized by an increase in the number of DC in the MLN that express CD134L

To further characterize where CD134$^+$ CD4$^+$ T cells interact with CD134L$^+$ cells, the expression of CD134 and CD134L in the MLN and colon was investigated by flow cytofluorography and immunohistochemistry. Immunohistochemical analysis of MLN revealed significant numbers of CD134L$^+$ cells only in mice with colitis, with few positive cells detectable in BALB/c mice. Significantly, there was a reduction in the number of CD134L$^+$ cells in mice protected from colitis by transfer of CD45RB^{high} and Treg cells (Fig. 4*A*) or after treatment with OX89 (data not shown). Lack of detectable expression of CD134L in the MLN of OX89-treated mice was not a result of blockade due to bound mAb, as addition of a secondary anti-Rat Ig reagent failed to reveal positive cells.

Flow cytofluorography allowed us to quantitate the number of CD134L$^+$ cells in the MLN as well as identify their phenotype. Consistent with immunohistochemical results, MLN from mice with colitis contained a 2- to 3-fold higher frequency of CD134L$^+$ cells compared with MLN from mice that did not develop colitis. This latter group comprised SCID mice reconstituted with both CD45RB^{high} and CD25$^+$ CD4$^+$ Treg cells (protected SCID), normal BALB/c mice, and unreconstituted SCID mice (Fig. 5*A*). In all cases, the CD134L$^+$ cells were CD11c positive, suggesting that these cells are DC. They were also found to be CD11b$^+$ and CD8α^-, suggesting that they are of myeloid origin (49).

Although the frequency of CD11c$^+$ cells in the MLN was similar in T cell-restored (CD45RB^{high} CD4$^+$ alone or in combination with Treg cells) and unreconstituted SCID mice (16–19%; Fig. 4*A*, Fig. 5 legend), the cellularity of the MLN was very different between groups. Thus, the total cell number in colitic mice ranged from 2 to 11 $\times$ 10^6, whereas MLN from unrestored SCID mice or SCID mice protected from colitis by transfer of Treg cells contained ~10-fold fewer cells (0.2–0.6 $\times$ 10^6). Therefore, not only was there an increase in the proportion of DC that were CD134L$^+$, there was also a significant increase in the total number of CD11c$^+$ DC in the MLN of colitic mice. As a consequence of these two factors, it follows that there was a 24- to 36-fold (compared with SCID mice given a mixture of CD45RB^{high} CD4$^+$ + Treg cells)

FIGURE 3. OX89 treatment reduces T cell proliferation in the MLN and prevents T cell accumulation in the colon. *A*, The frequency of CD4$^+$ T cells in the spleen of T cell-restored mice was determined by flow cytofluorography. Data represent the mean plus SEM of 24 mice per group. The frequency of CFSE$^+$ (shown as representative FACS plot or summary bar graph) (*B*) or $\alpha_4\beta_7^+$ (*C*) CD4$^+$ T cells in the MLN of T cell-restored mice was determined by flow cytofluorography 8 days after reconstitution. Data represent the mean plus SEM of five mice per group. OX89-treated mice had reduced T cell proliferation in the MLN (more CFSE$^+$ cells) ($p < 0.01$) and a lower frequency of cells expressing $\alpha_4\beta_7$ ($p < 0.001$). Two additional experiments gave similar results. *D*, Lamina propria cells were isolated from the colon 12 days after reconstitution (pooled from three to four mice per group), and the number of CD4$^+$ cells was determined. Data were pooled from four independent experiments. Isotype control-treated mice had significantly more T cells in their colons compared with OX89-treated mice ($p < 0.03$).

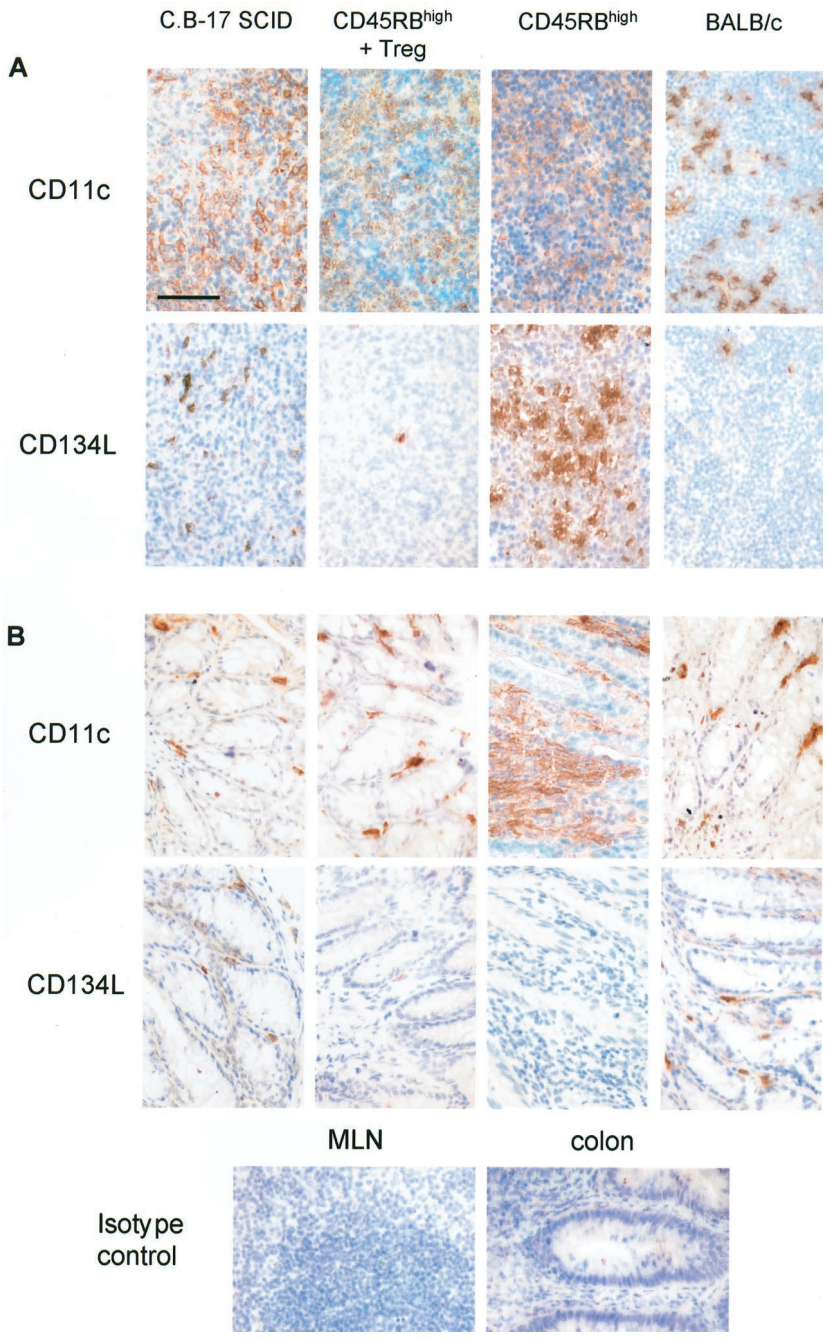


FIGURE 4. CD11c and CD134L expression in MLN and colon sections. Frozen sections of MLN (*A*) or colon (*B*) were stained with anti-CD134L, anti-CD11c, or isotype control Ab, and positive cells were detected, as described in *Materials and Methods*. *A*, Abundant CD134L expression (brown staining) in the MLN is a characteristic of mice with colitis, with fewer positive cells present in naive BALB/c and SCID mice as well as in mice reconstituted with both $CD45RB^{high}$ $CD4^+$ and $CD25^+$ $CD4^+$ Treg cells. Representative sections are shown; at least three mice per group were investigated in two or more experiments. *B*, $CD11c^+$ cells (brown) are increased in the colons of colitic mice, but there are few detectable $CD134L^+$ cells. All images are at the same magnification. Bar represents 100 μm.

or 15-fold (compared with BALB/c) increase in the total number of $CD134L^+$ $CD11c^+$ DC in these mice. Expression of CD134L on DC is a marker of DC activation (50). So our data suggest that there is an abnormal accumulation of activated DC in the MLN of colitic mice. Consistent with this, $CD134L^+$ DC from MLN of colitic mice also expressed CD40 and CD80, both markers of activation (data not shown). Flow cytometry also revealed detectable numbers of $CD134L^+$ $CD11c^+$ DC in unmanipulated SCID mice, albeit 12-fold lower than in mice with colitis. It should be noted that while there are detectable $CD134L^+$ cells in the MLN of SCID mice (Fig. 4*A*), these MLN are very leukopenic, meaning that the total numbers of $CD134L^+$ $CD11c^+$ cells are low (Fig. 5*A*). The fact that mice protected from colitis by transfer of both $CD45RB^{high}$ $CD4^+$ and $CD25^+$ $CD4^+$ Treg cells had a similar number of $CD134L^+$ $CD11c^+$ DC to that found in unreconstituted SCID mice indicates that transfer of Treg cells prevents the accumulation of activated DC in the MLN.

Immunohistochemical analysis revealed scattered $CD11c^+$ cells in the lamina propria of colons from unmanipulated BALB/c and CB.17-SCID mice as well as in mice reconstituted with $CD45RB^{high}$ $CD4^+$ and $CD45RB^{low}$ $CD4^+$ or $CD25^+$ $CD4^+$ Treg cells. In contrast, colitic mice, reconstituted with $CD45RB^{high}$ $CD4^+$ cells only, had a very cellular lamina propria with an abundance of $CD11c^+$ cells (Fig. 4*B*). Despite this accumulation of DC in the colon of colitic mice, there was undetectable CD134L expression (Fig. 4*B*). Consistent with the lack of CD134L expression in the colon after T cell reconstitution, there were no detectable $CD134^+$ $CD4^+$ cells either assessed by both immunohistochemistry and flow cytofluorography (data not shown). In contrast, and a good positive control for the reagents, $CD134^+$ $CD4^+$ T cells

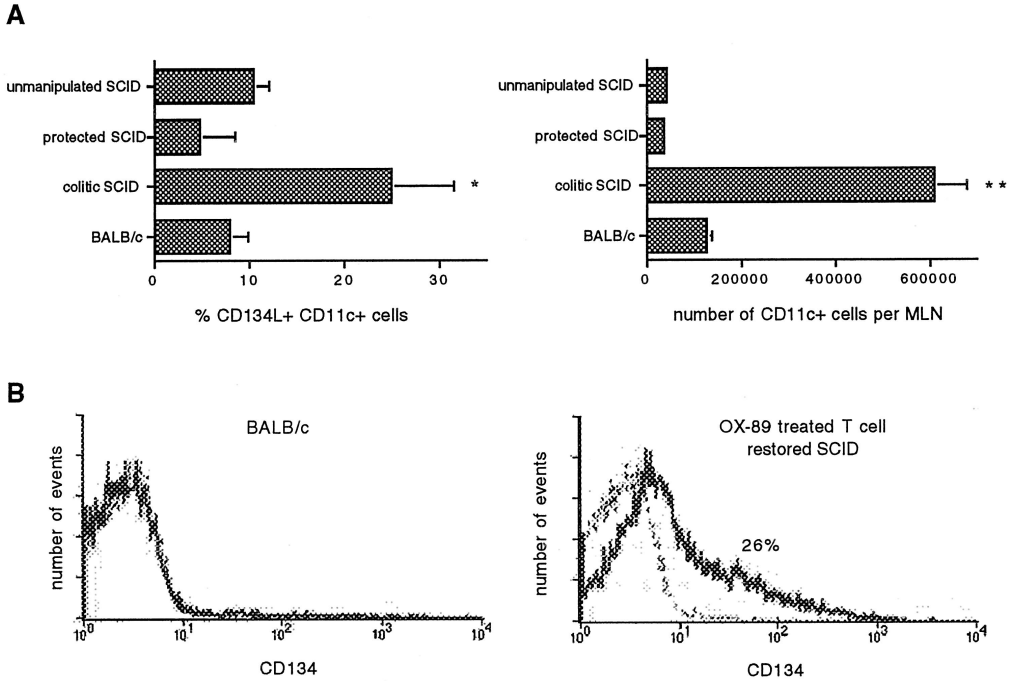


FIGURE 5. Colitis is characterized by an increase in the number of CD134L$^+$ DC in the MLN. *A*, Flow cytofluorography of MLN cells revealed that colitic mice had an increase in the frequency of CD11c$^+$ cells that expressed CD134L (*, $p < 0.05$) as well as an increase in the total number of CD11c$^+$ cells (**, $p < 0.01$) compared with protected (restored with CD45RB^{high} CD4$^+$ in combination with CD25$^+$ CD4$^+$ Treg cells) and naive animals. Cell yields from MLN of BALB/c mice ranged from 6 to 36 $\times$ 10^6 cells per mouse, ~1% of which were CD11c$^+$ cells. In contrast, SCID mice had 16–19% CD11c$^+$ cells in their MLN irrespective of reconstitution, but cell yields from colitic mice were between 2 and 11 $\times$ 10^6 cells per mouse compared with 0.2–0.6 $\times$ 10^6 per mouse from protected or unreconstituted SCID mice. Data represent the mean plus SEM of 5–11 mice per group. *B*, MLN from BALB/c or T cell-restored SCID mice were stained with anti-CD4 PerCP and anti-CD134 FITC (bold line) or isotype control FITC Ab (dashed line) and analyzed by flow cytofluorography. CD134$^+$ CD4$^+$ cells were undetectable in the MLN of BALB/c mice, whereas reconstituted SCID mice had readily detectable numbers of CD134$^+$ CD4$^+$ cells, as illustrated by an OX89-treated mouse. The frequency of CD134$^+$ CD4$^+$ cells in T cell-restored SCID mice varied from 5 to 30%. Similar frequencies were found in SCID mice with colitis (restored with CD45RB^{high} cells) or those that did not develop colitis (restored with CD45RB^{high} cells and treated with OX89, or given a mixture of CD45RB^{high} cells and CD25$^+$ CD4$^+$ Treg cells). Histograms are gated on CD4$^+$ cells and are representative of individual analyses from three to five mice per group.

were readily detectable in the MLN of T cell-restored SCID mice (ranging from 5 to 30% of CD4$^+$ T cells), but not in the MLN of normal immune-competent mice (Fig. 5*B*). The frequency of CD134$^+$ CD4$^+$ cells did not correlate with colitis, as similar frequencies of positive cells were found in the MLN of colitic mice as were found in T cell-restored SCID mice that did not develop colitis, including OX89-treated mice and mice that received a mixture of CD45RB^{high} CD4$^+$ cells plus Treg cells.

Results of flow cytofluorography and immunohistochemistry suggest that interaction between CD134L$^+$ APC and CD134$^+$ CD4$^+$ T cells most likely occurs in the MLN rather than in the colon of T cell-restored immune-deficient mice. These results taken together with the finding that OX89 treatment impedes T cell activation in the MLN and prevents the development of colitis suggest that T cell encounter with CD134L$^+$ DC in the MLN is crucial for the pathogenesis of intestinal inflammation in T cell-restored SCID mice.

Discussion

Transfer of peripheral CD4$^+$ T cells to immune-deficient recipients in the absence of Treg cells results in the development of a dysregulated Th1 response in the colon (7–11). In this study, we show that colitis is characterized by a 15- to 36-fold increase in the number of activated CD134L$^+$ DC in the MLN compared with mice that do not develop colitis. Expression of CD134L is functionally important, as administration of an anti-CD134L mAb (OX89) impeded T cell activation in the MLN and inhibited the development of colitis. These results identify the dysregulated expression of CD134L by DC in the MLN as one factor that drives the pathogenic process in T cell-restored SCID mice. Importantly, cotransfer of Treg cells prevented the increase in CD134L$^+$ DC in the MLN, indicating that inhibition of DC activation is one mechanism by which Treg cells prevent immune pathology.

The mechanism of action of OX89 is not known, but it could act in one or more of three possible ways: first, it could block the interaction of CD134 and CD134L and prevent signal transmission. Although the mAb did not block the interaction between purified recombinant proteins, it gave about 70% inhibition in an assay designed to mimic the multivalent interaction between cells (Fig. 1). Second, the OX89 mAb could act by down-regulating CD134L, and indeed treated animals had few detectable CD134L$^+$ cells in their MLN (data not shown). Third, it is possible that the OX89 could also give signals to the DC, as CD134L has been shown to interact with signaling proteins through its cytoplasmic domain (51).

CD134L is reported to be expressed by a variety of cell types after activation, including B cells, DC, and vascular endothelium (27–31). This makes it possible that blockade of CD134-CD134L interactions may affect both T cell priming and migration. The finding that CD134L was expressed predominantly by DC in the MLN of mice with colitis and that OX89 treatment led to retarded T cell proliferation and expression of activation Ags in the MLN, suggests that CD134L expression on DC plays an important role in driving the T cell response in this model. These data are consistent

with a number of recent studies that have highlighted the important role that CD134L expression by DC plays in the development of optimal T cell responses. Mice with a targeted disruption of CD134L mounted impaired contact hypersensitivity reactions, and DC from these mice were found to be deficient in inducing T cell proliferation and cytokine secretion in vitro (37, 38). Although treatment with OX89 inhibited expression of CD134L on DC in the MLN, there was no alteration in CD134 expression on T cells in the MLN of treated vs untreated mice. As expression of CD134 is restricted to activated T cells, these data are consistent with previous findings, suggesting that CD134-CD134L interactions are important for the amplification of primed cells as opposed to their initial priming (24).

Transfer of T cells to a lymphopenic environment leads to substantial expansion of the transferred T cells (1–3). Recent studies have shown that expansion is dependent on MHC class I and class II molecules, suggesting that the process is driven by recognition of Ag (52–54). T cell expansion and immune pathology in SCID mice are driven by intestinal bacteria, as transfer of $CD45RB^{high}$ $CD4^+$ cells to SCID mice raised under germ-free conditions or under conditions of reduced bacterial flora led to substantially reduced T cell expansion and no colitis (13, 14, 55). Accompanying the dysregulated T cell expansion in mice with colitis was a 5-fold expansion in the number of DC in the MLN. These DC were highly abnormal, as 20–30% expressed CD134L, a molecule present on activated DC, which is present at lower levels on DC present in the MLN (3–8%) or spleen of normal mice. Somewhat paradoxically, despite there being abundant $CD134L^+$ cells in the MLN of mice with colitis, there were few, if any, in the colon. It is possible that in mice with colitis, $CD134L^+$ DC migrate from the colon to the MLN, where they present intestinal Ags to T cells. Such DC migration has been observed after intestinal Ag delivery, and is enhanced by endotoxin and TNF (56, 57).

Although this whole process is dependent on intestinal bacteria, it remains to be established whether they provide peptide Ags or act to enhance the costimulatory capacity of DC that present endogenous Ags, or both. Surprisingly, 9–11% of DC in the MLN of unrestored SCID mice expressed CD134L. Induction of CD134L on DC in vitro has been shown to require CD40 signaling (50). However, the finding that CD134L was expressed on DC in the MLN of unreconstituted SCID mice indicates that in vivo the requirement for T cell-dependent CD40 signaling can be circumvented. Although these activated DC were present in significantly reduced number in SCID mice compared with SCID mice restored with $CD45RB^{high}$ $CD4^+$ cells, they may provide the initial costimulatory signals that drive the differentiation of $CD45RB^{high}$ cells into pathogenic Th1 cells. Importantly, mice protected from colitis by transfer of Treg cells had similar numbers of $CD134L^+$ DC in the MLN as unrestored SCID mice, suggesting that inhibition of the accumulation of activated $CD134L^+$ DC in the MLN is a feature of the immune-suppressive properties of Treg cells.

Blockade of CD134-CD134L interactions led to amelioration of EAE and of colitis in a trinitrobenzene sulfonic acid-induced model, in IL-$2^{-/-}$ mice, and in mice with acute graft-versus-host disease (30, 40, 19). Although these results identify this pathway as important in gastrointestinal immune pathology, they do not identify whether this pathway is involved in T cell costimulation or migration of T cells into the intestine. $CD134^+$ cells have been found in the brain in mice with EAE and in the colon of mice with colitis (30, 40). In the former case, $CD11b^+$ microglia were shown to express CD134L, suggesting that CD134L present on APC in inflammatory lesions may be an important costimulatory molecule driving proliferation of $CD134^+$ effector cells at sites of inflammation. Somewhat at odds with these findings, our studies failed to reveal CD134L expression on cells in the colon of mice with colitis by flow cytofluorography or in situ by immunohistochemistry. Furthermore, despite the presence of substantial T cell infiltration, there were few $CD134^+$ T cells in the inflamed colon, but abundant expression of CD134 on T cells in the MLN, further supporting the idea that it is DC expression of CD134L in the MLN that drives the pathogenic process. However, lack of detectable expression of CD134 or CD134L in the colon does not rule out the possibility that this pathway provides T cell costimulation in the colon in addition to in the MLN, and additional experiments are required to address this. It has been argued that the inhibition of Th1 inflammatory responses via blockade of this pathway involves effects on CD134L on the vascular endothelium and inhibition of migration and not costimulation (58). Consistent with this, analysis of CD134L expression using CD134Ig revealed CD134L expression on the inflamed vascular endothelium in patients with IBD (59). However, our results are at odds with this hypothesis, as in colitic mice, there was no detectable CD134L in the colon or on the vascular endothelium. However, the finding that the frequency of T cells expressing the gut-homing molecule $\alpha_4\beta_7$ was significantly reduced in OX89-treated mice suggests that an impairment in costimulation can also affect migration as a result of reduced expression of cell adhesion molecules. Indeed, interaction between $\alpha_4\beta_7$ and its ligand mucosal addressin cell adhesion molecule 1 has been shown to be essential in the development of colitis in the SCID model (60), and it seems likely that part of the ability of OX89 to inhibit colitis involves reduced expression of this molecule.

Based on these data, we propose a model in which inflammation in the intestine, probably triggered by bacteria, leads to an increase in the recruitment of DC or their precursors from the blood into the lamina propria. In the intestinal environment, these DC sample intestinal Ags (bacterial and/or self) become activated, and migrate to the MLN, where they activate T cells. In the absence of Treg cells, $CD134L^+$ DC drive uncontrolled T cell expansion, which, in the presence of IL-12, leads to the differentiation of Th1 cells. These cells home to the intestine, where, after secondary stimulation, they mediate their effector function. This increases the level of inflammation, which in turn increases DC recruitment to the lamina propria. Based on this model, immune interventions that disrupt this positive feedback loop should inhibit colitis. Consistent with this, in addition to OX89 treatment shown in this study, disruption of IL-12 signaling (7, 11) or of CD154-CD40 interactions (61) also prevented colitis in T cell-restored SCID mice. Normally, Treg cells interrupt this feedback by preventing the accumulation of activated DC in the MLN. Whether this is a result of effects on DC migration or on their activation and survival in the MLN remains to be established.

It is a possibility that dysregulated DC activation and expression of CD134L is a feature of immune pathology that accompanies lymphopenia in a number of models, and that regulation of CD134L expression on DC by Treg cells is one mechanism by which these cells actively control peripheral T cell responses. Based on these studies, targeting of CD134-CD134L interactions may be efficacious for the prevention of a number of T cell-mediated immune pathologies, including IBD and organ-specific autoimmune diseases.

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