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Polyclonal Tregs Modulate T Effector Cell Trafficking

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Abstract

In this study, we have analyzed the *in vivo* dynamics of the interaction between polyclonal Foxp3⁺ Tregs, effector T (Teff) cells, and DCs in order to further our understanding of the mechanisms of Treg-mediated suppression. Co-transfer of polyclonal activated Tregs into normal mice attenuated the induction of EAE. Suppression of disease strongly correlated with a reduced number of Teff cells in the spinal cord, but not with Treg-mediated inhibition of Th1/Th17 differentiation. Co-transfer of Tregs with TCR Tg Teff cells followed by immunization by multiple routes resulted in an enhanced number of Teff cells in the lymph nodes draining the site of immunization without an inhibition of Teff cell differentiation. Fewer Teff cells could be detected in the blood in the presence of Tregs and fewer T cells could access a site of antigen exposure in a modified delayed type hypersensitivity assay. Teff cells recovered from LN in the presence of Tregs expressed decreased levels of CXCR4, syndecan, and the sphingosine phosphate receptor, S1P1. Thus, polyclonal Tregs influence Teff cell responses by targeting trafficking pathways, thus allowing immunity to develop in lymphoid organs, but limiting the number of potentially auto-aggressive cells that are allowed to enter the tissues.

Keywords

Treg; Trafficking; EAE; Immunosuppression

Introduction

Numerous mechanisms exist to both activate and dampen immune responses. A primary cell type involved in immune suppression is the thymic-derived regulatory T cell (Treg) defined by the expression of the transcription factor Foxp3. Mutations in Foxp3 lead to severe defects of immunological homeostasis in both mouse and human [1]. Treg have also been shown to play a pivotal role in numerous disease settings, including autoimmunity, infection and tumor progression [2]. Multiple mechanisms have been proposed for suppressor function of Tregs including the secretion of suppressive cytokines, direct cytotoxicity of T effector (Teff) cells, metabolic disruption through tryptophan catabolites, adenosine or IL-2 deprivation, and direct interference of co-stimulation via expression of CTLA-4 [3]. Given the obvious interest in targeting Tregs in various disease settings through pharmacological intervention, a more definitive understanding of their mechanism of action is warranted. In order to achieve this, the *in vivo* dynamics of the interaction between Tregs, Teff, and DC needs to be more thoroughly evaluated.

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Conflict of interest

The authors declare no financial or commercial conflict of interest

Upon immunological challenge, DCs capture antigen and migrate to draining LNs where they present the antigen to Teff [4]. The Teff then become activated, undergo several rounds of division during which time they differentiate. After this has occurred, Teff leave the LN, enter the circulation and ultimately enter tissues. All of these steps represent potential checkpoints where Tregs may exert their influence. Both polyclonal [5,6] and antigen-specific Tregs [7, 8] are capable of inhibiting the development of autoimmune disease although the latter appear to be more effective [7]. Here, we more closely evaluate, in an *in vivo* setting in immunocompetent mice, the checkpoints at which polyclonal Tregs exert their inhibitory function.

Results

Enhancement of Treg numbers inhibits the induction of autoimmune disease without inhibiting Teff differentiation

We evaluated the role of Tregs in the well-characterized model of myelin oligodendrocyte glycoprotein (MOG)-induced EAE. As previous studies [9] have shown that administration of polyclonal Treg to normal mice can partially inhibit the development of EAE, we transferred into recipient mice either Tregs that had been purified from normal mice and expanded *in vitro* by stimulation with anti-CD3 and IL-2 or Tregs that had been generated from Foxp3⁺ T cells by stimulation *in vitro* with TGF- β . One day following transfer, the mice were immunized for the induction of EAE. Both groups of Treg treated mice displayed significantly reduced clinical severity as compared to the control group (Fig. 1A, right panel). Endogenous Tregs also control the development of EAE as mice treated with a partially depleting or inactivating anti-CD25 [10] three days prior to immunization consistently exhibited an exacerbated disease course (Fig. 1A, left panel).

Taken together, these studies demonstrate that merely altering the number of Tregs *in vivo* can dramatically alter the course of an autoimmune disease. To more thoroughly understand the mechanism(s) for the reduction of disease severity by enhancement of Treg numbers, we evaluated the phenotype of the Teff cells that had trafficked into the brain. We isolated the cellular infiltrate from the spinal cords of mice with EAE that had either received or had not received Tregs, re-stimulated them *in vitro* with PMA/ionomycin, and evaluated cytokine production by intracellular staining. Mice that had received Tregs had a two-fold reduction in the percentage of central nervous system infiltrating CD4⁺ Teff (Fig. 1B, top), but on a per cell basis, the cytokine profile of these cells was almost identical between the two groups (Fig. 1B, bottom; the two fold difference in IFN- γ +IL-17⁺ cells was not a consistent reproducible result). No differences were observed in the production of IL-2, IL-4, or TNF- α , or in the expression of memory/activation markers such as CD44, CD25, or CD69 (data not shown). Thus, the reduced clinical disease most strongly correlates with the reduced percentage of Teff cells that invade the CNS rather than Treg-mediated inhibition of Th1/Th17 differentiation or induction of immune deviation leading to the development of a less pathogenic Th2 phenotype.

Co-transfer of polyclonal Treg results in accumulation of fully differentiated Teff in the LNs draining the site of immunization

To more precisely characterize the effects of polyclonal Tregs on the expansion/differentiation of Teff cells *in vivo*, we developed a model in which congenically marked CFSE-labeled TCR transgenic Foxp3⁺ T cells were adoptively transferred to normal recipients in the presence or absence of congenically distinct pre-activated polyclonal Tregs (Fig. 2A). The following day, the mice were immunized with their cognate peptide in CFA, and the numbers and activation status of transferred Teff cells were analyzed at various time points. As our studies in the EAE model demonstrated that fewer Teff cells were present in

the target organ, we hypothesized that in the presence of Tregs, a decrease in Teff cell proliferation would be observed. Surprisingly, Tregs had no effect on Teff proliferation as measured by CFSE dilution and a two-fold increase in the percentage and absolute number of Teff cells present in the draining LN was observed (Fig. 2B, D; Supporting Information Fig. S1A). Further analysis of the transferred T cells demonstrated that there was no difference in the percentage of cells differentiating into either Th1 or Th17 lineages, nor were there differences in the level of expression of the activation marker CD44 (Fig. 2C). As it remained possible that potential suppressive effects of Treg were blocked by the use of CFA as an adjuvant, we also immunized the mice with peptide-pulsed splenic DC. The results were identical to those observed in the presence of CFA. Teff cell proliferation was not blocked, and there was a greater than two-fold increase the total number of the Teff cells in the spleen in the presence of Tregs (Fig. 2D). Although the experiments in Fig. 2D were performed with CD4⁺CD25⁻ T cells from 2D2 mice that might contain a small number of CD25⁻Foxp3⁺ T cells, identical results were observed when Foxp3⁻ Teff were purified from TCR Tg mice on a RAG^{-/-} background (Supporting Information Fig. S1A and S1B). Similar results were observed when we immunized the mice with PCC protein i.v. or transferred cytochrome specific T cells to mice that transgenically expressed pigeon cytochrome C (PCC) (Supporting Information Fig. S2). Taken together, these studies demonstrate the effects of polyclonal Treg under immunization strategies ranging from highly immunogenic (CFA) to tolerogenic (i.v. antigen or endogenous expression of antigen) all resulted in an amplification of the total number of Teff at the site of immunization.

The protocol used in the previous experiments had the disadvantage of only being able to track one cell population at a time. We were therefore limited in our ability to track the relative dynamics of Teff and Tregs at the same time. We addressed this issue by co-transferring CFSE labeled CD45.2⁺Thy1.1⁻ 2D2 TCR Tg (specific for MOG₃₅₋₅₅) Teff cells in the presence or absence of CFSE-labeled CD45.2⁺Thy1.1⁺ Tregs into CD45.1⁺ recipients at a Teff to Treg ratio of 1:4. The ratio of Teff to Treg was chosen on the basis of previous experiments that demonstrated that the engraftment efficiency of Treg is far lower than that of Teff. Therefore, the Teff:Treg ratio injected does not equal the Teff:Treg ratio that engrafts. Three days after immunization with MOG-pulsed splenic DCs, total donor cells were differentiated from host cells based on CD45.2 expression (Fig. 3) and Tregs were distinguished from Teff on the basis of Thy1.1 expression. As seen previously, no difference in CFSE profiles were observed between the two groups, but the total number of Teff in the spleen was greater in the presence of Tregs. There was appreciable proliferation of the Tregs, but they did not divide to the same extent, as did the Teff. Teff expansion greatly outpaced Treg expansion, becoming 97% of the total transferred CD4⁺ population. Although recent reports [11] have suggested that during inflammatory conditions Tregs downregulate the expression of Foxp3, the levels of Foxp3 expression were almost identical to pre-transfer levels (Fig. 3 and data not shown).

Polyclonal Treg inhibit Teff cell egress from the site of immunization and limit access of Teff to tissues

The increase in the number of antigen-specific T cells in the LN following priming in the presence of polyclonal Tregs is in apparent conflict with our studies in EAE that demonstrated a decreased number of Teff cells in the target organ in the presence of an excess of Tregs. However, the total number of T cells in the LN is determined not just by *in situ* proliferation and expansion, but also by the relative contribution of entry and exit from the LN. We therefore determined the relative proportions of transferred T cells in the LN and the blood. In mice that had received Teff in the absence of Treg, 8.63% of the total LN CD4⁺ cells were of donor origin seven days following immunization (Fig. 4, top panels). At

the same time point, 4.13% of the CD4⁺ cells in the blood were of donor origin. In contrast, in mice that had received Treg in addition to Teff, 11.6% of the LN CD4⁺ cells were of donor origin, but only 1.3% of the CD4⁺ cells in the blood were of donor origin. In multiple experiments, we consistently found a greater number of cells in the LN, and fewer cells in the blood of mice that had received Tregs at multiple time points (Fig. 4, lower panels; Supporting Information Fig. S1C).

To determine if Treg altered the trafficking of Teff cells, we used a modified delayed type hypersensitivity model in which we could control the timing and location of a tissue dwelling antigen. CD45.1⁺ 5CC7 TCR-Tg T cells (specific for PCC), were adoptively transferred into CD45.2⁺ recipients in the presence or absence of Treg. The following day the mice were immunized in the hind flank with PCC in CFA. Seven days later, the mice were challenged in the ear with PCC peptide in PBS. The next day the ears were removed, dissociated, and the total number of Teff cells enumerated (Fig. 5). As seen previously, there was an increase in the percentage and absolute numbers of Teff cells in the LN, and a decreased number of Teff cells in the blood of mice that had received Tregs. We also observed a greatly decreased number of donor cells in the ears of mice that had received Tregs (Fig. 5, bottom panel; Supporting Information Fig. S1D). This result is consistent with the hypothesis that in the presence of polyclonal Tregs fewer cells leave the LN to enter the circulation, and fewer cells are therefore available to respond to antigen at a distant site.

Potential mechanisms used by Tregs to retain Teff at site of immunization

To begin to explore potential mechanisms by which Tregs might inhibit T cell trafficking from the site of immunization, we initially compared the phenotype of Teff primed in the presence or absence of Tregs. There were no differences between the two groups for a variety of markers tested. A summary of various markers, cytokines and chemokine/chemokine receptors that was consistently found to be unaltered between the two groups can be found in Supporting Information Table 1. These results suggested to us that the presence of a higher number of Tregs does not result in global and dramatic alterations to the immune response, but influences immunological outcomes by targeting very specific pathways. To elucidate these pathways, we purified Teff cells from mice that had been immunized in the presence or absence of Tregs and subjected mRNA from these cells to microarray analysis. Remarkably, very few genes were found to be up or downregulated by more than 3-fold between the two groups (data not shown), further confirming the notion that Tregs do not induce global changes. Notably, two of the genes that were found to be different between the two groups were involved in cell migration and trafficking. CXCR4 was found to be decreased over 4-fold in the presence of Tregs. We confirmed this observation both at the protein and at the mRNA level (Fig. 6). We also confirmed at the protein level decreased expression of Syndecan-4, a molecule involved in cell motility [11]

An additional molecule that has been well characterized as being important in the trafficking of T lymphocytes is the sphingosine 1-phosphate receptor 1, S1P1 [12]. S1P1 levels are rapidly downregulated on T cells following entry into the LN. As T cells are primed and differentiate, they upregulate S1P1 allowing the cell to respond to high levels of S1P in the circulation and exit the LN in response to the concentration gradient [13, 14]. We observed a dramatic decrease in S1P1 expression at the mRNA level in Teff cells that had been primed in the presence of Tregs. This observation provides a potential mechanistic explanation for the retention of Teff cells in the LN. By altering the expression of S1P1 on Teff cells, Tregs would affect the ability of these cells to migrate out of the LN and into the circulation. It remains to be determined whether Treg-mediated suppression of S1P1 upregulation on Teff is direct or indirect.

Discussion

Both polyclonal and antigen-specific Treg are capable of suppressing immune responses in vitro and in vivo. A number of studies have shown that antigen-specific Tregs inhibit the expansion and/or the differentiation of Teff cells [15, 8] in vivo presumably by downregulating DC function [16–18] or by direct killing of antigen presenting cells [19]. Much less is known concerning the suppressive mechanisms of polyclonal Treg. Previous studies in the EAE model [9] demonstrated that augmentation of Treg numbers in normal recipients by 50–75% resulted in marked attenuation of disease activity accompanied by normal activation of Th1 cells, enhanced production of Th2 cytokines, and decreased infiltration into the CNS. The induction of autoimmune gastritis following transfer of gastric antigen-specific Teff cells to *nu/nu* mice could be inhibited by co-transfer of polyclonal Treg [6]. The Treg did not inhibit the expansion of the Teff cells at the site of inflammation (gastric LN or stomach), but appeared to inhibit the induction of Th1 cytokine production. Sarween et al [6] in a TCR-Tg transfer model of diabetes observed modest effects of Treg on the expansion of effector cells, but marked effects on the ability of the effectors to enter the target tissue. Here, we have re-examined potential mechanisms of suppression by polyclonal Treg and have performed all experiments in immunologically intact recipients and carefully monitored the fate and differentiation of the Teff on a single cell basis. Our results clearly indicate that rather than altering priming, expansion, or differentiation, Treg primarily functioned by altering the trafficking potential of Teff. These data are supported not only by the combined results of figures 2 and 4, but also with the EAE data which demonstrated that fewer cells arrived in the CNS, but those that did were phenotypically indistinguishable from Teff in non-Treg treated mice. Thus, by trapping effector cells in the LN, Tregs would limit the number of potentially auto-aggressive T cells that would be available to migrate into tissues where they would subsequently cause damage.

It should be noted that we have performed the majority of our studies with polyclonal Treg populations that have been activated via their TCR and expanded in IL-2. The primary reason for this approach was to obtain sufficient numbers of Treg for use in our transfer protocols. It is widely accepted that once activated Treg exert their suppressive function in a non-antigen-specific fashion, at least in studies performed in vitro [20]. However, due to their polyclonal nature, it remains unclear how, or even if, these cells were re-activated in vivo. Several hypotheses might account for the effect that we have observed, including re-activation of a sub-population of antigen specific Treg within the polyclonal pool, activation on a self-antigen (s) unrelated to the immunizing antigen, or no need for re-activation as a result of their pre-activation in vitro. In a limited number of studies we have observed similar results following transfer of freshly explanted Treg and presumably under these conditions, the Treg are activated in vivo by recognition of their target self-antigens. Our data cannot distinguish these possibilities and further studies will be required to resolve these issues. Yet, the transfer of pre-activated Tregs resulted in a demonstrable effect on the trafficking capabilities of Teff. Understanding the dynamics of this interaction is important as transferred, pre-activated polyclonal Tregs are the most likely to be used in clinical situations. The mechanisms by which Treg inhibit Teff cell trafficking remain to be fully elucidated. The decrease in S1P1 expression at the mRNA in Teff that had been primed in the presence of Tregs is an attractive mechanism for the retention of the Teff in the LN, but other effects of Treg on chemokine expression [6] or on adhesion molecule expression [9] must also be considered. Although our studies were performed in a model system using TCR transgenic Teff cells, recent studies have shown that polyclonal Treg cells may also regulate trafficking of CD8⁺ T effector cells in vivo during acute infection with respiratory syncytial virus [21].

It is clear from these studies that polyclonal Tregs do not influence the immune response by simply “shutting down” immunity. In fact, it has recently been shown that polyclonal Treg enhance antibody responses when mice are immunized intranasally in the presence of the cholera toxin potentially by promoting Teff cell retention in the LN and promoting T-dependent B cell responses [22]. It would therefore be expected that the therapeutic administration of polyclonal Tregs would not necessarily lead to global immunosuppression or the inhibition of responses to all antigens or pathogens. Rather, they influence the Teff cell responses by specifically targeting trafficking pathways, thus allowing immunity to develop in lymphoid organs, but limiting the number of potentially auto-aggressive cells that are allowed to enter tissues.

Materials and Methods

Mice

C57BL/6 and B10.A mice were obtained from DCT, NIH. C57BL/6 CD45.1⁺ and CD45.1⁺ 5CC7 TCR Tg mice on RAG^{-/-} background were obtained from Taconic Farms. 2D2 TCRTg and B6 Thy1.1 (B6.PL) mice were obtained from The Jackson Laboratory. 2D2-Thy1.1 mice were generated in house by crossing 2D2 TCR Tg mice with Thy1.1 (B6.PL) mice and screening the progeny by flow cytometry with anti-V β 11 and Thy1.1 antibodies.

EAE Induction

EAE was induced in C57BL/6 mice by subcutaneous immunization in the hind flank with 200 μ l of an emulsion containing 400 μ g of MOG₃₅₋₅₅ peptide and 400 μ g of *Mycobacterium tuberculosis* strain H37Ra in CFA (Difco). On days 0 and 2, the mice received an i.p. injection of 200 ng pertussis toxin (CalBiochem) dissolved in 100 μ L PBS. Clinical symptoms were assessed according to the following criteria: 0, no signs of disease; 1, complete tail paralysis; 2, hind limb weakness; 3, complete hind limb paralysis; 4, unilateral forelimb paralysis; and 5, moribund/death. Mice observed in a moribund state were euthanized. Data presented are the mean clinical scores of five mice per group.

Cells

Polyclonal Tregs were isolated on the basis of CD25 expression using the Treg isolation kit (catalog number 130-091-041) from Miltenyi Biotec according to the manufacturer's protocol. The purified Tregs were activated for three days on plate-bound anti-CD3 (Becton Dickinson) in 24 well plates (Falcon) at 2 μ g/well in complete medium with IL-2 100 IU/ml. Foxp3 purity was consistently 85–95%. CD4⁺ cells⁻ were isolated using the CD4⁺ T cell isolation kit (catalog number 130-090-860) from Miltenyi according to the manufacturer's instructions. CD4⁺CD25⁻ were purified on the AutoMacs. iTregs were induced from CD4⁺CD25⁻ precursors by three day incubation on plate bound anti-CD3 (2 μ g/well) and anti-CD28 (1 μ g/well) in 24 well plates in complete medium containing TGF- β (5 ng/ml) and IL-2 (100 IU/ml). Where indicated, cells were labeled with CFSE by incubation in 1 μ M CFSE in PBS for 8 minutes followed by a wash in complete medium, followed by an additional wash in PBS. DCs were obtained from collagenase (Liberase Blendzyme TH, Roche) digested spleens by incubation with CD11c beads (Miltenyi) followed by purification on the AutoMacs cell separator (Miltenyi) using the POSSELD2 program.

Immunization and cell analysis

For immunization in the flank, mice were injected with peptide (either PCC or MOG) emulsified in CFA. Cells from the draining inguinal LN were used for analysis. For immunization with peptide-pulsed DCs, mice were injected i.v. with both the DCs and the T cells, and cells from the spleen were used for analysis. Single cells suspensions, obtained by

mechanical disruption of the organ, were incubated with a combination of fluorochrome-labeled antibodies appropriate for the particular experiment, washed and subjected to flow cytometry on the LSRII instrument (BD). Cells from the ear dermis were obtained as previously described [23]. CD4-Pacific Blue (1:250), CD45.1-APC (1:250), CD45.2-APC-Alexa750 (1:250) and CD44-Alexa700 (1:250), IFN- γ -PECy7 (1:600), IL-17-PerCPCy5.5 (1:350) and FoxP3-PE were all obtained from eBioscience. The cells were first stained for surface markers in PBS containing 5% BSA and 2mM EDTA and washed. If intracellular staining was desired, the cells were then fixed and permeabilized with Fix/Perm buffer followed by staining in Perm buffer (FoxP3 staining buffer kit, eBioscience). Analysis was performed with FlowJo software (Treestar).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used

MOG	Myelin oligodendrocyte glycoprotein
PCC	pigeon cytochrome c
S1P1	sphingosine 1-phosphate receptor 1
Teff	T effector cell

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EAE induction and followed for symptoms. Data are representative of 3–5 experiments. (B) Mice that had received either Tregs (1×10^6) or left untreated were immunized to induce EAE. On day 21, the mice were euthanized and infiltrating leukocytes were isolated from the spinal column. The cells were restimulated with PMA/ionomycin, stained for the indicated markers, and analyzed by flow cytometry. Data are representative of at least five experiments.

Figure 2.

Co-transfer of polyclonal Tregs enhances Teff cell numbers in LN. (A) Schematic diagram illustrating the experimental approach. CD45.1⁺CD45.2⁻CD4⁺ TCR Tg cells from TCR Tg mice on a RAG^{-/-} background were transferred either alone or in combination with CD45.2⁺CD45.1⁻ Tregs into CD45.2⁺ recipients. The following day, mice were immunized with peptide in adjuvant. At various time points, the transferred Teff cells were identified as CD4⁺CD45.1⁺CD45.2⁻. (B), CD45.1⁺ 5CC7 TCR Tg T cells were transferred into naïve B10.A recipient mice, which were immunized the following day with PCC emulsified in CFA. (B, left panel) CD45.1 and CD45.2 staining of cells in the CD4⁺ gate. Gated cells are the Teff cells and the numbers indicate the percentage of transferred Teff cells within the CD4⁺ gate. (B, right panel) CFSE staining of the gated transferred Teff cells. Data are representative of 3–5 experiments. (C) Experimental design as in (B), except cells were analyzed on day 7, restimulated with PMA/ionomycin, and stained for surface CD44 expression in conjunction with intracellular cytokines. The result shown is representative of at least five experiments. (D) CD4⁺CD25⁻ 2D2-Thy1.1 cells were co-transferred with MOG-pulsed DCs into Thy1.2⁺ recipients. Three days later, the CFSE profiles and total number of CD4⁺Thy1.1⁺ cells in the spleen were determined. Data are representative of at least three experiments.

Figure 3.

Both Tregs and Teff cells proliferate in the draining LN. CD4⁺CD45.2⁺Thy1.1⁻ 2D2 TCR transgenic T cells were co-transferred with CD4⁺CD45.2⁺Thy1.1⁺ Tregs into CD45.1⁺ recipients. Mice were immunized by co-transfer of MOG-pulsed DCs. Splenic cells were analyzed three days later. Transferred Teff cells and Tregs were identified in the CD4⁺ gate as CD45.2⁺CD45.1⁻. Teff and Treg populations were distinguished on the basis of Thy1.1 positivity. CFSE profiles of the Teff cell population alone (top) and CFSE profiles of Teff cells co-transferred with Tregs (bottom) are depicted as well as the CFSE profile and Fopx3 staining of the transferred Treg population (bottom). Data are representative of at least three experiments.

Figure 4.

Fewer Teff cells are found in the blood of mice that receive Tregs (Top) CD4⁺CD45.1⁺ 5CC7 TCR-Tg cells were transferred alone or with Tregs into CD45.2⁺ recipients and immunized with PCC/CFA as described in Fig 1. Gated cells are the Teff cells from either LN or blood and the numbers represent the percentage of Teff cells in the CD4⁺ gate. (Bottom) Time course of appearance of Teff cells in the LN (left panel) and blood (right panel). Data are representative of at least three experiments.

Figure 5.

Polyclonal Treg prevent Teff from homing to the target organ. (Top) CD4⁺CD45.1⁺ 5CC7 TCR Tg T cells were transferred into CD45.2⁺ recipients either alone or in combination with Tregs. Mice were immunized with PCC/CFA in the flank. Seven days later, mice were challenged with PCC in PBS in the ear dermis. The following day, the ears, blood and the LN draining the site of PCC-CFA immunization were collected. Donor T cells were identified as CD45.1⁺CD45.2⁻ cells within the CD4⁺ gate. (Bottom) Total numbers of donor Teff cells in the LN and ears, and percentage of donor Teff cells in the blood. Data are representative of at least five experiments.

Figure 6.

Expression of CXCR4, syndecan, and S1P1 on Teff cells from mice immunized in the presence of absence of Tregs. (Top panels) Mice were immunized as described in Fig. 1. Seven days later, the surface expression levels of CXCR4 and Syndecan-4 were determined on donor Teff cells from mice that were transferred alone (unfilled histograms) or with Tregs (filled histograms). CXCR4 data are representative of at least four experiments. Syndecan-4 data are representative of three experiments. (Bottom panels) Transferred Teff cells were FACS purified on the basis of CD4⁺ and CD45.1 expression. mRNAs were isolated and used to determine CXCR4 and S1P1 expression. Data for both molecules are representative of three experiments.