

CD4⁺CD25⁺ regulatory T cells suppress contact hypersensitivity reactions through a CD39, adenosine-dependent mechanism

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Background: Injection of regulatory T (Treg) cells into sensitized mice abrogates the elicitation phase of contact hypersensitivity (CHS) reactions by blocking the adherence of leukocytes to vascular endothelium.

Objective: We set out to analyze whether adenosine, a suppressive factor recently described as produced by Treg cells, can account for the suppression of the effector T-cell–endothelial cell (EC) interaction.

Methods: T cells and ECs were cultured in the presence of adenosine, and expression of adhesion molecules and adhesion of T cells to ECs under shear stress were assessed. Furthermore, we injected Treg cells derived from ectonucleotidase-deficient (CD39^{−/−}) mice into sensitized mice and analyzed the sticking and rolling of leukocytes during a CHS response using intravital microscopy.

Results: Adenosine or Treg cells, respectively, abrogated the adherence of effector T cells to ECs *in vitro*. Likewise, injection of adenosine and Treg cells abrogated the ear-swelling reaction, indicating a role of adenosine during Treg cell–induced

suppression of CHS responses. As a source for Treg cell–derived adenosine, we identified the ectonucleotidase CD39 because CD39-deficient Treg cells did not prevent adhesion of leukocytes to the endothelium. Furthermore, we show that the impaired adhesion of effector T cells to inflamed endothelium was induced by adenosine-mediated downregulation of expression of E- and P-selectin on the vascular endothelium.

Conclusion: Adenosine release by Treg cells is essential to block leukocyte adhesion to endothelium, providing a novel mechanism by which Treg cells mediate immune suppression *in vivo*. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: Contact hypersensitivity, regulatory T cells, adenosine, endothelial cells, skinfold chamber

Naturally occurring CD4⁺CD25⁺ regulatory T (Treg) cells have the capacity to suppress proliferation of effector T cells and are essential for the maintenance of peripheral tolerance. When studied *in vitro*, Treg cells require cell-to-cell contact with effector T cells to convey suppressive action.^{1,2} However, under *in vivo* conditions, soluble factors seem to play a role in which release of IL-10 and IL-35 and secretion of TGF-β are crucially attributed to the suppressive effects.^{3–7}

Hapten-induced contact hypersensitivity (CHS) in mice has long served as a model for allergic skin reactions in human subjects. Using this model, we have previously shown that Treg cell injection suppressed the ear-swelling reaction that is induced after reapplication of the sensitizing hapten onto the ear (ie, the elicitation phase).⁸ During the elicitation phase, local antigen-presenting cells (APCs) recruit proinflammatory lymphocytes from the blood to the tissue. Analysis of the function of regulatory cells in this sequence of events has shown that Treg cells and the tissue culture supernatant thereof blocked the adherence of leukocytes to vascular endothelium.⁹ Others have described that Treg cells secrete the immunomodulatory factors TGF-β, IL-10, and adenosine.^{3,4,10} To investigate whether adenosine is involved in regulating the effector T-cell–endothelial cell (EC) interaction, we set up cocultures of ECs and effector T cells. Using this system, we show that adenosine and Treg cells were independently able to potentially suppress the adhesion of effector T cells to EC monolayers *in vitro* and to suppress the elicitation of an ear-swelling response. To further study how adenosine might mediate Treg cell–suppressive effects, we used *in vivo* microscopy to directly observe leukocyte adherence to inflamed vascular endothelium in a mouse model. Within this system, we further observed that injection of Treg cells derived from ectonucleoside triphosphate diphosphohydrolase 1-deficient (CD39^{−/−}) mice,¹¹ which have impaired adenosine production, did not

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Abbreviations used

AMP:	Adenosine monophosphate
APC:	Antigen-presenting cell
ATP:	Adenosine 5'-triphosphate disodium salt
CHS:	Contact hypersensitivity
EC:	Endothelial cell
NECA:	5'-(N-ethylcarboxamide) adenosine
TNCB:	2,4,6-Trinitro-1-chlorobenzene
Treg:	Regulatory T
WT:	Wild-type

prevent adhesion and extravasation of leukocytes. The suppressive effects of Treg cells were induced by adenosine-mediated downregulation of E- and P-selectin expression on the vascular endothelium, thus blocking adherence of effector T cells.¹² In aggregate, our data indicate that Treg cell release of adenosine is essential for preventing leukocyte adhesion to vascular endothelium and might provide a novel means by which Treg cells mediate immune suppression.

METHODS**Mice**

BALB/c and C57BL/6 mice originally purchased from Charles River (Sulzfeld, Germany) were housed at the central animal facility of the University of Heidelberg and cared for in accordance with the institutional guidelines for animal welfare. CD39^{-/-} mice were housed in the animal facility of New York University according to respective institutional guidelines.

Reagents and antibodies

The CD4⁺CD25⁺ Regulatory T Cell Isolation Kit and CD8 MicroBeads were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany); adenosine 5'-triphosphate disodium salt (ATP), adenosine, 8-(p-Sulfo-phenyl)theophylline, 5'-(N-ethylcarboxamide) adenosine (NECA), the PKH26 red fluorescent cell linker kit, and 2,4,6-trinitro-1-chlorobenzene (TNCB) were from Sigma-Aldrich (Taufkirchen, Germany). Monoclonal antibodies for flow cytometry were purchased from BD Biosciences (Heidelberg, Germany) and from Santa Cruz Biotechnology (Santa Cruz, Calif), and the fluorescein isothiocyanate forkhead box protein 3 staining set was from eBioscience (San Diego, Calif). Cell culture medium consisted of RPMI 1640 supplemented with 10% heat-inactivated FCS (both from PAA Laboratories GmbH, Cölbe, Germany).

Purification of Treg T-cell subpopulations

CD4⁺CD25⁺ Treg cells were always isolated from peripheral lymph nodes and spleens of naive mice by using magnetic bead separation (MACS, Miltenyi Biotec). The negatively selected CD4⁺ cells (average purity, >97%) were incubated with anti-CD25-phycoerythrin mAb followed by anti-phycoerythrin Microbeads. The CD4⁺CD25⁺ T-cell subpopulation was positively selected according to the manufacturer's instructions (average purity, >90%). The CD8⁺ T cells were isolated by using CD8-specific MicroBeads (average purity, >95%).

CHS

Mice were sensitized by painting 15 μ L of 1% TNCB dissolved in acetone/olive oil (4:1) on the shaved abdomen on day 0. On day 5, ear thickness was measured by using microcalipers (Oditest, Kroepelin, Germany), followed by the epicutaneous application of 0.5% TNCB solution, 10 μ L on each side of one ear. The other ear was treated with solvent only. After 24 hours, the ear thickness was measured again, and the difference was calculated as the change in ear response in millimeters $\times 10^{-2} \pm$ SD. The number of mice per group is

depicted in the figure legends as "n." For Treg and CD4⁺CD25⁺ cell treatment: 3×10^6 Treg cells were intravenously injected 15 minutes before challenge. For adenosine treatment, 1.25 mg adenosine/kg body weight was intravenously injected 15 minutes before challenge. For theophylline treatment, 30 mg of theophylline/kg body weight was intravenously injected 1 hour before injection of Treg cells.

In vitro proliferation assays

Freshly isolated CD4⁺CD25⁺ T cells (2×10^5 /well/200 μ L) were stimulated with anti-CD3/anti-CD28 (0.5 μ g/mL each) or allogeneic bone marrow-derived dendritic cells (1:100) in the presence of adenosine (10 μ mol/L) or the agonist NECA (10 μ mol/L). Bone marrow-derived dendritic cells were prepared according to standard methods.¹³ Briefly, bone marrow cells of mice were cultured for 6 days in complete RPMI supplemented with GM-CSF and IL-4 (100 U/mL; Promocell, Heidelberg, Germany) with intermittent washes. In functional assays freshly isolated Treg cells were added to the anti-CD3/anti-CD28-stimulated CD4⁺CD25⁺ T-cell cultures in different concentrations. Cell proliferation was determined by measuring tritiated thymidine incorporation.

Flow chamber

The murine EC line (bEND.3; ATCC, Berlin, Germany) was cultured on collagen-coated coverslips and stimulated with TNF- α (1 μ g/mL medium) alone or together with adenosine (10 μ mol/L) or Treg cells (5×10^5 /mL medium). Treg cells were activated with anti-CD3/anti-CD28 before addition to the ECs. After 24 hours, the coverslips with the differently treated ECs were placed into a heated flow chamber (Chromaphor, Ascheberg, Germany) and superfused with fluorescent dye (PKH-PE; Sigma, Deisenhofen, Germany)-labeled effector CD4⁺CD25⁺ T cells or CD8⁺ T cells, respectively. The number of rolling and adherent cells per visible field was determined by using video microscopy.

Analysis of ECs by means of flow cytometry

The murine EC line bEND.3 was cultured in tissue flasks and stimulated with TNF- α (1 μ g/mL medium) alone or together with adenosine (10 μ mol/L) or Treg cells (5×10^5 /mL medium). Unstimulated ECs served as negative controls. After 4 hours in culture, ECs were washed and analyzed by means of flow cytometry for E- and P-selectin expression.

Detection of adenosine by means of HPLC

A high-pressure liquid chromatograph (Dionex ICS-300; Dionex, Frankfurt, Germany) equipped with an Acclaim 120 C-18 reversed-phase column (Dionex) was used to measure adenosine. Standard adenosine and all other reagents were purchased from Sigma. Twenty-microliter aliquots of the samples were measured with a 0% to 7% acetonitrile/H₂O gradient mobile phase (1 mL/min over 15 minutes), and absorption was measured at 260 nm.

Skinfold chamber

For intravital microscopy, we used the dorsal skinfold chamber preparation, as described previously.⁹ Mice were injected with Rhodamine 6G (Sigma-Aldrich) to visualize endogenous leukocytes. When mice had recovered from the anesthesia, intravital microscopic images were recorded with the high-resolution black-and-white camera Spot RT KE Monochrome w/o IR Filter (Vision, Munich, Germany) and analyzed with Spot software. Sequences of 1 minute were recorded in defined microvessel segments per chamber at baseline (ie, before antigen re-exposure) and 30, 60, 120, and 240 minutes after challenge with 10 μ L of 0.1% TNCB dissolved in acetone/oil (1:4) on the skin on the back site of the observation window.

Analysis of mouse ears

Ear cells were obtained from mouse ear halves by means of trypsinization for 30 minutes at 37°C. Isolated cells were analyzed by means of flow

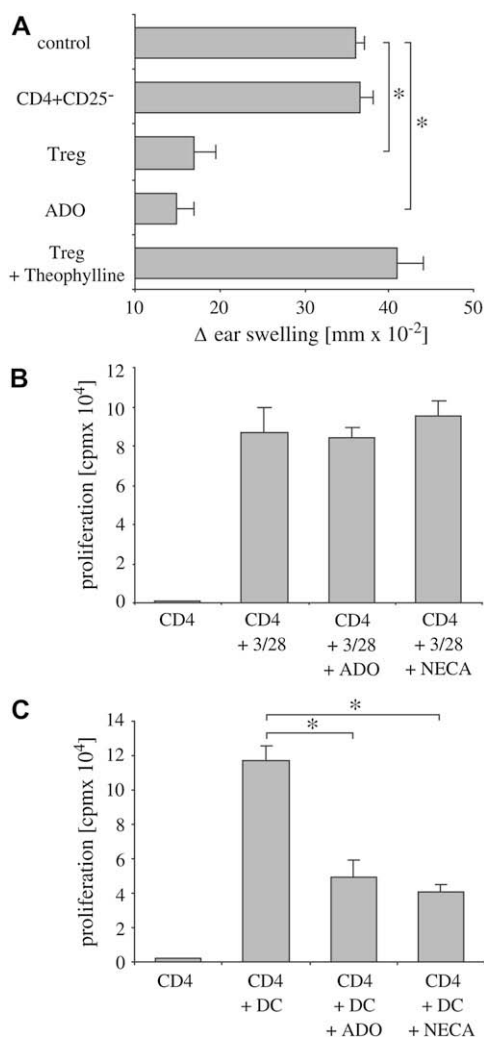


FIG 1. Adenosine (ADO) blocks the elicitation phase of CHS and abrogates T-cell proliferation *in vitro*. **A**, Control mice were sensitized and challenged with TNCB. The other experimental groups were additionally treated with CD4⁺CD25⁻ cells, Treg cells, adenosine, or Treg cells and theophylline. Shown is the mean increase of ear thickness $\pm$ SD of 3 independent experiments ($n = 5$; * $P < .005$). **B**, Proliferation of CD4⁺CD25⁻ T cells (CD4) stimulated with anti-CD3/anti-CD28. **C**, Proliferation of CD4⁺CD25⁻ T cells (CD4) stimulated with allogeneic dendritic cells (DC). Data in Fig 1, B and C, show the mean counts per minute $\pm$ SD from a typical experiment carried out in triplicates.

cytometry. Thin tissue sections from mouse ears of the different experimental groups were saturated with 20% sucrose mounted in Tissue-Tek mounting-freezing media (Miles, Torrance, Calif) and sectioned with a cryostat (Leica, Wetzlar, Germany). Sections were either stained with hematoxylin and eosin for morphologic evaluation or with fluorochrome-conjugated antibodies (Caltag Laboratories, Burlingame, Calif) for immunofluorescence analysis.

RESULTS

Adenosine blocks the ear-swelling reaction *in vivo* and abrogates T-cell proliferation in mixed leukocyte reactions *in vitro*

Because we and others have shown that Treg cells suppress the challenge phase of CHS reactions, we set out to investigate whether adenosine is involved in this process. During initial

experiments, we injected Treg cells or adenosine into TNCB-sensitized mice before challenge and found that Treg cells and adenosine were independently able to suppress the ear-swelling reaction induced by challenge (Fig 1, A). Moreover, theophylline, an adenosine receptor antagonist, prevented the suppressive capacity of the Treg cell (Fig 1, A). This further supports our notion that adenosine is involved in the Treg cell-mediated suppression of the ear-swelling response.

To test the direct effects of adenosine on T-cell proliferation *in vitro*, we stimulated CD4⁺CD25⁻ T cells with anti-CD3/anti-CD28 antibodies (Fig 1, B) or allogeneic APCs (Fig 1, C) in the presence of adenosine or the adenosine receptor agonist NECA. Here we show (Fig 1, B) that neither adenosine nor NECA was able to block proliferation of anti-CD3/anti-CD28-stimulated T cells. In contrast, NECA, as well as adenosine, significantly reduced T-cell proliferation when added to T-cell-dendritic cell cocultures (Fig 1, C). These results are in line with previous observations showing that the adenosine-suppressive effects present in APC-T-cell cocultures are overridden when effector T cells are directly activated through their CD3 and CD28 receptors.

Adenosine and Treg cells block adherence of effector T cells to activated ECs *in vitro*

Because Treg cells suppress the ear-swelling responses in a murine CHS model by inhibiting the adherence of effector T cells to vascular endothelium,⁹ we next set out to investigate whether Treg cell-derived adenosine is involved in this process. Therefore we set up *in vitro* cultures of the EC line bEND.3 and investigated the effects of Treg cells and adenosine on adherence of effector T cells to these stimulated ECs under shear-force conditions.

bEND.3 cells were cultured on collagen-coated coverslips; pretreated with TNF- α , adenosine, preactivated Treg cells, or combinations thereof as indicated; and placed in a heated flow chamber. Thereafter, the ECs were superfused with fluorescent dye-labeled effector T cells under constant observation by using video microscopy. Individual movies were later analyzed to determine the number of rolling and adherent cells per visible field.

As shown in Fig 2, A and B, CD4⁺CD25⁻ effector T cells adhere to TNF- α -stimulated ECs in significantly greater numbers compared with unstimulated ECs. Addition of adenosine or Treg cells, respectively, reduced CD4⁺CD25⁻ effector T-cell adherence to control levels (see videos E1-E4 in this article's Online Repository at www.jacionline.org). Because CD8⁺ T cells are the main effector cells in the murine CHS model, we also performed flow-chamber experiments by using fluorescent dye-labeled CD8⁺ T cells. Similar to CD4⁺CD25⁻ T cells, the adherence of CD8⁺ T cells on TNF- α -stimulated ECs was significantly diminished by pretreatment of the ECs with Treg cells or adenosine, respectively (Fig 2, C and D). Thus these data indicate that adenosine or Treg cells suppress adhesion of CD4⁺CD25⁻ and CD8⁺ T cells to the EC monolayer, supporting the conclusion that Treg cell-derived adenosine might be a key factor mediating suppression of CHS responses.

Treg cells produce adenosine through CD39 ectonucleotidases

To determine the source of adenosine, we next tested Treg cells for surface expression of CD39 and CD73, 2 ectoenzymes that serially dephosphorylate ATP to adenosine monophosphate

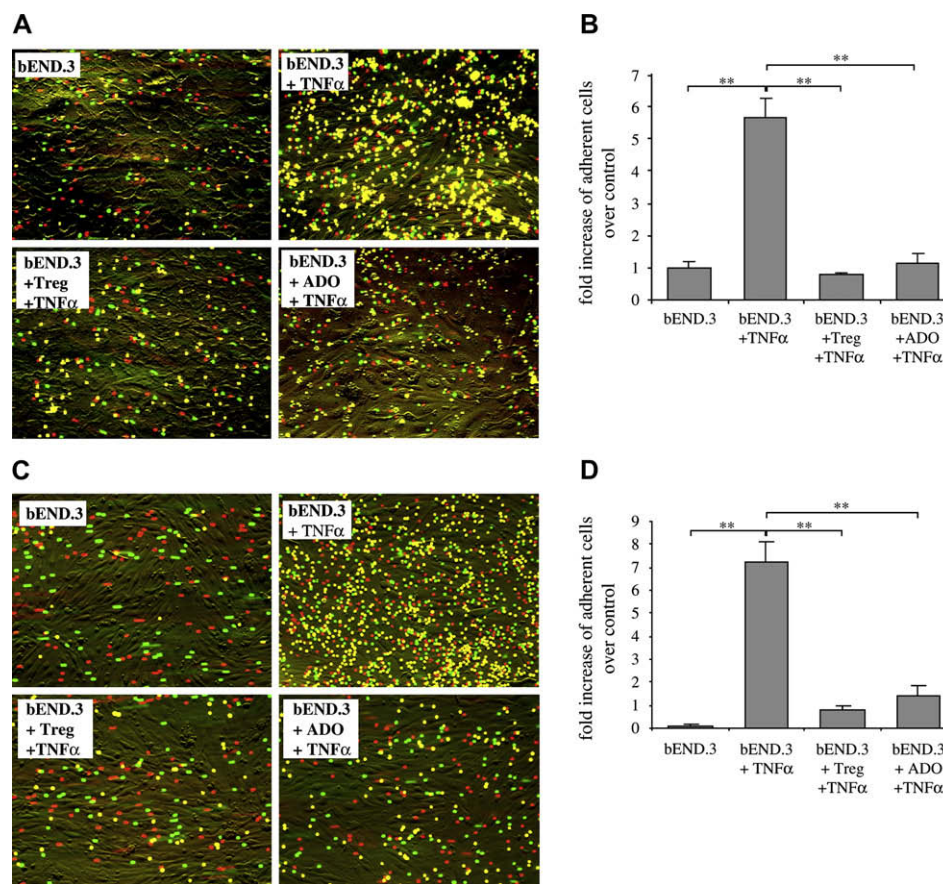


FIG 2. Adenosine (ADO) and Treg cells preactivated with anti-CD3/anti-CD28 block adherence of T cells to ECs *in vitro*. **A** and **C**, Merged pictures of flowing CD4⁺CD25⁻ T cells (**Fig 2, A**) and CD8⁺ T cells (**Fig 2, C**) after 10 minutes (red) and 11 minutes (green) of flow. Nonmoving adherent cells appear yellow. **B** and **D**, Number of adherent CD4⁺CD25⁻ T cells (**Fig 2, B**) and CD8⁺ T cells (**Fig 2, D**) on the ECs after 20 minutes of flow. Data show the fold increase of adherent cells on stimulated bEND.3 cells in comparison with that seen on unstimulated bEND.3 cells (** $P < .001$).

(AMP) and then to adenosine, respectively. Using flow cytometry, we show that Treg cells constitutively express both enzymes (**Fig 3, A**), with further enhanced expression after stimulation. In contrast, conventional CD4⁺CD25⁻ T cells did not express CD39 in a resting state, and activation by anti-CD3/anti-CD28 resulted in only modest CD39 expression. Similarly, CD73 was expressed at low levels in resting CD4⁺CD25⁻ T cells, with no change after activation. We next directly investigated Treg cell generation of adenosine by analyzing the tissue culture supernatant by means of HPLC. Our results demonstrate that Treg cells are indeed able to produce adenosine (**Fig 3, C**). When the cells were cultured in the presence of ATP, which is the substrate for adenosine production through the CD39/CD73 cascade, increased amounts of adenosine were noted that corresponded to the concentrations of ATP added to the cultures (**Fig 3, D**). These data indicate that Treg cells are capable of producing adenosine through the CD39/73 ectonucleotidase pathway.

Treg cells deficient in CD39 expression do not suppress the ear-swelling response in CHS reactions *in vivo*

To verify that CD39-derived adenosine production by Treg cells mediates suppression of CHS reactions *in vivo*, we injected

wild-type (WT) mice before challenge with Treg cells isolated from CD39-deficient (CD39^{-/-}) mice and measured the ear thickness 24 and 48 hours later. These previously described mice lack the production of adenosine through the CD39 ectonucleotidase pathway.^{10,11}

We found that Treg cells derived from CD39^{-/-} mice were unable to suppress the ear-swelling reaction in contrast to their WT counterparts (**Fig 4, A**). Moreover, this effect was only apparent *in vivo*, in contrast to the comparable *in vitro* T-cell suppression induced by Treg cells derived from WT and CD39^{-/-} mice (**Fig 4, B**). Thus these findings support our results obtained with adenosine agonists depicted in **Fig 1** and indicate that adenosine does not directly affect anti-CD3/anti-CD28-induced proliferation of T cells *in vitro*. Instead, *in vivo* ECs are a possible target for Treg cell-derived adenosine.

To further investigate the *in vivo* relevance of our *in vitro* data shown in **Fig 2**, we used the skinfold chamber model. In this model we are able to visualize T-cell adherence to inflamed endothelium *in vivo* directly at the site of antigen challenge.

When sensitized mice were challenged with hapten, we observed substantially increased numbers of rolling and adhering effector T cells at the site of the inflamed endothelium (**Fig 4, C** and **D**, and see **videos E5-E8** in this article's Online Repository at www.jacionline.org). This adherence is abrogated when Treg cells

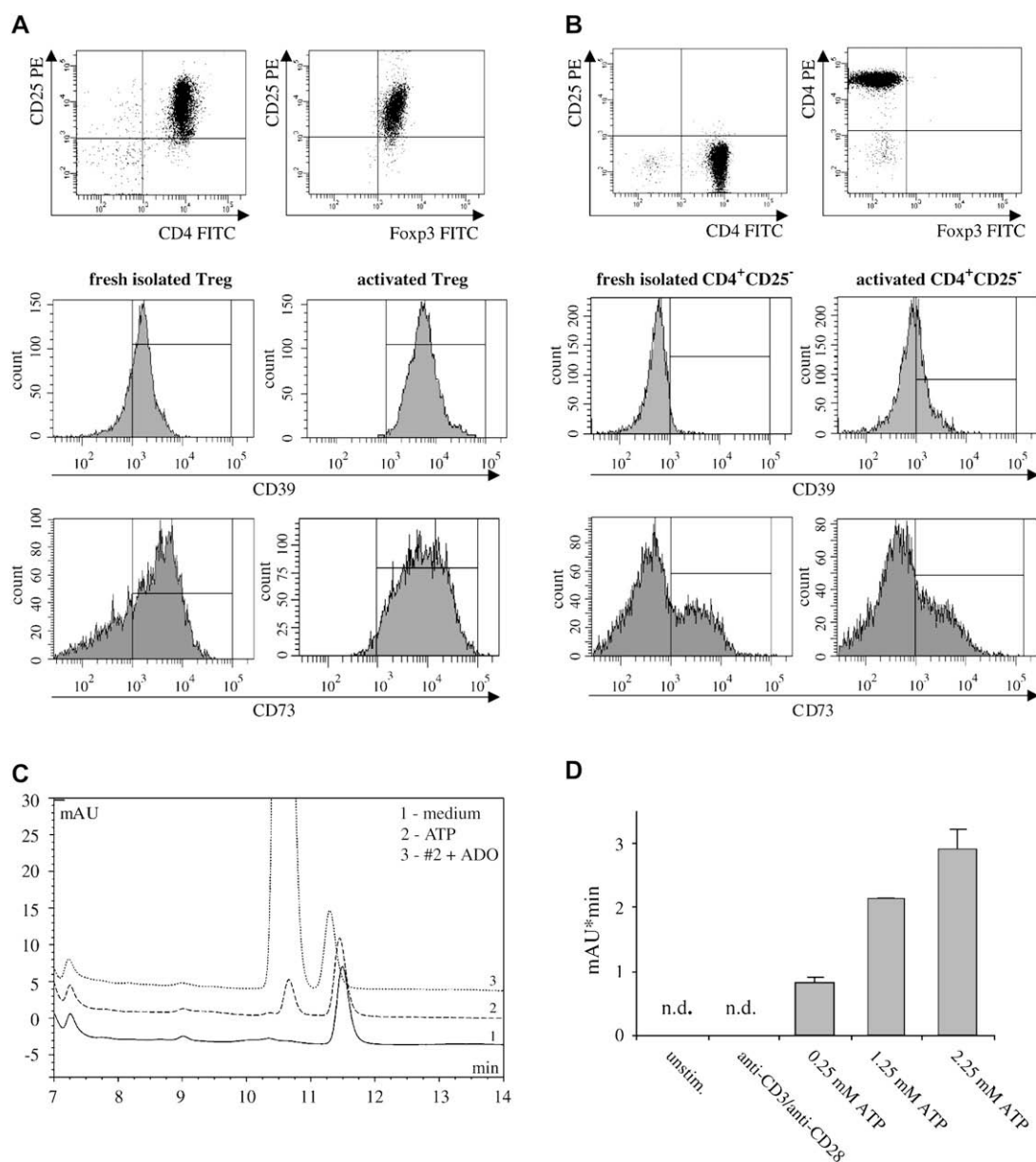


FIG 3. CD39⁺CD73⁺ Treg cells produce adenosine after activation with ATP. **A**, Expression of CD39 and CD73 on either freshly isolated or activated (anti-CD3/anti-CD28 0.5 μ g/mL each, 24 hours) CD4⁺CD25⁺ forkhead box protein 3 (Foxp3)⁺ Treg cells. PE, Phycoerythrin; FITC, fluorescein isothiocyanate. **B**, Expression of CD39 and CD73 on CD4⁺CD25⁻ Foxp3⁻ T cells. **C**, Analysis of adenosine (ADO) in culture supernatants. The chromatogram shows overlay of one typical experiment. Lane 1, Treg cells; lane 2, Treg cells cultured with 1.25 mmol/L ATP; lane 3, spiking sample no. 2 with 200 μ mol/L adenosine. **D**, Adenosine measured in the tissue culture supernatant of differently treated Treg cells.

were injected 15 minutes before antigen challenge. In contrast, Treg cells derived from CD39^{-/-} mice did not suppress adherence of effector T cells to vascular endothelium *in vivo*, thus indicating that Treg cell–derived adenosine generated through the CD39 ectonucleotidase pathway is essential for mediating the suppressive effects on effector T-cell–endothelium interaction.

Treg cells and adenosine downregulate E- and P-selectin on the surface of ECs

Our data indicate that Treg cell–derived adenosine targets the vascular endothelium, leading to downregulation of molecules

crucial for effector T-cell adherence and diapedesis into the inflamed tissues. ECs were stimulated with TNF- α in the presence of either adenosine or preactivated Treg cells and analyzed for adhesion molecule expression by means of flow cytometry to investigate possible targets among adhesion molecules. Among the molecules investigated, we identified significant effects on E- and P-selectin expression (Fig 5, A and B) in which addition of either Treg cells or adenosine blocked both E- and P-selectin up-regulation in TNF- α –stimulated ECs. Thus these data indicate that Treg cell–derived adenosine downregulates E- and P-selectin on endothelial surfaces, consequently leading to abrogation of adherence and extravasation of effector T cells.

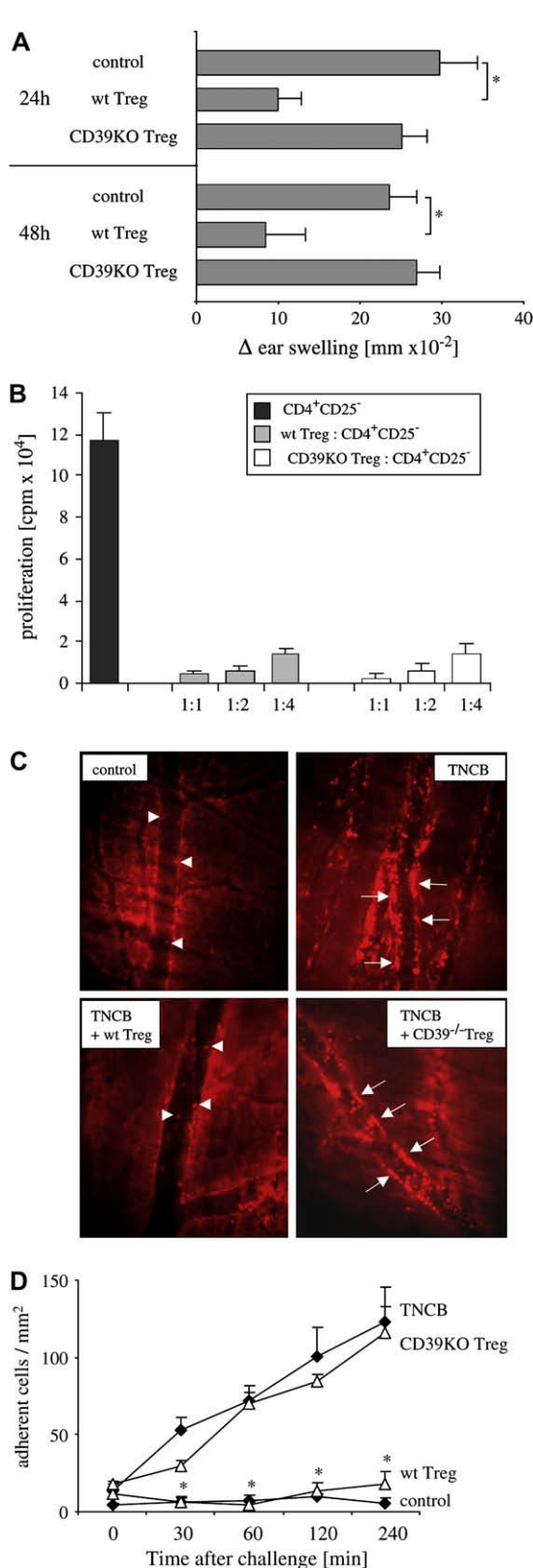


FIG 4. Expression of CD39⁺ on Treg cells is essential for the *in vivo* suppression of CHS. **A**, Mean ear swelling $\pm$ SD of 2 independent experiments ($n = 5$, $*P < .005$). **B**, Suppressive activity of WT Treg cells and CD39 knock-out (CD39KO) Treg cells *in vitro*. Proliferation was measured by means of tritiated thymidine incorporation. Shown is the mean proliferation in triplicates $\pm$ SD of one characteristic experiment of 3. **C**, Leukocyte-endothelium

Treg cells and adenosine suppress E- and P-selectin expression on ECs *in vivo*

To verify that Treg cells affect modulation of adhesion molecules *in vivo*, we first prepared single-cell suspensions of ears from WT mice that had been sensitized and challenged with or without prior injection of Treg cells or adenosine. Flow cytometric analysis of cell-surface marker expression revealed upregulation of E- and P-selectin on CD31⁺ ECs in the ears of challenged mice compared with that seen in unchallenged control mice (Fig 6, A). However, upregulation of E- and P-selectin was attenuated in WT mice receiving Treg cells or adenosine before challenge. Similar results were apparent when analyzing surface marker expression by using immunofluorescence staining (Fig 6, B). Here also the downregulation of E- and P-selectin expression was detectable in mice injected with Treg cells or adenosine compared with that seen in uninjected control animals.

DISCUSSION

In this study we found that Treg cell-derived adenosine plays a major role in preventing the elicitation of CHS reactions by blocking the interaction of effector T cells with the vascular endothelium. The well-documented anti-inflammatory effects of adenosine have earned it the description of "retaliatory metabolite,"¹⁴ with reported suppressive effects, including direct inhibition of colitis and ileitis,^{15,16} amelioration of type I diabetes,¹⁷ stimulation of IL-10 release by macrophages,¹⁸ and blockade of T-cell activation.¹⁹ Moreover, numerous studies have demonstrated that the anti-inflammatory effects of methotrexate, a commonly used and effective agent for the treatment of rheumatoid arthritis, are mediated by extracellular adenosine production.^{20,21} Conversely, eliminating adenosine-mediated suppression blocked the anti-inflammatory effects of methotrexate in experimental arthritis and led to increased antitumor immunity in a murine lymphoma model.^{21,22}

Although adenosine can be produced intracellularly and released through gap junctions or specialized transporters,²³ an equally efficient mechanism of adenosine production is the extracellular conversion of adenine nucleotides to adenosine by ectonucleotidases. In this pathway ATP is first degraded by CD39 to adenosine diphosphate and then to AMP, which is then dephosphorylated by CD73 to adenosine. Conventional T cells have only a low basal expression of CD39,²⁴ which is in stark contrast to our demonstration that Treg cells are strongly positive for cell-surface expression of CD39 and CD73. Moreover, Treg cells further upregulated CD39 expression after activation, and this activation is a prerequisite for Treg cells to acquire their suppressive capacity. Thus this functional connection of acquiring immunosuppressive functions and augmented expression of CD39 further strengthens the role of adenosine as a soluble mediator of suppression conveyed by Treg cells. Other soluble factors, such as IL-10 and TGF- β , are also released by activated Treg cells and predominantly mediate suppression *in vivo*. Treg cell-derived IL-10, in particular, is crucial to (1) prevent colitis,⁴ (2) prevent allergic reactions in the lung and skin,^{9,25} and (3) prevent the

interaction 30 minutes after challenge. Arrows indicate examples of leukocytes attached to the vessel wall. Arrowheads indicate free-flowing leukocytes and vessel walls devoid of attached leukocytes. **D**, Amount of adherent cells over a period of 4 hours after challenge (mean $\pm$ SD, $n = 3$, $*P < .005$).

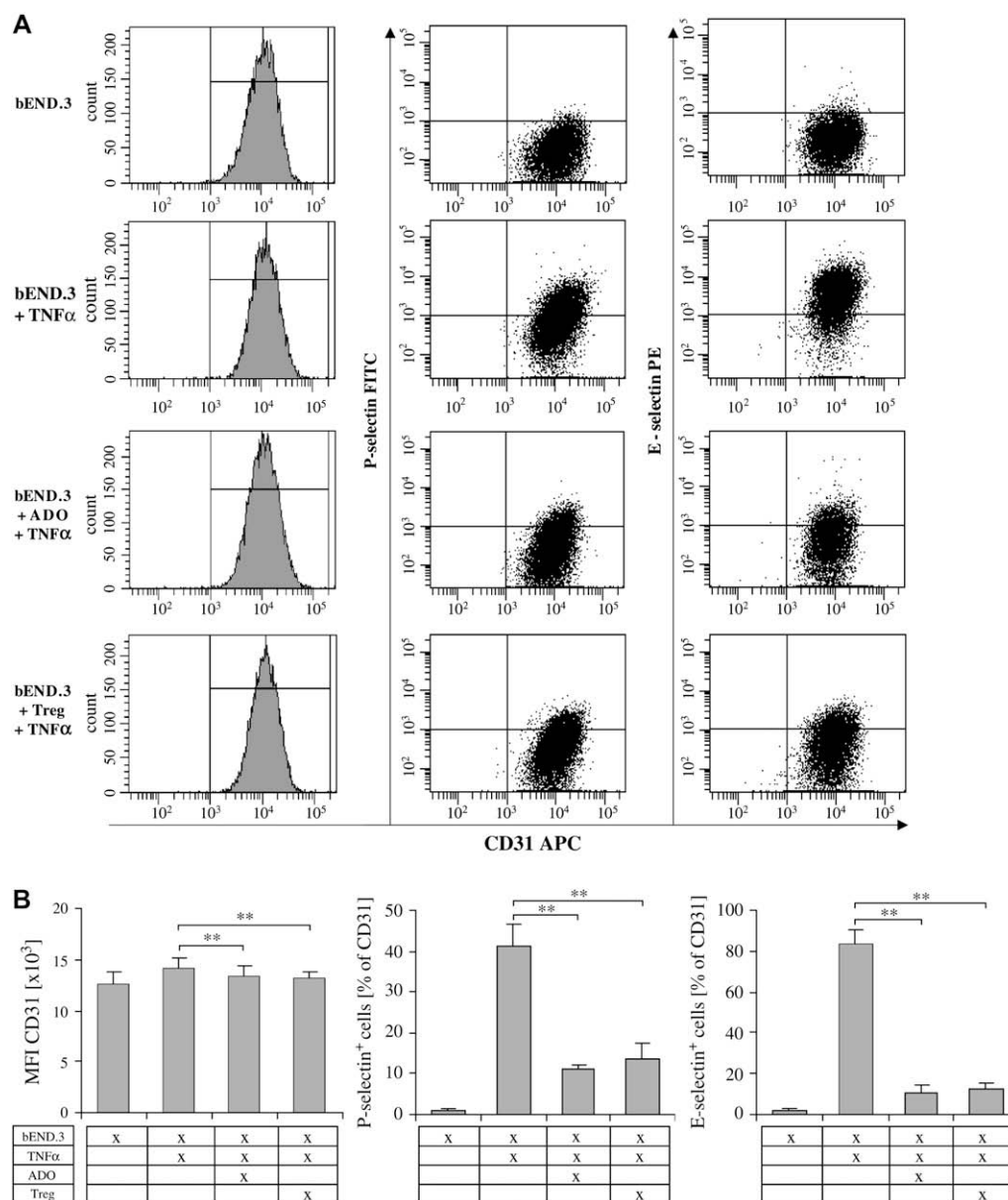


FIG 5. Prevention of upregulation of adhesion molecules on ECs by preactivated Treg cells and adenosine (ADO) *in vitro*. **A**, Expression of CD31 and the adhesion molecules P- and E-selectin were detected by means of flow cytometry. FITC, Fluorescein isothiocyanate; APC, allophycocyanin. **B**, Mean fluorescence intensity (MFI) of CD31 and percentages of P- and E-selectin in percentages of CD31⁺ cells $\pm$ SD (n = 5, **P < .001).

effective clearance of pathogens from the body.^{26,27} IL-10 and adenosine might also interact with each other. For example, it has been shown that adenosine stimulates the release of IL-10 by macrophages.¹⁷ Conversely, our own preliminary results indicate (data not shown) that IL-10 augments adenosine production by Treg cells. Therefore both adenosine and IL-10 might act synergistically to augment the suppressive effects of Treg cells by different means.

In the CD39/CD73 ectonucleotidase cascade, ATP is the crucial substrate for adenosine production, and therefore access of Treg cells to ATP at inflamed tissue sites is mandatory. Indeed, ATP is released during inflammation, and it is considered a sign for “dangerous” invasion.²⁸ The resulting increased levels of ATP and adenine nucleotides present in inflamed tissues can thus serve

as substrates for Treg cell-associated ectonucleotidases. ATP has both direct Treg cell-activating capacities²⁴ and proinflammatory activities, including stimulation of IL-1 β release by ECs²⁹ and maturation induction of dendritic cells,³⁰ which are critical for stimulating immune responses.

In aggregate, the Treg cell–ATP–adenosine pathway might be crucial for the downregulation of inflammation through several means. First, ATP activates Treg cells at the site of inflammation. Second, ATP provides the substrate for the production of Treg cell–derived immunosuppressive adenosine. Third, CD39/CD73-expressing Treg cells degrade and clear the proinflammatory agent ATP from the site of inflammation. In addition to Treg cells, other cells residing in the skin might also contribute to the adenosine generation. For example, it has been shown that

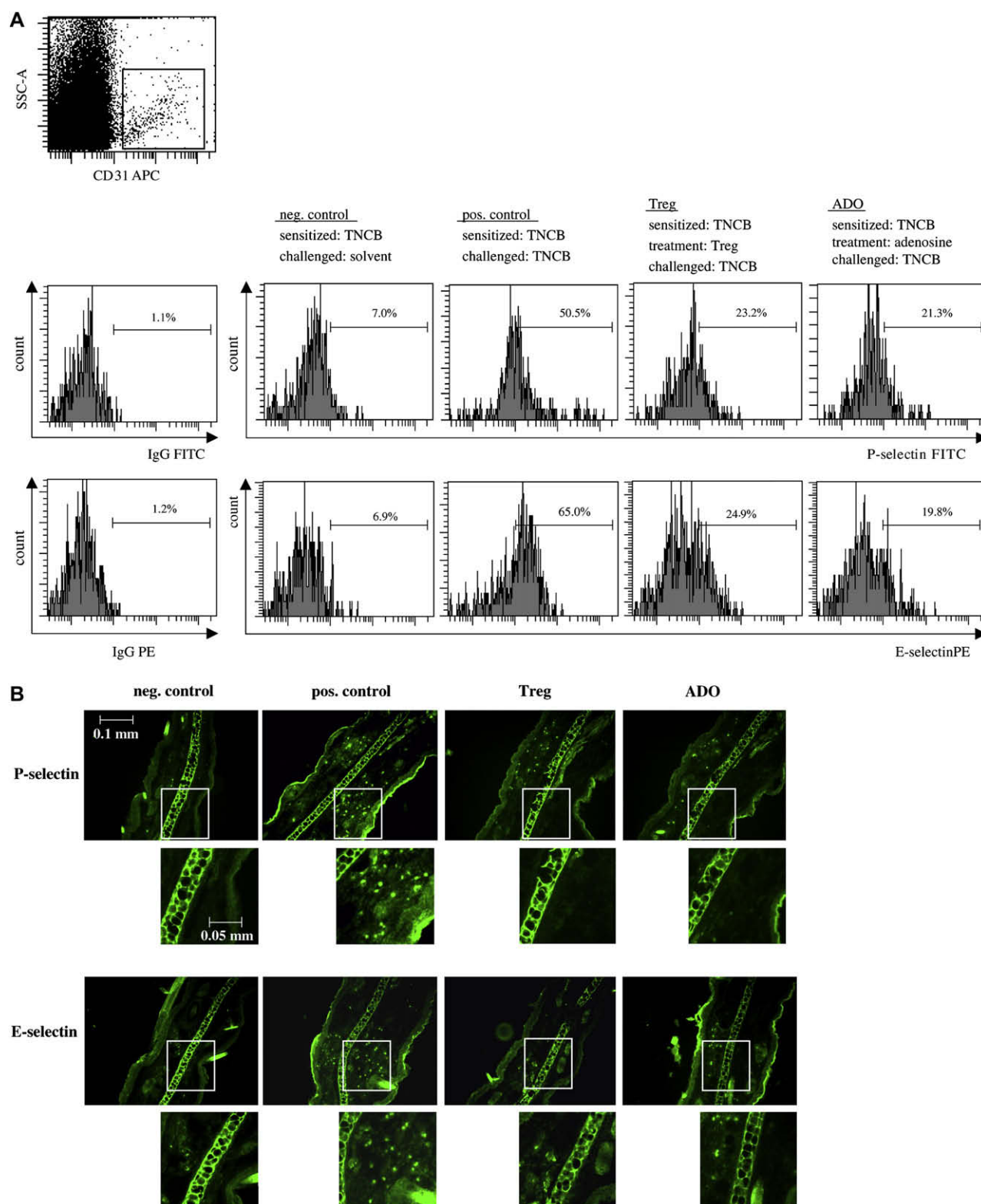


FIG 6. Inflammation-induced upregulation of adhesion molecules is abrogated by Treg cell-derived adenosine (ADO). **A**, Expression of P-selectin and E-selectin in percentages of the CD31⁺ ECs in the ears of differently treated mice analyzed by means of flow cytometry. *SSC-A*, sideways scatter; *APC*, allophycocyanin; *FITC*, fluorescein isothiocyanate; *PE*, phycoerythrin. **B**, Cryosections of the ears from the same experimental groups depicted in Fig 6, A, were stained with P- or E-selectin-specific antibodies and appropriate fluorescein isothiocyanate-labeled secondary reagents. *Inserts* show high-power magnifications.

Langerhans cells and ECs³¹ also express CD39, and keratinocytes sequester ATP in response to skin irritants.³² Thus Treg cells, together with other tissue-resident cells, might produce sufficient adenosine to prevent exacerbated tissue damage during ongoing immune reactions.

In our study we have identified the vascular endothelium as a possible *in vivo* target of the Treg cell–derived adenosine. However, other mechanisms after initial adhesion, such as leukocyte activation or their transendothelial migration, might also be influenced by adenosine. During inflammation, the endothelium acts as a gatekeeper for inflammatory cells. In this process adenosine has a substantial effect because it reduces vascular leakage,^{33,34} downregulates several adhesion molecules on human ECs,³⁵ blocks β_2 -integrin and very late antigen 4–mediated adhesion of neutrophils to vascular endothelium,^{36,37} and prevents the $\alpha 4\beta 1$ –vascular cell adhesion molecule interaction of monocytes with endothelium.³⁵ In line with our notion that IL-10 and adenosine release might interconnect, it has been shown that IL-10 downregulates adhesion of monocytes³⁸ and T lymphocytes to ECs that have been activated with LPS.³⁹ Therefore adenosine and IL-10 might have synergistic effects on the modulation of EC expression of adhesion molecules during infection. Even under physiologic conditions, adenosine plays a role in maintaining the unobstructed circulation of blood cells. This has been demonstrated by genetic disruption of the CD73 ectonucleotidase, which resulted in decreased adenosine production and led to increased leukocyte adherence to the vascular endothelium.⁴⁰ Thus adenosine-mediated effects on leukocyte adhesion to endothelium are established in several experimental settings, and our results indicate that Treg cells substantially contribute to increased adenosine levels in the vasculature.

Our data indicate that the presence of Treg cells in the affected skin is not required to suppress the ear-swelling response. This is in line with other observations showing that Treg cells are able to suppress colitis⁴¹ or allograft rejection,⁴² although they were unable to accumulate in the gut, in the graft, or both, respectively. However, others report that Treg cells can be primed to express organ-selective homing receptors, such as E- and P-selectin ligand, for skin homing and $\alpha 4\beta 7$ integrin for gut tropism.⁴³ These Treg cells were very efficient in entering the skin and gut, respectively, but their ability to suppress delayed-type hypersensitivity reactions in the skin or colitis in the gut was not strictly correlated with the expression of the respective skin- or gut-homing receptors.⁴³ More recently, in the model of scurfy mice, it was shown that skin-homing Treg cells are mandatory for maintenance of long-term tolerance in the skin, with animals devoid of Treg cells in the skin having spontaneous cutaneous inflammation over a period of 3 weeks.⁴⁴ Thus these data might indicate that homing of Treg cells to peripheral tissues is required for maintenance of the basal levels of suppression required for the upkeep of peripheral tolerance, and the production of soluble factors, such as adenosine, is an immediate response to infection and inflammation.

On the whole, our data corroborate recent findings showing that Treg cells are producers of adenosine and yet add another soluble factor produced by Treg cells to their arsenal of inhibitory mechanisms. This is in line with a recently developed concept of “metabolic disruption.”¹ Here mechanisms involving IL-2 consumption, transfer of cyclic AMP, and generation of pericellular adenosine are described. The functional advantages of such suppressive means are obvious because the use of soluble factors relieves Treg cells from the necessity to enter inflamed tissue sites

or lymph nodes to curb T-cell proliferation. Moreover, by having several redundant mechanisms at hand, Treg cells might be able to “select” an appropriate suppressor mechanism for targeted suppression of effector T cells. Finally, a full arsenal of different inhibitory mechanisms might be required to ensure that a small population of Treg cells can adequately curb a large pool of effector T cells at different sites of the body.

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Clinical implications: Modification of the adenosine turnover by Treg cells caused by specific drugs might provide a tool to augment Treg cell–mediated immune suppression during allergic and autoimmune diseases.

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METHODS

Mice

C57BL/6 mice purchased from Charles River were housed at the central animal facility of the University of Heidelberg and cared for in accordance with the institutional guidelines for animal welfare. CD39^{-/-} mice were housed in the animal facility of New York University, according to the respective institutional guidelines.

Detection of the interaction between effector cells and ECs under shear-stress conditions

The murine EC line (bEND.3, ATCC) was cultured on collagen-coated coverslips and stimulated with TNF- α (1 μ g/mL medium) alone or together with adenosine (10 μ mol/L) or Treg cells (5×10^5 /mL medium). ECs were left unstimulated as a control. ECs were washed after 4 hours, placed into a heated flow chamber (Chromaphor, Ascheberg, Germany), and superfused with fluorescent dye-labeled, activated, effector CD4⁺ T cells. Video microscopy was used to determine the number of rolling and adherent effector T cells.

Skinfold chamber

For intravital microscopy, we used the dorsal skinfold chamber preparation, as described previously.^{E1} Mice were sensitized with 15 μ L of 1%

TNCB on day 0, and 5 days later, the skinfold chambers were implanted. After 24 hours, the mice were treated directly on the skinfold chamber with either vehicle only, with 10 μ L of 0.1% TNCB dissolved in acetone/olive oil (1:4), with 3×10^6 Treg cells followed by TNCB application, or with 1.25 mg adenosine/kg body weight 15 minutes before challenge. Mice were injected with Rhodamine 6G (Sigma-Aldrich) to visualize endogenous leukocytes. When mice had recovered from anesthesia, intravital microscopic images were recorded with the high-resolution black-and-white camera Spot RT KE Monochrome w/o IR Filter (Visitron) and analyzed with Spot software. Blood flow was analyzed immediately thereafter for 1 hour by means of fluorescence video microscopy. Shown is a video sequence depicting leukocyte-endothelium interactions in the vessels 30 minutes after challenge.

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VIDEO E1. Effector T cells do not adhere to nonactivated ECs *in vitro*. Shown are the rolling and adherent red-labeled CD4⁺ T cells 30 minutes after starting a constant flow.

VIDEO E2. Effector T cells adhere to TNF- α -activated ECs *in vitro*. Shown are the rolling and adherent red-labeled CD4⁺ T cells 30 minutes after starting a constant flow.

VIDEO E3. Adenosine blocks adherence of effector T cells to TNF- α -activated ECs *in vitro*. Shown are the rolling and adherent red-labeled CD4⁺ T cells 30 minutes after starting a constant flow.

VIDEO E4. Treg cells block adherence of effector T cells to TNF- α -activated ECs *in vitro*. Shown are the rolling and adherent red-labeled CD4⁺ T cells 30 minutes after starting a constant flow.

VIDEO E5. Leukocytes do not adhere to the endothelium after application of solvent. Shown is a video sequence depicting leukocyte-endothelium interactions in the vessels 30 minutes after challenge.

VIDEO E6. Leukocytes adhere to the endothelium after challenging with TNCB. Shown is a video sequence depicting leukocyte-endothelium interactions in the vessels 30 minutes after challenge.

VIDEO E7. Injection of WT Treg cells prevents adherence of leukocytes to the endothelium after challenge with TNCB. Shown is a video sequence depicting leukocyte-endothelium interactions in the vessels 30 minutes after challenge.

VIDEO E8. Injection of CD39^{-/-} Treg cells does not suppress adherence of leukocytes to the endothelium after challenge with TNCB. Shown is a video sequence depicting leukocyte-endothelium interactions in the vessels 30 minutes after challenge.