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Regulatory T Cells Dynamically Regulate Selectin Ligand Function during Multiple Challenge Contact Hypersensitivity

Latasha D. Abeynaike,* James A. Deane,* Clare L. V. Westhorpe,* Zachary Chow,* Maliha A. Alikhan,* A. Richard Kitching,*†‡ Andrew Issekutz,§‖ and Michael J. Hickey*

Regulatory T cells (Tregs) play critical roles in restricting T cell–mediated inflammation. In the skin, this is dependent on expression of selectin ligands required for leukocyte rolling in dermal microvessels. However, whether there are differences in the molecules used by Tregs and proinflammatory T cells to undergo rolling in the skin remains unclear. In this study, we used spinning disk confocal microscopy in Foxp3-GFP mice to visualize rolling of endogenous Tregs in dermal postcapillary venules. Tregs underwent consistent but low-frequency rolling interactions under resting and inflamed conditions. At the early stage of the response, Treg adhesion was minimal. However, at the peak of inflammation, Tregs made up 40% of the adherent CD4⁺ T cell population. In a multiple challenge model of contact hypersensitivity, rolling of Tregs and conventional CD4⁺ T cells was mostly dependent on overlapping contributions of P- and E-selectin. However, after a second challenge, rolling of Tregs but not conventional CD4⁺ T cells became P-selectin independent, and Tregs showed reduced capacity to bind P-selectin. Moreover, inhibition of E-selectin at this time point resulted in exacerbation of inflammation. These findings demonstrate that in this multiple challenge model of inflammation, Treg selectin binding capacity and the molecular basis of Treg rolling can be regulated dynamically.


Contact sensitivity (CS) is a T cell–mediated form of inflammation in which leukocyte recruitment to the affected site is essential (1–4). Adhesion molecules expressed by leukocytes and endothelial cells play central roles in mediating this leukocyte recruitment (5–10). Circulating leukocytes initiate contact with the endothelial surface via the processes of tethering and rolling, interactions predominantly dependent on the selectin family of adhesion molecules. Inhibition or deletion of selectins or their ligands severely limits development of CS (7, 11). Furthermore, T cell transfer studies have demonstrated that this requirement extends specifically to the proinflammatory T cells, which drive the CS response (4, 10). What is less well understood is whether adhesion molecule usage differs between the various T cell subsets that contribute to this response. This is particularly important in that one of the populations present in inflamed skin, the CD4⁺ regulatory T cell (Treg), acts to restrict the development of inflammation and resolve established inflammatory responses (12–14).

Tregs are a subpopulation of CD4⁺ T cells that suppress the immune system, restricting organ damage stemming from inappropriate inflammatory responses (15–17). A growing body of evidence indicates that Tregs must be able to home to peripheral sites to be effective in controlling local inflammation. For example, in the skin, only Tregs that express functional selectin ligands used for rolling in the dermal microvasculature are able to efficiently regulate inflammation (18, 19). Conversely, Treg-restricted deficiency in selectin ligands and other skin-homing molecules is sufficient to result in spontaneous skin inflammation (20, 21). Given the evidence that conventional effector T cells use similar rolling-associated adhesion molecules, these findings raise the question as to whether there is any distinction between the molecular pathways used by effector T cells and Tregs in undergoing rolling in the inflamed microvasculature. The ideal approach for addressing this issue is the direct visualization of T cell subsets as they undergo rolling interactions in vivo. Bonder et al. (22, 23) used passive transfer of in vitro–activated CD4⁺ T cells to demonstrate that in the intestinal microvasculature, these cells used P-selectin, CD44, and E-selectin for rolling interactions. A similar approach has been used to demonstrate the capacity of in vitro–induced Tregs to undergo rolling interactions in postcapillary venules of the inflamed cremaster muscle (24). However, these experiments did not allow assessment of endogenous T cell subsets, an important consideration given the potential differences in homing molecules expressed by T cells activated ex vivo and endogenously activated T cells (25, 26). An alternative approach has been the examination of transgenic mice expressing GFP in CD4⁺ and CD8⁺ T cells (27, 28).

The advent of rapid frame rate, multichannel forms of in vivo imaging has facilitated the analysis of the interactions of multiple low-frequency endogenous leukocyte subsets in the microvasculature (29–31). These studies used i.v. administered Abs against subset-restricted markers to differentiate CD4⁺ T cells from neutrophils and demonstrated that CD4⁺ T cells undergo
infrequent but consistent interactions in activated postcapillary venules. Nacher et al. (30) found CD4+ T cell rolling in inflamed skin to be entirely dependent on endothelial selectins and leukocyte-expressed selectin ligands. Extending this approach to Tregs has been hampered by the lack of a specific cell surface marker for this subset. To overcome this, we have recently used Foxp3-GFP mice, in which Tregs express GFP, to investigate adhesion of endogenous Tregs in the inflamed dermal microvasculature (32). However, the microscopy technique used in this study was incapable of analysis of Treg rolling. Therefore, the aim of the current study was to use in vivo spinning disk confocal microscopy to characterize rolling and arrest of Tregs and proinflammatory T cells during a multiple challenge model of CS. These experiments revealed that Tregs undergo a low but consistent level of constitutive rolling in uninflamed skin and that during inflammation, alterations in Treg rolling do not correlate with increases in adhesion. Furthermore, this work demonstrates that, in response to local Ag challenge, circulating Tregs can rapidly modulate their capacity to roll via P-selectin such that selectin usage can differ between Tregs and conventional CD4+ T cells.

Materials and Methods

Mice

Foxp3-GFP mice (C57BL/6 background, provided by Dr. A. Rudensky, Memorial Sloan Kettering Cancer Center, New York, NY) mice (33) or C57BL/6 mice (Monash Animal Research Platform), aged 7–10 wk, were used for all experiments. All animal experiments were approved in advance by the Monash Medical Centre Animal Ethics Committee B.

Contact sensitivity

CS was induced as described previously (29, 32, 34, 35). In brief, mice were sensitized by the application of 50 μl 5% oxazolone (4-ethoxymethylene-2-phenyl-2-oxazolin-5-one) (Sigma-Aldrich, St. Louis, MO) in an acetone/olive oil vehicle (4:1) to a shaved region on the back. Five to 10 d later, mice were challenged with 50 μl 1% oxazolone solution on the flank. In some experiments, mice underwent a second challenge with 1% oxazolone on the same region 48 h after the first challenge. In control experiments, mice sensitized in the same manner were challenged with vehicle alone. In some experiments, mice underwent a second challenge with 1% oxazolone solution. In these experiments, the contralateral ear, challenged with vehicle alone, served as control. Ear thickness was measured using a micrometer. Data are expressed as the contralateral ear, challenged with vehicle alone, served as control. In some experiments, mice underwent sensitization and challenge as already described. Subsequently, mice were injected with the CCR4 antagonist, C21 (also known as RME-1; R&D Systems, via Bio-Scientific, Gymea, NSW, Australia) (3 mg/kg in DMSO/saline s.c.), or as control, DMSO/saline alone. Four h after imaging at the 24-h time point (35, 37). In experiments to examine the effect of administration of anti–E-selectin during the second challenge, mice underwent sensitization and initial challenge of both the skin and ear. Forty-eight hours after the first challenge, mice received either RME-1 or control mouse IgG (100 μg i.v.) and then immediately underwent the second challenge. Four hours later, leukocyte adhesion in the dermal microvasculature, and ear thickness were assessed as already described. To assess the impact of this protocol on Treg suppressive function, separate groups of mice underwent the same procedure. Thirty minutes after the second challenge, splenocytes were removed, and splenic Tregs were isolated and used in suppression assays as described below.

Flow cytometric assessment of selectin ligand binding function and marker expression

Leukocyte selectin binding function was assessed by flow cytometry using a modification of a previously described technique (38). Briefly, blood (1 ml) was collected from Foxp3-GFP mice and the erythrocytes lysed (NH4Cl for 10 min at room temperature). The pellet was resuspended in 1% BSA/HBSS with CaCl2/MgCl2 (Invitrogen, Carlsbad, CA). Aliquots of cells were then treated with mouse P-selectin (2.5 μg/100 μl) or E-selectin (1.25 μg/100 μl) Fc Chimera (R&D Systems) for 30 min. Cells were washed and stained with PE-conjugated goat anti-human IgG secondary Ab (1:50; Jackson ImmunoResearch Laboratories, West Grove, PA) for 30 min. Cells were washed again and then stained using anti-CD3 eFluor 450 (1:100; eBioscience), anti-CD4 allophycocyanin-Cy7 (1:200; BD Biosciences), and anti-CD44 allophycocyanin (1:400; BD Biosciences) for 30 min. All experiments included an irrelevant secondary Ab control. Samples were run on a FACSCanto II Analyzer (BD Biosciences), and data were analyzed using FlowJo (Tree Star, Ashland, OR). In separate samples, CD4+ T cell enumeration was performed using flow cytometry analysis of P- and E-selectin expression on Foxp3+CD4+ T cells, and these gates were subsequently applied to the CD4+GFP (Treg) population.

Experimental protocol

Oxazolone-sensitized mice were examined via spinning disk confocal microscopy 1, 4, 8, 16, 20, 24, and 48 h after a first oxazolone challenge, or 4 h after a second challenge. To assess the molecular basis of rolling, function-blocking Abs against P-selectin (RB40.34, 20 μg/mouse; BD Biosciences), rat/mouse E-selectin-1 (RME-1, 100 μg/mouse), or P-selectin glycoprotein ligand-1 (PSGL-1) (2PH1, 2 mg/kg; BD Biosciences) were used. Previous studies have demonstrated these Abs to functionally inhibit their target molecules at these doses (36). Abs were administered i.v. in 100 μl saline 15 min prior to imaging. In some experiments, anti–P-selectin and anti–E-selectin were administered together. In experiments to examine the role of CCR4 on Treg adhesion, mice underwent sensitization and challenge as already described. Subsequently, mice were injected with the CCR4 antagonist, C21 (also known as RME-1; R&D Systems, via Bio-Scientific, Gymea, NSW, Australia) (3 mg/kg in DMSO/saline s.c.), or as control, DMSO/saline alone. Four h after imaging at the 24-h time point (35, 37). In experiments to examine the effect of administration of anti–E-selectin during the second challenge, mice underwent sensitization and initial challenge of both the skin and ear. Forty-eight hours after the first challenge, mice received either RME-1 or control mouse IgG (100 μg i.v.) and then immediately underwent the second challenge. Four hours later, leukocyte adhesion in the dermal microvasculature, and ear thickness were assessed as already described. To assess the impact of this protocol on Treg suppressive function, separate groups of mice underwent the same procedure. Thirty minutes after the second challenge, splenocytes were removed, and splenic Tregs were isolated and used in suppression assays as described below.
Leukocytes were detected in ear sections using a two-layer staining protocol. Ears were fixed in paraformaldehyde (4%, 4 h) and cryoprotected in 20% sucrose, and 8-μm cryostat sections were prepared. Sections were blocked with Dako serum-free protein block and then stained with anti-CD4 (clone 30-F11, 1:100 dilution, 1 h at room temperature; eBio-science), followed by detection with goat anti-rat IgG Alexa Fluor 594 (ab150160, 1:500, 30 min at room temperature; Abcam, Cambridge, MA). Hoechst 33258 was used for nuclear staining. Coverslips were mounted using Dako fluorescence mounting medium before being examined on a Nikon C1 inverted confocal microscope. To quantitate the degree of leukocyte infiltration, images of sections were analyzed using Image J, using pre-determined intensity thresholds to define 1) the section area and 2) the area occupied by CD45 staining. Typically, five to nine regions were examined from each ear section. Data were expressed as percent skin area occupied by CD45 staining.

**Treg suppression assay**

To assess Treg suppressive capacity, a CFSE dilution-based T cell proliferation assay was used (39). Initially, CD4+ T cells were isolated from spleens of mice via MACS using mouse CD4 (L3T4) MicroBeads, according to the manufacturer’s instructions (Miltenyi Biotec, Macquarie Park, NSW, Australia). To purify CD4+CD25+ T cells (Tregs) and CD4+CD25+ (T responders [resp]), the enriched CD4+ T cells were incubated for 20 min at 4°C with anti-CD4 allophycocyanin (1:100; BD Biosciences) and anti-CD25 PE (1:100; BD Biosciences). Dead cells were excluded via propidium iodide staining. Cells were then sorted on a MoFlo XDP cell sorter (Beckman Coulter, Mt. Waverley, VIC, Australia). Purified CD4+CD25+ T cells were labeled with 10 μM CFSE (Life Technologies, Carlsbad, CA) in PBS supplemented with 0.1% BSA for 10 min at 37°C and then washed in complete medium (RPMI 1640 medium containing 10% FCS, 2 mM t-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-ME). CFSE-labeled CD4+CD25+ T cells (10^5/well) were cocultured with CD4+CD25+ Tregs at ratios from 1:16-1:1 in 96-well round-bottom plates precoated with anti-mouse CD3 (2.5 μg/ml; eBio-science) and anti-mouse CD28 (5 μg/ml; BioLegend, San Diego, CA). Cells were harvested after 72 h, and CFSE dilution was analyzed on a FACSCanto II. Proliferation was defined as the percentage of cells, which had undergone at least one round of CFSE dilution. Flow cytometric data were analyzed using FlowJo (Tree Star, Palo Alto, CA).

**Statistical Analysis**

For statistical comparisons, either a one-way ANOVA with a post hoc test (Dunnet’s) or Student t test was used. A p value < 0.05 was defined as statistically significant.

**Results**

**T cell rolling and adhesion in CS are regulated independently**

In initial experiments, spinning disk confocal microscopy was used to examine rolling of endogenous T cell subsets in dermal postcapillary venules under resting conditions and at various stages of the two challenge CS response. For assessment of CD4+ and CD8+ T cells, experiments were performed in wild-type C57BL/6 mice administered fluorochrome-conjugated anti-CD4/anti-CD8 Abs (Fig. 1A, Supplemental Video 1). In uninflamed mice, T cell rolling was infrequent: approximately one CD4+ and CD8+ T cells was observed rolling every 4 and 10 min, respectively (Fig. 1B, 1D). Comparable levels of CD4+ and CD8+ T cell rolling were observed in dermal venules in the ear (data not shown), suggesting that the T cell rolling observed in flank venules was not an artifact associated with surgical exteriorization of the flank microvasculature.

Within 1 h of the first CS challenge, CD4+ T cell rolling flux increased significantly, peaking 4 h after challenge. However, even at the peak of the response, rolling of CD4+ T cells was <1.5 cells/min (Fig. 1B). By 8 h after challenge, CD4+ rolling flux had returned to basal levels and subsequently remained stable out to 48 h. No change in rolling was observed 4 h after an additional hapten challenge (Fig. 1B). Inflammation-associated changes in rolling in the dermal microvasculature can be manifest as reductions in leukocyte rolling velocity (30, 40). We also examined the effect of CS on rolling velocity. Following a single challenge, rolling velocity of CD4+ T cells was unchanged from baseline at 1, 4, and 48 h, with the only change observed being a significant elevation at 24 h (Table I). In contrast, 4 h after a second challenge, a significant reduction in CD4+ T cell rolling velocity was observed (Table I). Examination of rolling of CD8+ T cells revealed contrasting results in that CD8+ T cell rolling flux and velocity showed no significant alterations at any time point examined following either a single hapten challenge or in response to a second challenge (Fig. 1D, Table I).

In contrast to rolling, induction of CS resulted in substantial increases in T cell adhesion. CD4+ T cell adhesion increased gradually after a single challenge, with peak levels sustained between 8 and 20 h. CD4+ T cell adhesion remained elevated at 48 h and following a second challenge (Fig. 1C). Adhesion of CD8+ T cells also increased gradually, peaking 24 h after challenge, and increased further following a second challenge (Fig. 1E). For all T cell subsets examined, adhesion was minimal in mice challenged with vehicle alone, indicating that re-exposure to the sensitizing agent was required for induction of T cell adhesion. Taken together, these data indicate that adhesion of CD4+ and CD8+ T cells in CS is regulated independently from alterations in T cell rolling.

**Treg rolling and adhesion in CS**

We next examined rolling of endogenous Tregs in dermal venules, identifying this low frequency CD4+ T cell subset in Foxp3-GFP mice on the basis of GFP expression (Fig. 2A, Supplemental Video 2 [no anti-CD4], Supplemental Video 3 [plus anti-CD4]). In the absence of anti-CD4 staining, Tregs were found to undergo a consistent but low level of rolling of 0.1 cells/min, findings stemming from analysis of >50 uninflamed dermal postcapillary venules in seven animals (Fig. 2B). Following induction of CS, Treg rolling flux was significantly elevated above basal levels 4 and 20 h after challenge, while remaining extremely infrequent, at <0.25 cells/min (Fig. 2B). Treg rolling flux was unaltered following a second challenge. Treg rolling velocity differed from that of total CD4+ T cells, in that 24 h after challenge, Treg rolling velocity was significantly reduced relative to uninflamed conditions (Table I). No adherent Tregs were detected in uninflamed dermal postcapillary venules (Fig. 2C). Upon induction of CS, however, Treg adhesion gradually increased, peaking 20–24 h after CS challenge. Taken together with our analysis of total CD4+ T cell adhesion (Fig. 1C), these data demonstrate that in the first 16 h of the response, adherent CD4+ T cells were predominantly Foxp3 negative, whereas after 20–24 h, 40–50% of adherent CD4+ T cells were Foxp3+

The capacity to detect rolling of GFP+ Tregs in Foxp3-GFP mice allowed investigation of whether the anti-CD4 Ab used to label CD4+ T cells was modulating the endothelial interactions of this subset. Therefore, we compared rolling of GFP+ Tregs (sensitized mice, 4 h after oxazolone challenge) in the presence and absence of anti-CD4. Treg rolling was significantly reduced in mice that received anti-CD4 (no Ab, 0.070 ± 0.01 cells/min; anti-CD4, 0.019 ± 0.02 cells/min; n = 7 mice/gp, p < 0.01 no Ab versus anti-CD4). This finding indicated that in experiments where Abs were used to detect specific leukocyte subsets, it is likely that the number of rolling leukocytes detected for each subset was lower than that occurring in the absence of Ab staining. Although this effect did not eliminate rolling or prevent adhesion of CD4+ T cells, under these circumstances it is appropriate to avoid comparisons of the number of rolling interactions between labeled and nonlabeled subsets, specifically total CD4+ T cells and Tregs (nonlabeled).
Comparison of molecular pathways of adhesion in CD4+ T cells and Tregs

We noted from analysis of adhesion of CD4+ T cells and Tregs that although adhesion of CD4+ T cells was significantly increased within 8 h of challenge, adhesion of Tregs was not significantly elevated until 20–24 h after initiation of the response. These findings indicate that during the development of CS, adhesion of proinflammatory CD4+ T cells is regulated differently from that of Foxp3+ Tregs. Furthermore, these findings raised the possibility that adhesion of these two subsets was regulated via distinct mechanisms. Therefore, to assess potential mechanisms underlying these differences, we used flow cytometry to compare expression of candidate molecules responsible for T cell arrest, LFA-1 and C5aR, on conventional CD4+ T cells and Tregs, but observed minimal differences between the subsets (Fig. 3A–C). CCR4 is a further receptor potentially involved in CD4+ T cell interactions with the endothelium (20). However, CCR4 inhibition failed to inhibit adhesion of either CD4+ T cell subset at the 24 h time point (Fig. 3D), indicating that this pathway is not critical for CD4+ T cell arrest under these conditions. These findings indicate that the difference in timing of adhesion of total CD4+ T cells and Tregs does not stem from differences in expression of LFA-1 and C5aR, or function of CCR4.

Dermal T cell subset rolling is modulated by dynamic changes in the roles of P-selectin and E-selectin

It is unknown whether selectin usage varies among conventional CD4+ T cells, Tregs, and CD8+ T cells. Therefore, we examined the roles of P-selectin and E-selectin in T cell subset rolling at various stages in the CS response. In uninflamed mice, anti-P-selectin eliminated CD4+ and CD8+ T cell rolling, whereas blocking E-selectin had no effect on rolling flux or rolling velocity (Fig. 4A, 4C, Table I). Similarly, T cell rolling was minimal in P-selectin–deficient mice (Fig. 4A, 4C), findings consistent with T cell rolling in uninflamed skin being solely dependent on P-selectin.

A different pattern was observed in skin undergoing CS. At the early stage of the response (1 and 4 h), P-selectin inhibition eliminated most CD4+ T cell rolling, with the residual rolling being eliminated by subsequent E-selectin inhibition (Fig. 4B). However, 24 h after the first challenge, substantial CD4+ T cell rolling persisted following administration of anti-P-selectin. Both at 1 and 24 h, the residual P-selectin–independent rolling showed a significantly slower rolling velocity typical of that mediated by E-selectin (Fig. 4B, Table I). Furthermore, at all time points examined, combined blockade of E- and P-selectin eliminated rolling of CD4+ T cells. The slow increase in the level of E-selectin–dependent

Table I. Rolling velocities of T cell subsets in unsensitized mice and at various stages during the two-challenge CS response, with and without selectin blockade

<table>
<thead>
<tr>
<th>Subset</th>
<th>Unsensitized</th>
<th>Ch1 1 h</th>
<th>Ch1 4 h</th>
<th>Ch1 24 h</th>
<th>Ch1 48 h</th>
<th>Ch2 4 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+</td>
<td>20.9 ± 4.7 (8)</td>
<td>19.0 ± 2.4 (6)</td>
<td>29.5 ± 3.4 (8)</td>
<td>45.7 ± 7.7* (7)</td>
<td>21.7 ± 1.4 (4)</td>
<td>9.8 ± 2.6* (6)</td>
</tr>
<tr>
<td></td>
<td>+ anti–P-selectin</td>
<td>ND (3)</td>
<td>7.3 ± 0.9 (7)</td>
<td>11.4 ± 5.6 (4)</td>
<td>7.6 ± 2.1 (7)</td>
<td>NE</td>
</tr>
<tr>
<td></td>
<td>+ anti–E-selectin</td>
<td>23.5 ± 4.4 (4)</td>
<td>17.0 ± 1.7 (4)</td>
<td>1.7 ± 0.3 (4)</td>
<td>17.9 ± 1.8 (6)</td>
<td>NE</td>
</tr>
<tr>
<td>CD8+</td>
<td>16.8 ± 3.5 (8)</td>
<td>15.7 ± 3.8 (6)</td>
<td>13.1 ± 4.7 (8)</td>
<td>21.6 ± 7.5 (7)</td>
<td>20.3 ± 5.9 (4)</td>
<td>12.4 ± 3.2 (6)</td>
</tr>
<tr>
<td></td>
<td>+ anti–P-selectin</td>
<td>ND (3)</td>
<td>11.5 ± 4.8 (7)</td>
<td>— (4)</td>
<td>10.9 ± 6.3 (7)</td>
<td>NE</td>
</tr>
<tr>
<td></td>
<td>+ anti–E-selectin</td>
<td>28.0 ± 6.3 (4)</td>
<td>26.3 ± 10.3 (4)</td>
<td>1.8 ± 0.4 (4)</td>
<td>19.6 ± 5.3 (6)</td>
<td>NE</td>
</tr>
<tr>
<td>Treg</td>
<td>29.9 ± 5.5 (7)</td>
<td>31.2 ± 4.7 (5)</td>
<td>25.3 ± 7.4 (4)</td>
<td>17.8 ± 3.4* (12)</td>
<td>15.7 ± 8.5 (3)</td>
<td>12.6 ± 4.4 (10)</td>
</tr>
<tr>
<td></td>
<td>+ anti–P-selectin</td>
<td>12.2 ± 4.8* (6)</td>
<td>57.6 ± 8.7 (4)</td>
<td>15.8 ± 6.3 (5)</td>
<td>— (9)</td>
<td>NE</td>
</tr>
<tr>
<td></td>
<td>+ anti–E-selectin</td>
<td>38.7 ± 11.8 (3)</td>
<td>28.4 ± 6.4 (4)</td>
<td>18.5 ± 4.0 (5)</td>
<td>30.9 ± 6.7 (3)</td>
<td>NE</td>
</tr>
</tbody>
</table>

Velocity data are expressed as micrometers per second and shown as mean ± SEM of n mice per time point. —, insufficient cells for analysis; ND, none detected; NE, not examined.

*p < 0.05 versus unsensitized control. †p < 0.05 versus Ch1 48 h, ††p < 0.001 versus no treatment.
rolling during the course of CS is consistent with the delayed and prolonged increase in E-selectin expression previously documented in this model (41, 42). Following a second challenge, CD4+ T cell rolling showed a similar phenotype, being significantly reduced by anti–P-selectin but displaying residual E-selectin–dependent rolling (Fig. 4B). CD8+ T cell rolling displayed a similar pattern of selectin usage in that, at all time points examined, combined blockade of P- and E-selectin was required to eliminate rolling, with the level of E-selectin–dependent, P-selectin–independent rolling peaking 24 h after the first challenge (Fig. 4D).

Treg rolling becomes P-selectin–independent in response to a second hapten challenge

We next examined selectin usage by Tregs. The low level of Treg rolling in uninflamed animals was almost eliminated by P-selectin inhibition, leaving only a minimal level of residual E-selectin–dependent rolling (Fig. 5A). Interestingly, during the first and second challenges, Treg usage of E-selectin differed from that of total CD4+ T cells. Four hours after CS induction, P-selectin inhibition only eliminated ∼50% of Treg rolling, with the remainder being mediated by E-selectin. In contrast, at 24 h, Treg rolling was almost entirely P-selectin dependent, when at the same time point, E-selectin makes a significant contribution to total CD4+ T cell rolling (Fig. 5B). Furthermore, 4 h after a second challenge, Treg rolling was unaffected by P-selectin inhibition, whereas E-selectin inhibition alone was sufficient to almost eliminate it (Fig. 5B). These data demonstrate that during multiple challenge CS, Tregs and conventional CD4+ T cells can use different combinations of selectins at different times to undergo rolling in the dermal microvasculature. To identify the ligand used by Tregs to bind P-selectin, we examined the role of PSGL-1 in Treg rolling. Inhibition of PSGL-1 eliminated Treg rolling in four of five mice examined (Fig. 5B), results comparable to those achieved by inhibition of P-selectin, indicating that PSGL-1 is the major functional P-selectin ligand expressed by Tregs.

T cell selectin binding capacity is dynamically regulated during CS development

The changes observed in T cell selectin usage during CS raised the possibility that CD4+ T cell subsets were dynamically altering their capacity to bind selectins over the course of the CS response. To assess this possibility, we measured P- and E-selectin binding by circulating CD4+Foxp3+ and CD4+Foxp3− T cells from Foxp3-GFP mice (Fig. 6A). Under basal conditions, 5–10% of conventional (Foxp3−) CD4+ T cells were capable of binding P-selectin (Fig. 6B, 6C). Following sensitization and challenge, this was predominantly unchanged, although a significant reduction in the percentage of cells binding P-selectin was observed 4 h after a second challenge (Fig. 6C). The pattern of P-selectin binding by Tregs slightly differed from that of CD4+Foxp3− cells. Tregs also showed a significant drop in P-selectin binding 4 h after a second challenge (Fig. 6B, 6C). However, 24 h after the first challenge, the percentage of Tregs capable of binding P-selectin was significantly greater than that of CD4+Foxp3− cells (Fig. 6C). Analysis of E-selectin binding by circulating CD4+ T cells demonstrated that 10–15% of CD4+Foxp3− cells bound E-selectin at most time points, although this was significantly reduced in mice that had undergone sensitization alone (Fig. 6B, 6D). In contrast, ∼20% of Tregs were positive for E-selectin binding, and this was unchanged throughout sensitization and challenge (Fig. 6B, 6D).

One possible explanation for the observed changes in Treg P-selectin binding was that the number of selectin binding Tregs present in the circulation was undergoing dynamic changes during sensitization and challenge. The capacity of Tregs to bind selectins is indicative of a postactivation effector phenotype (18, 19, 24). Furthermore, these effector Tregs have been shown to express CD44 at high levels (18). Therefore, to assess the proportion of effector Tregs in the circulation, we assessed the proportion of CD44hi Tregs during sensitization and challenge. Under resting conditions, ∼30% of circulating Tregs were CD44hi, and this population did not significantly change at any stage examined (Fig. 6E). Furthermore, the percentage of Tregs capable of binding E-selectin remained unchanged at the same time as the changes in P-selectin binding occurred (Fig. 6C, 6D). Taken together, these observations support the concept that the observed changes in P-selectin–dependent Treg rolling did not stem from major reductions in the circulating effector Treg population.

Given these findings, we hypothesized that the decrease in Treg P-selectin ligand binding activity was due to reduced expression of
PSGL-1, the ligand we previously demonstrated to be required for Tregs to interact with P-selectin (Fig. 5B). Leukocytes have been reported to shed PSGL-1 under activating conditions (43, 44). We therefore examined PSGL-1 expression by circulating T cells and Foxp3+CD4+ T cells as determined using flow cytometry in unsensitized mice, sensitized mice, and 4 and 24 h after first oxazolone challenge, and 4 h after second oxazolone challenge (n = 5 in each group). Data represent mean fluorescence intensity (MFI) and are shown as mean ± SEM. *p < 0.05, **p < 0.01 versus unsensitized, ***p < 0.001 between T cell subsets. (B) Representative histogram showing data 24 h after oxazolone challenge for unstained cells (as control) and Foxp3+ and Foxp3+CD4+ T cells as determined using flow cytometry. (C) C5aR expression on Foxp3+ and Foxp3+CD4+ T cells as determined using flow cytometry. (D) Role of CCR4 in T cell subset adhesion in dermal venules during CS; 24 h after a single challenge. Mice received either CCR4 antagonist, C021, or vehicle DMSO as control (n = 4/group) s.c. 4 h prior to imaging, and CD4+ T cell or Foxp3+ Treg adhesion was quantified via spinning disk confocal microscopy at the 24-h time point. All data are shown as mean ± SEM.

Discussion
Several studies have used in vivo imaging to examine rolling and adhesive interactions of T cell subsets, including Tregs, in the inflamed microvasculature. In most cases, these experiments have made use of ex vivo–activated T cells in transfer studies to facilitate analysis of these normally low-frequency interactions (22–24). However, studies have identified differences in homing molecule expression on CD4+ T cells activated in vivo versus ex vivo, indicating that assessment of endogenous T cells may provide a more accurate analysis of the molecular basis of recruitment of activated T cells (25). To this end, more recently, rapid frame-rate multichannel imaging has allowed analysis of endogenous CD4+ T cell rolling in the inflamed skin, revealing the contributions of endothelial selectins and their ligands to CD4+ T cell rolling at the peak of the CS response (29, 30). However, these studies did not extend to analysis of Tregs or CD8+ T cells or examine T cell interactions throughout the entire CS response. In this study, we examined rolling and adhesion of endogenous
conventional CD4+ T cells, Tregs, and CD8+ T cells, allowing comparison of intravascular interactions of these subsets. These experiments demonstrated dynamic, subset-specific changes in selectin usage by these subsets at different stages of the CS response. It should be noted that the current experiments were all performed using oxazolone, and it is unclear whether comparable alterations in Treg behavior would be observed during responses to other haptens or during other forms of skin inflammation.

In the current study, we observed differences in the pattern of T cell adhesion in that adhesion of conventional CD4+ T cells and CD8+ T cells was most prominent 8–16 h after CS challenge, whereas Treg adhesion only increased 24 h after induction of the response. Consistent with this, in the first 16 h of the response, CD4+ T cell adhesion was significantly elevated whereas Treg adhesion remained minimal. In contrast, at the peak of Treg adhesion (24 h), Tregs represented 40–50% of all adherent CD4+ T cells, findings similar to our previous observations (32). These findings are consistent with a model in which selective recruitment of proinflammatory T cells early in the response enables efficient stimulation of the inflammatory response, whereas later in the response, the ratio of Tregs to effector T cells is higher, facilitating Treg-mediated regulation of inflammation (4, 46). The mechanisms underlying the selective modulation of arrest of conventional and regulatory CD4+ T cells observed in this study is unknown. Examination of molecules potentially involved in attachment of CD4+ T cells to the endothelium, LFA-1, C5aR, and CCR4 (20, 29, 47), revealed no major differences in expression (LFA-1, C5aR) or functional role (CCR4) between Tregs and conventional CD4+ T cells. Further work is required to understand the mechanisms underlying this difference in arrest behavior. It should be noted that in previous work we have performed similar experiments examining the time course of lymphocyte adhesion in the oxazolone CS model (32). In these experiments, we observed that CD4+ T cell adhesion had returned to baseline by 48 h after challenge, whereas in the current study, adhesion of CD4+ T cells remained somewhat elevated at this timepoint (Fig. 1C). The reason for this discrepancy is not clear. It may stem from differences in the imaging systems used in the two studies (conventional confocal versus spinning disk confocal microscopy). Alternatively, it is possible that the low number of adherent T cells observed in individual vessels in these studies renders the data more susceptible to study-to-study variability, particularly at time points when the inflammatory response is waning.

FIGURE 4. Roles of P- and E-selectin in CD4+ and CD8+ T cell rolling during development of CS. Anti–P-selectin (αP) and anti–E-selectin Abs (αE) were administered prior to imaging, and rolling flux of CD4+ T cells (A and B) and CD8+ T cells (C and D) was measured in unsensitized mice (A and C) and at various time points following one or two hapten challenges (B and D). Rolling was also examined in uninflamed P-selectin−/− mice [PKO in (A) and (C)]. In (B) and (D), data are shown for mice 1, 4, and 24 h after first challenge and 4 h after second challenge. ND (none detected) indicates that no rolling cells were visualized. Data are shown as mean ± SEM of three to eight mice per group. *p < 0.05, **p < 0.01, ***p < 0.001 versus no selectin blockade at the same time point.

FIGURE 5. Roles of endothelial selectins and PSGL-1 in Treg rolling in CS. Anti–P-selectin (αP), E-selectin (αE), and anti–PSGL-1 (αPSGL1) Abs were administered prior to imaging, and rolling flux of Tregs was measured in unsensitized mice (A) and following one or two hapten challenges (B) (anti–PSGL-1 was only examined 24 h after first challenge). In (B), data are shown for 1, 4, and 24 h after first challenge and 4 h after second challenge. ND (none detected) indicates no rolling cells were visualized. Data are shown as mean ± SEM of 3–12 mice/group. *p < 0.05, **p < 0.01 versus no selectin blockade at the same time point.
We also observed dynamic changes in selectin usage by Tregs. Although at most time points, Treg rolling was mediated by P-selectin, with variable overlapping contributions from E-selectin, 4 h after a second challenge Treg rolling was P-selectin-independent, occurring entirely via E-selectin. Notably, at the same time point, rolling of conventional CD4+ T cells and CD8+ T cells required both P- and E-selectin. As such, we hypothesized that E-selectin inhibition during this phase of the response would limit recruitment of Tregs but not proinflammatory T cells. Consistent with this hypothesis, E-selectin blockade during the second challenge resulted in increased swelling and neutrophil adhesion in dural microvessels. Previous studies have demonstrated an unexpected anti-inflammatory role for E-selectin in specific phases of the CS response, although the mechanism underlying this finding was unclear (48). The present findings raise the possibility that exacerbated inflammation in the absence of functional E-selectin stems from selective inhibition of Treg adhesion and recruitment and ensuing loss of regulation of the local inflammatory response. In the present experiments, we stained for CD45 as a readout of total leukocyte infiltration and observed a similar density of leukocyte infiltration in control and anti-E-selectin–treated animals, under conditions where the skin volume was increased in mice that received anti–E-selectin. These findings are consistent with an overall increase in the level of leukocyte recruitment in anti–E-selectin–treated mice. However, further analysis is required to delineate whether the increased inflammation in these animals stemmed from an alteration in the balance of effector and Tregs in the skin infiltrate. An alternative hypothesis is that the increased number of adherent neutrophils in the microvasculature following E-selectin blockade was sufficient to mediate the observed exacerbation in endothelial barrier dysfunction in the absence of major changes in effector T cell recruitment.

It also remains to be determined how inhibition of Treg rolling resulted in increased neutrophil adhesion. Previous studies have shown that Tregs can reduce selectin expression by activated endothelial cells, demonstrating that endothelial selectin expression can be a target of the anti-inflammatory actions of Tregs (24, 49). However, in the present studies, neutrophil rolling flux was not increased in mice in which Treg–endothelial cell interactions were inhibited by anti–E-selectin. This indicates that under the conditions examined in this study, selectin-mediated rolling is not a major inhibitory target of Tregs. Conversely, in a previous study in which we reduced Treg adhesion via ICAM-1 blockade, we found that neutrophil adhesion became dependent on molecules other than ICAM-1 and the αβ integrin (32). This raises the possibility that the absence of Tregs leads to upregulation of a yet-to-be-identified alternative molecular pathway in endothelial cells capable of supporting neutrophil adhesion.

The observation that other T cell subsets continued to use P-selectin for rolling at the same time that Treg rolling became P-selectin–independent indicated that this change in Treg rolling was not due to changes in endothelial function. Therefore, in experiments investigating the temporary loss of P-selectin–dependent rolling by Tregs, we examined Treg selectin binding function in vitro. Following a second challenge, Tregs had significantly reduced capacity to bind P-selectin, supporting the concept that the loss of P-selectin–dependent rolling was an intrinsic response of Tregs. Notably, at the same time as the change in P-selectin binding capacity, Treg E-selectin binding function and the proportion of CD44hi Tregs in the circulation were unchanged, consistent with the idea that this alteration represents a cellular response of Tregs present in the circulation, rather than an alteration in the Treg populations in the circulation. Previous studies of Tregs in atherosclerotic lesions have demonstrated similar changes in Treg selectin binding function, associated with reduced Treg accumulation in the vascular wall and lesion pro-

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**FIGURE 6.** Dynamic regulation of selectin binding and CD44 expression by Tregs and effector CD4+ T cells. Selectin binding and CD44 expression of circulating T cell subsets in Foxp3-GFP mice in uninflamed, sensitized (unchallenged) and challenged mice. (A) Representative flow cytometry dot plots from 4 h after first challenge demonstrating gating performed to obtain T cell subsets. PBMCs were defined from scatter analysis of the whole blood following erythrocyte lysis. Subsequently CD4+ T cells were categorized as Tregs or conventional CD4+ T cells on the basis of Foxp3 (GFP) expression, and both populations were analyzed for selectin ligand binding. (B) Representative histograms for each CD4+ T cell subset for P-selectin binding (P-sel lig) and E-selectin binding (E-sel lig). Gray-shaded histograms represent negative control, the thin-lined histogram represents T cell selectin binding from unsensitized mice, and the thick-lined histogram represents T cells from 4 h after second challenge. (C and D) Mean percent positive for binding P-selectin (C) or E-selectin (D), shown for CD3+CD4+Foxp3+ (conventional) T cells and CD3+CD4+Foxp3+ Tregs. Data are shown for unsensitized, sensitized unchallenged, 4 and 24 h after first challenge, and 4 h after second challenge, and represent mean ± SEM of three to seven mice per group. (E) Proportions of Foxp3+ CD4+ T cells and Tregs expressing high levels of CD44 (CD44hi) in untreated mice and during CS sensitization and challenge. Data (shown as mean ± SEM) represent the proportion of CD44hi cells and are derived from analysis of five to seven mice per group. *p < 0.05 versus unsensitized. †p < 0.05, ††p < 0.01, †††p < 0.001 versus Foxp3+ CD4+ T cells.
FIGURE 7. PSGL-1 expression on Tregs and Foxp3-negative CD4+ T cells during the CS response. (A and B) PSGL-1 expression on Foxp3-CD4+ T cells and CD4+Foxp3+ Tregs. (A) Representative flow cytometry histograms showing PSGL-1 expression on Foxp3- and Foxp3+CD4+ T cells following sensitization. Gray histogram represents control (unstained) cells. (B) Mean PSGL-1 expression on CD4+ T cell subsets in unsensitized mice, sensitized mice, and 4 and 24 h following first challenge. (*p < 0.05 versus unsensitized.

FIGURE 8. E-selectin inhibition during second challenge increases swelling and neutrophil adhesion. Mice underwent sensitization and a first oxazolone challenge (flank/ear) and then received either control Ab (IgG) or anti-E-selectin (“Anti-E-sel” - RME-1, 100 µg) immediately prior to a second challenge (flank/ear). (A) Change in ear thickness 4 h after second challenge. Data represent the difference (micrometers) between the oxazolone-challenged ear and the contralateral vehicle-challenged ear. (B) Adhesion of Gr1+ leukocytes in the flank dermal microvasculature 4 h after second challenge, as assessed using spinning disk confocal microscopy. (C and D) Immunohistochemical analysis of CD45+ leukocyte infiltration in ears from experiments in (A). (C) Example confocal microscopy image of staining for CD45+ leukocytes (magenta) in inflamed ear. Nuclei (blue) are stained via Hoechst 33258, and cartilage and epithelial structures are visible via autofluorescence (green). Scale bar, 100 µm. (D) Quantitation of ear leukocyte infiltration from experiments in (A). Data represent percent area occupied by CD45+ staining and were derived from three to nine images per mouse. Data in (A), (B), and (D) are shown as mean ± SEM of n = 6/group in the swelling/immunohistochemistry experiments, and n = 5/group in the adhesion experiments. *p < 0.05 versus IgG control group. (E) Effect of anti-E-selectin on Treg suppressive capacity. Mice underwent oxazolone sensitization and two oxazolone challenges [as in (A and B)] and then received either control IgG or anti-E-selectin. Splenic CD4+CD25+ T cells (Tregs) were isolated from the mice and cocultured with CFSE-labeled responder CD4+CD25+ T cells (T resp) at different ratios. T resp proliferation was assessed via CFSE dilution. Data are shown as the mean ± SEM of two independent experiments, with each experiment consisting of five to seven mice per group. Data are also shown for T resp proliferation in the absence of Tregs.
extensive carbohydrate modification (54), it is conceivable that these changes are caused by a rapid reduction in the glycosylation of this molecule not detected by the Ab used in the present experiments. However, to our knowledge, such a change has not been reported.

Nevertheless, these observations demonstrate that Treg selectin ligand usage is complex and highly regulated. Although we identified PSGL-1 as the dominant functional P-selectin ligand on Tregs, consistent with observations in other leukocyte subsets (55, 56), identifying the functional E-selectin ligands on Tregs is likely to be more challenging. Several leukocyte-expressed E-selectin ligands, including PSGL-1, E-selectin ligand-1, CD44, and CD43, have been identified (9, 30, 56–58). Expression of E-selectin ligand-1 has not been reported on lymphocytes. However, investigation of oxazolone-induced contact sensitivity has shown that most CD4⁺ T cell rolling is mediated by PSGL-1 and CD44 in tandem (30). In the present experiments, neither CD44 expression on Tregs nor Treg E-selectin binding function changed markedly at different points in the CS response. So although the functional E-selectin ligands expressed by Tregs remain to be determined, the changes in the functional role of PSGL-1 on Tregs observed in this study were not associated with concurrent alterations in E-selectin binding capacity.

These studies also allowed the analysis of Treg interactions under basal conditions. In this study, we observed that Tregs undergo infrequent but consistent P-selectin–dependent rolling interactions in the uninflamed dermal microvasculature. Together with the observation that Tregs are present in healthy skin (12, 32, 59), these data raise the possibility that this rolling underlies constitutive Treg trafficking to the dermis. Indeed, findings of spontaneous skin inflammation in mice with Treg–restricted selectin ligand deficiency indicate that selectin–mediated Treg interactions are critical to the delivery of Tregs to the skin and their subsequent regulation of skin inflammation (21). To support this hypothesis, the expectation is that occa- sional Tregs would be observed undergoing adhesion in the microvasculature, as they undergo the sequence of interactions required for migration out of the bloodstream. However, we failed to observe Treg adhesion in uninflamed skin, despite analysis of 49 vessels in seven mice. This may simply reflect the extremely low frequency of these events. Conversely, an alter- native interpretation is that Treg rolling in peripheral micro- vessels serves as a form of ongoing immune surveillance, whereby adhesion only occurs upon induction of an appropriate arrest signal in response to initiation of skin inflammation, sub- sequently allowing Tregs to enter the skin.

In this study, we have demonstrated that, at least during repeat hapten exposure, Tregs and proinflammatory T cell subsets can be distinguished by their capacity to undergo P-selectin–dependent rolling. In the context of inflammatory disease, this makes E-selectin less attractive as a target for therapeutic intervention because blocking the function of this molecule would be expected to be more effective at inhibiting recruitment of anti-inflammatory Tregs versus disease-promoting T cells. Previous findings of a lack of efficacy of anti–E-selectin therapy in psoriasis support this concept (60). However, in the setting of skin cancer, removal or inhibition of Tregs to the skin. If this could be applied to tumor therapy, it raises the interesting prospect of allowing inhibition of Treg recruitment to tumors in the absence of effects on recruitment of effector T cells, a situation that should promote the local antitumor response.


