CCL-21 Conditioned Regulatory T Cells Induce Allotolerance through Enhanced Homing to Lymphoid Tissue

Sunil K. Chauhan, Daniel R. Saban, Thomas H. Dohlman and Reza Dana

*J Immunol* 2014; 192:817-823; Prepublished online 11 December 2013;
doi: 10.4049/jimmunol.1203469
http://www.jimmunol.org/content/192/2/817
CCL-21 Conditioned Regulatory T Cells Induce Allotolerance through Enhanced Homing to Lymphoid Tissue

Sunil K. Chauhan, Daniel R. Saban, Thomas H. Dohlman, and Reza Dana

Regulatory T cells (Tregs) are instrumental in the induction and maintenance of tolerance, including in transplantation. Tregs induce allotolerance by interacting with APCs and T cells, interactions that require their proper homing to the lymphoid tissues. Using a well-characterized model of corneal allotransplantation, we demonstrate in this study that Tregs in the draining lymph nodes (LN) of allograft acceptors, but not rejectors, colocalize with APCs in the paracortical areas and express high levels of CCR7. In addition, we show that Treg expression of CCR7 is important not only for Treg homing to the draining LN, but also for optimal Treg suppressive function. Finally, we show that Tregs augmented for CCR7 expression by their ex vivo stimulation with the CCR7 ligand CCL21 show enhanced homing to the draining LN of allograft recipients and promote transplant survival. Together, these findings suggest that CCR7 expression is critical for Treg function and migration and that conditioning of Treg for maximal CCR7 expression may be a viable strategy for promoting allotrogenic survival.


R egulatory T cells (Tregs) are master regulators of the immune system that have been shown to impact nearly every aspect of T cell function by interacting with both T cells and APCs (1–4). CD4+CD25+Foxp3+ Tregs (which constitute 5–10% of CD4+ T cells) have shown considerable promise in strategies for the induction of allospecific tolerance in a number of settings including skin, cardiac, kidney, and islet transplantation, with most experimental strategies focused on Treg expansion and perioperative transfer to grafted hosts (5–7).

The current paradigm of Treg function proposes that these cells function in two principal locations: secondary lymphoid tissues, where they suppress induction of immunity by regulating T cell priming and expansion, and inflamed peripheral tissues, where they suppress effector immune cell functions (8–10). Appropriate Treg homing in secondary lymphoid tissues is required for the functional clustering of Tregs with APCs and T cells, which is needed for the induction and maintenance of immunological tolerance (11, 12). This Treg localization does not occur randomly, but is a highly coordinated process involving chemokines and adhesion molecules. A majority of Tregs found in the secondary lymphoid compartment preferentially express L-selectin (CD62L) and CCR7 that regulate Treg extravasation across the high endothelial venules and homing to the lymphoid tissues (9, 13–15).

Using a well-characterized mouse model of corneal transplantation (16, 17), we recently provided new insights on Treg localization and function in transplantation immunity (18). We reported that Tregs are crucial in promoting corneal transplant (the most common form of tissue allografting) survival primarily by suppressing the induction of alloimmunity in the draining lymph nodes (LN). In addition, we demonstrated that allograft outcome was closely correlated to Treg suppressive function rather than Treg frequencies; indeed, whereas Treg frequencies in the draining LN were found to be similar in both corneal allograft acceptors and rejectors, only Tregs in the LN of acceptors were capable of preventing allograft rejection (18). Importantly, however, the mechanisms that determine why Tregs in the LN of allograft acceptors are more functionally effective than those in allograft rejectors are not well understood.

In the current study, we investigated the localization of Tregs in the draining LN of allograft recipients and evaluated the expression patterns of different homing receptors by these Tregs. Our data demonstrate that Treg expression of CCR7 is most critical in regulating both their homing and suppressive function. Moreover, we provide novel evidence that ex vivo conditioning of Tregs with CCL21 leads to significant upregulation of Treg expression of LN homing receptors, including CCR7, and these CCR7-amplified Tregs home more efficiently to the reactive LN of corneal allograft recipients to promote graft survival.

Materials and Methods

Mice

Eight-week-old male BALB/c and C57BL/6 (Taconic Farms, Germantown, NY) mice were used in all experiments. CCR7−/− C57BL/6 mice were provided by Andrew Luster (Massachusetts General Hospital). Mice were housed in a specific pathogen-free environment at the Schepens Eye Research Institute animal facility. All procedures were approved by the Institutional Animal Care and Use Committee, and all animals were treated according to the Association for Research in Vision and Ophthalmology statement for the Use of Animals in Ophthalmic and Vision Research.

Corneal transplantation

Standard protocol for murine orthotopic corneal transplantation was used, as described previously (16–18). Briefly, donor center corneas (2-mm diameter) were excised from C57BL/6 mice and sutured onto recipient graft beds prepared by excising a 1.5-mm site in the central cornea of BALB/c mice. Simultaneously, some BALB/c mice received syngeneic (BALB/c) grafts to control for the nonallospecific effects of surgery. The corneal sutures were removed 7 d after surgery. All grafts were evaluated using slit-lamp biomicroscopy at weekly intervals. Grafts were defined as rejected when they became opaque and the iris details could not be
recognized clearly using a standardized opacity grading (range 0–5) scheme (16–18). In this model, ~50% of allografts are rejected within 3 wk of transplantation, whereas the remaining half enjoys indefinite survival. Compared with other transplant systems (e.g., skin, heart, kidney, etc.) in which a 100% graft rejection rate is observed in unmanipulated recipients, this model provides an exceptional opportunity to study why some allografts are spontaneously accepted and to evaluate the role of Tregs in this high frequency of transplant survival.

### Treg isolation and culture

CD4+CD25+ Tregs from the LN were isolated by magnetic separation using a regulatory T cell isolation kit (Miltenyi Biotec). Purity of sorted cells was >97% as confirmed by Foxp3 staining using flow cytometry. Sorted Tregs were cultured in complete RPMI 1640 media under standard culture conditions. To stimulate Tregs, chemokine ligands or recombinant murine CCL19 or CCL21 (PeproTech) were added at a concentration of 10 ng/ml. Sorted Tregs were cultured in complete RPMI 1640 media under standard culture conditions. To stimulate Tregs, chemokine ligands or recombinant murine CCL19 or CCL21 (PeproTech) were added at a concentration of 10 ng/ml to either media alone or with conditioning mixture (CD3 Ab, CD28 Ab, and IL-2) for Treg expansion (20).

### Immunohistochemistry

Draining submandibular LN of different transplant recipients were harvested at 3 wk posttransplantation, and acetone-fixed cryosections were prepared for immunostaining. The sections were first blocked with 2% BSA and anti-Fcγ Ab (eBioscience) and then immunostained with primary anti-mouse Foxp3 and anti-mouse CD11c or isotype-matched control Abs (Santa Cruz Biotechnology) overnight at 4°C and then with Alexa Fluor 350- and rodamine-conjugated secondary Abs (Invitrogen) for 1 h at room temperature. The stained cross-sections were mounted using Vector Shield mounting medium (Vector Laboratories) and examined under the epifluorescence microscope (Nikon).

### Flow cytometry

Draining LN were harvested from different transplant recipients, and single-cell suspensions were prepared (18). The isolated cells were stained with the following Abs: anti-CD4 FITC, anti-Foxp3 PECy5, and anti-CCR7, -CD62L, or -CD103 PE (eBioscience). CD4+Foxp3+ Tregs were gated first, and expression of different receptors was analyzed using an EPICS XL flow cytometer (Beckman Coulter).

### In vitro Treg suppression assay

Naive CD4+CD25+ Tregs from the LN of wild-type and CCR7 knockout (KO) mice and CD4+CD25+ effector T (Teff) cells from the LN of wild-type were isolated using a magnetic separation kit (Miltenyi Biotec). As described previously (18), CD4+CD25+ Teff cells (1 × 10⁵) were cocultured with CD4+CD25+ Tregs (5 × 10⁵). T cell–depleted syngeneic splenocytes (1 × 10⁵), and 1 μg/ml anti-CD3 Ab for 3 d. Proliferation of CD3 stimulated Teff cells without the addition of Tregs was considered as control proliferation with 0% suppression. Proliferation was measured using the BrdU incorporation assay (Millipore), and percent suppression was calculated using the following formula: % suppression = ([Teff cell proliferation without Tregs − Teff cell proliferation with Tregs]/Teff cell proliferation without Tregs) × 100 (18). In addition, culture supernatants were collected to evaluate the expression of immunoregulatory cytokines IL-10 and TGF-β using commercially available ELISA kits (R&D Systems).

### Adoptive transfer of Tregs to allograft recipients

Tregs were labeled with PKH-26 vital dye (Sigma-Aldrich) as per the manufacturer’s instructions. PKH-26–labeled Tregs (2 × 10⁶ cells/mouse) were transferred i.v. to the allograft recipients at 24 h postsurgery. Allograft survival rate in each group (n = 8/group) was monitored up to 8 wk posttransplantation.

### Results

#### Tregs of allograft acceptors colocalize with APCs in the paracortical areas of draining LN and express higher levels of CCR7

In order to investigate why Tregs are functionally more effective in allograft acceptors than rejectors, we first investigated the localization and cellular interactions of Tregs in the regional LN of graft-hosted allografts. Draining LN from syngeneically grafted recipients (BALB/c → BALB/c) and fully disparate allograft (C57BL/6 → BALB/c) acceptors and rejectors were harvested and immunostained for Foxp3+ Tregs and CD11c+ APCs (Fig. 1A). In allograft acceptors, Foxp3+ Tregs were localized in focal clusters in the paracortical region, primarily in close proximity to CD11c+ APCs of draining LN. Levels of CCR7, CD62L, and CD103 expressions by CD4+CD25+Foxp3+ Tregs were analyzed by measuring MFI using flow cytometry, showing that acceptor Tregs express very high levels of CCR7 compared with those expressed by rejector and syngeneic Tregs. Each transplant group consists of six mice, and data from a representative experiment of two performed are shown.

**FIGURE 1.** Localization and homing receptor expression by Tregs in the draining LN of syngeneically grafted recipients (BALB/c → BALB/c) and fully disparate allograft (C57BL/6 → BALB/c) acceptors and rejectors. The draining LN were harvested 3 wk after corneal transplantation. In this model of corneal transplantation, ~50% of allografts are rejected within 3 wk of transplantation, whereas the remaining half enjoys indefinite survival. (A) Confocal micrographs of LN show that only the acceptor Tregs (Foxp3, blue) are focally distributed in the paracortical area of the LN (circled with white dotted line; original magnification ×10) and colocalize with APCs (CD11c, red) (marked with white arrows; original magnification ×60). (B) Levels of CCR7, CD62L, and CD103 expressions by CD4+CD25+Foxp3+ Tregs were analyzed by measuring MFI using flow cytometry, showing that acceptor Tregs express very high levels of CCR7 compared with those expressed by rejector and syngeneic Tregs. Each transplant group consists of six mice, and data from a representative experiment of two performed are shown.

**Student t test was used for comparison of mean between the groups. Kaplan-Meier analysis was adopted to construct survival curves, and the log-rank test was used to compare the rates of corneal graft survival. Data are presented as mean ± SEM and considered significant at p < 0.05.**
APCs. In contrast, in the LN of allograft rejectors and syngeneically grafted recipients, Foxp3⁺ Tregs were found evenly distributed throughout the paracortex and were rarely in contact or close proximity with CD11c⁺ APCs.

To further understand why Tregs show different patterns of distribution and cellular interaction, we hypothesized that Tregs of allograft acceptors express high levels of homing receptors that enhance their homing and migration to the site of incoming APCs in the reactive LN. We thus investigated Treg expression of CCR7, CD62L, and CD103 homing receptors (Fig. 1B). Single-cell suspensions prepared from the reactive draining LN of different transplant recipients were analyzed using flow cytometry to determine the frequencies of CCR7⁺, CD62L⁺, and CD103⁺ Tregs, which were calculated by gating on CD4⁺Foxp3⁺ Tregs. In addition, analysis of mean fluorescence intensity (MFI) provided levels of receptor expression by these Tregs. There was no difference in the frequencies of any of the homing receptor–expressing Tregs in any of the transplant recipient groups, approximately all of the LN Tregs (>95%) expressed CCR7 and CD62L, and only 14–16% of Tregs expressed CD103. Interestingly, however, the Treg expression levels of CCR7 were substantially different among groups. More than 55% of Tregs from the LN of allograft acceptors expressed higher levels of CCR7 (MFI 320) compared with the Tregs of allograft rejectors and syngeneically grafted recipients, which expressed lower levels of CCR7 (MFI 230). In contrast to CCR7 expression, no difference in CD62L expression level was observed between Tregs from allograft acceptors, and syngeneically grafted recipients, which expressed relatively low levels of CD62L. Thus, the differential expression of these Treg homing receptors suggested to us that CCR7 could be the critical homing receptor regulating Treg migration toward APCs in the LN.

**CCR7 expression affects Treg phenotype and function**

It is still not clear whether, in addition to affecting Treg migration (15, 21), CCR7 expression itself affects Treg suppressive function. Using the in vitro suppression assay, we compared the suppressive function of Tregs isolated from CCR7-KO (C57BL6) and wild-type (C57BL6 and BALB/c) mice (Fig. 2A). CCR7-KO Tregs demonstrated a 40% reduction in the suppression of anti-CD3–stimulated CD4⁺ T cell proliferation compared with wild-type Tregs (p = 0.001). Similarly, culture supernatants from CCR7-KO Tregs showed significantly lower levels of immunoregulatory cytokines IL-10 and TGF-β compared with wild-type (C57BL6 and BALB/c) Tregs (Fig. 2B). Moreover, a 40–50% decrease in the expression levels of Foxp3 (Fig. 2C) and CD25 (Fig. 2D) were observed in CCR7-KO Tregs compared with wild-type Tregs. Together, these findings suggest that CCR7 expression on Tregs is capable of affecting both their phenotype and suppression potential.

**CCL21 signaling amplifies Treg expression of CCR7**

Next, we determined whether naive Tregs could be induced to express high levels of CCR7, which could in turn enhance both their homing to the reactive draining LN as well as their suppressive function. Because binding of cytokine or chemokine ligands to their receptors normally leads to cell activation and increased expression of receptors, we first stimulated purified naive Tregs in vitro with CCR7 ligands for 48 h to upregulate Treg expression of CCR7 (Fig. 3A). CCR7 has two chemokine ligands, CCL19 and CCL21, which are normally expressed in the T cell–rich areas of the LN (15). In vitro stimulation with CCL21 upregulated Treg expression of CCR7 substantially (~1.5–2-fold; MFI 38) compared with media only (MFI 21), whereas CCL19 stimulation led to downregulation of Treg expression of CCR7 (MFI 14).

![FIGURE 2. Effect of CCR7 expression on Treg function and phenotype. (A) Treg suppression function. Naive wild-type (WT) CD4⁺ T cells were stimulated with CD3 Ab for 3 d in the presence of different Tregs isolated from CCR7-KO, WT-C57BL6/6 (B6), and WT-BALB/c. CCR7-KO Tregs show a 40% reduction in the suppression of CD4⁺ T cell proliferation compared with WT Tregs. The activity of Tregs is measured at Treg/Teff cell ratio of 1:2. Proliferation was measured using the BrdU incorporation assay and compared with the proliferative responses of respective CD3-stimulated naive T cell in the absence of Tregs and percent suppression was calculated as described in Materials and Methods. (B) Expression of immunoregulatory cytokines TGF-β and IL-10 at protein levels in the culture supernatants from Treg suppression assays were analyzed using ELISA. Foxp3 (C) and CD25 (D) expression by CD4⁺CD25⁺Foxp3⁺ Tregs were analyzed by measuring MFI using flow cytometry. Each group consists of three mice, and data from a representative experiment of three performed are shown. p values are calculated using Student t test, and error bars represent SEM. *p < 0.02, **p < 0.01, ***p < 0.001.](http://www.jimmunol.org/DownloadedFrom/2224578/FIG2.jpg)
The application of Tregs as a cellular therapeutic to promote transplant survival requires their isolation and massive in vitro expansion. We thus next investigated if the addition of CCL21 during in vitro Treg expansion could lead to increased expression of...
the LN homing receptor CCR7 (Fig. 3B, 3C). Conditioning media consisting of CD3 agonist Ab in conjunction with IL-2 (T cell growth factor) and CD28 (costimulatory factor) agonist Ab were used to expand Tregs in vitro for 5 d (20), with or without addition of CCL21 (Fig. 3B, 3C). Tregs cultured and expanded in the presence of CCL21 expressed increased levels of the LN homing receptors CCR7 and CD62L and decreased levels of CD103 compared with those Tregs that were cultured in Treg expansion mixture or media only. Furthermore, the addition of CCL21 to Treg expansion media led to increased expression of Foxp3 by Tregs. However, as shown in the Supplemental Material, stimulation of CCR7-KO Tregs with CCL21 alone or CCL21 and Treg expansion media did not show any direct effect of CCL21 function on Treg upregulation of CCR7 and Foxp3 expression (Supplemental Fig. 1). Together, these findings confirm that addition of the CCR7 ligand CCL21 to Treg expansion media increases Treg activation and expression of CCR7 and CD62L, resulting in an amplified LN homing Treg phenotype (CCR7 hiCD62L hiCD103 lo).

**CCR7-amplified Tregs acquire maximal LN homing in allograft recipients and promote transplant survival**

To determine the in vivo function of Tregs amplified for LN homing in corneal transplant hosts, purified Tregs from naive BALB/c mice were cultured with CCL21 (CCR7 hi Tregs) or without CCL21 (CCR7 lo Tregs) and then labeled with vital cell tracking dye PKH-26. The i.v. infusion of labeled Tregs into corneal allograft recipients was performed 24 h after transplantation, a time point based on the observation of considerable numbers of graft-derived APCs in the draining LN of recipients 24 h postgrafting (16) and our previous work on Treg adoptive transfer in corneal transplant recipients (18). Three mice from each group were sacrificed 48 h post–Treg infusion to localize Tregs in the LN, spleen, and blood, whereas the remaining mice (n = 8–10/group) were used to compare allograft survival up to 8 wk posttransplantation.

CCR7 hi Tregs showed efficient homing to the lymphoid tissues, whereas CCR7 lo Tregs were primarily retained in the peripheral blood of allograft recipients (Fig. 4A). CCR7 hi Tregs demonstrated a 4-fold increase in the rate of homing to the reactive ipsilateral draining LN, as compared with CCR7 lo Tregs. In addition, the allograft recipients who received CCR7 hi Tregs demonstrated a significant increase in graft survival rate and reduced graft opacity scores compared with allograft recipients receiving either CCR7 lo Tregs or no Tregs (Fig. 4B). The allograft survival rate in recipients receiving CCR7 lo Tregs was similar to that of recipients receiving no Tregs, suggesting that CCR7 hi Tregs were unable to promote transplant survival. Together, these experiments demonstrate that Tregs that are ex vivo amplified for CCR7 expression acquire maximal homing to the draining LN in allograft recipients and promote transplant survival.

**Discussion**

In the current study, we show that Tregs of allograft acceptors but not rejectors colocalize with APCs in the paracortical areas of the draining LN and express high levels of CCR7. In addition, our data provide novel evidence that CCR7 expression affects Treg suppressive function and that increased CCL21 signaling in Tregs can amplify their expression of CCR7 and thus promote their tolerogenic function and promotion of transplant survival.

Previous studies localizing Foxp3 + Tregs in steady-state normal (ungrafted) lymphoid tissue have shown that they are evenly
CCR7 and CD62L work in concert to promote Treg homing to express higher levels of LN homing receptors, particularly CCR7, compared with Tregs of rejectors. There is evidence indicating that CCR7 and CD62L work in concert to promote Treg homing to and migration within the LN (8, 13). Conversely, CCL10 (integrin αE–E-cadherin binding) has been implicated in Treg retention in peripheral tissues (10, 23). Together, these findings indicate that Tregs in the LN of allograft acceptors are better able to migrate toward APCs, promoting immunoregulatory function at the immune synapse and that CCR7 could be the most critical homing receptor regulating Treg migration toward APCs in the LN. Furthermore, our data evaluating the function and phenotype of CCR7-KO and wild-type Tregs demonstrate that deletion of CCR7 leads to a significant 40% reduction in Treg suppression potential as well as decreased expression of Foxp3 and immunoregulatory cytokines IL-10 and TGF-β compared with wild-type Tregs, clearly suggesting that in addition to affecting Treg migration (15, 21), CCR7 expression by Tregs is capable of influencing both their phenotype and suppression potential.

To determine the therapeutic implication of these findings, we next investigated whether naive Tregs could be induced to express high levels of CCR7, which would in turn enhance both their homing to the reactive draining LN and their suppressive function. In vitro stimulation of Tregs with the CCR7 ligand CCL21, but not CCL19, led to an upregulation of Treg expression of CCR7. The chemokines CCL19 and CCL21, which are normally expressed in the T cell–rich areas of the LN (15), have previously been reported to be indistinguishable in their binding affinities for CCR7 and their ability to regulate chemotaxis and migratory speed (24, 25). However, they significantly differ in their regulation of cytoarchitecture following receptor binding; indeed, it has been observed that CCL19, but not CCL21, induces internalization of CCR7 and desensitizes receptor-expressing T cells and mature dendritic cells to a second stimulus of the chemokine (24, 25). Together, these observations corroborate our findings and suggest that CCL21 (but not CCL19) signaling can optimally upregulate Treg expression of CCR7. In addition, our data demonstrate that CCL21 is primarily expressed at high levels in the LN of allograft acceptors, whereas CCL19 is highly expressed in the LN of allograft rejectors (Supplemental Fig. 2), which further support our findings showing increased expression of CCR7 by allograft acceptor Tregs, but not by allograft rejector Tregs (Fig. 1B).

The use of Tregs as a cell-based therapy to promote transplant survival requires their in vitro expansion. We show in this study that the addition of CCL21 during in vitro Treg expansion leads to increased expression of the LN homing receptors CCR7 and CD62L. Interestingly, CCL21 signaling decreases the Treg expression of CD103, which has been shown to play a critical role in Treg retention in peripheral tissues, reducing their homing to the lymphoid tissues (10, 26). A previous study has reported that after ex vivo expansion of Tregs, Foxp3 expression and suppressive activity are maintained only in Tregs that express LN-homing CCR7 and CD62L molecules, data that are in accord with our present findings (27). Finally, the functional relevance of ex vivo CCR7-amplified Tregs in promoting transplant survival is fundamentally confirmed by our novel finding that adoptive transfer of CCR7hi Tregs to allograft recipients results in markedly increased homing to the reactive draining LN, which is paralleled by an increased rate of allograft survival. In contrast, CCR7lo Tregs could not preferentially home to the lymphoid tissues, and the allograft survival rate in recipients receiving CCR7lo Tregs was similar to that of recipients receiving no Tregs, suggesting that CCR7lo Tregs are unable to promote transplant survival. A small number of both CCR7hi- and CCR7lo-expressing Tregs were also detected in transplanted corneas but not the contralateral ungrafted cornea (Supplemental Fig. 3). These data corroborate our previous findings (18) showing the presence of Tregs in the cornea of both allograft acceptors and allograft rejectors and the draining LN as the primary site where Tregs exert their suppressive function and promote allograft survival.

In summary, our study provides new insights on the role of Tregs and their mechanism of action in the suppression of alloimmunity in transplantation. CCR7hi Tregs comprise the most functional subset of Tregs in the lymphoid tissues where they interact with incoming graft-derived APCs and regulate induction of alloimmune responses. By guiding a variety of immune cells to and within the lymphoid tissues, CCR7 thus contributes to both immunity and tolerance (15, 28) because this receptor is also involved in LN homing of naive T cells and tissue-derived dendritic cells. The novel findings presented in this study demonstrate that in addition to its role in homing, CCR7 expression by Tregs also regulates their suppressive function, suggesting that CCR7-associated impaired migration has functional implications related to allograft rejection. Further work is needed to understand the underlying mechanisms of how the CCR7 signaling pathway in Tregs is linked to Foxp3 and CD25 expression. Importantly, the current study describes a novel strategy of enhancing CCL21-mediated CCR7 signaling in Tregs during ex vivo expansion to augment their LN-homing characteristics (CCR7hiCD62LloCD103lo) without losing their suppressive phenotype. These findings may have important implications for future translational studies using Tregs as a potential means of promoting allograft survival and treating other T cell–mediated diseases.

Disclosures
The authors have no financial conflicts of interest.

References


Supplementary Text and Figures

**FIG. S1. Effect of CCL21 stimulation on CCR7-KO Tregs.** In addition to CCL21 stimulation of wild type Tregs (as shown in Fig 3), we stimulated CCR7-KO Treg with CCL21 alone or with CCL21 and Treg expansion cocktail to serve as a control. Our flow cytometry data demonstrate that i) there is no expression of CCR7 on these Tregs in any culture conditions; and ii) CCL21 along with polyclonal activation leads to upregulation of Treg expression of Foxp3 while CCL21 alone is not capable of promoting Foxp3 expression. These data suggest that CCL21 stimulation has no functional effect on CCR7-KO Tregs.
FIG. S2. Expression levels of CCR7 ligands CCL19 and CCL21 in the draining lymph nodes of corneal allograft recipients. Draining LN from corneal allograft acceptors and rejectors were harvested 3 weeks post transplantation. LN were homogenized to separate the leukocyte population from stromal tissue. Thereafter, stromal tissue was collagenase-digested to prepare single cell suspensions. Purity of CD45+ leukocyte and CD45– stromal cell populations was determined by flow cytometry. Whole LN single cell suspension and purified leukocyte and stromal cell populations were cultured for 24h with PMA+Ionomycin. After 24h culture, supernatants were collected and levels of CCL21 and CCL19 were evaluated using ELISA. Our data demonstrate that [A] CCL21 is primarily expressed at high levels in the LN of allograft acceptors, whereas CCL19 is highly expressed in the LN of allograft rejectors. [B] In addition, the major source of CCL21 in acceptor-LN is stromal cells (CD45– population) and the major source of CCL19 is the CD45+ leukocyte population.
**FIG. S3. Homing of CCR7^{high} versus CCR7^{low} Tregs to cornea.** In addition to tracking of PKH26-labeled Tregs to the lymphoid tissues, we examined the corneas of transplant recipients 48h after Treg adoptive transfer. By visualizing corneal wholemounts under an epifluorescence microscope, a small number of both CCR7^{high} and CCR7^{low} expressing Tregs were detected in transplanted corneas but not the contralateral ungrafted cornea. These data corroborate our previous findings (18) that showed i) the presence of Tregs in the cornea of both allograft acceptors and allograft rejectors; and ii) that the draining LN is the primary site where Tregs exert their suppressive function and promote allograft survival. Solid red color lines or circles in transplanted corneas represent autofluorescence from suture material.