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ATP Activates Regulatory T Cells In Vivo during Contact Hypersensitivity Reactions

Sabine Ring, Alexander H. Enk, and Karsten Mahnke

CD4⁺CD25⁺Foxp3⁺ regulatory T cells (Tregs) require activation to develop their full suppressive capacity. Similar to conventional T cells, Tregs can be activated via their TCRs; however, other means may be in place. We injected naive and nonactivated Tregs, being CD69[−]CD44^{low}CD62L⁺ into mice, and analyzed their phenotype after sensitization or challenge with the contact sensitizer 2,4,6-trinitro-1-chlorobenzene. We found that Tregs acquired an activated phenotype (CD69⁺CD44^{high}CD62L[−]) in the draining lymph node after sensitization. In contrast, Ag challenge activated Tregs in the blood. This tissue-specific activation was induced by ATP, which was released at the respective tissue sites after sensitization or challenge, respectively. To demonstrate that activation was also essential for the induction of the suppressive function of Tregs, Tregs were treated with ATP receptor antagonists. In this study, we show that ATP receptor antagonists abrogated the suppressive effects of injected naive Tregs in contact hypersensitivity reactions. Thus, these data indicate that activation of Tregs via ATP in vivo provides a novel pathway of stimulating the suppressive function of Tregs. *The Journal of Immunology*, 2010, 184: 000–000.

Naturally occurring CD4⁺CD25⁺Foxp3⁺ regulatory T cells (Tregs) are able to suppress proliferation of effector T cells and are essential for the maintenance of peripheral tolerance. Their regulatory function has been investigated during autoimmunity, tumor growth, and graft-versus-host disease; however, the underlying mechanisms of action are still not completely understood (1). For instance, several in vitro studies demonstrate the necessity of cell-to-cell contact of Tregs and effector T cells to convey suppressive action (2, 3). In contrast, under in vivo conditions, soluble factors seem to play a role, as release of IL-10 and IL-35 and secretion of TGF- β are crucially attributed to the suppressive effects (4–6).

Irrespective of the mode of action, it has been shown that Tregs require activation to act suppressive. In in vitro suppression assays, the activation is accomplished by cultivating Tregs with APCs together with anti-CD3 Abs or by incubating Tregs with a mixture of anti-CD3 and anti-CD28 Abs, respectively. These experimental settings mimic engagement of the TCR together with activation of coreceptors and trigger full activation of Tregs. Likewise in vivo, MHC–peptide complexes presented by professional APCs engage the TCR and activate Tregs in vivo. Because the TCR is specific for defined MHC–peptide complexes, this mechanism would imply that primarily Tregs, which express the matching Ag-specific TCRs become activated by APCs.

However, this pathway may not be exclusive, as previous results have shown that injection of freshly isolated and thus nonactivated Tregs into 2,4,6-trinitro-1-chlorobenzene (TNCB)-sensitized mice suppressed the ear-swelling reaction, which was induced after reapplication (challenge phase) of the Ag (7, 8). These data suggest that polyclonal, nonactivated Tregs become activated in vivo after application of contact sensitizers like TNCB.

To investigate the underlying mechanisms of the activation of Tregs, we first activated Tregs in vitro by anti-CD3/anti-CD28 Abs and showed upregulation of CD69, CD44, and CD73, indicating activation of the Tregs. Similar results were obtained in vivo. After injection of naive, dye-labeled Tregs into sensitized mice, we assessed the activation status of the labeled Tregs after sensitization or challenge. In this study, we show that CD69 and CD44 were upregulated, and simultaneously, CD62L was downregulated, indicating activation of Tregs. As a possible means of this activation, we identified ATP. ATP acts as a potent activator of Tregs and is produced at the tissue sites upon sensitization or challenge. Notably, blocking of ATP receptor(s) on Tregs abrogated their suppressive function in vivo. Thus, our data indicate that naive Tregs become activated in vivo during contact hypersensitivity (CHS) responses via ATP, which is released at the sites of hapten application.

Materials and Methods

Mice

BALB/c mice were purchased from Charles River Laboratories (Sulzfeld, Germany) and were housed at the central animal facility of the University of Heidelberg (Heidelberg, Germany). All experiments were performed in accordance with the governmental guidelines.

Reagents and Abs

Isolation kits for CD4⁺ T cell and Tregs were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany); anti-CD3 (145-2C11), anti-CD28 (37.51), and all fluorescent-labeled mAb for flow cytometry, except CD39, were obtained from BD Biosciences (Heidelberg, Germany); and PE-Cy7-conjugated anti-mouse CD39 were from eBioscience (San Diego, CA). PKH26 red fluorescent cell linker kit, TNCB, ATP disodium salt (ATP), and pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid) tetrasodium salt hydrate (PPADS) were purchased from Sigma-Aldrich (Taufkirchen, Germany); Luminescence ATP Detection Assay system (ATPlite) was from PerkinElmer (Waltham, MA). Cell culture medium consisted of RPMI 1640 supplemented with 10% heat-inactivated FCS (both PAA Laboratories, Cölbe, Germany).

Department of Dermatology, University Hospital Heidelberg, Heidelberg, Germany
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Address correspondence and reprint requests to Dr. Sabine Ring, Department of Dermatology, University of Heidelberg, Voßstrasse 11, 69115 Heidelberg, Germany. E-mail address: sabine.ring@med.uni-heidelberg.de

The online version of this article contains supplemental material.

Abbreviations used in this paper: CHS, contact hypersensitivity; dLN, draining lymph node; LN, lymph node; MFI, mean fluorescence intensity; ndLN, nondraining lymph node; PPADS, pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid) tetrasodium salt hydrate; TNCB, 2,4,6-trinitro-1-chlorobenzene; Treg, CD4⁺CD25⁺Foxp3⁺ regulatory T cell.

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Purification of T cell subsets and cell labeling

CD4⁺T cell subpopulations were isolated from pooled lymph node (LN) and spleens of naive mice using the "CD4 untouched" magnetic bead separation kit according to the manufacturer's protocol. To obtain CD4⁺CD25⁺ Tregs, the CD4⁺ T cells were stained with anti-CD25 PE mAb, followed by incubation with anti-PE MicroBeads and passed through separation columns placed in a magnetic field. After removal from the magnetic field, the CD25⁺ cells were eluted from the column and analyzed by FACS.

The purity of the isolated cell populations ranged from 90 to 95%. The suppressive capacity of the isolated Tregs was always assessed by standard suppression assays.

Freshly isolated Tregs were labeled with the fluorescent dye PKH26, which inserts into the cell membrane. Therefore, 2×10^7 serum-free cells were resuspended in 1 ml diluent C before adding 1 ml freshly prepared PKH26 (4 μ M). After 3 min of incubation at room temperature, the reaction was stopped by adding 2 ml FCS for 1 min. Thereafter, 4 ml medium was added, and the labeled cells were collected by centrifugation ($400 \times g$, 10 min, 25°C).

In vitro activation of cells

A total of 2×10^5 CD4⁺CD25⁺ T cells or Tregs were cultured in 200 μ l/well in 96-well round-bottom plates and stimulated either with anti-CD3 and anti-CD28 (0.5 μ g/ml each) or with different amounts of ATP (25, 75, 125, and 200 μ M). Activation statuses of the cells were analyzed after 24 h by flow cytometry.

Proliferation assays

Function of ATP preactivated Tregs: CD4⁺CD25⁺ T cells and Tregs were stimulated separately with either anti-CD3 and anti-CD28 (0.5 μ g/ml each) or ATP (25, 75, 125, and 200 μ M) for 24 h. Cells were washed thoroughly and cocultured in different ratios without further stimulation. After 24 h, 0.5 μ Ci/ml [³H]thymidine (Amersham Biosciences, Frankfurt, Germany) was added for 18 h, cells were harvested, and the thymidine incorporation was determined using a PerkinElmer scintillation counter.

Function of anti-CD3/anti-CD28-activated Tregs: CD4⁺CD25⁺ T cells and Tregs or Tregs 2 h preincubated with 20 μ M PPADS, respectively, were cocultured in 96-well round-bottom plate (1×10^6 cells/ml) in ratios

as indicated and stimulated with anti-CD3 and anti-CD28 (0.5 μ g/ml each). After 2–3 d, 0.5 μ Ci/ml [³H]thymidine was added for 18 h, cells were harvested, and the thymidine incorporation was determined.

Contact hypersensitivity

Mice were sensitized by painting 15 μ l of 1% TNBC dissolved in acetone/olive oil (4:1) or 15 μ l solvent only on the shaved abdomen on day 0. On day 5, blank values were determined by measuring the ear thickness using a caliper rule (Oditest, Kroepelin, Schlüchtern, Germany). Afterward, mice were challenged by epicutaneously application of 0.5% TNBC solution, 10 μ l on each site of the right ear. The left ear was treated with the same amounts of solvent only. After 24 h, the ear thickness was measured again and difference calculated as: Δ ear response (in mm) $\times 10^{-2} \pm$ SD.

A total of 3×10^6 PKH-PE-labeled Tregs, isolated from LNs and spleens of naive mice, were injected either 2 h before sensitization or 15 min before challenge of the mice. For the ATP receptor blocking experiments, Tregs were preincubated with 20 μ M PPADS for 2 h before injection.

Mice were sacrificed 24 h after Treg injection, and activation statuses of the Tregs were analyzed in the cell suspension of different organs by flow cytometry.

Detection of ATP in different organs

Mice were sensitized or sensitized and challenged, control mice were left untreated. Cell suspensions were prepared from non-draining (nd) LNs, draining (d)LNs, spleens, and ear tissue, and additionally, serum was taken 24 h after the respective treatment, and the amount of ATP was detected with the ATPlite luminescence assay system. Briefly, cells were cultured in a black 96-well culture plate (2×10^5 cells/well; 100 μ l serum/well), D-luciferin and luciferase were added and produced a luminescent signal, caused by the reaction of ATP with D-luciferin and luciferase, which was measured in the MicroBeta TriLux from PerkinElmer.

Results

Tregs constitutively express ectonucleotidases CD39 and CD73 become activated by ATP in vitro

Recent results show that Tregs produce immunosuppressive adenosine via the ectonucleotidases CD39 and CD73 (8–11). Therefore,

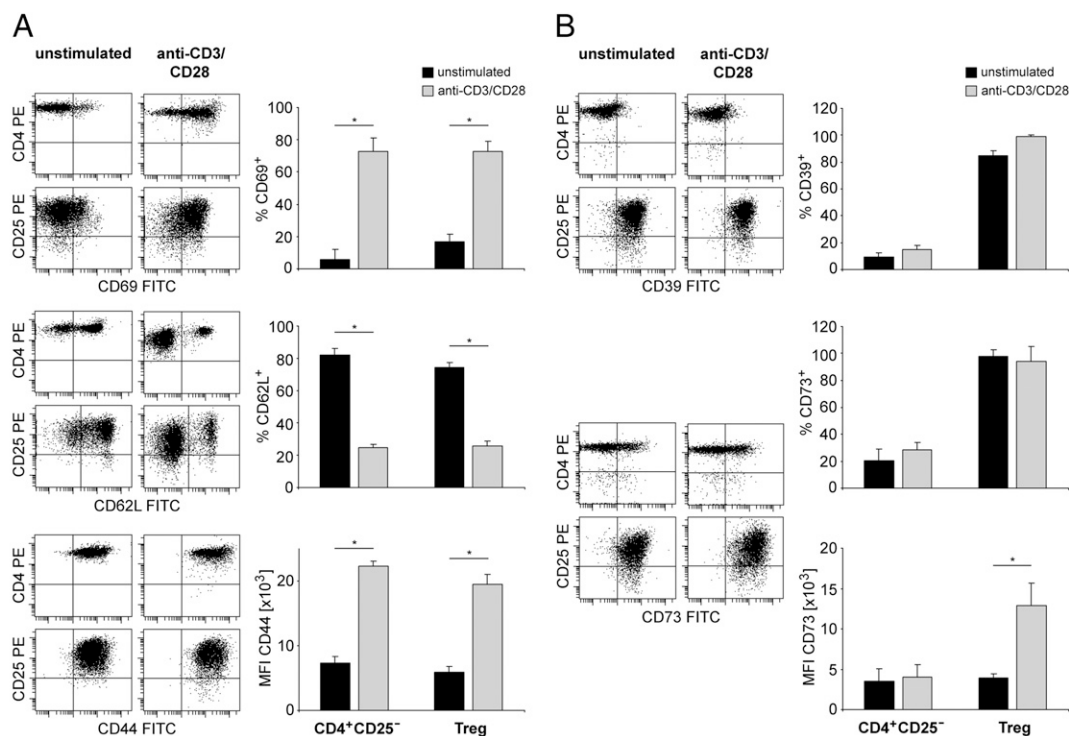


FIGURE 1. Upregulation of ectonucleotidases on activated Tregs. *A*, Tregs and CD4⁺CD25⁺ T cells were isolated from LN and spleens of naive mice and stimulated with anti-CD3 and anti-CD28 (0.5 μ g/ml each) or left untreated (unstimulated). Different activation markers were analyzed by flow cytometry after 24 h of stimulation. *B*, Expression of ectonucleotidases CD39 and CD73 on Tregs and on CD4⁺CD25⁺ T cells treated like described in *A*. Data show the means $\pm$ SD from five independent experiments. Significant differences ($p < 0.001$) between unstimulated and anti-CD3/CD28-treated cells were determined by Student *t* test.

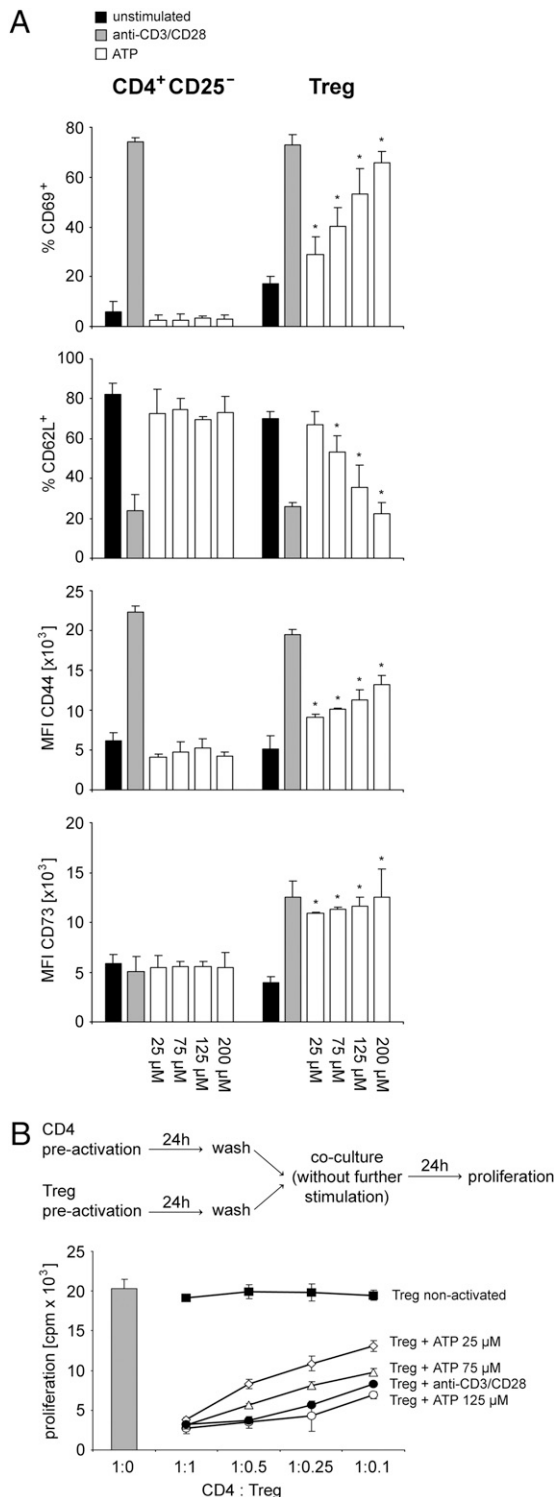


FIGURE 2. ATP activates Treg in vitro. **A**, CD4⁺CD25⁻ T cells and Tregs were isolated from LN and spleens of naive mice and stimulated with anti-CD3 and anti-CD28 (0.5 μ g/ml each), ATP (25, 75, 125, and 200 μ M), or left untreated. Different activation markers and MFI of ectonucleotidase CD73 were analyzed by flow cytometry after 24 h of culture. Data show the mean of percentage of positive cells or of the MFI, respectively, $\pm$ SD of three independent experiments. Significant differences ($*p < 0.05$) between unstimulated and ATP-treated cells were determined by Student *t* test. **B**, Isolated CD4⁺CD25⁻ T cells were stimulated with anti-CD3 and anti-CD28 (0.5 μ g/ml each), and Tregs were treated with anti-CD3 and anti-CD28 (0.5 μ g/ml each), ATP (25, 75, 125, and 200 μ M), or left untreated (non-activated). After 24 h in culture, both cell populations were washed, and CD4⁺CD25⁻ T cells were cocultured with the differently treated Tregs in the

we set out to investigate whether activation of Tregs regulates surface expression of these enzymes, which are crucial for adenosine production. We cultured isolated Tregs and conventional CD4⁺CD25⁻ T cells overnight with anti-CD3/anti-CD28 Abs and show that activation markers, such as CD69 and CD44, were upregulated on both cell types. Likewise, CD62L expression was reduced, indicating successful activation of the T cells (Fig. 1A).

Expression of the ectonucleotidases CD39 as well as CD73 was not upregulated on conventional CD4⁺CD25⁻ T cells upon activation and remained low, with approximately only 20% of the cells being positive for CD39 and CD73 (Fig. 1B). In contrast, $\sim 100\%$ of the Tregs stably expressed both markers, irrespective of whether the cells were activated with Abs or left untreated. However, the mean fluorescence intensity (MFI) of CD73 significantly increased after activation indicating an upregulation of this molecule, whereas the mean fluorescence of CD39 remained stable (data not shown).

Because ATP serves as substrate for adenosine production via the CD39/CD73 cascade, we next asked whether ATP itself exerts stimulatory activity on Tregs. Therefore, we cultured Tregs and conventional T cells overnight with graded doses of ATP and determined the surface expression of respective activation markers. As shown in Fig. 2A, Tregs became activated by ATP in a dose-dependent manner, as indicated by the upregulation of CD69 and CD44 and downregulation of CD62L. Of note, expression of CD73 was very sensitive to ATP as 25 μ M, which was not able to substantially increase CD69 expression, was readily able to induce profound CD73 expression (Fig. 2A, Supplemental Fig. 1). However, similar to results obtained with anti-CD3/anti-CD28 stimulation, CD39 was unaffected by ATP stimulation (data not shown). Similar results (i.e., the upregulation of CD69, CD44 and the downregulation of CD62L) were obtained with TGF- β -induced Tregs (Supplemental Fig. 2). In contrast to Tregs, conventional T cells were not activated by ATP, as indicated by unchanged surface expression of respective activation markers (Fig. 2A, Supplemental Fig. 1).

To further investigate whether ATP activation also results in increased suppressive capacity of Tregs, we established a novel suppression assay in which we cocultured anti-CD3/anti-CD28-preactivated responder T cells with differently pretreated Tregs. This assay is devoid of any T cell stimulatory Abs or APCs during the coculture and thus enables us to compare the suppressive capacity of unstimulated with preactivated Tregs. We show (Fig. 2B) that Tregs activated with 125 μ M ATP display similar suppressive activity as compared with anti-CD3/anti-CD28-activated Tregs. This suppressive capacity declined when lower concentrations of ATP (25 μ M) were used for activation. In contrast, nonactivated Tregs did not suppress the proliferation of prestimulated CD4⁺CD25⁻ responder T cells. Thus, these data indicate that ATP activates Tregs and stimulates their suppressive function in vitro.

Tregs become activated in vivo at different tissue sites during sensitization and challenge

Several therapeutic procedures in animal models use injection of freshly prepared Tregs for the treatment of medical conditions. That raises the question by which means the naive Tregs become activated in vivo.

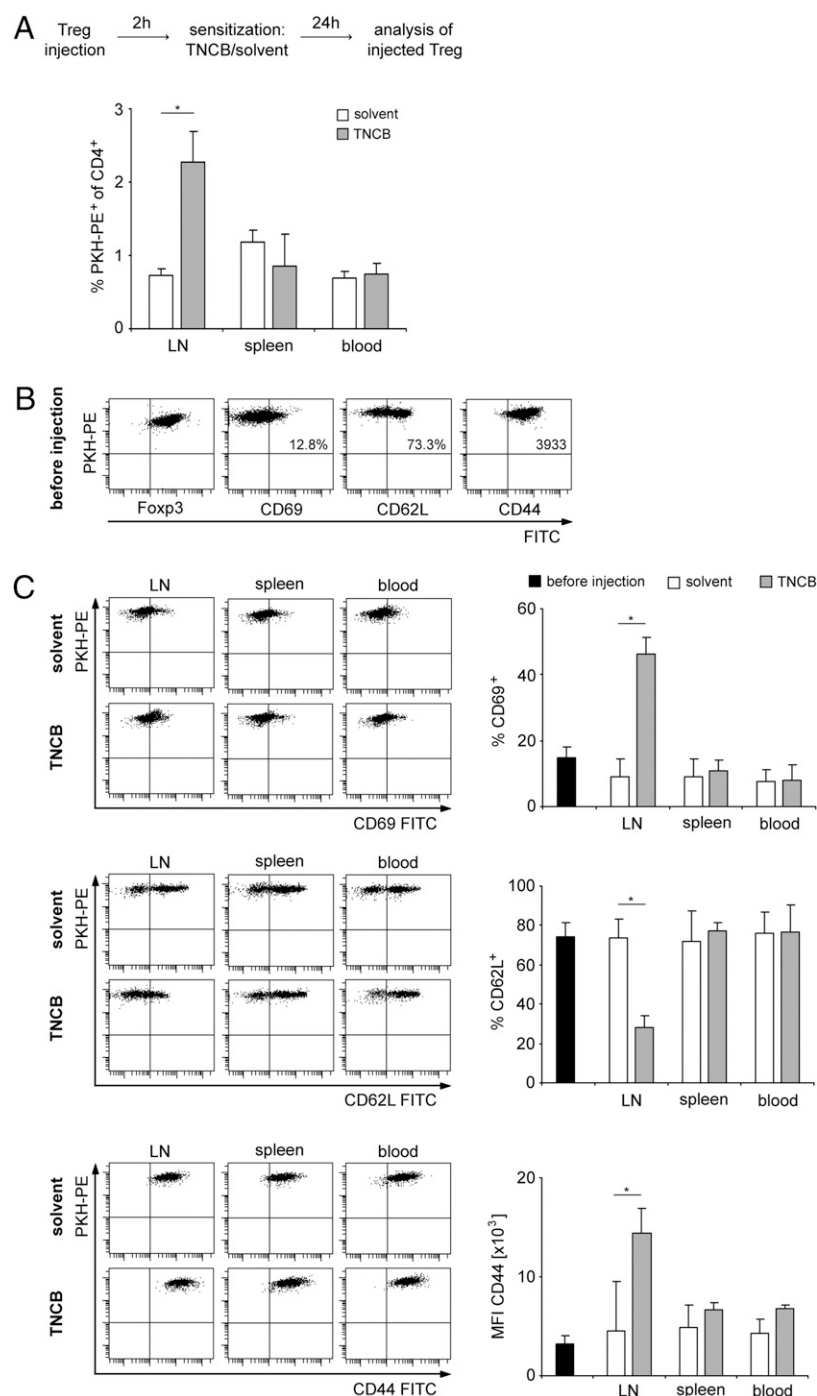
stated ratios. Proliferation was measured by [³H]thymidine incorporation. Data show the mean proliferation of CD4⁺CD25⁻ T cells $\pm$ SD of three independent experiments. Significant differences ($p < 0.05$) between the proliferation of CD4⁺CD25⁻ T cells cocultured with nonactivated Tregs and CD4⁺CD25⁻ T cells cocultured with Tregs pretreated with anti-CD3/CD28 or ATP were obtained in all dilutions by Student *t* test.

To first investigate the site of activation of Tregs in vivo during both phases of CHS reactions, we determined the homing of freshly prepared and dye-labeled Tregs during the sensitization phase as well as during the elicitation phase of CHS reactions. In this study, we show (Fig. 3A) that following sensitization of naive animals injected Tregs mainly accumulated in the dLN, whereas in the spleen and the blood, only reduced numbers of dye-labeled Tregs were detectable. Notably, when the mice were treated with solvent only, the preferential homing of Tregs to dLNs was abolished. Thus, these data indicate that hapten application drives Treg migration to dLNs.

Analyzing the activation status of the Tregs before and after injection by measuring the expression of respective markers CD69, CD62L, and CD44, we detected that directly after isolation from

naive mice, only 12.8% of the Tregs expressed CD69 (Fig. 3B). Therefore, the cells can be considered as “nonactivated.” These cells were injected into mice, which were subsequently treated with hapten or with solvent only. After 24 h, blood, spleen, and dLN were analyzed for presence of dye-labeled Tregs, and their activation status was reassessed by flow cytometry. As shown in Fig. 3C, the number of activated, CD69⁺ Tregs was greatly enhanced in the dLN of sensitized mice. Concomitantly, expression of CD62L was downregulated, and the MFI of CD44 was increased (Fig. 3C), indicating an activated phenotype of Tregs. In contrast, Tregs detected in spleens or blood displayed no signs of activation. Likewise, Tregs in “solvent only”-treated mice showed no signs of activation in each of the organs investigated and displayed similar activation levels as compared with “before”

FIGURE 3. During the sensitization phase of CHS reactions, Tregs get activated in the dLN. Tregs were isolated from LN and spleen of naive mice, labeled with PKH-PE, analyzed by flow cytometry, and injected i.v. into naive mice (3×10^6 cells/mouse). Two hours after injection, one experimental group was sensitized with 1% TNCB, and the other group was treated with solvent only. After 24 h, cell suspensions of dLN, spleen, and blood were prepared and analyzed. **A**, Numbers of immigrated injected PKH-PE⁺ Tregs in the different organs. Data show the mean of PKH-PE⁺ cells in percent of CD4⁺ cells $\pm$ SD of three independent experiments ($*p < 0.001$). **B**, Freshly isolated, PKH-PE-labeled cells were stained with anti-CD69, anti-CD62L, or anti-CD44 and examined by flow cytometry. Dot plots show the expression of the respective markers and numbers given in each dot plot indicates the percentages of CD69⁺ or CD62L⁺ cells or the MFI of CD44. **C**, Expression of CD69, CD62L, and MFI of CD44 on the injected Tregs in the different organs of the two experimental groups. Data show the mean of percentage of positive cells or of the MFI, respectively, $\pm$ SD of three independent experiments. Significant differences ($*p < 0.001$) between solvent-treated and TNCB-treated groups were determined by Student *t* test.



injection. Thus, these data indicate that Tregs become activated in vivo in the dLN during sensitization.

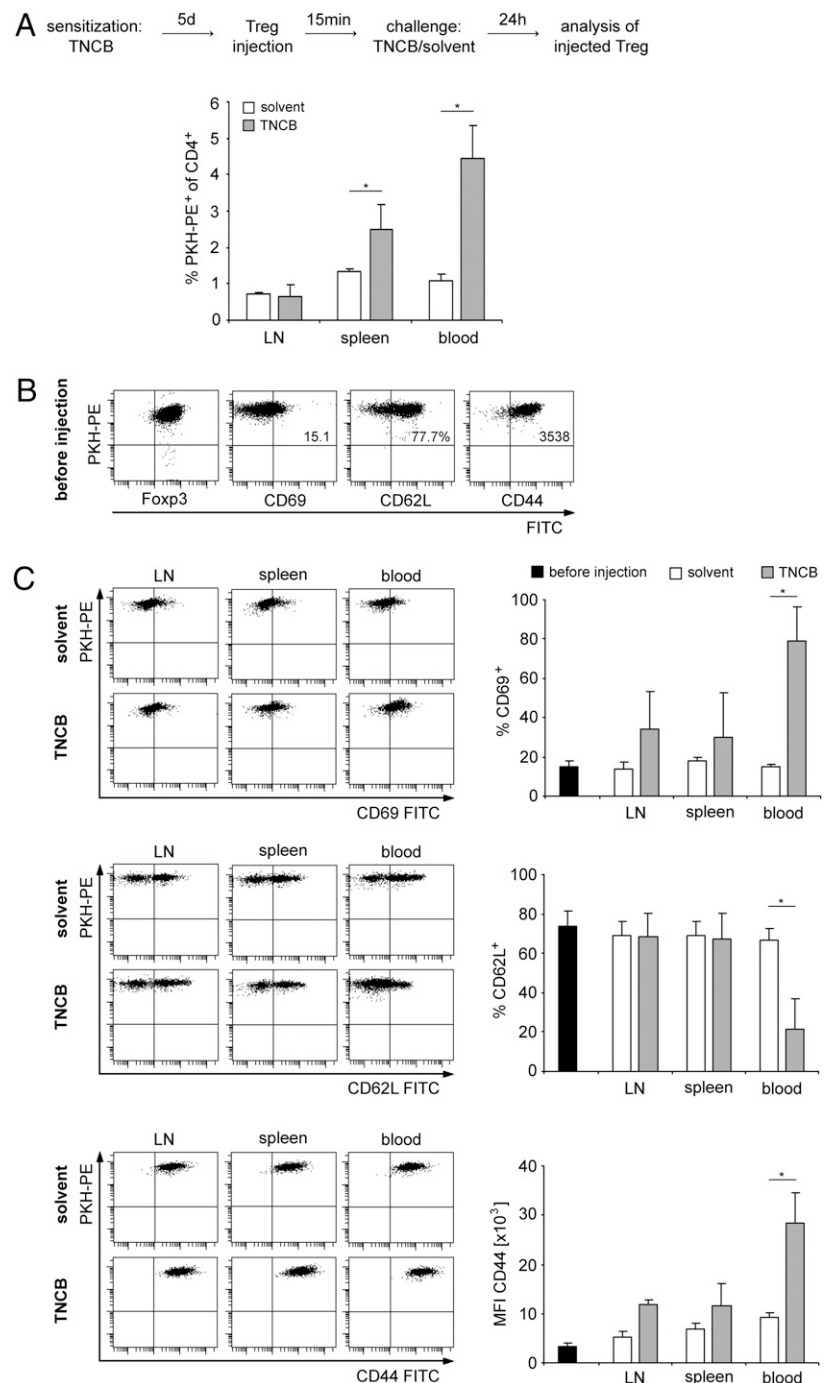
To investigate the effect of the challenge phase on the homing and the activation status of Tregs, freshly isolated and dye-labeled Tregs were injected into sensitized mice, which were challenged 15 min later. Fig. 4A shows that Tregs were primarily detected in the blood, and only declining numbers of Tregs migrated into the spleen and dLN. In contrast, after omitting the challenge (solvent only group), no accumulation of Tregs in the blood could be observed, and Tregs were nearly equally distributed among dLN, spleen, and blood (Fig. 4A).

Thereafter, we also assessed whether the activation status of the Tregs changed in vivo after Ag challenge in the different organs. In this study, we show that Tregs upregulated CD69 expression most vigorously in the blood (Fig. 4C), whereas in dLN and spleens only modest expression occurred. In parallel, downregulation of

CD62L and upregulation of CD44 were recorded exclusively in blood-derived Tregs (Fig. 4C), indicating that only Tregs in the blood become activated by the challenge reaction. Treatment of the mice with solvent only did not result in activation of the Tregs in the respective organs. In aggregate, these data indicate that Tregs upregulate markers, which are indicative for activation during the sensitization phase of CHS reactions in the dLN. In contrast, during the elicitation phase, activation of Tregs is exclusively detected in the blood.

As activation of T cells may also result in T cell proliferation, we determined the proliferation of CFSE-labeled Tregs in vivo in the sensitization and challenge phase (Supplemental Fig. 3). We observed approximately one round of proliferation in 5–10% of the injected Tregs in dLN (10% of the cells) and in spleens (5% of the cells). However, this proliferation was neither specific for Tregs

FIGURE 4. During the elicitation phase of CHS reactions, Tregs get activated in the blood. Tregs were isolated from LN and spleen of naive mice, labeled with PKH-PE, analyzed by flow cytometry, and injected i.v. into sensitized mice (3×10^6 cells/mouse). Fifteen minutes after injection, one experimental group was challenged with 0.5% TNCB on one ear, and the other group was treated with solvent only. After 24 h, cell suspensions of dLN, spleen, and blood were prepared and analyzed. **A**, Numbers of immigrated injected PKH-PE⁺ Treg in the different organs. Data show the mean of PKH-PE⁺ cells in the percentage of CD4⁺ cells $\pm$ SD of five independent experiments ($*p < 0.01$). **B**, Freshly isolated, PKH-PE-labeled cells were stained with anti-CD69, anti-CD62L, or anti-CD44 and examined by flow cytometry. Dot plots show the expression of the respective markers, and numbers given in each dot plot indicate the percentages of CD69⁺ or CD62L⁺ cells or the MFI of CD44. **C**, Expression of CD69, CD62L, and MFI of CD44 on the injected Tregs in the different organs of the two experimental groups. Data show the mean of percentage of positive cells or of the MFI, respectively, $\pm$ SD of five independent experiments. The significance of differences ($*p < 0.001$) between solvent-treated and TNCB-treated groups were determined using the Student *t* test.



(as control $CD4^+CD25^-$ T cells proliferated to a similar extent) nor dependent on elevated ATP levels (as proliferation also took place during challenge in the dLN, which does not display increased ATP content after challenge). Thus, we conclude that ATP does not drive proliferation of Tregs in vivo, and the insignificant proliferation is induced by hapten application to the skin.

ATP is elevated in dLN after sensitization and in serum after challenge

Because we have shown that Tregs become activated in different tissues after sensitization or challenge, respectively, we further hypothesized that ATP release at different body sites may activate Tregs. Therefore, we measured the ATP content of different organs in response to sensitization or challenge.

After sensitization at the ventral abdominal skin (Fig. 5A), ATP levels were significantly elevated in the respective dLN, whereas the serum, the spleen (data not shown), and distant ndLN, such as the mandibular LN, displayed control levels of ATP. By contrast, challenging the mice with hapten at the ear resulted in augmented levels of ATP in serum, whereas ATP remained unaltered in the proper draining and ndLN.

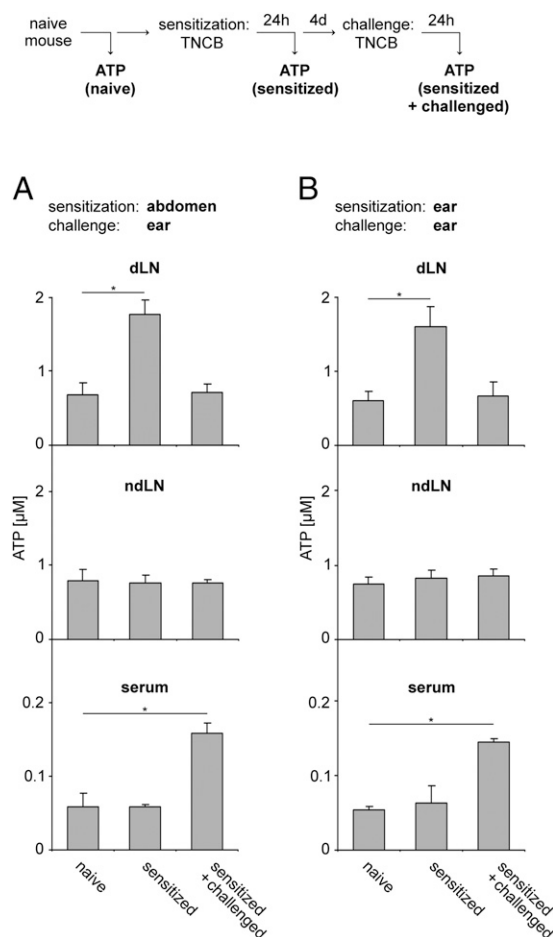


FIGURE 5. Increasing amounts of ATP at the site of hapten application in vivo. BALB/c mice were sensitized either on the abdomen (A) or on the ear (B) with 1% TNCB (sensitized), sensitized and challenged 5 d later with 0.5% TNCB (sensitized + challenged) or left untreated (naive). Twenty-four hours after treatment, ndLN, dLN, and serum were taken, and the amounts of ATP were detected with the ATPlite luminescence assay system. Data show the mean of ATP amount in the different organs $\pm$ SD of six independent experiments. The significance of differences ($*p < 0.01$) between the ATP amount in the organs of naive mice and of sensitized or sensitized + challenged mice was determined using the Student *t* test.

As sensitization and challenge were carried out at different tissue sites (i.e., the abdominal skin and the ear, respectively), we reasoned that the differences in ATP production may be due to different capacities of the tissues to release ATP in response to hapten application. Therefore, we next sensitized mice at one ear and measured the ATP content of the dLN and in the serum. In this study, we show (Fig. 5B) that ATP was only elevated in the respective dLN but not in serum and ndLN, confirming our previous results, showing elevated ATP after sensitization exclusively in the dLN. Likewise, the challenge of these ear-sensitized mice yielded results similar to that obtained after abdominal sensitization. That is, after challenge at the ear, we observed augmented ATP in the serum only, whereas the ATP levels in dLN and ndLN remained unaffected. These data indicate that during sensitization ATP is selectively elevated in the proper dLN, independent from the tissue used for sensitization, whereas challenge leads to increased levels of ATP in the serum. To further substantiate the role of ATP during CHS reactions, we systemically applied ATP before sensitization or challenge, respectively, and measured the ear-swelling reaction thereafter (Supplemental Fig. 4). In this study, we show that administration of ATP before challenge lead to a reduced ear-swelling reaction, whereas the sensitization remained unaffected by ATP. These results are in line with our in vivo observation (i.e., elevated levels of ATP in the blood/serum are effective in activating Tregs at vascular sites), which results in blockade of the elicitation of a CHS response. In contrast, the sensitization takes place in dLN, and i.v. injected ATP may not reach the LN residing Tregs. Consequently, activation of the suppressive function of the Tregs is not induced and sensitization takes place.

Blocking of ATP receptors on Tregs prevents their function in vivo

Our data suggest that ATP activates Tregs in vivo, which is a prerequisite for their suppressive activity. Therefore, we next wanted to test whether blockade of the ATP receptors on Tregs abrogates their suppressive capacity in vivo. To this end, we tested the recently described ATP receptor antagonist PPADS at first in vitro by incubation of the Tregs with PPADS prior to stimulation with ATP. Fig. 6A shows that this preincubation with PPADS abrogated the ATP-induced upregulation of the activation marker CD69 in Tregs, indicating blockade of activation by ATP. In addition, we performed suppression assays, where control Tregs or Tregs preincubated with PPADS were cocultured with responder T cells in presence of anti-CD3/anti-CD28 Abs. These data show that Tregs activated independently from ATP receptors are still suppressive and that the ATP receptor antagonist PPADS does not abrogate the suppressive function of Tregs unselectively for example by inducing cells death. It also demonstrates that several redundant mechanisms of Treg activation exist. That is, the blockade of the ATP receptors on the membrane of Tregs abrogates the ATP-induced Treg activation but still leaves the possibility open to activate Tregs by anti-CD3/anti-CD28 via their TCRs.

Because these in vitro assays established the selective function of PPADS, we next investigated the effects of ATP receptor blockade on the activity of Tregs in the CHS model (Fig. 6C, 6D). To this end, we injected normal Tregs or Tregs that had been preincubated with PPADS into mice 2 h before sensitization. Control mice did not receive any cells before sensitization. After 5 d, mice were challenged, and ear swelling was determined. Analysis of the mice showed that blockade of the ATP receptor(s) by PPADS abrogated the suppressive function of the Tregs (Fig. 6C). Similar results were obtained when Tregs were injected before challenging. In

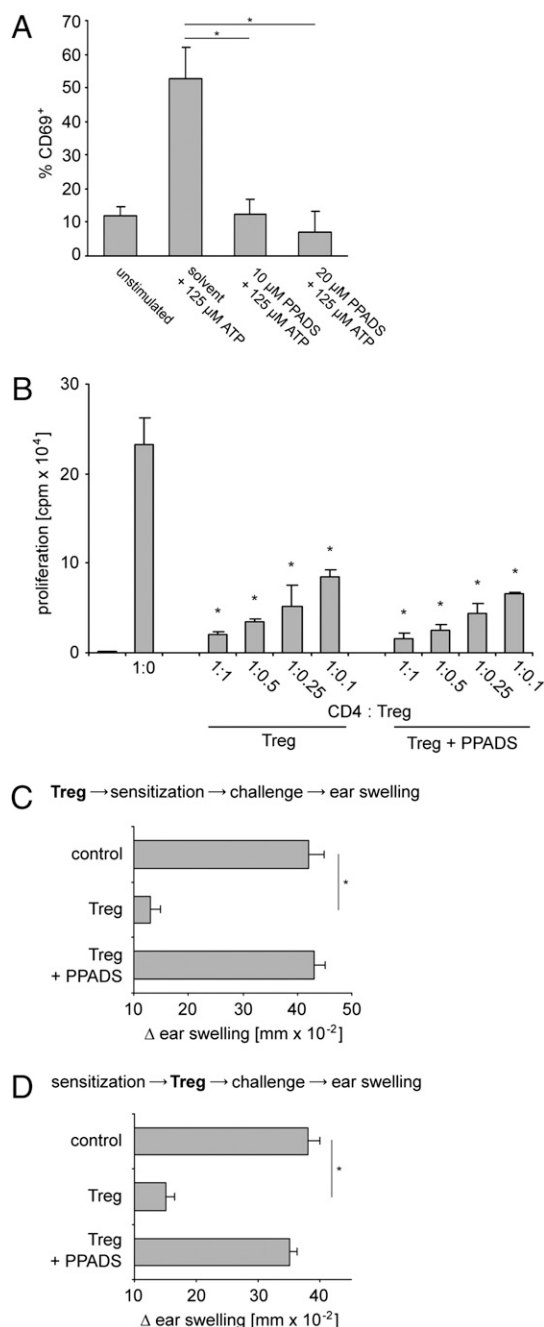


FIGURE 6. Blocking of ATP receptors on Tregs abrogates ATP-mediated activation and suppressive function of Tregs in vivo. **A**, Tregs were isolated from LN and spleen of naive mice, and one part was incubated with ATP receptor blocker PPADS (10 or 20 μ M; 2 h) or solvent only before stimulation with ATP (125 μ M). After 24 h of stimulation, Tregs were stained with activation marker anti-CD69 and analyzed by flow cytometry. Data show the mean of CD69 expression on Treg $\pm$ SD of four independent experiments. The significance of differences ($*p < 0.001$) between the solvent and ATP-treated cells and the cells, which were treated with PPADS prior to the ATP stimulation, were determined using the Student *t* test. **B**, Isolated CD4⁺CD25⁻ T cells were cocultured with Tregs or Tregs, which were preincubated with PPADS (20 μ M; 2 h), in the stated ratios and stimulated with anti-CD3 and anti-CD28 (0.5 μ g/ml each). After 3 d of culture, 0.5 μ Ci/ml [³H]thymidine was added for 18 h, cells were harvested, and the thymidine incorporation was determined. Data show the mean proliferation of CD4⁺CD25⁻ T cells $\pm$ SD of four independent experiments. Significant differences ($*p < 0.001$) between the proliferation of CD4⁺CD25⁻ T cells alone and CD4⁺CD25⁻ T cells cocultured with Tregs were determined by Student *t* test. A total of 3×10^6 Tregs or Tregs, which were preincubated with PPADS (20 μ M; 2 h), were

these experiments (Fig. 6D), Tregs or PPADS-pretreated Tregs were injected into sensitized mice 15 min before challenge. In this study, PPADS-treated Tregs failed to suppress the ear-swelling reaction as compared with their untreated counterparts. Thus, these data indicate that in vivo activation of naive Tregs by ATP release induced by hapten sensitization or challenge, respectively, is mandatory for generating their suppressive function.

Discussion

ATP is widely known for its role as energy carrier in almost all cells. However, increasing evidence established that ATP also acts as a second messenger during inflammatory and allergic diseases (12, 13). In line with these observations, our report shows that ATP is able to activate Tregs and to increase their suppressive capacity. We further show in a model of murine contact hypersensitivity (CHS) that application of hapten(s) to the ventral skin of mice (sensitization phase) augments ATP concentrations exclusively in skin dLNs, resulting in activation of naive Tregs. On the contrary, application of the hapten in a challenge reaction augments ATP content primarily in the serum. As a consequence, during sensitization, Tregs become activated in the dLN, whereas Ag challenge activates Tregs in the blood. Thus, these data indicate that ATP may act as a tissue site-specific activator of the suppressive capacity of Tregs in vivo.

It has been established that Tregs require activation to convey their suppressive capacity. Similar to conventional T cells, Tregs harbor a functional TCR and engagement of the TCR by respective MHC–peptide complexes, together with IL-2/IL-4 secretion, are able to induce activation of the suppressor function (3). However, recent results by Szymczak-Wortmann et al. (14) indicate that Tregs do not need to be activated through their TCRs. Our results corroborate these findings and describe a novel pathway of Treg activation by ATP, which is able to augment and/or to induce Treg-mediated suppression during inflammatory responses, independently from the specificity of the TCR(s) expressed by the Tregs. Although the exact means of ATP signaling in Tregs are not clear yet, ATP normally conveys its effects via a multitude of P2x and P2y receptors (12, 15). Both receptor types convey intracellular signaling by different means (i.e., the P2x receptors are ionotropic, and the P2y receptors are G-protein coupled). Especially the role of P2x receptors in signaling in T lymphocytes has been investigated, and recent results indicate that CD4⁺ effector T cell activation is maintained by binding of autocrinely produced ATP to P2x receptors (16, 17). Moreover, ATP induces a calcium influx in leukocytes by binding to a P2x family receptor. This ATP-induced calcium influx may then provide an interconnection where TCR- and ATP-induced T cell activation join a common pathway, because the “classical” TCR activation also induces elevated calcium levels, which are essential for further downstream signaling resulting in T cell activation. It has also been established that cytokines, such as IL-2, IL-4, IL-7, and IL-15, which trigger the common γ -chain and thus activate PI3K/AKT, are crucial for maintaining the suppressive function of Tregs (18, 19). Likewise, ATP receptors have been shown to interfere with the PI3K

injected i.v. into naive mice, followed by sensitization with 1% TNBC 2 h later and challenge with 0.5% on day 5 (sensitization phase) (C) or into sensitized mice 15 min before challenge (elicitation phase) (D). Control mice were not treated with cells at all. Ear-swelling reaction was measured 24 h after challenge. Data show in each case the mean increase of ear thickness $\pm$ SD of three independent experiments. Significant differences ($*p < 0.001$) between the ear thickness of control mice and of mice treated with Tregs or Tregs + PPADS were determined by Student *t* test.

pathway. Thus, it is conceivable that ATP may augment the suppressive activity of Tregs via the PI3K pathway.

ATP belongs to a network of several mediators that alert our body of “danger” that is also referred to as “damage-associated molecular pattern” (20). ATP is an ideal candidate to serve as a danger signal, because it is abundantly present in almost all cells, but its extracellular concentration is kept at very low levels (<20 nM). This level is maintained by ATP-degrading ectonucleotidases expressed by cells in the tissue. Therefore, passive release of small amounts of ATP induced by trauma, cell death, and bacterial infections in response to injury is easy to detect by leukocytes and may serve as an activating trigger for proinflammatory cells. For example, maturation of dendritic cells (21–25), stimulation of IL-1 β , IL-6, and IL-18 secretion by monocytes (26, 27), as well as activation of the inflammasome (20) by ATP, has been reported.

In line with these proinflammatory effects on cells of the innate immune system, ATP has also been shown to exert mitogenic effects on conventional CD4 $^{+}$ and CD8 $^{+}$ T cells, respectively (16, 17), as well as on the induction of Th17 T cells (28). These effects are mainly driven by the P2X $_7$ receptor, which acts via pore formation and induction of a calcium influx into cells. Similar to conventional T cells, Tregs have been shown to express functional P2X $_7$ receptors (29), and our data corroborate previous observations showing that engagement of this receptor leads to down modulation of CD62L (30), which is one indicator of T cell activation. But beyond that, the effects of ATP on Tregs are controversially discussed. It has been demonstrated that Tregs are in particular sensitive to ATP and may be driven into apoptosis by high amounts of ATP (31). In the investigations reporting apoptosis of Tregs after exposure to ATP, concentrations of >300 μ M ATP were used. In contrast, we show that concentrations of as low as 25 μ M are able to activate Tregs, without triggering apoptosis. Thus, ATP may execute different effects on Tregs, depending on the time and concentration used for stimulation. Our results further support the current hypothesis, that the magnitude of the P2X $_7$ -triggered calcium influx is crucially involved in decision making on whether activation or death of the respective T cells is induced (32). A low ATP stimulation causes only transient opening of calcium channels and thus induces activation of cells. In contrast, massive ATP concentrations induce prolonged influx of calcium, which eventually causes cell death.

According to its function as damage-associated molecular pattern, our data show that ATP was augmented in the dLN after topical application of a contact allergen and irritant to the skin. This observation is corroborated by previous results, showing that the ATP content of LN cultures is strongly elevated after application of skin sensitizing chemicals. This increase in ATP release is even considered a surrogate marker for estimation of the “sensitizing potential” of newly developed chemicals in the pharmaceutical industry (33, 34).

Our data show bisectonal ATP production during CHS reactions, with 1) ATP being produced in the dLN after sensitization, and 2) ATP being elevated in serum after challenge. These data strictly correlate with the observed pattern of Tregs activation (i.e., during sensitization Tregs were selectively activated in dLN, whereas Ag challenge induced activation of Tregs in the blood). The sensitization phase and the challenge phase differ in that sensitization takes place in the respective dLN, whereas challenge elicits an inflammatory reaction at the tissue site after Ag encounter. During sensitization, APCs transport Ags from the skin to the dLN and present Ags to respective T cells. This Ag presentation has recently been shown to trigger ATP release from the engaged T cells (17). Thus, as first-time contact of hapten-loaded APCs with reactive T cells during sensitization occurs in particular in the

dLN, augmented ATP release is specifically triggered in this study and may contribute to activation of Tregs residing in the dLN.

In contrast, during elicitation of CHS responses, the inflammatory reaction readily starts in the skin, where interaction of skin-residing APCs and effector T cells initially induces ATP release from the effector T cells. These elevated levels of ATP in the skin can further be augmented by keratinocytes and endothelial cells, which both have been shown to be a source for ATP (35–37). Thus, this localized immune reaction is able to produce enough ATP, which may reach the serum causing activation of Tregs in the blood.

Alternatively, a so-far unknown messenger may induce the release of ATP at a site distant from the hapten application. Such a mechanism has been demonstrated during spinal cord injury (38). In that model, ATP is not produced in the actual spinal cord region where injury occurs. Instead, it is rather produced in a perilesional area, which is distant from the tissue site that was traumatized. This would correspond to our observation that the contact sensitizer applied to the skin induces ATP production in a distant dLN. However, the mechanism how ATP release is “remote”-controlled in tissues far from the actual tissue damage is not known yet.

In this sequence of events, the “blood-borne” ATP serves as activator of blood-residing Tregs, which eventually migrate to tissue sites. However, direct chemotactic effects of ATP on T and B cell recruitment to tissue sites have not been observed (39). In particular, in CHS responses, we have clearly shown that Tregs do not enter the inflamed ear (7) but suppress the ear-swelling reaction by production of adenosine via degradation of ATP by CD39/CD73 ectonucleotidases (8). Thus, elevated levels of ATP, besides activating Tregs, also serve as substrate for the production of immunosuppressive adenosine by Tregs in the blood, bypassing the necessity for Tregs to home to inflamed tissues for restraining inflammation.

So far ATP has predominantly been attributed to proinflammatory actions, but in this study, we present novel data indicating that ATP induces also anti-inflammatory pathways by activating Tregs. This activation of counterbalancing mechanisms is typical for any homeostatic system, in which “activating” signals simultaneously trigger also “dampening” of activation, to prevent overboarding inflammation. In line with these anti-inflammatory functions, ATP has been shown to shift the development of dendritic cells into a Th2 T cell-inducing and thus less proinflammatory phenotype (11, 40). ATP also tightens the junctional permeability during acute lung injury (41), and high concentrations of it induce apoptosis in intestinal CD8 $^{+}$ T cells (42). Moreover, free ATP in tissues is rapidly degraded to adenosine, which has profound inhibitory effects on several immune reactions (43). Our report showing activation of Tregs by ATP adds a novel mechanism to the anti-inflammatory action of ATP and its metabolites. According to our data, ATP may possess a dual function during interaction with Tregs: 1) Tregs become activated by exposure to ATP leading to development of the full arsenal of inhibitory mechanisms, and 2) activated Tregs show enhanced activity of CD39/CD73 ectonucleotidases, resulting in augmented adenosine generation. As a consequence, Tregs clear the tissue environment from the “danger signal” ATP and simultaneously produce a metabolite (i.e., adenosine) with strong anti-inflammatory effects.

In conclusion, our data define a novel pathway of Treg activation, and directed manipulation of ATP/adenosine interaction with Tregs may provide a novel tool to tune the suppressive capacity of Tregs during autoimmune and allergic diseases.

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Disclosures

The authors have no financial conflicts of interest.

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Supplemental Figure Legends

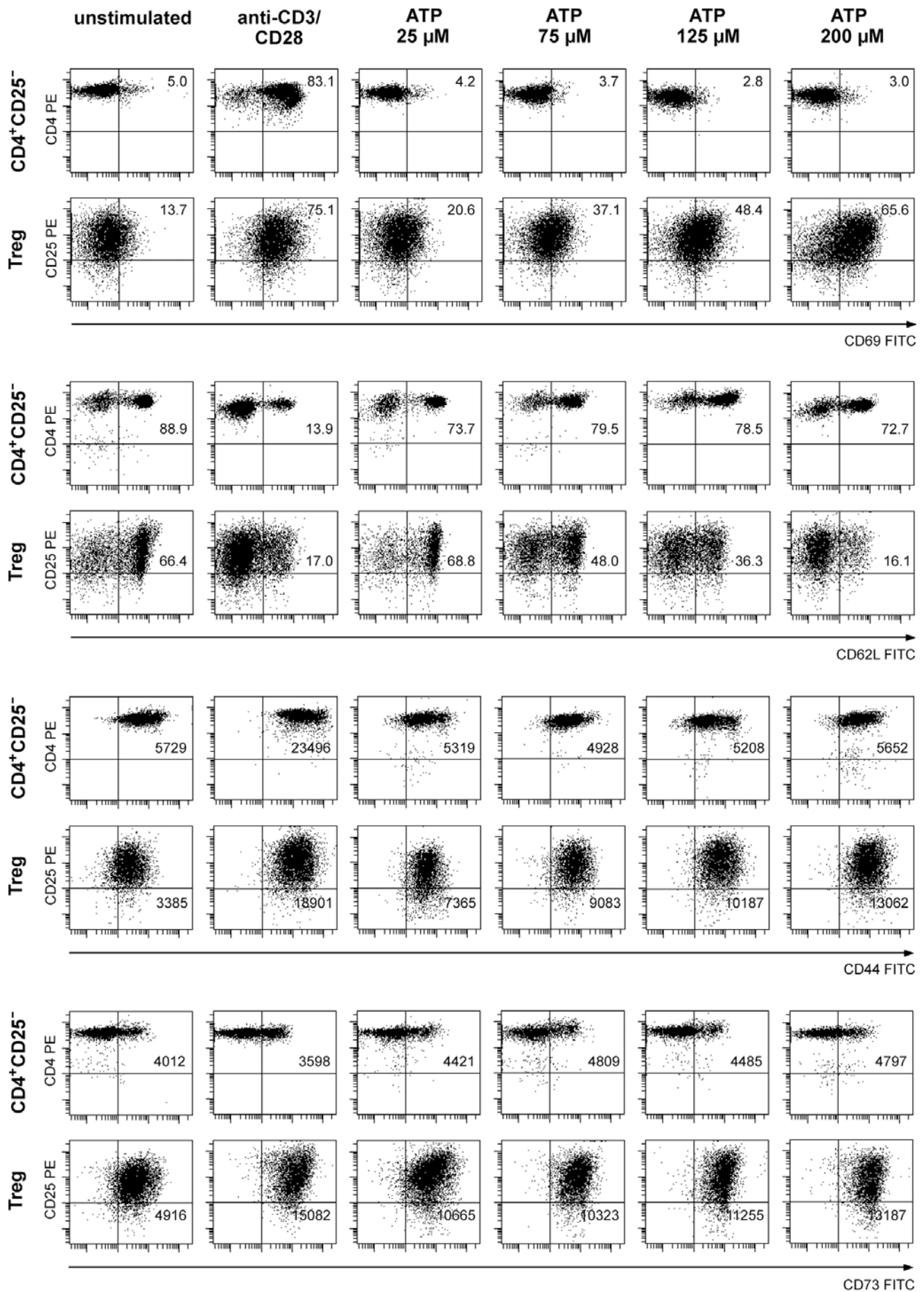
Figure S1: ATP activates Treg in vitro. CD4⁺CD25⁻ T cells and Treg were isolated from LN and spleens of naïve mice and stimulated with anti-CD3 and anti-CD28 (0.5µg/ml each), ATP (25µM, 75µM, 125µM, 200µM) or left untreated (unstimulated). Expression of the activation marker CD69, CD62L and MFI of CD44 and of ectonucleotidase CD73 was analyzed by flow cytometry after 24h in culture. Shown are the dot plots from one typical experiment out of three independent experiments.

Figure S2: ATP activates induced Treg in vitro. CD4⁺CD25⁻ T cells were isolated from LN and spleens of naïve mice and cultured in a 24well plate with plate-bound anti-CD3 (5µg/500µl/well), anti-CD28 (1µg/ml), TGF-β (20ng/ml) and IL-2 (20U/ml) for two days. Cells were washed and cultured for additionally three days with TGF-β and IL-2. (A) Induced Treg were purified using CD25 antibodies and magnetobeads and activation status was determined by flow cytometry. (B) Induced and purified Treg were cultured with anti-CD3/anti-CD28 or ATP (25µM, 75µM, 125µM, 200µM) or left unstimulated for 24h. Activation status was determined using flow cytometry. Shown are the means of the results from three independent experiments +/-SD. Significant differences (*p<0.001) between unstimulated and ATP treated cells were determined by Student's t-test.

Figure S3: In vivo proliferation of Treg and CD4⁺CD25⁻ T cells during sensitization and challenge phase. Freshly isolated Treg (A) or CD4⁺CD25⁻ T cells (B) were labeled with CFSE and 3x10⁶ cells were injected either 2h before sensitization (cells injected → sensitization) or 15min before challenge (cells injected → challenge) the mice. 48h after injection cell suspensions from spleen, lymph nodes (LN) and blood were prepared and analysed by flow cytometry. Histograms show the gated CFSE labeled Treg (A) or CD4⁺CD25⁻ T cells (B) in the different organs. Percentages shown in the histograms depict the dilution of the CFSE dye.

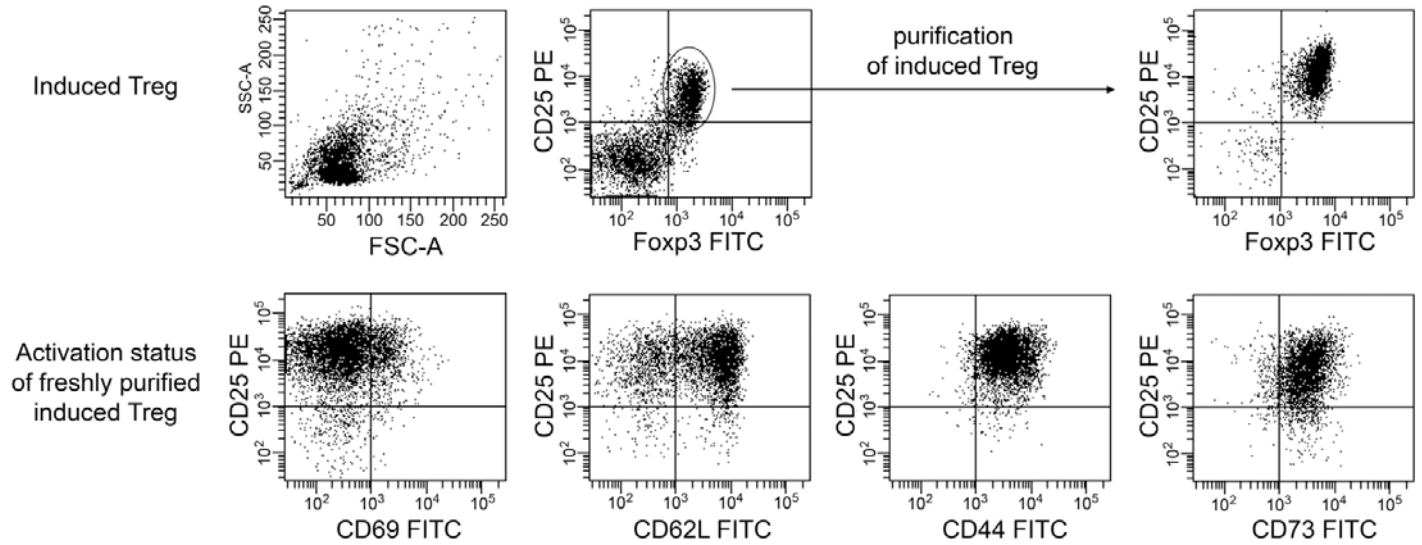
Figure S4: Mice were injected intravenously with ATP (100 mg/Kg bodyweight) either 2h before sensitization (ATP before sensitization) or 15min before challenge (ATP before challenge) the mice (equivalent to the experimental protocol for the Treg injection). Control mice were not treated with ATP (control). Ear swelling response was measured 24h after challenge, according to the standard protocol. Data show the means of the ear swelling responses of three independent experiments (each with three mice/group) $\pm$ SD (* $p < 0.001$).

Supplemental Figure S1

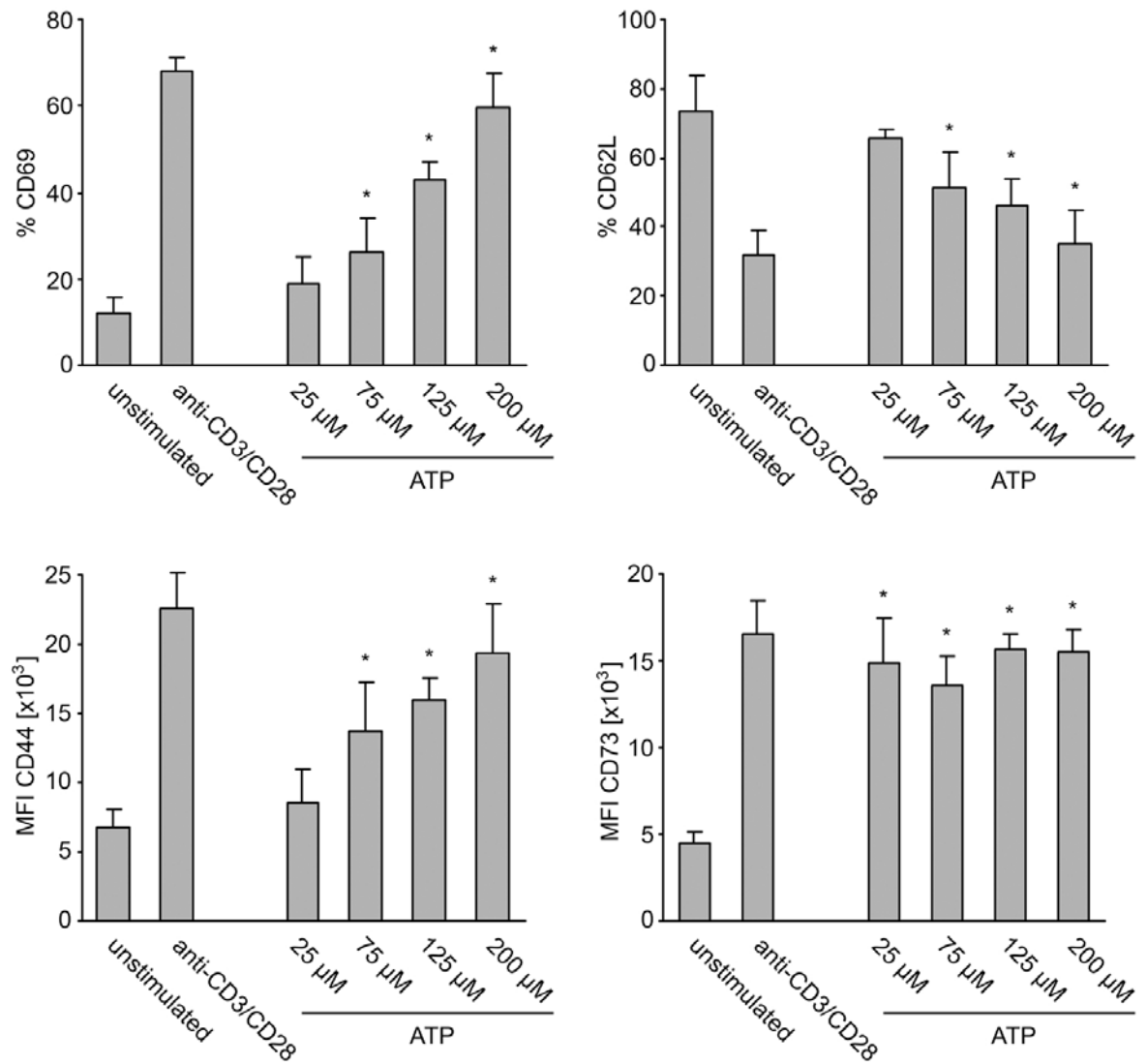


Supplemental Figure S2

A



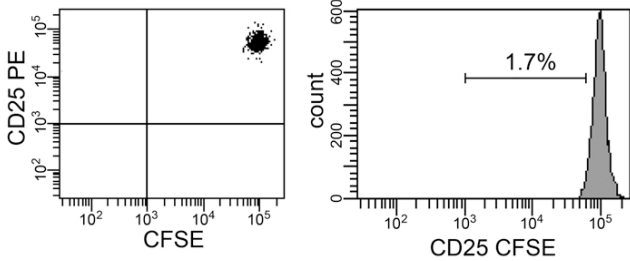
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Supplemental Figure S3

A

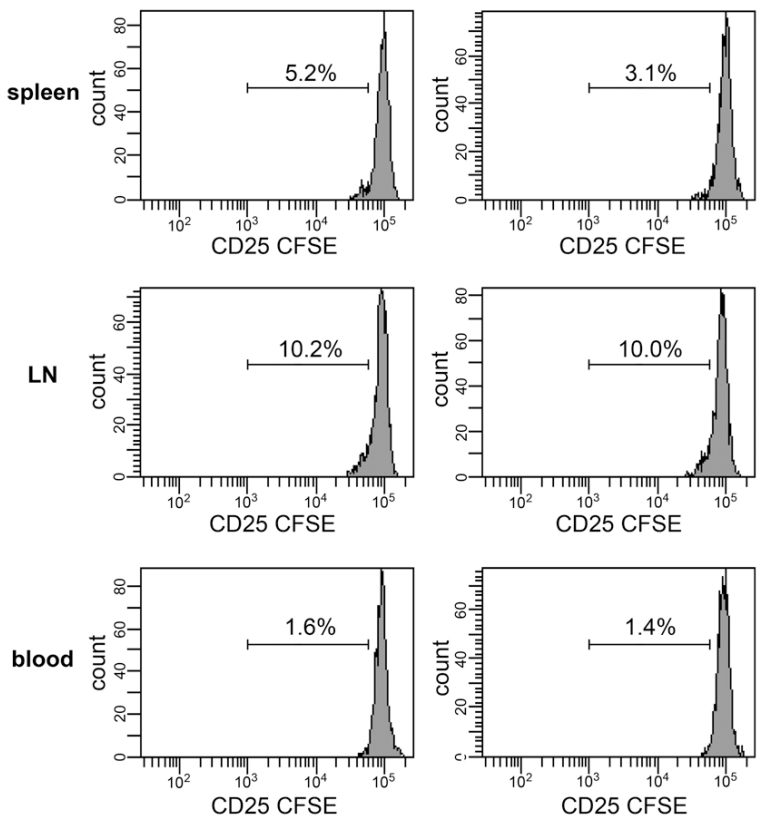
Freshly isolated, CFSE labeled
Treg before injection



Treg 48h after injection

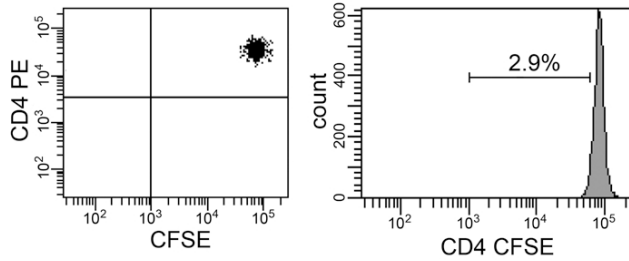
cells injected → sensitization

cells injected → challenge



B

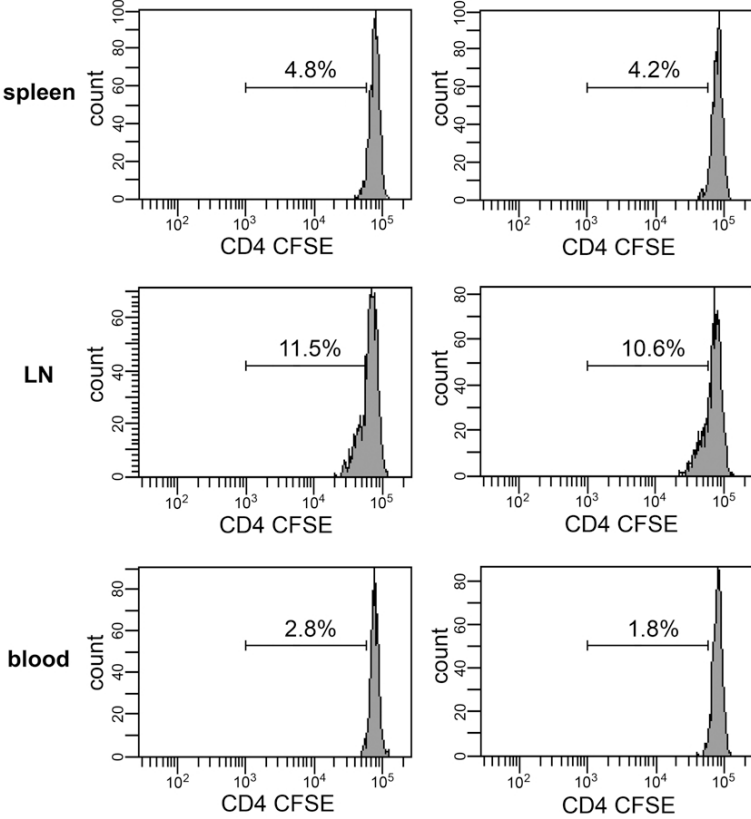
Freshly isolated, CFSE labeled
CD4⁺CD25⁻ T cells before injection



CD4⁺CD25⁻ T cells 48h after injection

cells injected → sensitization

cells injected → challenge



Supplemental Figure S4

