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Foxp3⁺-Inducible Regulatory T Cells Suppress Endothelial Activation and Leukocyte Recruitment

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The ability of regulatory T cells (Treg) to traffic to sites of inflammation supports their role in controlling immune responses. This feature supports the idea that adoptive transfer of in vitro expanded human Treg could be used for treatment of immune/inflammatory diseases. However, the migratory behavior of Treg, as well as their direct influence at the site of inflammation, remains poorly understood. To explore the possibility that Treg may have direct anti-inflammatory influences on tissues, independent of their well-established suppressive effects on lymphocytes, we studied the adhesive interactions between mouse Treg and endothelial cells, as well as their influence on endothelial function during acute inflammation. We show that Foxp3⁺ adaptive/inducible Treg (iTreg), but not naturally occurring Treg, efficiently interact with endothelial selectins and transmigrate through endothelial monolayers in vitro. In response to activation by endothelial function or immobilized anti-CD3ε, Foxp3⁺ iTreg suppressed TNF-α- and IL-1β-mediated endothelial selectin expression and adhesiveness to effector T cells. This suppression was contact independent, rapid acting, and mediated by TGF-β-induced activin receptor-like kinase 5 signaling in endothelial cells. In addition, Foxp3⁺ iTreg adhered to inflamed endothelium in vivo, and their secretion products blocked acute inflammation in a model of peritonitis. These data support the concept that Foxp3⁺ iTreg help to regulate inflammation independently of their influence on effector T cells by direct suppression of endothelial activation and leukocyte recruitment. The Journal of Immunology, 2011, 187: 000–000.

Regulatory T cells (Treg) have a critical role in protecting against autoimmunity and regulating immune responses by suppressing T cell proliferation (1). The ability of Treg to migrate to sites of inflammation is an important factor for their suppressive function (2); however, their direct influence at the site of inflammation is still unknown. The two main types of Treg are naturally occurring Treg (nTreg) and adaptive/inducible Treg (iTreg). nTreg develop in the thymus, where they acquire expression of the transcription factor Foxp3 in response to self-Ag presentation. Fully mature nTreg then migrate to the secondary lymphoid organs, where they suppress the expansion of self-reactive T cells (3). In contrast, Foxp3⁺ iTreg develop from naive CD4⁺ T cells in the lymphoid tissues in response to environmental Ags presented in association with TGF-β but without the influence of “danger signals,” such as pathogen- or damage-associated molecular patterns, or inflammatory cytokines that stimulate APCs, such as IFN-γ, IL-1, IL-6, and IL-12 (4). Ag presentation in the absence of these products, often referred to as tolerogenic, occurs with self-Ags and environmental Ags collected by APCs around the mucosal lining of the nasal passages, the lung, and the gut. Tolerogenic Ag presentation is a central mechanism in suppressing undesired immune reactivity against nonharmful material such as airborne particles, food, and commensal bacteria. In vitro studies show that TGF-β1 and IL-2 are critical factors for developing Foxp3⁺ iTreg (5), and that IL-4 and IFN-γ antagonize the acquisition of Foxp3 expression that orchestrates these iTreg functions. Moreover, the vitamin A metabolite, retinoic acid (RA), that is naturally synthesized by tolerogenic APCs, stabilizes Foxp3⁺ iTreg by blocking these antagonistic effects of IL-4 and IFN-γ (6, 7). In addition, RA promotes TGF-β1–mediated Foxp3 expression during in vitro differentiation of iTreg and preferentially supports Foxp3⁺ iTreg formation over Th17 (8). Interestingly, both nTreg and iTreg seem to have similar capacities to suppress T cell proliferation in vitro when using anti-CD3ε as a TCR stimulus. Nevertheless, the overall contributions of nTreg and iTreg to immune tolerance may be quite different with respect to mechanism and Ag specificities (9). iTreg are essentially non–self-reactive and respond to foreign antigenic stimulation, whereas nTreg have self-reactive TCR specificities that suppress autoimmune responses (4). Therefore, the presence of each Treg subset at the site of inflammation may have different implications.

Interestingly, Treg are found at the sites of inflammation in patients with various types of inflammatory conditions and in murine models of these diseases, including inflammatory bowel disease (10), lung injury (11), atherosclerosis (12), experimental autoimmune encephalomyelitis (13), and transplantation (14). Moreover, it was demonstrated that the ability of Treg to migrate to sites of inflammation is a prerequisite for their subsequent...
suppressive function in the draining lymph nodes (2). We have recently shown that Treg migrate into atherosclerotic lesions, and that persistent hypercholesterolemia leads to a reduction in Treg migration and an associated increase in disease progression (15). Nonetheless, despite growing interest in the therapeutic potential of in vitro expanded nTreg and in vitro generated iTreg (16, 17), the migratory characteristics of “inflammation visiting” Treg and the specific nature of their activity at the site of inflammation remain unclear. In this article, we characterize the adhesiveness of Foxp3+ iTreg and nTreg with inflamed endothelium. We demonstrate that only Foxp3+ iTreg adhere to cytokine-stimulated endothelial cells (ECs) and transmigrate in vitro at similar levels to Th1 effector cells. On contact with ECs in vitro, Foxp3+ iTreg respond to endothelial Ag presentation and suppress TNF-α- and IL-1β-mediated leukocyte recruitment by regulating the expression of endothelial CD62E and CD62P (E- and P-selectins, respectively). We also found that TGF-β1, which is released by Foxp3+ iTreg on reactivation, mediates the suppressive effects of iTreg on endothelial activation in an AKL5-dependent manner. Using intravital microscopy of leukocyte rolling in the microvasculature of the mouse cremaster muscle, we also show that Foxp3+ iTreg have strong interactions with inflamed endothelium in vivo. Finally, we demonstrate that both TGF-β1 and iTreg secretion products are able to suppress leukocyte recruitment in a model of TNF-α-induced acute peritonitis. These data demonstrate the suppressive influences of Foxp3+ iTreg on endothelial activation and leukocyte recruitment, and provide a novel insight into their regulatory function at the site of inflammation.

Materials and Methods

Mice

C57BL/6 mice, referred to as wild type, used in the study were purchased from Jackson Laboratory (Bar Harbor, ME); Foxp3-eGFP knockin mice that express cytoplasmic GFP as reporter for Foxp3 expression (18) were kindly provided by Dr. Vijay Kuchroo (Center for Neurological Disease, Brigham and Women’s Hospital, Boston, MA); SMARTA TCR transgenic mice, which express lymphocytic choriomeningitis virus glycoprotein (LCMVgvp)-specific CD4+ T cells, were a kind gift from Dr. Hans Hengartner (Institute of Experimental Immunology, University Hospital Zurich, Zurich, Switzerland) and Dr. Pamela Ohashi (University of Toronto, Toronto, ON, Canada) (19); and the SMARTA × Foxp3-eGFP mice are the crossbreed of the above two strains on the C57BL/6 background. Mice were used for experiments at 8–12 wk of age in a sex-matched setup. Male and female mice were used in separate experiments, with no differences in response to our experimental treatments in this study. Mice were housed and bred in the pathogen-free facility at the New Research Building, Harvard Medical School (Boston, MA). All procedures done with animals were conducted in accordance with protocols approved and supervised by the Institutional Committee for Animal Research at the Harvard Medical School in accordance with the National Institutes of Health guidelines for animal research.

Isolation of nTreg and in vitro derivation of mouse iTreg and Th1 cells

nTreg were isolated from spleens of Foxp3-eGFP knockin mice as previously described (18). Foxp3+ iTreg were derived from naive CD4+ CD25- GFP+ cells purified from spleens of the SMARTA × Foxp3-eGFP mice using a combination of CD4-MACS magnetic beads and FACS for GFP+ cells. These cells were stimulated with plate-bound anti-CD3ε (5 μg/ml) in culture media with the addition of activating anti-CD28 (2 μg/ml), human rTGF-β1 (10 ng/ml), IL-2 (10 U/ml), anti-murine IL-4 (0.5 μg/ml), anti-murine IFN-γ (2 μg/ml) with or without the addition of RA at concentration of 100 nM. For more clarity, Foxp3+ iTreg generated in the presence of RA were marked as iTreg-RA. After 48 h of activation, cells were transferred from the anti-CD3ε–coated plates to a fresh tissue culture plates for another 3 d; then Foxp3–GFP+ iTreg were sorted by FACs, and expression of Foxp3 protein was verified by intracellular staining. Both nTreg and iTreg were sorted for high purity (>98%) and tested for suppression of responder T cell proliferation (Supplemental Figs. 1, 2). Similarly, Th1 cells were developed from naive CD4+ that were activated by plate-bound anti-CD3ε with the addition of activating anti-CD28 (2 μg/ml) in the presence of IL-4–blocking Ab and IL-12 (10 ng/ml). Th1 phenotype was confirmed by intracellular staining of IFN-γ, and typically >95% of the viable cells were IFN-γ+. Flow cytometry data were processed using FlowJo software (Tree Star, Ashland, OR).

iTreg supernatant

iTreg were generated in vitro as described earlier, purified at day 5 of culture, rested for 1 d in media containing IL-2 (50 U/ml), then washed and reactivated by transfer to a 24-well culture dish (2 × 10^5 iTreg in 1 ml/well) containing Ag-pulsed mouse heart ECs (MHECs) or plate-bound anti-CD3ε. Sixteen hours later, the supernatant was collected, centrifuged, and stored at −20°C. Supernatants were absorbed with magnetic beads coated with affinity-purified goat anti-mouse TNFRII IgG (R&D Systems), to block the effect of soluble TNFRII (sTNFRII).

Purification and use of MHECs

MHECs were prepared using MACS-Immunomagnetic beads (Miltenyi Biotech) specific for CD31 and ICAM-2, as previously described (20). In brief, sheep anti-rat IgG Dynal beads are coated with either anti–PECAM-1 (CD31) or anti–ICAM-2 (CD102) mAb. Hearts are harvested from 2 mice per preparation, minced, and then digested in collagenase (180–200 U/ml) at 37°C for 45 min. The digested tissue is filtered through a 70-μm cell strainer, washed, and then incubated with PECAM–1–coated beads. The bead-bound cells are magnetically recovered and cultured in DMEM containing 20% FCS, supplemented with 100 μg/ml porcine heparin, and 100 μg/ml EC growth supplement (ECGS; Biomedical Technologies) in gelatin-coated tissue culture flask. After reaching 70–80% confluence (4–6 d), cells are detached from the culture dish with trypsin-EDTA and subjected to a second selection step using anti–ICAM-2–coated beads. The bead-bound cells are then magnetically recovered. Confluent monolayers of MHECs are used for experiments at passages 1–3.

In vitro assay for leukocyte adhesion and transendothelial migration

T cell adhesion to EC monolayers and transendothelial migration were measured in vitro as previously described (21). In brief, sheep anti-rat IgG Dynal beads were coated with either anti–PECAM-1 (CD31) or anti–ICAM-2 (CD102) mAb. Hearts are harvested from 2 mice per preparation, minced, and then digested in collagenase (180–200 U/ml) at 37°C for 45 min. The digested tissue is filtered through a 70-μm cell strainer, washed, and then incubated with PECAM–1–coated beads. The bead-bound cells are magnetically recovered. Confluent monolayers of MHECs are used for experiments at passages 1–3.

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Treg binding to E-selectin under flow conditions

Interactions of T cells and Treg with recombinant mouse E-selectin Fc chimera-coated coverslips (R&D Systems) were examined under defined laminar flow conditions in a parallel plate flow chamber as described previously (22, 23, 26). T cells and Treg were preincubated in Dulbecco’s PBS containing 0.1% (v/v) BSA and 20 mM HEPES, pH 7.4, at 37°C (5 × 10^6 cells/ml) and perfused over the coated coverslips. T cell interactions were recorded with a phase-contrast objective (20×) and a video microscope connected.
to VideoLab software (Ed Marcus Laboratories) to record cell behavior (shear force of 0.8 and 1 dynes/cm²). Accumulation of the cells was determined after the initial time of each flow rate by counting cells in five different fields.

**T cell adhesion in vivo in the microvasculature of the mouse cremaster muscle**

Intravital microscopy studies of the mouse cremaster muscle microcirculation were performed as described previously (27, 28). Mice were anesthetized, and a microcatheter was introduced through the right femoral artery to enable retrograde injection of fluorescently labeled cells (28). Transmitted light and fluorescent cremaster muscle imaging was done with an Olympus FV1000 confocal intravital microscope using a 40× water immersion objective. Fluorescence imaging was done sequentially at 473 and 635 nM to reduce the potential for channel cross talk. The centerline RBC velocity in each venule was measured in real time with an optical Doppler velocimeter (Texas A&M, College Station, TX), and velocity was used to determine the wall shear rate and critical velocity (28). GFP-iTreg and Alexa 680-labeled GFP-naïve T cells were suspended at 30 × 10⁶ cells/ml, and small boluses (3 × 10⁶ of each cell type) of a mixture of both cells were injected retrograde into the femoral artery catheter. Rolling interactions of adoptively transferred T cells and postcapillary venules of the cremaster muscle were then visualized. MicrovesSEL images were analyzed off-line using Imaris software (Bitplane, South Windsor, CT).

**Peritonitis model**

Peritonitis was induced in C57BL/6 mice by a single i.p. injection of TNF-α (0.5 μg in 200 μl saline) was injected intracranially 1.5 h before cremaster exteriorization. Mice were anesthetized, and a microcatheter was introduced into the right femoral artery to enable retrograde injection of fluorescently labeled T cells (28). Transmitted light and fluorescent cremaster muscle imaging was done with an Olympus FV1000 confocal intravital microscope using a 40× water immersion objective. Fluorescence imaging was done sequentially at 473 and 635 nM to reduce the potential for channel cross talk. The centerline RBC velocity in each venule was measured in real time with an optical Doppler velocimeter (Texas A&M, College Station, TX), and velocity was used to determine the wall shear rate and critical velocity (28). GFP-iTreg and Alexa 680-labeled GFP-naïve T cells were suspended at 30 × 10⁶ cells/ml, and small boluses (3 × 10⁶ of each cell type) of a mixture of both cells were injected retrograde into the femoral artery catheter. Rolling interactions of adoptively transferred T cells in postcapillary venules of the cremaster muscle were then visualized. MicrovesSEL images were analyzed off-line using Imaris software (Bitplane, South Windsor, CT).

**Statistical analysis**

Plot charts and statistical analysis included Student t test for experiments with two groups or one-way ANOVA with Tukey multiple-comparison posttest for experiments with three or more groups. Statistical analysis was performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA; http://www.graphpad.com).

**Results**

**Treg interactions with endothelial monolayer and transmigration under shear flow conditions**

We tested the main subsets of Treg, Foxp3⁺ nTreg and Foxp3⁺ iTreg, for the ability to interact with TNF-α–stimulated ECs under shear flow conditions. To study the adhesion and migratory function of iTreg in a comparative manner, we used naïve T cells as a negative control and Th1 cells as a positive control, because these subsets have low and high capacities, respectively, to adhere to cytokine-activated endothelium and to migrate to sites of inflammation (29). MHECs were grown in monolayers, stimulated with TNF-α, and the subsequent adhesive interactions of Treg with the MHECs were measured in a flow chamber assay. In this model, Foxp3⁺ iTreg formed more adhesive interactions with TNF-α–stimulated MHEC (64.95 ± 1.18% of the number of Th1 interactions) as compared with nTreg and naïve T cells (29.2 ± 4.58 and 10.2 ± 0.11% of the number of Th1 interactions, respectively; Fig. 1A). Moreover, we found large differences in the ability of Foxp3⁺ iTreg and nTreg to transmigrate through the MHEC monolayer. Of the firmly adherent Th1 cells that were used as positive control for the assay, 49.02 ± 3% transmigrated, whereas 31.85 ± 5.9% of the adherent iTreg transmigrated. In contrast, only 4.7 ± 1.5% of the firmly adherent nTreg cells transmigrated, which was not significantly different from the transmigration observed in naïve T cells (1.3 ± 0.78%; Fig. 1B). Interestingly, reactivation of nTreg by different protocols as detailed in our later discussion did not change their adhesive or transmigration behavior on TNF-α– or IL-1β–stimulated endothelial monolayers (data not shown).

**Expression of chemokine receptors and selectin ligands in Th1 and Foxp3⁺ iTreg**

Given the enhanced adhesive and transmigration behavior of both Th1 effector cells and Foxp3⁺ iTreg, we also sought to determine the molecules expressed by these cells that supported their interactions with cytokine-activated endothelium. Specifically, we investigated the expression of chemokine receptors and sialyltransferases involved in the synthesis of selectin ligands. As anticipated, we found that Th1 effector cells and Foxp3⁺ iTreg have different expression patterns of chemokine receptors. Notably, CCR5 was expressed by Th1 cells but not by iTreg, whereas CCR6, CCR8, and CCR9 were expressed by iTreg but not by Th1 cells (Fig. 2A). CCR7 was also broadly expressed by Foxp3⁺ iTreg but only minimally expressed by Th1 cells. Furthermore, Foxp3⁺ iTreg and Th1 cells shared a high level of S1PR1 expression, which is required for lymphocyte egress from the lymph nodes en route to the blood and peripheral sites of inflammation (30). Foxp3⁺ iTreg also showed significantly more expression of α(1,3)-fucosyltransferase-IV and less expression of β(1,6)-N-acetyl glucosaminyltransferase (Core2 or C2GnT) mRNA compared with Th1 cells, whereas the levels of fucosyltransferase-IV were comparable. These differences in glycosyltransferase expression correlated with a marked increase of surface glycosylated CD43 in iTreg compared with Th1 (Fig. 2B). Furthermore, the synthesis of functional selectin ligands, which is also glycosylation dependent, was very strong in iTreg, and interactions with immobilized CD62E and CD62P under shear flow conditions were at levels comparable with those of Th1 (Fig. 2C). Interestingly, the addition of RA to iTreg differentiation cultures not only enhanced the differentiation and viability of Foxp3⁺ iTreg, as suggested by others (31, 32), but also enhanced their ability to bind CD62P. In contrast, neither naïve CD4⁺ T cells nor nTreg showed measurable

![Figure 1](http://www.jimmunol.org/DownloadedFrom/3040307/FIGURE-1.png)
expression of CCR5, CCR6, CCR8, and CCR9 genes. We did find low-level CCR7 expression in nTreg and high levels of S1PR1 in both naive CD4+ T cells and nTreg. Consistent with their poor adhesive and transmigration behavior (Fig. 1), neither naive CD4+ T cells nor nTreg expressed detectable levels of hyperglycosylated CD43 on their surface, and neither interacted with immobilized CD62E and CD62P under shear flow conditions (data not shown).

**F4x3** iTreg suppress endothelial functions

The robust adhesion of Foxp3+ iTreg to both TNF-α–activated ECs and purified selectins led us to predict that iTreg would also have a regulatory influence on endothelial function. To examine this possibility, we used two different in vitro systems to test the contact-dependent and -independent influences of iTreg on ECs. The contact-mediated influence of iTreg on ECs was tested by first allowing iTreg to respond to endothelial Ag presentation and then evaluating their regulatory influence on endothelial activation by proinflammatory cytokines. The contact-independent effects of iTreg on endothelial function were evaluated by exposing endothelial monolayers to TNF-α or IL-1β in the presence of supernatant taken from iTreg reactivated by endothelial Ag presentation or by TCR-complex cross-linking. During preliminary studies for the contact inhibition experiments, we established that pretreatment with IFN-γ caused MHEC to express I-A b (Supplemental Fig. 3) and efficiently present Ag to stimulated Foxp3+ iTreg (as we show later) and Th1 cells (data not shown).

**Foxp3** iTreg suppress endothelial adhesiveness for Th1 cells and selectin expression in response to contact with ECs

To determine the influence of iTreg contact on subsequent leukocyte–endothelial interactions, we added iTreg to TNF-α–stimulated ECs for 96 h. The robust adhesion of Foxp3+ iTreg to both TNF-α–activated ECs and purified selectins led us to predict that iTreg would also have a regulatory influence on endothelial function. To examine this possibility, we used two different in vitro systems to test the contact-dependent and -independent influences of iTreg on ECs. The contact-mediated influence of iTreg on ECs was tested by allowing iTreg to respond to endothelial Ag presentation and then evaluating their regulatory influence on endothelial activation by proinflammatory cytokines. The contact-independent effects of iTreg on endothelial function were evaluated by exposing endothelial monolayers to TNF-α or IL-1β in the presence of supernatant taken from iTreg reactivated by endothelial Ag presentation or by TCR-complex cross-linking. During preliminary studies for the contact inhibition experiments, we established that pretreatment with IFN-γ caused MHEC to express I-A b (Supplemental Fig. 3) and efficiently present Ag to stimulated Foxp3+ iTreg (as we show later) and Th1 cells (data not shown).

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ulated MHEC monolayers and then measured Th1 cell adhesion to the MHEC under shear flow conditions. Contact with iTreg reduced the numbers of Th1 cells that accumulated on MHEC monolayers by 55% (from 75 ± 0.5 to 33.5 ± 9.5% of Th1 cells loaded) and also suppressed the transmigration of accumulated Th1 by 69% (from 23.5 ± 0.5 to 7.45 ± 0.55% of the Th1 cells accumulated; Fig. 3A). Next, we measured the influence of iTreg contact on endothelial expression of surface selectins and found that iTreg suppressed the numbers of EC expressing CD62P by 85% in response to TNF-α (from 64 ± 3.8 to 9.6 ± 2% CD62P+ MHEC) and by 90% in response to IL-1β (from 53.6 ± 5.6 to 5.3 ± 1% CD62P+ MHEC; Fig. 3B). Similarly, the number of MHEC expressing CD62E was reduced by 83% in response to TNF-α (from 56 ± 7.2 to 9.6 ± 2% CD62E+ MHEC; p < 0.01) and by 86% in response to IL-1β (from 52 ± 4 to 7 ± 4.7% CD62E+ MHEC; Fig. 3B). These experiments demonstrated that contact with activated iTreg suppresses cytokine-mediated endothelial adhesiveness to Th1 cells, and that this response is associated with reduced endothelial surface expression of E- and P-selectin. In both experiments, we found no evidence that iTreg contact damaged ECs because there was no effect on the expression of I-Ak, H2-Kβ, ICAM-2 (CD102), or the apoptosis marker Annexin V after >8 h of coculture with iTreg (data not shown). In addition, iTreg differentiated without the addition of RA showed equal potency in contact-induced suppression of EC adhesiveness to Th1 cells and selectin expression as compared with iTreg-RA (data not shown).

FIGURE 4. Antigenic stimulation of iTreg induces the release of TGF-β1 and contact-independent suppression of endothelial function. LCMVgp p61-specific iTreg-RA were rested for 4 d in media containing IL-2. Restered iTreg were added to MHEC cultures that were pretreated with IFN-γ (to induce endothelial Ag presentation) and pulsed with LCMVgp p61 Ag or with control peptide Ag (OVA p323–339). Sixteen hours later, culture supernatants were harvested and TGF-β1 levels were evaluated by ELISA (A). Restered iTreg were treated for 16 h with plate-bound control-Ig or plate-bound anti-CD3, and TGF-β1 levels in the supernatant were evaluated by ELISA (B). Data represent TGF-β1 levels (mean ± SD) in four independent iTreg cultures in one of two experiments performed (A, B). Supernatant from the earlier described anti-CD3e reactivated iTreg (C) or iTGF-β1 at concentration of 1 ng/ml (D) was added to MHEC monolayers at the same time as TNF-α, and 4 h later, interactions with Th1 cells were evaluated in a flow chamber. Supernatant from iTreg stimulated with control Ag or with plate-bound control-Ig were used as control media; data represent the number of adherent Th1 cells (mean ± SD) in eight fields from two different coverslips per condition, and p values were calculated by one-way ANOVA; and data are from one representative experiment out of two experiments performed (C, D).

Foxp3+ iTreg secrete TGF-β1 in response to endothelial Ag presentation and suppress endothelial adhesiveness to Th1 cells independently of cell contact

Both contact-dependent and -independent mechanisms have been described for Treg suppression of T cell and dendritic cell function (33). We therefore tested whether the in vitro influences of iTreg on endothelial function as described earlier could be duplicated by exposure of EC to supernatants from activated Treg. For this purpose, iTreg supernatant was collected from reactivated iTreg, and levels of TGF-β1 were evaluated by ELISA to confirm activation. We found that Foxp3+ iTreg stimulated by either Ag-pulsed MHEC or plate-bound anti-CD3e readily secreted detectable amounts of TGF-β1 (335 ± 31 and 513 ± 61 pg/ml, respectively; Fig. 4A, 4B). Interestingly, contact with MHEC alone, even without antigenic stimulus, induced TGF-β1 secretion from activated iTreg (62 ± 16 pg/ml; Fig. 4A), whereas contact with the culture dish did not (Fig. 4B). Addition of iTreg supernatant to MHEC monolayers together with TNF-α resulted in the suppression of Th1 cell accumulation on the MHEC monolayer by 65% (from 1080 ± 192 to 378 ± 60 Th1 cells/mm²; Fig. 4C). iTreg developed without RA secreted TGF-β1 at similar levels to iTreg-RA, and the supernatant collected from these iTreg was equally suppressive for EC functions as supernatant from iTreg-RA (data not shown).

It is well documented that prolonged conditioning of ECs with TGF-β1 (12–24 h) leaves ECs refractory to stimulation by TNF-α (34–36), although there is no published evidence to suggest that TGF-β1 can actively block TNF-α stimulation of endothelium without extended preconditioning. Because we found that re-stimulated iTreg secrete high levels of TGF-β1, we tested whether TGF-β1 is the primary suppressive factor in Foxp3+ iTreg supernatant. Accordingly, we designed an experiment in which TGF-β1, instead of iTreg supernatant, was added to MHEC during activation with TNF-α. Surprisingly, we found that TGF-β1 treatment given concurrently with TNF-α suppressed MHEC adhesiveness and reduced the number of Th1 cells bound to the monolayer by 59% (from 930 ± 101 to 384 ± 127 Th1/mm²; Fig. 4D). This suppression was also associated with a significant reduction (~60–80%) in the number of selectin expressing EC (data not shown). These data show that the suppressive effects of iTreg and TGF-β1 on endothelial activation is rapid acting and does not require several hours of conditioning of ECs as previously described (36).
selectin expression by MHEC in response to TNF-α (from 100% to 31.7 ± 5.5%), which was also completely reversed by pretreatment with the ALK5 inhibitor (109.3 ± 8.5%, nonsignificant from TNF-α alone; Fig. 5B). These data indicate that the suppressive effect of iTreg was primarily mediated by secretion of TGF-β1 and inhibitory signaling through ALK5, which activates the Smad2/3 phosphorylation cascade (37–39). Given the fast action of the ALK5-related Smad2/3 signaling cascade, these results could explain how iTreg interfere with the rapid translocation of NF-κB prompted by treatment with TNF-α. Because ALK5/Smad2/3 activation has an obvious influence on transcriptional events, we further evaluated whether iTreg supernatant suppressed TNF-α-induced expression of selectin mRNA (Fig. 5C). We found that iTreg supernatant suppressed TNF-α-mediated induction of CD62E mRNA by 89% (a decrease from 4.9 ± 0.33- to 0.54 ± 0.04-fold induction) and CD62P mRNA by 53% (a decrease from 6.7 ± 0.2- to 3.12 ± 0.1-fold induction). Ad-

dition of ALK5 inhibitor alone or in combination with TNF-α did not increase surface expression of endothelial selectin (data not shown). These data indicate that iTreg supernatant acts at the transcriptional level to suppress endothelial selectin expression.

iTreg interact with inflamed endothelium in vivo and suppress leukocyte recruitment during acute peritonitis

To explore the in vivo significance of our findings, we used two different models of inflammation. First, interactions of iTreg with microvascular endothelium were studied by intravital microscopy of the mouse cremaster muscle after intrascrotal injection of TNF-α (27, 28). Adoptive transfer of fluorescently labeled iTreg into the femoral artery demonstrated that iTreg effectively migrate to postcapillary venules at the site of inflammation and exhibited robust rolling interactions relative to adoptively transferred naive T cells (3.67 ± 0.34 compared with 0.36 ± 0.082 rolling cells/min, respectively; n = 35 vessels in 5 mice; Fig. 6A). Interestingly, the number of rolling iTreg per minute was comparable with that observed with adoptive transfer of Th1 effectors (2.44 ± 0.35 rolling cells/min; n = 22 vessels in 4 mice) measured with identical activation conditions and experimental setup (P. Alcaide, unpublished observations). To determine whether iTreg secretion products could also suppress inflammation in vivo, we used a model of TNF-α–induced acute peritonitis (Fig. 6B). We pretreated mice (i.p.) with TGF-β1 or supernatant collected from reactivated iTreg-RA and then administered an i.p. injection of TNF-α to induce peritonitis. Peritoneal lavage fluid was then collected after 4 h and leukocyte counts were performed (Fig. 5B). Mice pretreated with iTreg-RA supernatant or TGF-β1 had a marked decrease in peritoneal leukocyte count (3.16 ± 0.33 × 10⁶ [n = 12] and 1.65 ± 0.33 × 10⁶ peritoneal leukocytes/mouse

FIGURE 5. iTreg suppression of endothelial functions is dependent on ALK5 signaling. MHEC monolayers were grown on coverslips and stimulated with TNF-α (50 ng/ml) in the presence of control media (black bars) or iTreg supernatant (white bars), or given a 40-min pretreatment with the ALK5 inhibitor SB431542 (500 mM) and then stimulated with TNF-α (50 ng/ml) in the presence of iTreg supernatant (gray bars). Four hours later, the MHEC-laden coverslips were placed in flow chamber, and Th1 adhesion to the monolayers was evaluated under flow conditions (A). Data represent the number of Th1 cells (mean ± SD) adherent to the MHEC in 10 fields from two different coverslips per condition. MHEC were detached from the monolayers, and the frequency of selectin-positive cells was evaluated by flow cytometry (B). Data pooled from three independent experiments represent the relative proportion of selectin-positive cells normalized to the number of selectin-positive cells detected in TNF-α–treated monolayers. RNA was isolated from the same MHEC monolayers and resuspended from the same MHEC monolayers (from 100% to 31.7 ± 5.5%), which was also completely reversed by pre-

FIGURE 6. iTreg interact with inflamed endothelium in vivo and on reactivation secrete anti-inflammatory factors. Mice were prepared for intravitral microscopy of the cremaster muscle, and adhesion of fluorescently labeled iTreg (white bars) and CD4+ naive T cells (gray bars) with the vessel wall was recorded by confocal intravitral microscopy (A). Mice were pretreated with i.p. injection of control media (Media), supernatant from reactivated iTreg (iTreg-RA), or with media supplemented with rTGF-β1 (4 ng/mouse). Thirty minutes later, peritonitis was induced by single injection of TNF-α (12.5 ng/mouse), and 4 h later, mice were sacrificed and peritoneal leukocytes were counted (B). Data represent the average number of rolling cells per minute counted by intravital microscopy in 35 different vessels in five mice (A). Plot represents data pooled from three experiments; each data point represents the number of leuko-

cytes in the peritoneal lavage taken from one mouse (B).
Discussion

Since the discovery of Treg, much research has focused on the mechanisms involved in their development and suppression of adaptive immune responses. The influence of Treg on non-hematopoietic cells, however, has not been well studied. Although it is clear that both effector T cells and Treg migrate to sites of inflammation, little is known about the direct regulatory effect of Treg on inflamed tissue. In this study, we investigated adhesive interactions between Treg and endothelium both in vitro and in vivo, and examined whether iTreg could suppress EC activation and leukocyte recruitment during acute inflammation.

Because of the widely reported presence of Foxp3+ cells in inflamed tissues of both human and mouse (10–14, 40), we hypothesized that Foxp3+ Treg may have direct anti-inflammatory effects on endothelial function. Although subpopulations of “memory-like” αEC-positive Treg were previously suggested to possess high CD62P/E-binding abilities (41), the defining migratory characteristics of these Treg are unclear. We used an in vitro flow assay to establish that Foxp3+ iTreg, but not nTreg, adhere and transmigrate efficiently across TNF-α-stimulated endothelial monolayers under physiological shear flow conditions. These results led us to believe that Foxp3+ iTreg were more likely to successfully migrate to sites of inflammation and were therefore more relevant to our study. Moreover, antigenic activation of Foxp3+ iTreg in the proximity of ECs triggers a regulatory response that rapidly suppresses endothelial activation by TNF-α and IL-1β, as evidenced by a decrease in endothelial selectin expression and effector T cell adhesion. We further demonstrated that this suppressive effect is contact independent and principally mediated by the TGF-β/ALK5 signaling pathway in EC.

EC responses to TGF-β are complex because they have two distinct signaling pathways that lead to different and competing outcomes. TGF-β signaling is initiated when the cytokine brings together two receptors, TGF-βRII and TGF-βRI (also known as ALK5). In most types of cells, the formation of the heteromeric complex TGF-βRII/TGF-β/ALK5 allows the constitutively active kinase in the cytoplasmic tail of TGF-βRII to phosphorylate/activate the cytoplasmic tail of TGF-βRI/ALK5, which, in turn, activates the Smad2/3 cascades (38, 39, 42). In addition to TGF-βRII and TGF-βRI, ECs express endoglin (CD105) that joins into an ALK5/Smad1/5/8 cascade (43, 44). The ALK5/Smad1/5/8 pathway promotes EC proliferation and migration, and is inhibited by signaling from the ALK5/Smad2/3 pathway (45). Moreover, endoglin expression is important for EC survival in the presence of TGF-β1 (46), and endoglin deficiency is the cause for vascular dysplasia and reoccurring hemorrhages that underlie hereditary hemorrhagic telangiectasia type 1 (47).

We found that MHEC expressed significant levels of ALK1, ALK5, and endoglin mRNA, and that expression levels were not significantly affected by exposure to iTreg supernatant or to TGF-β1 (Supplemental Fig. 4A–C). Notably, the levels of endoglin mRNA were very high (33 ± 1.6% of Actb expression), and surface endoglin was high and remained unchanged after stimulation with TNF-α (Supplemental Fig. 4D). It was recently suggested that TNF-α induces endoglin shedding in cultured HUVECs, and that TNF-α is an important mediator of the elevation of soluble endoglin during preclampsia (48). Nonetheless, this study demonstrates that ALK5 signaling has a dominant role in Foxp3+ iTreg suppression despite high levels of surface endoglin on ECs.

Another possible explanation for the suppressive effects of iTreg supernatant in our study is that sTNFRII (sCD120b) that is shed by activated Treg (49) blocked the soluble rTNF-α we used to stimulate ECs. There are several reasons, however, why this explanation is unlikely. First, it is established that sTNFRII binds mainly to membrane-bound TNF-α and has very low binding capacity for soluble TNF-α (50). Second, throughout our study, we stimulated MHEC with soluble TNF-α at a range of concentrations (25–100 ng/ml) that are 5- to 20-fold greater than the levels of sTNFRII reported in supernatants of activated Treg of both murine and human (5–6 ng/ml) (49). Third, in experiments where we used IL-1β to stimulate the endothelium, we found no reduction in iTreg capacity to suppress selectin expression and leukocyte recruitment. Lastly, the suppressive effect of iTreg supernatant was not affected after removal of sTNFRII via immunoprecipitation with magnetic beads labeled with goat anti-mouse sTNFRII capture IgG (data not shown).

The differences in activation status between nTreg and iTreg may underlie some of the observed differences in adhesion and transmigration behavior. iTreg are rapidly formed from activated naive T cells, proliferate as long as they are provided IL-2, and then disappear without leaving a known “rested” memory cell. In contrast, only a minute fraction of peripheral nTreg are recent thymus emigrants with an activated phenotype. To test whether recent activation promotes nTreg adhesion, we stimulated purified nTreg with either plate-bound stimulating anti-CD3ε Ab or with spleen-derived APCs in combination with stimulating anti-CD3ε Ab and tested adhesion after various time points. These experiments, which we performed as part of our preliminary studies, have demonstrated that TCR activation does not change the adhesion and transmigration behavior of nTreg. Moreover, the addition of cytokines such as IL-2 and TGF-β1 and supporting costimulation with agonistic anti-CD28 Ab did not change these outcomes, and again, restimulated nTreg showed very poor interactions with cytokine-stimulated EC or with immobilized selectin ligand (data not shown).

This study suggests that Foxp3+ Treg that form naturally in response to nonpathological oral and intranasal Ags (9, 51) interact with inflamed endothelium and suppress further endothelial activation and leukocyte recruitment. This view is complementary to the recent study by Clark and colleagues (52) that demonstrates that nTreg, but not iTreg, require TNF-α or reactivation in the presence of exogenous TGF-β1 to suppress CD4+ T cell-mediated colitis in the RAG-deficient mice. This study supports our observation that TGF-β1 released by reactivated iTreg (Fig. 3), but not nTreg (data not shown), mediated swift inhibition of endothelial activation.

The presence of Foxp3+ Treg at inflammatory sites in humans and mouse models has led to speculation about their possible function as direct suppressors of tissue inflammation. Although the ability to interfere with Ag presentation is likely to be one important function of Treg, the suppressive effect Treg have on nonhematopoietic cells during inflammation may be underappreciated. Because the endothelium is a major target for effector T cell-derived proinflammatory cytokines, it is plausible that Treg influence EC to control the spread and pathological side effects of inflammation. Nevertheless, it is technically intricate to isolate the influence of Treg on endothelium in vivo. In this work, we demonstrated that iTreg strongly interact with cytokine-stimulated endothelial monolayers in vitro and are capable of suppressing adhesive interactions with effector T cells. iTreg suppression was mediated by TGF-β in an ALK5-dependent manner and was
triggered either in response to antigenic stimulus by a third party, which we experimentally demonstrated by carrying over supernatant, or by direct contact and immune synapse formation with endothelial Ag presentation. The in vivo relevance of these findings was confirmed first by intravital microscopy of iTreg rolling on inflamed endothelium and then by demonstrating the robust suppressive capacity of iTreg secretion products on leukocyte recruitment during acute peritonitis. Taken together, our data suggest that Foxp3 Treg are capable of controlling inflammation through direct suppression of endothelial activation and leukocyte recruitment in a manner independent of their influence on effector T cell activation and proliferation.

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**Disclosures**

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**References**


