Endogenous Regulatory T Cells Adhere in Inflamed Dermal Vessels via ICAM-1: Association with Regulation of Effector Leukocyte Adhesion

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Regulatory T cells (Tregs) must express appropriate skin-homing adhesion molecules to exert suppressive effects on dermal inflammation. However, the mechanisms whereby they control local inflammation remain unclear. In this study we used confocal intravital microscopy in wild-type and Foxp3-GFP mice to examine adhesion of effector T cells and Tregs in dermal venules. These experiments examined a two-challenge model of contact sensitivity (CS) in which Treg abundance in the skin progressively increases during the course of the response. Adhesion of CD4+ T cells increased during CS, peaking 8–24 h after an initial hapten challenge, and within 4 h of a second challenge. At these time points, 40% of adherent CD4+ T cells were Foxp3+ Tregs. CD4+ T cell adhesion was highly dependent on ICAM-1, and consistent with this finding, anti–ICAM-1 prevented Treg adhesion. Skin TGF-β levels were elevated in skin during both challenges, in parallel with Treg adhesion. In the two-challenge CS model, inhibition of ICAM-1 eliminated Treg adhesion, an effect associated with a significant increase in neutrophil adhesion. Similarly, total CD4+ T cell depletion caused an increase in adhesion of CD8+ T cells. Because Treg adhesion was restricted by both of these treatments, these experiments suggest that adherent Tregs can control adhesion of proinflammatory leukocytes in vivo. Moreover, the critical role of ICAM-1 in Treg adhesion provides a potential explanation for the exacerbation of inflammation reported in some studies of ICAM-1–deficient mice. The Journal of Immunology, 2012, 188: 000–000.
work to the examination of Treg function has been hampered by their scarcity and lack of a specific cell surface marker differentiating them from other CD4+ T cells. The development of Foxp3-GFP mice, in which GFP is expressed exclusively in Tregs, may provide an approach to overcome this limitation (32). Therefore, the aim of this study was to use confocal intravital microscopy in Foxp3-GFP mice to investigate the adhesion of Tregs within the vasculature. We reasoned that this was best done in a model in which there is evidence of vigorous local immune regulation of a T cell-mediated inflammatory response. Therefore we examined a multiple challenge model of contact sensitivity (CS), in which the resolution of the inflammatory response is markedly accelerated after a second challenge (33, 34). These experiments revealed that Tregs undergo adhesion in inflamed dermal venules, and strategies that restrict Treg adhesion can promote adhesion of proinflammatory leukocytes. Moreover, this study demonstrates an essential role for ICAM-1 in Treg adhesion, providing a potential explanation for the exacerbation of inflammation observed in some studies of ICAM-1-deficient mice.

Materials and Methods

Mice
Eight- to 10-wk-old male wild-type C57BL/6 mice or Foxp3-GFP mice expressing GFP under the control of the Foxp3 promoter (C57BL/6 background) (32), provided by Dr. Alexander Rudensky (University of Washington), were used for CS experiments. All animal experiments were approved in advance by the Monash Medical Centre Animal Ethics Committee “B”.

Oxazolone-induced model of contact sensitivity
CS was induced as previously described (35). Briefly, mice were sensitized by the application of 50 μl 5% oxazolone (Sigma-Aldrich, St. Louis, MO) in an acetone/olive oil vehicle (4:1) to a shaved patch on the back. Five to 7 d later, a 10 × 20-mm area of the flank was shaved and challenged with 50 μl 1% oxazolone in vehicle. In experiments involving a second CS challenge, the skin was rechallenged with 1% oxazolone 48 h after the initial challenge.

Histology
Skin samples were immersion fixed in 10% formalin, processed, embedded in paraffin, sectioned, and stained with H&E using standard techniques.

Intravital microscopy of the dermal microvasculature of the flank
Skin on the flank was surgically prepared for standard epifluorescence and confocal intravital microscopy as previously described (35). Mice were anesthetized (ketamine hydrochloride, 150 mg/kg; xylazine hydrochloride, 10 mg/kg) and a heat pad was used to maintain body temperature. The left jugular vein was cannulated to allow the administration of fluorescent dyes and additional anesthetic. To expose the dermal vasculature for intravital microscopy, a midline skin incision was made and the flank skin and associated vasculature were separated from underlying connective tissue and extended over a heated pedestal using sutures attached to the margin. Loose dermal connective tissue was removed to allow a clear view of the intact dermal vasculature. The exposed area was then immersed in saline and sealed with a coverslip held in place with vacuum grease.

Total leukocyte adhesion. To examine total leukocyte adhesion via standard epifluorescence microscopy, leukocytes were stained with rhodamine 6G (Sigma-Aldrich, 50 μg/ml; BD Biosciences), CD4-FITC (2.5 μg/ml; eBioscience), and CD8-Alexa Fluor 750 (2.5 μg/ml; Invitrogen, Carlsbad, CA). To quantify the total number of T cells present, a standardized number of fluorescent latex beads was added to samples, allowing a defined portion of sample to be collected and referenced to the number of CD3+ cells present in the same sample. CD3 data are expressed as cells per gram of extrapilated skin dry weight (based on wet weight versus dry weight data from edema studies) to standardize for the variation in skin weight due to edema.

Skin cytokine expression
The challenged area of skin was removed from underlying connective tissue and stored at −70°C. Skin was ground in RPMI 1640 plus 10% FCS and 0.1 mg/ml DNAse (1 h, 37°C). Dissociated skin was filtered through a 70-μm sieve, washed with PBS plus 10% FCS, and labeled with cell surface Abs for 30 min on ice. Abs against cell surface molecules used were CD3-PE (30 μg/ml; BD Biosciences), CD4-FITC (2.5 μg/ml; eBioscience), and CD8-Alexa Fluor 750 (2.5 μg/ml; Invitrogen, Carlsbad, CA). To quantify the number of T cells present, a standardized number of fluorescent latex beads was added to samples, allowing a defined portion of sample to be collected and referenced to the number of CD3+ cells present in the same sample. CD3 data are expressed as cells per gram of extrapilated skin dry weight to facilitate comparison with cytokine data. To identify the Foxp3+ population, cells were fixed and permeabilized with Fix/Perm buffer (eBioscience) for 30 min on ice, washed, and then labeled with anti-Foxp3-allophyocyanin (5 μg/ml). Labeled cells were analyzed using a MoFlo cell sorter (Beckman Coulter). For experiments examining the CD4+Foxp3+ population, cells were first gated for CD3 to identify T cells.

Experimental protocol
Function-blocking Abs were used to assess the roles of ICAM-1, the α4 integrin, and the IL-10 receptor in CS-associated leukocyte adhesion. Abs used were: YN1/1.7.4 to inhibit ICAM-1 (200 μg/mouse/dose), PS2, to inhibit the α4 integrin (100 μg/mouse/dose), both grown from hybridoma, and 1B1.3A to inhibit the IL-10 receptor (250 μg/mouse, i.v.) (BioXCell, West Lebanon, NH). Rat IgG2b (300 μg/mouse/dose; LTF-2; BioXCell) was used as an isotype control. In mice undergoing a single CS challenge, an initial Ab dose was administered (i.p., in 200 μl saline) 4–6 h after the initial challenge and an additional dose of Ab was administered at 24 h after the initial challenge. In the two-challenge CS model, mice received the initial dose of Ab at the initiation of the second challenge, and they also received an additional dose during surgical preparation, except for mice treated with the anti–IL-10 receptor, which only received a single dose of Ab at the initiation of the second challenge. To assess a role for the CD44/hyaluronan pathway, mice were treated with PS2 or ω1 integrin to 24 h prior to imaging (37). In these experiments, mice also received Abs against ICAM-1 and the α4 integrin to assess the effect of combined inhibition of these pathways. Leukocyte adhesion in dermal venules was assessed using intravital microscopy as described above.

To assess the effects of CD4+ T cell depletion, mice underwent treatment with the CD4-depleting Ab GK1.5, as previously described (38). Anti-CD4 (1.5 mg, i.v.) was administered 24 h after the induction of an initial oxazolone challenge. Flow cytometric assessment of blood and skin-draining lymph nodes confirmed depletion of CD4+ cells 24 h after Ab administration. Rat IgG2b (LTF-2) was used as a control. Other Abs used included...
anti-CD25 (PC61, 1.5 mg/mouse, grown from hybridoma) and anti-FR4 (Th6, 100 μg/mouse; eBioscience). These Abs were administered as for GK1.5.

Statistical analyses

Data were compiled with Excel (Microsoft) and analyzed using Prism (GraphPad Software). ANOVAs including a Dunnett or Tukey (for inter-group comparisons) post hoc test, or a t test, were used to determine statistical significance. A p value of <0.05 was deemed significant.

Results

A second oxazolone challenge results in accelerated induction and resolution of inflammation and promotes a switch from Th1 to Th2 cytokine production

In previous work, we have used intravital microscopy of the dermal microvasculature in flank skin to demonstrate that CD4+ T cells represent only a minor proportion of the total number of interacting leukocytes during the CS response (35). In experiments focusing on recruitment of these infrequent leukocyte subsets, examination of the flank skin affords the advantage over the more common ear skin preparation of allowing visualization of at least 10 postcapillary venules in each preparation, facilitating robust quantitative analysis of adhesion of these low-frequency subsets. Image clarity is also superior in the flank preparation. Therefore, given that the focus of this study was T cell subset adhesion, this approach was also used in the present experiments.

Data from previous studies indicate that multiple exposures to hapten result in an alteration in the nature of the CS response consistent with more active immune regulation. This is apparent as an accelerated resolution of edema and an alteration in the cytokine milieu of the affected skin, favoring production of Th2 cytokines (33, 34). Although previous studies have examined up to 12 challenges, it was unclear whether one additional challenge was sufficient to induce these changes, and whether they also affected the pattern of leukocyte recruitment. Therefore, we first asked whether the pattern of leukocyte adhesion in the dermal microvasculature is altered in the two-challenge CS model. Using rhodamine 6G to stain all leukocytes, we found that in sensitized mice challenged once with oxazolone, adhesion followed an extended time course, peaking 8–24 h after challenge before returning to near basal levels by 48 h (Fig. 1A), observations consistent with previous findings (35, 39). In contrast, after a second challenge, leukocyte adhesion peaked sharply at 4 h and then rapidly declined such that by 8 h, adhesion had returned to basal levels, where it remained over the subsequent 40 h (Fig. 1A). In mice challenged with vehicle alone, only a basal level of adhesion was observed, indicating that the adhesion response was specific to the presence of the sensitizing Ag, and that surgical exposure of the skin did not induce leukocyte adhesion. Assessment of the time course of edema development revealed a similar pattern. After an initial challenge, edema followed the kinetics of a typical delayed response, gradually increasing to a peak at 24 h and subsequently declining, whereas after a second challenge, the peak of swelling occurred substantially earlier, within only 8 h of challenge (data not shown).

Histological comparison of skin undergoing the one or two-challenge model of CS also revealed pertinent differences in the pattern of leukocyte recruitment. Four hours after a single oxazolone challenge, the skin was not markedly altered from baseline (data not shown), reflecting the delayed time course of the CS inflammatory response. However, 24 h after a single challenge, extensive dermal leukocyte infiltration was apparent, associated with edema, epithelial injury, abscess formation, and leukocyte infiltration, which persisted to 48 h (Fig. 1B–D). It was at this point in the dual-challenge model used in this study that the skin was rechallenged with hapten. Four hours after this second challenge, numerous leukocytes were present in the dermis, in contrast to the minimal leukocyte infiltration observed at this point during the first challenge. Leukocyte infiltration remained substantial 24 h after the second challenge, although by 48 h, this had largely abated (Fig. 1E–G).

Multiple hapten challenges have also been shown to alter cytokine production during the CS response. Therefore, we compared cytokine levels in the skin after one and two challenges. After one challenge, production of the type 1 cytokine IFN-γ was robust, peaking after 24 h. However, following a second challenge, IFN-γ levels were lower, particularly 24 h after challenge, and not significantly elevated relative to uninfamed control skin (Fig. 1H). In contrast, IL-4 and IL-10 showed minimal expression after an initial challenge, but were expressed at significantly elevated levels following a second challenge (Fig. 1I, 1J). Taken together, these findings indicate that one additional challenge is sufficient to change the dermal microenvironment such that the induction and resolution of the response are markedly accelerated and type 2 cytokines are more prevalent. Moreover, the changes seen after only two challenges were similar to those seen previously after further additional challenges (33, 34). In the context of this two-challenge model, it is notable that, at the time of initiation of the second challenge, numerous newly infiltrated leukocytes were present in the skin.

Neutrophil and CD8+ T cell adhesion are blunted in multiple-challenge contact sensitivity

We have recently used confocal intravital microscopy to enable identification of neutrophils and CD4+ T cells undergoing rolling and adhesive interactions in the inflamed dermal microvasculature (35). This work demonstrated that the CD4+ T cell population critical for the initiation of a CS response represents only a small proportion of the total number of adherent leukocytes in the inflamed dermal microvasculature. However, CD8+ T cells have also been shown to play important effector roles in CS (40, 41). Therefore, in this study we used this approach to examine adhesion of CD8+ T cells in addition to CD4+ T cells and neutrophils in the two-challenge CS model (Fig. 2A). In unchallenged skin, no adherent CD4+ or CD8+ T cells were observed (Fig. 2B, 2C). After the first challenge, adhesion of CD4+ and CD8+ T cells peaked at 8–24 h, with some residual adhesion at 48 h (Fig. 2B, 2C, Supplemental Video 1). During the second challenge, given the accelerated time course of total leukocyte adhesion (Fig. 1A), it was only necessary to assess leukocyte subset adhesion at 4 and 24 h after challenge. In response to a second challenge, CD4+ cells continued to adhere at a level comparable to that of the first challenge, and a similar level of adhesion was observed when the second challenge was applied at a remote naive site (Fig. 2B). Adhesion of CD8+ T cells was lower than that seen during the first challenge both at 4 h, when the total leukocyte adhesion response was peaking, and at 24 h (Fig. 2C). However, when the second challenge was applied to a region of naive skin, CD8+ T cell adhesion was similar to that seen in the first challenge, indicating that this abatement of CD8+ T cell adhesion was specific to the site of initial challenge (Fig. 2C).

We next assessed adhesion of Gr-1+ neutrophils under the same conditions. Following a single oxazolone challenge, neutrophil adhesion was significantly increased within 2 h and remained elevated for at least 24 h before returning to basal levels by 48 h (Fig. 2D). At the peak of neutrophil adhesion at 8 h, the number of adherent neutrophils was ~10-fold greater than that of CD4+ and CD8+ T cells combined (Fig. 2B, 2C), indicating that our previous
finding relating to the relative scarcity of CD4+ cells also applies to CD8+ cells (35). Following a second challenge, neutrophil adhesion was of a similar magnitude but peaked at 4 h and subsided more rapidly to near basal levels by 24 h (Fig. 2D). Changes to the pattern of neutrophil adhesion were specific to the site of the initial challenge, as for the CD8+ T cells, with neutrophil adhesion remaining elevated after 24 h when the second challenge was applied to an area of naive skin (Fig. 2D). These data indicate that during a second oxazolone challenge to the same area of skin, adhesion of CD8+ T cells and neutrophils is attenuated relative to the pattern seen during the first challenge, but that, in contrast, the pattern of CD4+ T cell adhesion is similar in both challenges.

Infiltration of Tregs increases progressively in multiple challenge sensitivity

Examination of leukocyte adhesion and edema formation in this two-challenge model of CS indicated that upon a second hapten challenge, these aspects of the inflammatory response were being terminated more rapidly. These altered kinetics suggested that inflammation during the second challenge was being limited by more active immune regulation. Given that Tregs have been shown to be capable of limiting CS responses (42–44), this raised the possibility that Tregs contribute to the accelerated resolution of inflammation during the second challenge. To understand the contribution of Tregs in this response, we therefore examined the time course of Treg accumulation in the two-challenge CS model. Because Tregs form a subset of the total T cell population, we initially examined the accumulation of all T cells in the inflamed skin. Using flow cytometry to detect CD3+ cells in dissociated skin samples, we found substantial T cell accumulation within 24 h of the first challenge (Fig. 3A). Notably, T cell accumulation remained significantly elevated at 48 h, consistent with our histological assessment (Fig. 1F), at a time when leukocyte adhesion and edema had resolved. Application of an additional oxazolone challenge at this time point caused no further increase in T cell accumulation. In contrast, T cell numbers in the skin progressively declined to basal levels by 48 h after the second challenge. In uninflamed skin, and throughout both first and second challenges, CD4+ T cells represented just >50% of the total T cells present in the skin (data not shown).

We next delineated the proportion of the CD4+ T cells that were Tregs, using intracellular staining for Foxp3 (Fig. 3B). In the absence of inflammation, ~8% of the resident CD4+ population in the skin was Foxp3+ (Fig. 3C). Forty-eight hours after a single challenge, this proportion increased significantly to 20% (Fig. 3B, 3C). However, the highest Treg proportion was observed during the second challenge phase, at which point >25% of the CD4+ cells were Foxp3+ (Fig. 3C). Comparison of the 4 h time points illustrates a key difference in the process of Treg recruitment between first and second challenges. In the first challenge, T cell adhesion was only just above basal levels (Fig. 2), indicating that T cell (and Treg) recruitment was in its initial stages. In contrast, at the same stage in the second challenge, Tregs represented >25% of the large number of CD4+ T cells present in the tissue.
FIGURE 2. A second CS challenge induces subset- and site-specific changes in leukocyte adhesion. Confocal intravital microscopy and in vivo Ab labeling were used to identify adherent CD4+ T cells, CD8+ T cells, and Gr-1+ neutrophils in the vasculature of the skin. (A) Image shows a dermal venule (wall denoted by a dotted line) in CS-inflamed skin. Neutrophils are visible as red cells, CD4+ T cells as green, and CD8+ cells as blue (see also Supplemental Video 1). Scale bar, 30 μm. (B–D) Assessment of adhesion of CD4+ T cells (B), CD8+ T cells (C), and neutrophils (D) at various time points after one and two CS challenges. Data are shown for untreated (0 h) and 2, 4, 8, 24, and 48 h after the first challenge, and 4 and 24 h after the second challenge. Also shown are adhesion data 24 h after application of the second challenge at a different site from the initial challenge (B–D, “naive site”). Data are shown as means ± SEM of 4–10 mice per time point. *p < 0.05, **p < 0.01, ***p < 0.001 versus control.

Tregs form a large proportion of adherent CD4+ T cells in CS

Given that the number of CD4+ Tregs present in the skin increased markedly during both the single- and dual-challenge CS responses, we next investigated whether this was associated with Treg adhesion in inflamed dermal venules. This was achieved using Foxp3-GFP mice to enable detection of endogenous Tregs via confocal intravital microscopy. In inflamed dermal venules of Foxp3-GFP mice, adherent GFP+ Tregs could be observed infrequently but consistently during the CS response (Fig. 3D). The combined expression of intracellular GFP and surface CD4, as detected via in vivo anti-CD4 staining (Fig. 3D, Supplemental Video 2), confirmed the identity of these cells. At the time of peak adhesion during the first challenge (24 h), ∼40% of adherent CD4+ T cells were Foxp3+ Tregs (Fig. 3E), whereas only 6% of circulating CD4+ T cells were Foxp3+ (data not shown). An almost identical response was observed at the peak of the second challenge (4 h) (Fig. 3E). Given that adhesion of CD4+ T cells was minimal 4 h after the first challenge (Fig. 2B), this demonstrates that intravascular adhesion of Tregs is induced much more rapidly in response to a second challenge. Moreover, these findings demonstrate that during the CS inflammatory response, Tregs form a markedly greater proportion of adherent CD4+ T cells relative to their proportion within the circulating CD4+ population.

ICAM-1 has a dominant role in mediating adhesion of both effector T cells and Tregs

The capacity to identify adherent CD4+ and CD8+ T cells and Tregs allowed us to compare the molecular mechanisms of adhesion of these infrequent leukocyte subsets. To achieve this aim, we administered function-blocking Abs against candidate adhesion molecules during each challenge phase and examined T cell adhesion via confocal intravital microscopy (Fig. 4). Previous evidence of marked upregulation of ICAM-1 and VCAM-1 during the CS effector phase (45), we elected to investigate the ICAM-1/β2 integrin and α4 integrin/VCAM-1 pathways, using anti–ICAM-1 and anti–α4 integrin, respectively. Inhibition of ICAM-1 significantly reduced adhesion of CD4+ and CD8+ T cells during both the first and second challenges (Fig. 4A–D). In contrast, inhibition of α4 integrin did not alter T cell adhesion, and combined blockade of α4 integrin and ICAM-1 was no more effective in preventing adhesion than was inhibition of ICAM-1 alone (Fig. 4A–D). These findings indicate that T cell adhesion in response to both the first and second challenges is largely ICAM-1–dependent and α4 integrin-independent.

Given the dominant role of ICAM-1 in mediating CD4+ T cell adhesion, we next specifically examined the role of this pathway in Treg adhesion, making use of Foxp3-GFP mice. Twenty-four hours after the first challenge, and 4 h after the second challenge, anti–ICAM-1 almost completely eliminated Treg adhesion (Fig. 4E, 4F), indicating that under these conditions, ICAM-1 is critically important in Treg adhesion. Furthermore, this applies equally after first and second challenges, despite the marked acceleration in induction of Treg adhesion during the second challenge phase.

Reduction of Treg adhesion via ICAM-1 inhibition is associated with increased neutrophil adhesion in multiple-challenge CS

Given the marked enrichment of Tregs adherent within the vasculature relative to their proportions in the circulation and within the tissue, this raised the intriguing possibility that the vasculature is an important site of the anti-inflammatory actions of Tregs. We reasoned that using our knowledge of the adhesive mechanisms used by endogenous Tregs, we could interfere with adhesion and recruitment of Tregs and assess the effect on the activation state of the dermal vasculature by examining neutrophil adhesion (42, 46). During the first challenge, in mice in which Treg adhesion was blocked by anti–ICAM-1, neutrophil adhesion was not significantly altered (Fig. 5A). However, during the second challenge, neutrophil adhesion was significantly increased in mice in which Treg adhesion was prevented by ICAM-1 inhibition (Fig. 5B). Why this effect was seen after the second but not the first challenge is unclear, but the different cytokine microenvironments present during the two challenge phases (Fig. 1) may underlie differences in tissue responses at the two time points.

These experiments also enabled the assessment of the molecular basis of neutrophil adhesion. During the first challenge, inhibition of ICAM-1 or the α4 integrin alone failed to alter adhesion, and only combined inhibition of ICAM-1 and the α4 integrin reduced neutrophil adhesion significantly (Fig. 5A), indicating that the β2 and α4 integrin pathways have overlapping roles in mediating
neutrophil adhesion at this point. In contrast, during the second challenge, even combined inhibition of ICAM-1 and α4 integrin failed to block neutrophil adhesion (Fig. 5B), suggesting the up-regulation of additional adhesive pathways under these conditions. The possibility that this function was mediated by the CD44/hyaluronan pathway, which we have previously identified as an alternative neutrophil adhesion pathway under some circumstances (37), was excluded via examination of mice treated with hyaluronidase to eliminate endothelial hyaluronan (data not shown).

Next, we reasoned that using inhibition of ICAM-1 to eliminate Tregs from the vasculature precluded the assessment of changes in adhesion of the CD8+ T cell subset, for which ICAM-1 is also a key adhesion molecule. As an alternative approach to examining the actions of Tregs, we tested previously published Treg depletion strategies (anti-CD25, anti-FR4) (47, 48). However, when these treatments were initiated prior to the second challenge in the dual-challenge CS model used in this study, they were ineffective in selective Treg depletion (data not shown). Therefore, we used the alternative approach of depleting all CD4+ T cells using an anti-CD4 Ab, commencing 24 h after the initial challenge (38). Administration of anti-CD4 resulted in >90% depletion of both intravascular and extravascular CD4+ cells within 24 h, that is, prior to initiation of the second challenge (data not shown). In mice treated in this manner, we observed a robust neutrophil adhesion response in the second challenge, consistent with our previous findings that the critical ‘pioneer’ CD4+ effector T cells required for initiation of the CS response enter the skin within the first 2 h after the initial challenge (39). Examination of the dermal microvasculature in CD4-depleted mice demonstrated a significant, 5-fold increase in adhesion of CD8+ T cells during the second challenge, relative to nondepleted (isotype control) mice (Fig. 5C–E, Supplemental Video 3). The increase in CD8+ T cell adhesion was eliminated by anti–ICAM-1, demonstrating that ICAM-1 retained a dominant role in adhesion of this subset under these conditions. However, in contrast to the effects of anti–ICAM-1 on neutrophil adhesion during the second challenge (Fig. 5B), CD4 depletion, which reduced both circulating and tissue-resident CD4+ T cells, did not cause any change in neutrophil adhesion during the second challenge (Fig. 5F). Notably, the exacerbation of neutrophil adhesion seen in response to ICAM-1 inhibition was eliminated in CD4-depleted mice (Fig. 5F), demonstrating that the proadhesive effect of ICAM-1 inhibition required the presence of CD4+ T cells. Taken together, these data illustrate the complexity of the regulation of leukocyte adhesion in CS, showing that CD4+ T cells control adhesion of neutrophils and CD8+ T cells via distinct mechanisms, with potentially different roles for effector and regulatory cells in the vascular and tissue compartments.

**Potential mechanisms of regulation of endothelial adhesive function in CS**

The aim of the next series of experiments was to address potential mechanisms underlying the control of endothelial adhesive function in the two-challenge model of CS. IL-10 is a prominent Treg product with established roles in regulation of inflammation. To examine the role of IL-10 in the two-challenge model of CS, we used a function-blocking Ab against the IL-10 receptor. In mice treated with this Ab, neutrophil adhesion during the second challenge was unaltered relative to the response seen in isotype control Ab-treated mice (Fig. 5B), in marked contrast to the effect of ICAM-1 inhibition. Similarly, inhibition of the IL-10 receptor failed to modulate the level of adhesion of CD4+ and CD8+ T cells during the second challenge (Fig. 4B, 4D). These findings argue against a role for IL-10 in controlling endothelial adhesive function in this model. Another product of Tregs, TGF-β, has been shown capable of restricting endothelial adhesion molecule expression (49, 50). Therefore, we measured TGF-β production in skin samples from different stages of the two-challenge CS model (Fig. 6). Four hours after a first challenge, skin TGF-β was not significantly different from that in unchallenged skin, whereas by 24 h, a significant increase was detected. Assessment of the sec-
of adherent Tregs observed at the corresponding time points. Notably the levels of TGF-β were significantly elevated above control levels 4 h after challenge. Paired comparison of skin TGF-β expression in the same mice, 4 h after a second challenge at the original site of challenge or an alternative, naive site, demonstrated significantly greater TGF-β in the sites challenged a second time (Fig. 6). Notably the levels of TGF-β show correlation with the numbers of adherent Tregs observed at the corresponding time points (Fig. 3E).

Discussion

The skin represents a key site of Treg action, as shown by spontaneous dermal inflammation in patients and mice deficient in functional Tregs (15–17). Moreover, mice in which Tregs lack either functional selectin ligands or CCR4 have greater susceptibility to dermal inflammation, indicating that Tregs must have the capacity to respond to adhesive signals in dermal venules to control local inflammation (22, 23). Despite these studies, little is known about the mechanisms whereby Tregs achieve these effects. In this study, we show that Tregs undergo ICAM-1–dependent adhesion in dermal venules in a multiple-challenge model of CS, and that they represent ∼40% of all adherent CD4+ T cells. Preventing their adhesion within the dermal vasculature was associated with increased adhesion of other leukocyte populations. Notably, this effect was only apparent during a second challenge, when the dermal microenvironment was markedly different from that existing at the time of initial challenge, despite abundant Treg adhesion during both the first and second challenges. These data demonstrate the potential for blockade of adhesion molecules during the inflammatory response to lead to unexpected effects via interfering with Treg/endothelial cell interactions. Moreover, they demonstrate that findings in acute single-challenge models of T cell-dependent skin inflammation may not be representative of those that occur when skin is exposed to multiple challenges. A caveat associated with these findings is that the methods used to implicate a role for Tregs were not entirely Treg-specific. CD4 depletion reduced both effector and regulatory populations, and as such could have unanticipated effects. Similarly, ICAM-1 inhibition has the potential to affect a wide range of cells. As such, these findings require further investigation using more selective approaches to eliminate Tregs.

In general, investigation of adhesion molecule-deficient mice in inflammatory models has demonstrated reduction in inflammation, consistent with the idea that adhesion molecules act to recruit leukocytes to affected tissues, resulting in tissue damage. However, several studies have observed the opposite effect of exacerbation of inflammation in adhesion molecule-deficient mice (9–12). One
potential explanation for these unexpected findings is that the adhesion molecule deficiency in question causes a significant reduction in recruitment of Tregs, with the associated loss of local immune regulation causing exacerbation of inflammation. Alternative hypotheses, such as loss of protective effects of soluble adhesion molecules, increased chemokine expression, and altered type 1/type 2 cytokine balance, have also been proposed, with varying levels of supporting evidence. In this study, we show that ICAM-1 is critical in recruitment of Tregs to inflamed skin. Moreover, ICAM-1 inhibition during the effector phase of the second challenge prevented Treg adhesion, resulting in significant exacerbation of adhesion of neutrophils. These findings could explain the exacerbation of CS-associated inflammation recently observed in ICAM-1–deficient mice in a Th2-dependent model of CS (12).

In addition to its role in Treg recruitment, our findings demonstrate that ICAM-1 is also the major adhesion molecule involved in adhesion of effector CD4+ and CD8+ T cells. This indicates that ICAM-1 is not selective in recruitment of CD4+ Tregs over CD4+ effector T cells, and it raises the question as to why ICAM-1 inhibition has a proinflammatory effect when it is used by both pro- and anti-inflammatory T cell populations. These findings are consistent with a model in which the dynamics of the T cell response after a second challenge are distinct from those of the first challenge. Prior to the initial challenge, steady-state skin contains only a small population of T cells. Our previous studies have demonstrated that the key leukocytes that drive the oxazolone CS response are recruited during the first 2 h after challenge, consistent with the pioneer T cell concept (39). However, at the time of second challenge, 48 h after the initial challenge, T cells (both effectors and Tregs) are abundant in the challenged skin. Under these conditions, numerous hapten-specific effector T cells are presumably present in the skin, where they are sufficient to initiate a response to a second exposure to hapten, a concept supported by our finding of a robust second-challenge response in mice in which ICAM-1 inhibition prevented recruitment of additional effector T cells during the second response. Under these conditions, recruitment of additional effector T cells may have minimal effect on induction of inflammation, and, as such, inhibition of their recruitment would not alter the response. In contrast, our data suggest that recruitment of Tregs during this second challenge has an important function in controlling leukocyte recruitment in the dermal vasculature.

One of the advantages of the approach used in this study was that it allowed the mechanisms of adhesion specific to CD4+ and CD8+ T cells and neutrophils to be examined individually. These studies demonstrated that neutrophil adhesion in CS is regulated via mechanisms distinct from those of CD8+ cells, at a molecular and cellular level. ICAM-1 was the dominant adhesion molecule for CD8+ T cells in CS, even under conditions of exaggerated leukocyte recruitment in the absence of Tregs (Fig. 5E). In contrast, neutrophil adhesion was mediated by the combined effects of ICAM-1 and the α4 integrin, and, when Treg adhesion was inhibited by anti–ICAM-1, by an additional unidentified molecular pathway.

Comparison of the effects of ICAM-1 inhibition versus total CD4+ T cell depletion on adhesion of neutrophils and CD8+ T cells revealed further differences. To interpret the results from these two treatments, note that while they do not have differential effects on Tregs versus effector CD4+ T cells, they differ in their ability to reduce T cell populations in different compartments. ICAM-1 inhibition was initiated only at the commencement of the second challenge, and as such could only affect CD4+ T cells undergoing intravascular adhesion in the 4-h period prior to imaging. In contrast, CD4+ T cell depletion affected both intra- and extravascular CD4+ T cells. Adhesion of CD8+ T cells during the second challenge was increased by CD4 depletion, consistent with a model in which Treg-derived inhibitory effects on CD8+ T cell adhesion dominate over any proinflammatory effects derived from effector CD4+ T cells. In contrast, neutrophil adhesion was increased by ICAM-1 inhibition but not total CD4 depletion. Given that the latter depletion strategy reduced extravascular effector CD4+ T cells presumably to a greater extent than did brief ICAM-1 inhibition, these findings suggest that effector CD4+ T cells present in the skin increase neutrophil adhesion via promotion of endothelial expression of an adhesion molecule used by neutrophils but not CD8+ T cells. Further experiments will be required to identify this pathway.

Although studies now support the concept that Tregs must have the capacity to roll in dermal venules and respond to chemotactic stimuli in the skin to control local inflammation, the mechanism whereby they achieve their regulatory effect has not been defined. Ring et al. (42, 46) showed that transfer of exogenous CD25+ Tregs resulted in a reduction in leukocyte adhesion in dermal venules at the site of hapten challenge, with evidence supporting a role for Treg-derived IL-10 and adenosine in inhibiting endothelial adhesion molecule expression. However, in these experiments, transferred Tregs were not found in the challenged skin, an observation at odds with previous findings that Treg homing to the skin is required for effective regulation of inflammation (21–23). There are potential limitations in T cell transfer studies, for example in the isolation of Tregs of high purity, with appropriate tissue-homing characteristics to mimic endogenous skin-homing Tregs. In this study, we demonstrate that endogenous Tregs form a large proportion of the CD4+ T cells that adhere in the inflamed dermal microvasculature. Indeed, Tregs were markedly overrepresented within the adherent CD4+ T cell population relative to their proportion in the circulation or the inflamed skin. This raised the intriguing possibility that the vasculature represents a key site of Treg action in inflamed skin. Preventing Treg adhesion to the endothelial surface was associated with significant increases in adhesion of effector leukocyte populations, consistent with the concept that one mechanism whereby Tregs control inflammation is by restricting adhesion of proinflammatory leukocytes in the vasculature.

This concept is further supported by recent studies in which application of Tregs to cultured endothelial cells reduced adhesion and transmigration of Th1 effector cells, as well as endothelial adhesion molecule expression (51). Tregs possess a wide variety of...
anti-inflammatory molecules and mechanisms with the potential to mediate this effect, including IL-10, TGF-\(\beta\), adenosine, and IL-35 (13, 18). Maganto-Garcia et al. (51) provided evidence that this effect was due to Treg-derived TGF-\(\beta\). This is consistent with several earlier studies demonstrating the ability of TGF-\(\beta\) to reduce adhesiveness of human endothelial cells via effects on endothelial adhesion molecule expression (49, 50). In contrast, other studies have demonstrated that Tregs can mediate this effect via a CD39/adenosine-dependent mechanism (46). In this study, we addressed this point first by examining the role of the IL-10 pathway. Blockade of the IL-10 receptor had no effect on leukocyte adhesion in dermal vessels, eliminating this as a potential mechanism for the in vivo response observed. Second, we measured TGF-\(\beta\) in inflamed skin at various stages in the response. TGF-\(\beta\) was found to gradually increase during the first challenge, but during the second challenge to be significantly increased within 4 h. Of note, the amount of TGF-\(\beta\) detected correlated with the level of Treg adhesion observed previously. This raises the possibility that Tregs are affecting endothelial adhesion function in vivo in this model via release of TGF-\(\beta\).

In T cell-mediated diseases such as psoriasis and allergic contact dermatitis, inhibition of leukocyte entry into skin has emerged as a viable therapeutic strategy (52). This study raises two issues that experimental work is required to support this hypothesis. First, the authors addressed this point first by examining the role of the IL-10 pathway. Blockade of the IL-10 receptor had no effect on leukocyte adhesion in dermal vessels, eliminating this as a potential mechanism for the in vivo response observed. Second, we measured TGF-\(\beta\) in inflamed skin at various stages in the response. TGF-\(\beta\) was found to gradually increase during the first challenge, but during the second challenge to be significantly increased within 4 h. Of note, the amount of TGF-\(\beta\) detected correlated with the level of Treg adhesion observed previously. This raises the possibility that Tregs are affecting endothelial adhesion function in vivo in this model via release of TGF-\(\beta\). However, more experimental work is required to support this hypothesis.

In T cell-mediated diseases such as psoriasis and allergic contact dermatitis, inhibition of leukocyte entry into skin has emerged as a viable therapeutic strategy (52). This study raises two issues that warrant consideration in regard to use of such approaches. Inhibition of the \(\beta_2\) integrin LFA-1 has resulted in significant but modest clinical improvement in psoriasis patients (52). Given the present findings that indicate that both effector T cells and Tregs use the major LFA-1 ligand, ICAM-1, as a critical adhesion pathway, it is conceivable that inhibition of recruitment of Tregs could limit the efficacy of anti-LFA-1 in this condition. Alternative approaches that reduce effector T cell recruitment but do not interfere with Treg recruitment could potentially be more effective. Additionally, patients typically present with active skin inflammation, indicating that effector T cells are already present in the skin. It is clear from this study that skin affected by ongoing inflammation is very different from uninflamed, steady-state skin in terms of the cellular and cytokine milieu. Under these conditions, blockade of entry of additional T cells may only be minimally efficacious or even harmful, requiring alternative approaches to be devised.

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Disclosures

The authors have no financial conflicts of interest.

References

Tregs AND EFFECCTOR LEUKOCYTE ADHESION


ON-LINE SUPPLEMENTAL MATERIAL

Video 1: Adhesion of CD4+ and CD8+ T cells and neutrophils in a CS-inflamed venule. Venule in the dermal microvasculature 24 h after a single CS challenge in a C57BL/6 wild-type mouse, as imaged via confocal intravital microscopy. Subset-specific antibodies have been used to label neutrophils (red), CD4+ T cells (green) and CD8+ T cells (blue). Numerous neutrophils are visible undergoing interactions, whereas only occasional CD4+ and CD8+ T cells are observed.

Video 2: Tregs undergo adhesion in the inflamed dermal microvasculature. Dermal venule in a Foxp3-GFP mouse, 4 h after a second CS challenge, imaged by confocal microscopy. CD4+ T cells (red) and a CD8+ T cell (blue) are visible undergoing interactions in the dermal microvasculature. An adherent CD4+ is also labeled intracellularly in yellow, representing the merging of the green GFP (Foxp3) signal and the red CD4 signal, indicating this is a CD4+ Foxp3+ Treg.

Video 3: CD4 depletion increases adhesion of CD8+ T cells during a second CS challenge. Dermal venule in a CD4-depleted C57BL/6 wild-type mouse, 4 h after a second CS challenge, imaged by confocal microscopy. Subset-specific antibodies have been used to label neutrophils (red), CD8+ T cells (blue), CD4+ T cells (green – note, no CD4+ cells are visible due to their depletion). The number of adherent CD8+ T cells is markedly increased relative to the number seen in a normal CS response.