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Down-Regulation of CD62L Shedding in T Cells by CD39⁺ Regulatory T Cells Leads to Defective Sensitization in Contact Hypersensitivity Reactions

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Abstract

Injection of regulatory T cells (Tregs) followed by sensitization with 2,4,6-trinitrochlorobenzene induced a transient increase in size and cellularity of skin-draining lymph nodes (LNs) in mice. This led us to hypothesize that Tregs may affect the trafficking of T cells from and to peripheral LNs. Two to three hours after sensitization, we found fewer CD8⁺ T cells expressing CD62L in LNs compared with untreated controls. Injection of wild-type Tregs prevented this down-regulation of CD62L. In contrast, Tregs devoid of the adenosine triphosphate (ATP)-degrading ecto-enzyme CD39 were unable to do so. As for the mechanism of CD62L regulation, we found that ATP, which is released in skin upon hapten-exposure, is inducing the protease ADAM17 in LN-residing T cells via engagement of P₂X₇ ATP receptors. ADAM17 cleaves CD62L from the surface of CD8⁺ T cells, which in turn provide a signal for T cells to leave the LNs. This regulation of CD62L is disturbed by the presence of Tregs, because Tregs remove extracellular ATP from the tissue by activity of CD39 and, therefore, abrogate the shedding of CD62L. Thus, these data indicate that the regulation of ATP turnover by Tregs in skin and LNs is an important modulator for immune responses.

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CONFLICT OF INTEREST

The authors state no conflict of interest.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at <http://dx.doi.org/10.1016/j.jid.2016.08.023>.

INTRODUCTION

In the murine contact hypersensitivity (CHS) model, allergic contact dermatitis can be induced by small (<1,000 Da) chemical compounds (haptens). Upon penetration of the skin, haptens interact with proteins and develop into full antigens. The antigens are taken up by dendritic cells (DCs) and are transported to peripheral lymph nodes (LNs) (Honda et al., 2013). Here priming and activation of T cells ensues. The role of proinflammatory DCs in this process is essential; therefore, activating stimuli provided by the innate immune system, such as toll-like receptor ligands and adenosine triphosphate (ATP) are mandatory for the induction of CHS reactions (Burnstock et al., 2012; Weber et al., 2010).

Counter regulatory mechanisms, provided by Langerhans cells and naturally occurring CD4⁺CD25⁺ regulatory T cells (Tregs), have been identified recently and are attributed to their secretion of IL-10 and transforming growth factor- β (Kaplan et al., 2012; Vignali et al., 2008). Beyond that, Tregs interact with DCs in peripheral LNs by means of gap junctions (Ring et al., 2010b) restraining the priming of effector T cells, and as a consequence the sensitization phase of CHS reactions is abrogated. However, these effects are operative at the earliest 6 hours after application of the respective haptens, because skin-derived DCs have to reach draining LNs first before being targeted by LN-residing Tregs (Tomura et al., 2014).

Much earlier, that is, along with application of the respective haptens to skin, ATP is released into the extracellular space in skin by pannexin channels in keratinocytes and by apoptotic cells (Mizumoto et al., 2003; Onami et al., 2014). It serves as a potent “danger” signal providing the necessary stimulation of inflammasomes and creating a proinflammatory environment (Di Virgilio, 2007). To compensate for this proinflammatory action, Tregs are able to degrade extracellular ATP by action of the ectonucleotidases CD39 and CD73 (Deaglio et al., 2007; Ring et al., 2009). Both molecules are constitutively expressed by Tregs, and several reports have proven that production of adenosine is relevant for the suppressive action of Tregs during tumor growth, autoimmunity, and suppression of CHS reactions (Antonioli et al., 2013; Challier et al., 2013; Linden and Cekic, 2012; Ring et al., 2009, 2010a). In CHS, production of adenosine seems critical for the outcome of the disease, because blocking of the adenosine-producing enzyme CD39 or genetic ablation of CD39 in Tregs abrogates their suppressive functions in CHS responses (Ring et al., 2009).

However, the function of CD39 and CD73 in Tregs during the sensitization phase is less understood. Given the potent proinflammatory activities of ATP, we hypothesize that Tregs by means of ATP turnover may influence the induction of immune responses. Here we show that extracellular ATP stimulates shedding of CD62L by activating ADAM17 via P₂X₇ ATP receptors in T cells, and this shedding is prevented by Tregs, because they constitutively express the ATP-decomposing enzymes CD39 and CD73. As a consequence of the reduced down-regulation of surface CD62L, egress of T cells from LNs is impaired, eventually leading to defective sensitization.

RESULTS

Tregs degrade extracellular ATP and induce accumulation of CD8⁺ T cells in draining lymph nodes

Sensitization with haptens induces release of ATP in skin (Onami et al., 2014; Weber et al., 2010). To assess the effects in LN cells after sensitization, mice were sensitized with 2,4,6-trinitrochlorobenzene (TNCB) or solvent, respectively, and were killed at different time points (Figure 1a). Whole LNs were removed and immediately disrupted with tweezers in 100 μ l of medium, followed by quantification of ATP in tissue culture supernatants. Figure 1a shows significantly elevated levels of ATP 2 to 4 hours after sensitization. However, after 6 hours, the ATP concentration was reduced to levels obtained after 1 hour of culture.

To investigate whether Tregs are involved in regulating extracellular ATP content in LNs upon sensitization, we intravenously injected animals with wild-type (wt) Tregs or CD39-deficient (CD39^{-/-}) Tregs (3×10^6 cells/mouse), respectively, before sensitizing with TNCB and prepared LN cells 3 hours later. This injection led to accumulation of Tregs in LNs and spleens (see Supplementary Figure S1 online). In these experiments, typically 1.5% to 2.0% of CD4⁺ T cells in LNs are derived from injection. In the supernatants of the LN cultures, elevated levels of ATP were detected after sensitization of uninjected control mice (Figure 1b). In contrast, no elevation of ATP concentrations was detectable in LN-culture supernatants from wt Treg-injected mice. Injection of CD39^{-/-} Tregs did not prevent the hapten-induced increase of extracellular ATP in LN tissue cultures. Therefore, we conclude that CD39-expressing Tregs are able to modulate the concentration of extracellular ATP in LNs.

To assess the effects of ATP turnover by Tregs on LN cells, we analyzed LN cells by flow cytometry. We found increased sizes of LNs and higher total cell numbers in animals injected with wt Tregs (Figure 1c). However, after injection of Tregs alone, no significant changes of T-cell numbers occurred. In particular, increased numbers of CD8⁺ T cells (Figure 1d) were recorded after injection of wt Tregs and subsequent sensitization. At the same time, we observed slightly reduced numbers of CD4⁺ T cells. However, this down-regulation, although significant, was negligible compared with CD8⁺ T cells. In contrast, uninjected control mice and mice injected with CD39^{-/-} Tregs showed no significant changes in total cell numbers and in the frequency of CD8⁺ T cells. However, in all experimental groups, other cell populations such as CD11c⁺ DCs, CD11b⁺ monocytes, and B220⁺ B cells did not vary in frequencies (data not shown).

To analyze the phenotype of the CD8⁺ T cells after Treg injection and sensitization, we tested isolated LN cells for surface marker expression and found that, among all markers examined (CD49d/e, CD69, LFA-1, CCR7, not shown), CD62L is down-regulated 3 hours after sensitization (Figure 2a). Injection of wt Tregs before sensitization prevented this down-regulation, whereas CD39^{-/-} Tregs had no effect on CD62L expression. Therefore, we concluded that Tregs interfere with regulation of surface expression of CD62L by means of ATP degradation.

Because CD62L serves as an LN-homing molecule, we investigated whether Tregs prevent the egress of T cells from lymphoid organs by suppressing the ATP-induced down-regulation of CD62L. We isolated CD8⁺ T cells from LNs and spleens of untreated animals, labeled the cells with the fluorescent dye PKH-PE, and injected them into naive hosts. After 24 hours, animals were injected with wt or CD39^{-/-} Tregs, and sensitization was performed. Thereafter, LN single-cell suspensions were analyzed for CD8⁺PKH-PE⁺ cells by flow cytometry. These experiments (Figure 2b) showed that in control animals (no injection of Tregs), the number of CD8⁺PKH-PE⁺ T cells is reduced after application of TNCB. In contrast, injection of wt Tregs suppressed this effect. Animals injected with CD39^{-/-} Tregs showed a decreased number of CD8⁺PKH-PE⁺ T cells, similar to controls. Moreover, we analyzed the ear skin, spleens, and blood of sensitized animals for presence of PKH-PE⁺CD8⁺ T cells (see Supplementary Figure S2 online). Here we found increased frequencies of PKH-PE⁺CD8⁺ T cells after sensitization in skin. Injection of wt Tregs prevented this accumulation, whereas CD39^{-/-} Tregs failed to suppress the accumulation of the PKH-PE⁺ cells at the site of sensitization. In spleens only insignificant changes in the frequencies of PKH-PE⁺ cells in response to sensitization were recorded, and in blood we detected only slightly increased numbers of PKH-PE⁺ cells (see Supplementary Figure S2c). Therefore, we hypothesize that down-regulation of CD62L expression in LN CD8⁺ T cells and their accumulation in skin after hapten application is regulated by Tregs.

Degradation of extracellular ATP by Tregs down-regulates IFN- γ secretion by CD8⁺ T cells

To characterize the effects of Treg-mediated ATP degradation on CD8⁺ T cells, we cultivated CD8⁺ T cells from LNs of the differently treated mice with 2,4,6-trinitrobenzenesulfonic acid solution (TNBS)-pulsed DCs. After 48 hours, tissue culture supernatants were analyzed for IL-10 and IFN- γ . Here we found elevated concentrations of IFN- γ in supernatants of CD8⁺ T cell from sensitized mice and in mice injected with CD39^{-/-} Tregs (Figure 3a) before sensitization. In contrast, injection of wt Tregs decreased secretion of IFN- γ by CD8⁺ T cells. However, secretion of IL-10 remained unaltered in all groups. Therefore, we concluded that degradation of extra-cellular ATP by wt Tregs down-regulates production of IFN- γ by CD8⁺ T cells directly after sensitization.

To further investigate whether CD62L, which has been shown to induce proinflammatory signaling events in leukocytes, is instrumental for this modulatory effect, CD8⁺ T cells isolated from sensitized mice were co-cultured with hapten-pulsed DCs in presence of anti-CD62L antibodies. After 48 hours, tissue culture supernatants were analyzed for IFN- γ . We found decreased concentrations of IFN- γ in cultures treated with anti-CD62L versus with isotype controls or untreated samples (Figure 3b). Thus, in addition to serving as an adhesion molecule, CD62L is involved in regulating IFN- γ production of CD8⁺ T cells.

ATP-induced shedding of CD62L is prevented by Tregs

Our data indicate that Tregs modulate expression of CD62L on CD8⁺ T cells by degrading extracellular ATP. To test this hypothesis in detail, we incubated freshly isolated CD8⁺ LN T cells with graded doses of ATP and found a dose-dependent down-regulation of CD62L expression in CD8⁺ T cells by ATP (Figure 4a). CD62L expression by CD4⁺ T cells was less sensitive to ATP treatment. To further confirm involvement of ATP and its receptor P₂X₇, we

added either the ATP-degrading enzyme 5'-ectonucleotidase (i.e., ATPase) or P₂X₇ antagonist (i.e., KN-62) to the cultures (Figure 4b). In these experiments, the down-regulatory effect of ATP on CD62L expression was abolished.

To analyze whether Tregs are able to prevent ATP-mediated down-regulation of CD62L, wt Tregs were added in graded doses to tissue cultures of CD8⁺ T cells supplemented with ATP. Here, wt Tregs abolished the reduction of surface expression of CD62L by ATP in a dose-dependent manner (Figure 4c). The maximal reduction of CD62L expression in CD8⁺ T cells was obtained at a Treg-to-CD8 ratio of 1:4. In contrast, CD39^{-/-} Tregs (in the same 1:4 ratio) (Figure 4c, right panel) were unable to prevent this ATP-induced effect, supporting our notion that extracellular ATP and its degradation by Tregs is an important modulator of CD62L expression. To test whether haptens are able to induce extracellular ATP directly in LNs, we applied TNBS to LN cell cultures and analyzed the content of ATP in supernatants 2 hours later. Figure 4d shows elevated levels of extracellular ATP after treatment of LN cells with different doses of TNBS. Higher doses of TNBS lead to increased apoptosis and were therefore not used further (data not shown). Moreover, when using TNBS at 50 µmol/L, the ATP-inducing effect was abrogated after adding wt Tregs (Treg:LN cells in a 1:4 ratio) to the cultures as opposed to CD39^{-/-} Tregs, which failed to ameliorate ATP content in culture supernatants. In these Treg-supplemented cultures we also analyzed expression of CD62L and found increased frequencies of CD8⁺CD62L⁺ cells in co-cultures with wt Tregs (Figure 4d). In contrast, CD39^{-/-} Tregs failed to maintain CD62L expression on CD8⁺ T cells.

Because CD62L can be removed from the surface of cells by proteolytic cleavage (shedding), we tested the tissue culture supernatants of LN cultures supplemented with ATP for soluble CD62L. We detected sizable amounts of soluble CD62L in the tissue culture supernatant of ATP-treated T cells, whereas no soluble CD62L was detectable in controls (Figure 4e). The amount of soluble CD62L was substantially reduced after adding wt Tregs to the cultures. Thus, these data indicate that ATP induces shedding of CD62L and that Tregs abolish this shedding of CD62L by degrading extracellular ATP. Because our experiments focused on the surface expression and shedding of CD62L and not on mRNA data, we cannot exclude that CD62L is also regulated on a transcriptional level.

Tregs regulate ADAM17 expression by CD39-induced ATP degradation

As for the mechanism of the ATP-induced CD62L shedding, we investigated whether ADAM17 is involved in cleavage of surface-bound CD62L. We incubated isolated CD8⁺ LN cells with graded doses of ATP and found up-regulation of ADAM17 expression and increased phosphorylation of extracellular signal-regulated kinase (Figure 5a). Moreover, down-regulation of CD62L by ATP in CD8⁺ T cells was blocked by the ADAM17 antagonist TAPI-1 to cell cultures (Figure 5b). These results showed that ATP induces increased expression of ADAM17, leading to cleavage of CD62L.

To prove this mode of action in vivo, animals were intravenously injected with wt or CD39^{-/-} Tregs and were subjected to sensitization. When analyzing ADAM17 expression in CD8⁺ T cells isolated from skin-draining LNs, we found increased amounts of ADAM17 after TNCB treatment (Figure 5c). Injection of wt Tregs ameliorated induction of ADAM17

expression. In contrast, CD39^{-/-} Tregs were unable to suppress ADAM17 up-regulation after sensitization. However, these data rely on Western blots and do not exclude the possibility that ADAM17 may also be regulated by Tregs on a transcriptional level or that Tregs are able to affect the stability and degradation of ADAM17 in CD8⁺ T cells. However, even with the exact mechanism being unclear, the results support our notion that Tregs by means of ATP degradation are able to down-regulate ADAM17 expression in T cells during CHS reactions.

DISCUSSION

Trafficking of T cells is a tightly regulated process involving the coordinated action of adhesion molecules, chemokines, and their respective receptors (Grailer et al., 2009). CD62L has an important role in guiding T cells to LNs, because high endothelial venules constitutively express CD62L ligand(s) (Venturi et al., 2003; Yang et al., 2011). Therefore, regulation of CD62L expression by T cells provides a mechanism to influence their migration into and out of lymphatic organs and to regulate inflammatory reactions (Harp et al., 2010; Savage et al., 2002; Subramanian et al., 2012). When we analyzed CD62L expression as early as 3 hours after sensitization, we found that Tregs were able to maintain CD62L expression by CD8⁺ T cells, and we recorded elevated frequencies of CD8⁺ T cells in the respective LNs. Other molecules classically involved in regulating T-cell trafficking from and to LNs, such as S1P receptor-1 and CCR7 (Maeda et al., 2007; Shioh et al., 2006), were not changed.

Given that CD62L functions in recruitment of lymphocytes to LNs, we hypothesized that prolonged expression of CD62L prevents egress of T cells from the LNs or shifts their trafficking from peripheral tissues toward LNs (Chung et al., 1986; Shimizu et al., 2013; Zhi et al., 2011). As a consequence, a rather down-regulated immune response is observed.

In the sensitization phase, CD62L expression and, thus, the keeping of T cells in the draining LNs is mandatory to facilitate the effective priming by incoming skin DCs. However, after application of haptens, DCs arrive earliest after 6 hours in the draining LN (Tomura et al., 2014). At this time point, however, elevated ATP concentrations have already ceased in the LN. Thus, under normal conditions, the early peak of ATP leading to egress of T cells may not interfere with priming taking place later on. Because 3 hours after sensitization no antigen-bearing DCs are present in the LN, one can only speculate on the effects of an antigen-unspecific activating process induced by ATP. For instance, the ATP-induced activation of T cells may drive the cells out of the LN to provide space for skin-derived T cells and/or DCs. Moreover, the antigen-unspecifically activated T cells may provide cytokines important to promoting the subsequent antigen-specific CHS response in peripheral tissues.

We observed accumulation of CD8⁺ T cells in the skin as soon as 2 hours after hapten application. Therefore, activated CD8⁺ T cells and the cytokines produced thereof may be present in the tissue to pave the way for further inflammation. We also directly used CD39^{-/-} animals for sensitization experiments and analyzed expression of CD62L by CD8⁺ T cells 2 hours after sensitization compared with wt controls (see Supplementary Figure S3

online). Here no apparent differences in CD62L down-regulation between the mouse strains were recorded. This may be explained by the fact that CD39^{-/-} animals lack ATP-degrading enzymes, which led us to expect increased down-regulation of CD62L in the first place. However, the constantly elevated ATP levels render the P₂X₇ ATP receptors hyporesponsive (Mizumoto et al., 2003). Therefore, ATP-driven activation of lymphocytes is impaired in CD39^{-/-} animals, and the expected increased shedding of CD62L is counterbalanced by decreased sensitivity of the cells to ATP. Both effects may be active in CD39^{-/-} mice, and thus no significant changes in the frequency of CD62L⁺ cells in LNs would be the result.

CD62L binds to protein kinase C, interacts via α -actinin, and activates signaling via the GTPase Ras (Brenner et al., 1997; Chen et al., 2007). Because actin remodeling and Ras signaling regulates cell motility, it is conceivable that extended expression of CD62L may block the locomotion of T cells in the LNs. Moreover, signals induced by CD62L increase expression of CCR7 (Moore et al., 2011), which is normally down-regulated in lymphocytes before LN egress. Therefore, prolonged surface expression of CD62L by LN-residing leukocytes can contribute to up-regulation of CCR7 and may stall CD62L⁺ T cells in LNs.

CD8⁺ T cells have to be induced for IFN- γ production for successful sensitization, because in the absence of IFN- γ ⁺ CD8⁺ T cell sensitization is ameliorated, and regulatory CD4⁺ T cells prevail in LNs (Okazaki et al., 2002; Xu et al., 1996). In this regard, we show decreased frequency of IFN- γ ⁺ T cells in mice injected with Tregs before sensitization, which depends on CD39 expression by Tregs. This may be explained by the lack of production of immunosuppressive adenosine by CD39^{-/-} Tregs. However, engagement of CD62L in vitro by antibodies also leads to decreased IFN- γ production in DC–T-cell cultures, suggesting a role for CD62L in modulating IFN- γ secretion. The exact means are not clear, but our results are in agreement with observations showing that CD62L engagement induces rather stimulatory pathways, that is, mitogen-activated protein kinase and c-Jun N-terminal kinase signaling (Brenner et al., 1997). In our scenario we can only speculate that the initial expression of CD62L is mandatory to induce priming and IFN- γ production of T cells; however, prolonged CD62L engagement, as induced by antibody treatment or by tempering with the egress of T cells from LNs, may reduce their IFN- γ production.

During T-cell activation, CD62L is down-regulated by shedding of the L-selectin ectodomain. The primary “shed-dase” is ADAM17 (Li et al., 2006; Venturi et al., 2003). As for the mechanism of reduced CD62L shedding during CHS reactions after Treg injection, we found that ADAM17 activity is regulated by extracellular ATP (Foster et al., 2013; Gu et al., 1998; Scheuplein et al., 2009). ATP is released into extra-cellular space by keratinocytes after application of haptens (Onami et al., 2014), and it functions as a crucial activator of skin DCs and T cells in LNs (Burnstock et al., 2012; Ohara et al., 2010; Weber et al., 2010). ATP can engage P₂X₇ receptors and stimulate extracellular signal-regulated kinase–dependent signaling, a major pathway of ADAM17 activation (Díaz-Rodríguez et al., 2002; Fan et al., 1999). Therefore, ADAM17 activity can be up-regulated by extra-cellular ATP. Because Tregs express ATP-degrading ectonucleotidases CD39 and CD73 (Mizumoto et al., 2002), they are able to dampen ADAM17 activity in effector cells.

The importance of ATP release during CHS responses was recognized by attempts to assess the sensitizing potency of newly developed drugs by testing their capacity to induce release of ATP in keratinocyte cultures (Mizumoto et al., 2003). That is, the higher the sensitizing potency of a chemical, the more ATP was released. In turn, these results support our notion that modulation of the ATP content in skin by either cells and/or drugs may be a tool to modulate inflammatory skin reactions. Generally, extracellular ATP concentrations are kept low in tissues ($\mu\text{mol/L}$ range) (Adinolfi et al., 2005). Therefore, even small changes of extracellular ATP concentrations can be recognized by “sentinel” cells, such as Tregs. Thus, the regulation of free ATP by Tregs might be a fine-tuned mechanism to control inflammation.

Apart from inflammasome activation, ATP also activates Tregs, as indicated by increased IL-10 production, augmented CD69 expression, and increased suppressive functions (Ring et al., 2010a). Therefore, even scattered numbers of Tregs in skin and LNs may at first be activated by extracellular ATP, and once activated, the cells may produce several inhibitory means (not just production of adenosine via ATP degradation). This concerted action can then successfully suppress immune reactions.

Beyond inflammatory reactions such as CHS, ATP degradation may also be one mechanism by which Tregs are involved in maintaining tolerance in the peripheral tissue. For instance, after apoptosis or limited tissues destruction, only trace amounts of ATP are released into the tissue/lymphoid environment. Henceforth, only immunosuppressive cells such as Tregs are activated, because they become already activated by low concentrations of ATP (Ring et al., 2009). Thus, immune suppression will prevail and (auto-)immunity will be prevented. In contrast, in a severe infection and during massive tissue destruction, that is, when a potent immune reaction is needed, extracellular ATP increases massively, and its proinflammatory actions, mediated by inflammasome activation, rule over the degradation to adenosine by Tregs (Di Virgilio, 2007).

In summary, our data show that Tregs are able to interfere with the trafficking of CD8⁺ T cells in and out of skin-draining LNs by suppressing the ATP-induced shedding of CD62L. Thus, detoxifying tissues from extracellular ATP Tregs might ameliorate inflammatory reactions.

MATERIALS AND METHODS

A detailed description is available in the Supplementary Materials online.

Mice and reagents

C57BL/6N mice were from Janvier Labs (Saint Berthevin, France). CD39-deficient (CD39^{-/-}) mice were bred under specific pathogen free conditions in the central animal facility of the University of Heidelberg. All experiments were approved by the state of Baden Württemberg.

Complete medium was RPMI with 10% fetal calf serum, Pen/Strep, HEPES, and glutamine (GE-Healthcare, Freiburg, Germany). Standard chemicals were purchased from Carl Roth,

Karlsruhe, Germany. The P₂X₇ antagonist KN-62 was from Tocris (Bio-Techne, Wiesbaden, Germany) and was used at a final dilution of 10 µmol/L (Ring et al., 2011). The ADAM17 antagonist TAPI-1 (10-µmol/L final dilution) was from EMD (Merk-Millipore, Schwalbach, Germany). The purified ATPase was purchased from Roche (Mannheim, Germany) and used in a concentration of 2 U (Yip et al., 2009).

Induction of CHS

Mice were sensitized by painting 15 µl of 1% TNCB (Sigma-Aldrich, Germany) dissolved in acetone/olive oil (4:1) or 15 µl of solvent on the abdomen or ears.

Preparation of single-cell suspensions, purification of T-cell subsets, and labeling

CD4⁺CD25⁺ T cells (Tregs) and CD4⁺ and CD8⁺ T cells were prepared from a single-cell suspension of spleens and LNs using commercially available enrichment kits (Miltenyi, Bergisch Gladbach, Germany) according to the manufacturer's protocol. Cell labeling with PKH-PE was performed according to the manufacturer's protocol (PKH26 red fluorescent cell linker kit; Sigma-Aldrich, Taufkirchen, Germany). 3 × 10⁶ cells/mouse (Tregs, CD8⁺ T cells) were injected in 100 µl of phosphate buffered saline intravenously into the tail vein (Ring et al., 2010a, 2010b). Numbers of PKH-PE⁺ cells were analyzed by flow cytometry.

Flow cytometry and functional assays

Cell suspensions were stained with the following antibodies: CD4-FITC (1 µg/ml), CD8-APC (0.4 µg/ml), CD62L-PE (0.4 µg/ml), CD69-PE (0.4 µg/ml) (all from eBioscience, Frankfurt, Germany), and CD49e-PE (0.4 µg/ml) (Biolegend, Fell, Germany) and analyzed by flow cytometry. For detection of ATP, draining LNs were removed from sensitized mice, disrupted with tweezers, and assayed for ATP (ATPlite, Perkin Elmer). For DC-T-cell co-cultures, DCs were prepared as previously described (Ring et al., 2010b). Co-cultures of 1 × 10⁶ T cells and 1 × 10⁵ DCs or 5 × 10⁴ DCs were set up in 1 ml complete medium, and tissue culture supernatant was assessed for IL-10 and IFN-γ by ELISA (Ready-SET-Go, eBioscience, Frankfurt, Germany).

Western blotting

Cell suspensions were lysed in phosphate buffered saline/1% Triton X-100/protease inhibitor cocktail (Roche, Mannheim, Germany). 10 µg of was loaded on 7.5% standard polyacrylamide gels and transferred onto polyvinylidene fluoride PVDF membranes (all reagents from Peqlab, Erlangen, Germany). ADAM17 (Santa Cruz Biotech, Heidelberg, Germany) antibodies were diluted 1:500, and extracellular signal-regulated kinase/phosphorylated extracellular signal-regulated kinase antibodies (Cell Signaling, Frankfurt, Germany) were used at 1:600. Staining with anti-actin (1:500; Santa Cruz Biotechnology, Heidelberg, Germany) served as loading control. Blots were visualized by horseradish peroxidase-labeled secondary reagents (Dianova, Hamburg, Germany) and chemiluminescence (ECL; GE-Healthcare, Freiburg, Germany).

Statistics

For testing of significance, GraphPad Prism software (Statcon, Witzenhausen, Germany) was used.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ATP	adenosine triphosphate
CHS	contact hypersensitivity
DC	dendritic cell
LN	lymph node
TNBS	2,4,6-trinitrobenzenesulfonic acid solution
TNCB	2,4,6-trinitrochlorobenzene
Treg	regulatory T cell
WT	wild type

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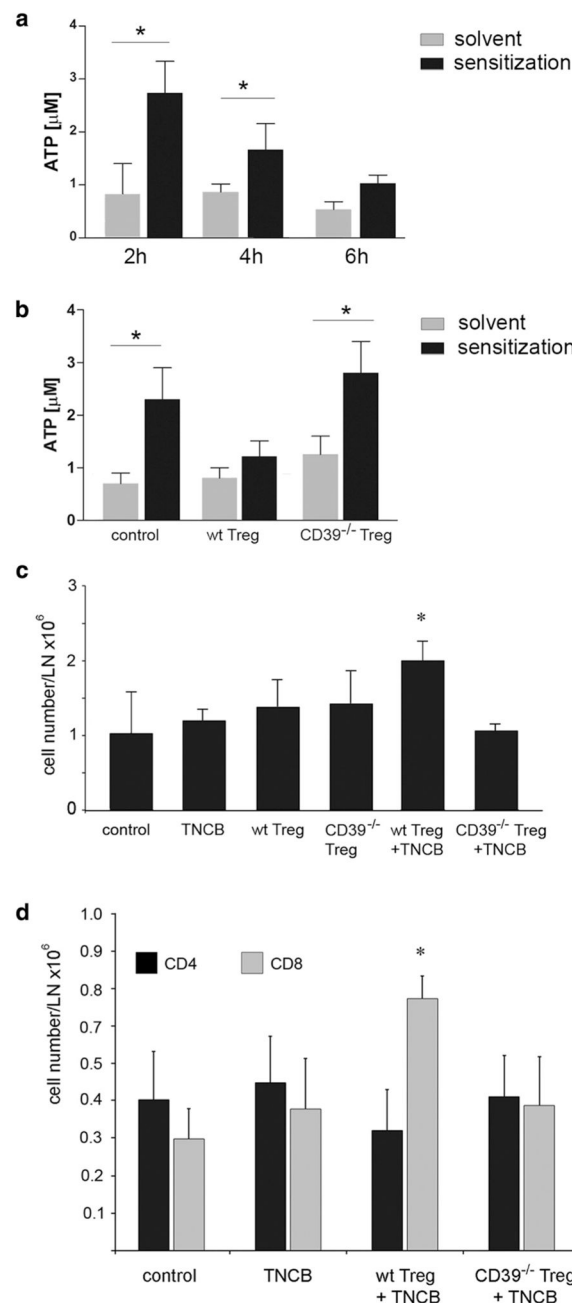


Figure 1. Tregs degrade extracellular ATP and induce accumulation of CD8^+ T cells in draining LNs

(a) LNs were isolated from mice sensitized at the abdomen (15 μl ; 1% TNCB) and killed after indicated time points. Single-cell suspensions were prepared by disrupting the LNs with tweezers, and ATP was measured immediately $*P < 0.05$ (t test). (b) Mice were intravenously injected with 3×10^6 Treg and sensitized with TNCB at the abdomen. After 2 hours, LNs were treated as in a. $*P < 0.05$ (t test). (c) Mice were treated with Tregs and sensitized at the ears (10 μl ; 1% TNCB). After 3 hours, ear-draining LNs were removed, and cells were counted. $*P < 0.05$ (analysis of variance). (d) Cells prepared as in c were

analyzed by FACS. $*P < 0.05$ (analysis of variance). All experiments were carried out five times. ATP, adenosine triphosphate; h, hours; LN, lymph node; M, mol/L; TNCB, 2,4,6-trinitrochlorobenzene; Treg, regulatory T cell; wt, wild type.

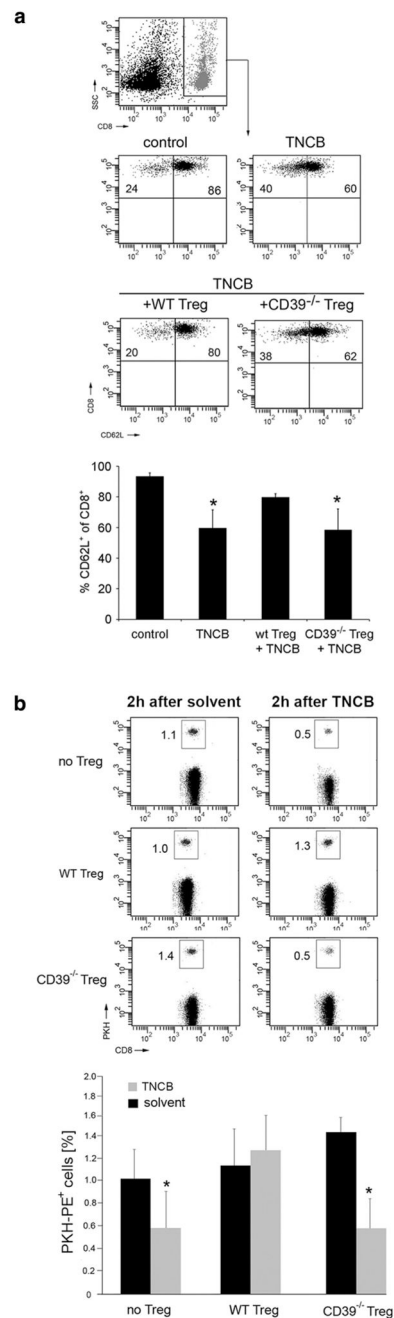


Figure 2. CD39^{-/-} Tregs fail to block down-regulation of CD62L in CD8⁺ T cells and suppress egress of CD8⁺ T cells

(a) LN cells from mice treated with 3×10^6 Tregs as indicated and sensitized at the ears (10 μ l; 1% TNCB) were analyzed for CD8 and CD62L expression by flow cytometry. Numbers in quadrants depict percentages of CD62L⁺ cells among CD8⁺ cells. Bar graph depicts the mean values of five independent experiments. * $P < 0.05$ (t test). (b) A total of 3×10^6 CD8⁺PKH-PE⁺ T cells were intravenously injected into recipients, followed by intravenous injection of Tregs and sensitization at the ears. 2 hours later, CD8⁺PKH-PE⁺ T cells were analyzed in the LNs. Numbers reflects percentages of PKH-PE⁺ cells among all CD8⁺ T

cells. Bar graph shows mean values of four independent experiments. $*P < 0.05$ (t test). h, hours; TNCB, 2,4,6-trinitrochlorobenzene; Treg, regulatory T cell; WT, wild type.

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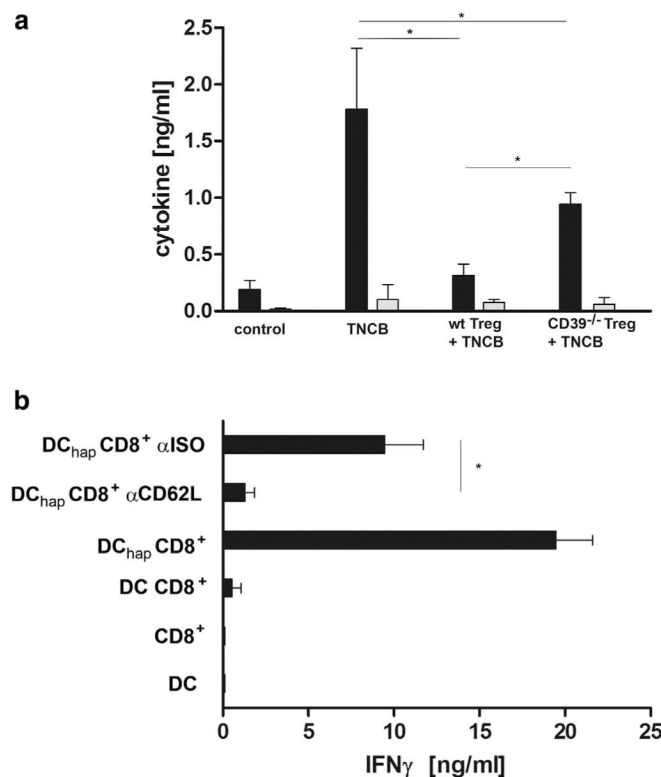


Figure 3. Degradation of extracellular ATP by Tregs down-regulates IFN- γ secretion by CD8⁺ T cells

(a) Animals ($n = 4$) were injected with Tregs and sensitized with TNCB. After 3 hours, CD8⁺ LN cells were prepared, and 1×10^6 T cells were co-cultured with 5×10^4 hapten-pulsed DCs in 1 ml complete medium. Tissue culture supernatants were tested for IL-10 and IFN- γ by commercial ELISA after 48 hours. Shown are means $\pm$ standard deviations of four independent experiments. $*P < 0.05$ (t test). (b) 1×10^6 CD8⁺ T cells from sensitized animals ($n = 4$) were isolated and restimulated with 5×10^5 hapten-pulsed DC_{hap} or control DCs in 1 ml of complete medium. To some cultures anti-CD62L or isotype antibodies (10 μ g/ml) were added as indicated. Tissue culture supernatant was tested for IFN- γ after 48 hours. Shown are means $\pm$ standard deviations of four independent experiments. $*P < 0.05$ (t test). ATP, adenosine triphosphate; DC, dendritic cell; h, hours; LN, lymph node; TNCB, 2,4,6-trinitrochlorobenzene; Treg, regulatory T cell; wt, wild type.

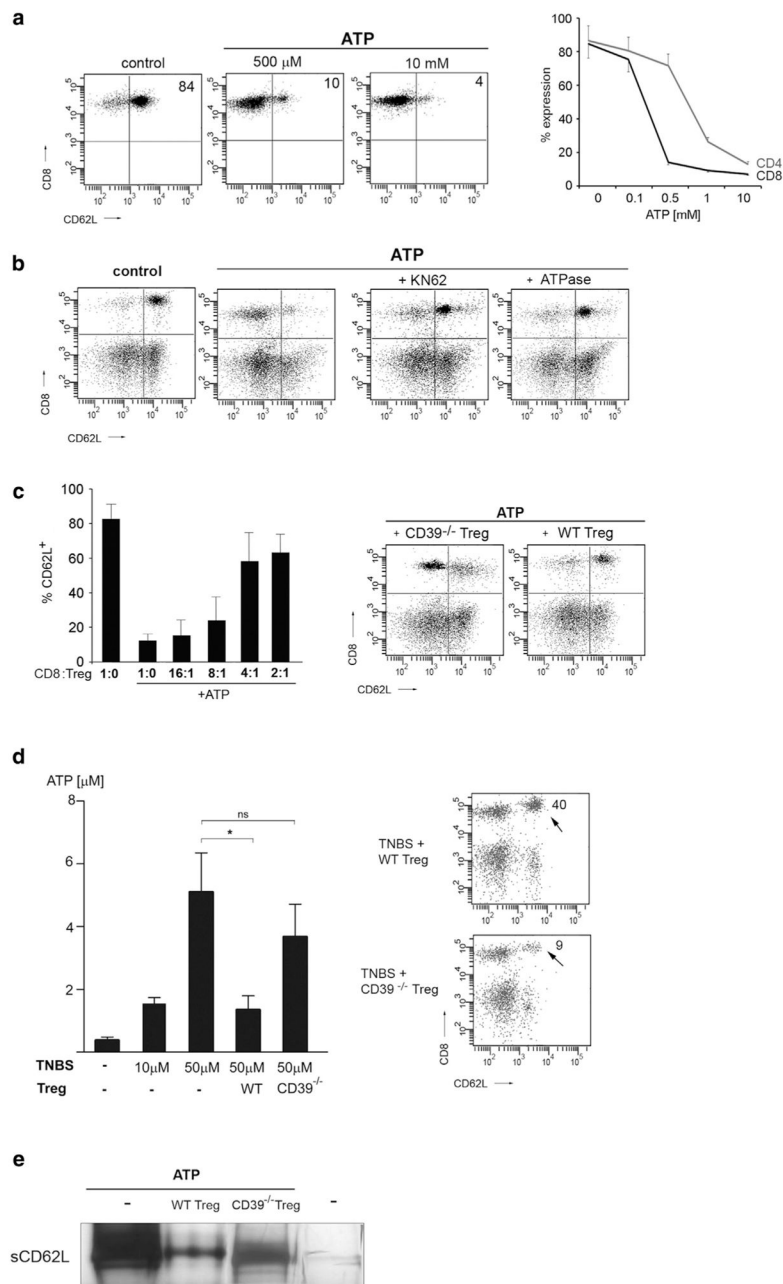


Figure 4. ATP induces shedding of CD62L that is blocked by Tregs

(a) CD8⁺ cells were cultivated with ATP (3 hours) and CD62L expression was determined. Shown are means $\pm$ standard deviations of four experiments. (b) LN cells were cultured with 500 μ mol/L ATP, Tregs, KN62 (10 μ mol/L), or ATPase (2U/ml) (3 hours), and CD8/CD62L expression was analyzed. (c) Ratios of CD8⁺ T cells and Tregs (left) and LN cells and Tregs (4:1; right) were cultivated with 500 μ mol/L of ATP (3 hours), and CD62L⁺ expression was analyzed. Means $\pm$ standard deviations of four experiments are shown. (d) LN cells were incubated with TNBS and Tregs as indicated, and ATP was determined. Dot plots show CD8 and CD62L expression, and numbers indicate percentage of CD62L⁺CD8⁺ cells. (e) Tissue

culture supernatants of 5×10^6 control and/or ATP-treated LN cells were immunoprecipitated with anti-CD62L antibodies followed by SDS-PAGE. ATP, adenosine triphosphate; LN, lymph node; M, mol/L; ns, not significant; TNCB, 2,4,6-trinitrochlorobenzene; Treg, regulatory T cell; WT, wild type.

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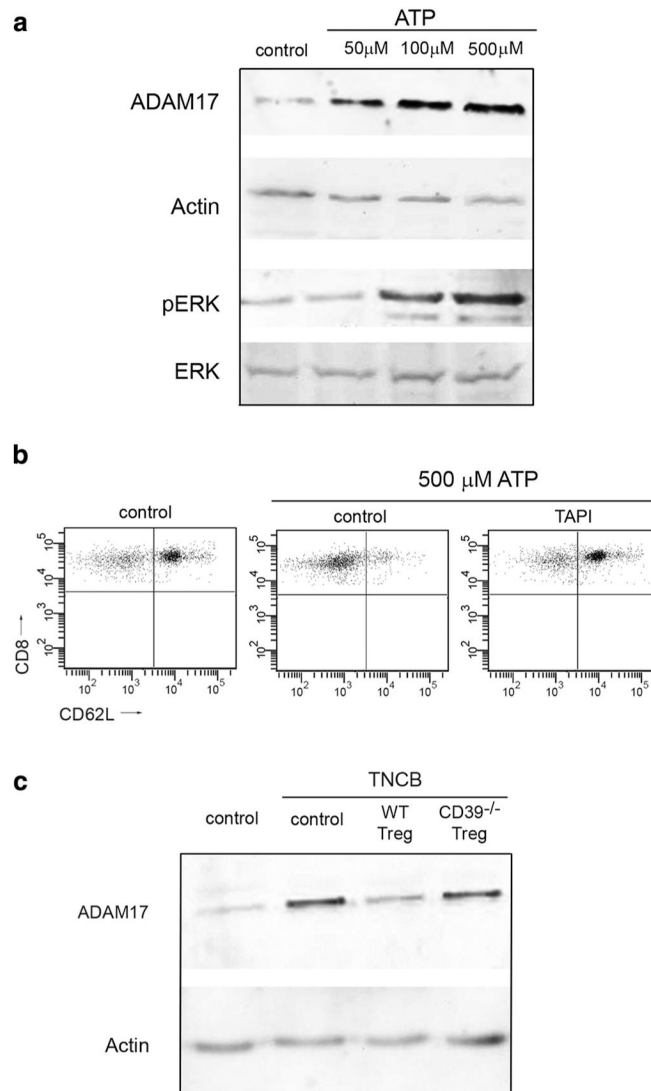


Figure 5. Sensitization induces phosphorylation of ERK and increased activation of ADAM17
(a) CD8⁺ T cells isolated from LNs were treated with graded doses of ATP for 3 hours as indicated. Thereafter, cell lysate was analyzed for ADAM17, actin, ERK, and pERK expression by Western blot (n = 3; shown is a typical result). **(b)** CD8⁺ T cells isolated from LNs were treated with ATP and the ADAM17 antagonist TAPI for 3 hours as indicated. Thereafter, expression of CD62L by CD8⁺ T cells was analyzed (n = 3). **(c)** Mice were intravenously injected with 3×10^6 Tregs or phosphate buffered saline and sensitized (10 μl; 1% TNCB) at the ear. 3 hours later, CD8⁺ T cells were isolated from LNs, and ADAM17 and actin expressions were determined by Western blot (n = 3; shown is a typical experiment). ATP, adenosine triphosphate; ERK, extracellular signal-regulated kinase; LN, lymph node; M, mol/L; pERK, phosphorylated extracellular signal-regulated kinase; TAPI, N-(R)-[2-(hydroxyaminocarbonyl) methyl]-4-methylpentanoyl-L-naphthylalanyl-L-alanine, 2-aminoethyl amide; TNCB, 2,4,6-trinitrochlorobenzene; WT, wild type.