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CHAPTER 4

CD39 is involved in mediating suppression by *Mycobacterium bovis*
BCG-activated human CD8\(^+\)CD39\(^+\) regulatory T-cells

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Abstract

Regulatory T (Treg) cells can balance normal tissue homeostasis by limiting inflammatory tissue damage, e.g. during pathogen infection, but on the other hand can also limit protective immunity induced during natural infection or following vaccination. Because most studies have focused on the role of CD4\(^+\) Treg cells, relatively little is known about the phenotype and function of CD8\(^+\) Treg cells, particularly in infectious diseases. Here, we describe for the first time the expression of CD39 (E-NTPDase1) on Mycobacterium-activated human CD8\(^+\) T-cells. These CD8\(^+\)CD39\(^+\) T-cells significantly co-expressed the Treg markers CD25, Foxp3, lymphocyte activation gene-3 (LAG-3), and CC chemokine ligand 4 (CCL4), and suppressed the proliferative response of antigen-specific CD4\(^+\) T helper-1 (Th1) cells. Pharmacological or antibody mediated blocking of CD39 function resulted in partial reversal of suppression. These data identify CD39 as a novel marker of human regulatory CD8\(^+\) T-cells and indicate that CD39 is functionally involved in suppression by CD8\(^+\) Treg cells.
**Introduction**

*Mycobacterium tuberculosis* was responsible for 1.4 million deaths in 2010 and is estimated to have infected one-third of the world population [1]. There is extensive evidence suggesting that *M. tuberculosis* strongly modulates the immune response, both innate and adaptive, to infection, with an important role for regulatory T (Treg) cells [2]. In mice, *M. tuberculosis* infection triggers antigen-specific CD4+ Treg cells that delay the priming of effector CD4+ and CD8+ T-cells in the pulmonary lymph nodes (LNs) [3], suppressing the development of CD4+ T helper-1 (Th1) responses that are essential for protective immunity [4]. Thus, these CD4+ Treg cells delay the adequate clearance of the pathogen [5] and promote persisting infection.

*M. tuberculosis* - as well as *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) - have been found to induce CD4+ and CD8+ Treg cells in humans [6-8]. CD4+ and CD8+ Treg cells are enriched in disseminating lepromatous leprosy lesions, and are capable of suppressing CD4+ Th1 responses [9;10]. Naive CD8+CD25+ T-cells can differentiate into CD8+CD25+ Treg cells following antigen encounter [11]. In *M. tuberculosis*-infected macaques, IL-2-expanded CD8+CD25+Foxp3+ Treg cells were found to be present alongside CD4+ effector T-cells *in vivo*, both in the peripheral blood and in the lungs [12]. In human *Mycobacterium*-infected LNs and blood, a CD8+ Treg subset was found expressing lymphocyte activation gene-3 (LAG-3) and CC chemokine ligand 4 (CCL4, macrophage inflammatory protein-1β). These CD8+LAG-3+CCL4+ T-cells could be isolated from BCG-stimulated PBMCs, co-expressed classical Treg markers CD25 and Foxp3, and were able to inhibit Th1 effector cell responses. This could be attributed in part to the secretion of CCL4, which reduced Ca2+ flux early after T-cell receptor triggering [8]. Furthermore, a subset of these CD8+CD25+LAG-3+ T-cells may be restricted by the HLA class-Ib molecule HLA-E, a non-classical HLA class-I family member. These latter T-cells displayed cytotoxic as well as regulatory activity *in vitro*, lysing target cells only in the presence of specific peptide, whereas their regulatory function involved membrane-bound TGF-β [13]. Despite these recent findings, the current knowledge about CD8+ Treg cell phenotypes and functions is limited and fragmentary when compared with CD4+ Treg cells [6;14].
CD39 (E-NTPDase1), the prototype of the mammalian ectonucleoside triphosphate diphosphohydrolase family, hydrolyzes pericellular adenosine triphosphate (ATP) to adenosine monophosphate [15]. CD4+ Treg cells can express CD39 and their suppressive function is confined to the CD39+CD25+Foxp3+ subset [16;17]. Increased in vitro expansion of CD39+ regulatory CD4+ T-cells was found after M. tuberculosis specific ‘region of difference (RD)-1’ protein stimulation in patients with active tuberculosis (TB) compared with healthy donors. Moreover, depletion of CD25+CD39+ T-cells from PBMCs of TB-patients increased M. tuberculosis specific IFNγ production [18]. CD39 expression on CD8+CD25+Foxp3+ Treg cells has been described in simian immunodeficiency virus infection [19], but has not been studied in mycobacterial infections in humans.

ATP and other nucleotides can induce an array of intercellular signals, depending on the receptor subtype and pathways involved [20]. In damaged tissues, ATP is released in high concentrations, and functions as chemoattractant, generating a broad spectrum of pro-inflammatory responses [21]. ATP can also trigger mycobacterial killing in infected macrophages [22-24], can stimulate phagosome-lysosome fusion through P2X7 receptor activation [25], and can drive Th17 cell differentiation in the murine lamina propria [26]. In a study focusing on the novel M. tuberculosis vaccine MVA85A, a drop in extracellular ATP consumption by PBMCs from subjects 2 weeks after vaccination corresponded with a decrease in CD4+CD39+ Treg cells and a concomitant increase in the co-production of IL-17 and IFNγ by CD4+ T-cells [27]. Further hydrolysis of adenosine monophosphate by ecto-5’-nucleotidase (CD73) generates extracellular adenosine [20], which modulates inflammatory tissue damage, among others by inhibiting T-cell activation and multiple T-cell effector functions through A2A receptor-mediated signaling [28].

BCG, the only currently available vaccine for TB, fails to protect adults adequately and consistently from pulmonary TB [29], and part of this deficiency may be explained by induction of Treg cells by the BCG-vaccine [7;30;31]. In this study, we have used live BCG to activate CD8+ Treg cells, and demonstrate that these CD8+ T-cells express CD39, and co-express the well-known Treg markers CD25, Foxp3, LAG-3, and CCL4. Finally, we describe involvement of CD39 in suppression by CD8+ T-cells.
Materials and Methods

Blood samples. Anonymous buffy coats were collected from healthy adult blood bank donors that had signed consent for scientific use of blood products. PBMCs were isolated by density centrifugation and cryopreserved in fetal calf serum supplemented medium. Cells were counted using the CASY cell counter (Roche, Woerden, the Netherlands). Recognition of mycobacterial PPD was tested by assessing IFNγ production in vitro. PBMCs were stimulated with 5 μg/mL PPD (Statens Serum Institute, Copenhagen, Denmark) for 6 days and supernatants were tested in IFNγ ELISA (U-CyTech, Utrecht, the Netherlands). Positivity was defined as IFNγ production ≥ 150 pg/mL.

Cell cultures. PBMCs were cultured in Iscove’s modified Dulbecco’s medium (Life Technologies-Invitrogen, Bleiswijk, the Netherlands) supplemented with 10% pooled human serum. BCG (Pasteur) was grown in 7H9 plus ADC, frozen in 25% glycerol and stored at -80°C. Before use, bacteria were thawed and washed in PBS/0.05% Tween 80 (Sigma-Aldrich, Zwijndrecht, the Netherlands). Infections were done at an MOI of 1.5. IL-2 (25 U/mL; Proleukin; Novartis Pharmaceuticals UK Ltd., Horsham, UK) was added after 6 days of culture.

Restimulation of cell lines was done in 96-well round-bottom plates (1 x 10^5 cells/w) with αCD3/CD28 beads (Dynabeads Human T-activator, Life Technologies-Invitrogen), IL-2 (50 U/mL), IL-7, and IL-15 (both 5 ng/mL, Peprotech, Rocky Hill, NJ, USA); pooled, irradiated (30 Gy) PBMCs were added as feeders. Cells were maintained in IL-2 (100 U/mL).

FACS analysis. T-cell lines were incubated overnight with αCD3/28 beads, for the last 16h Brefeldin A (3 μg/mL, Sigma-Aldrich) was added. Following the labeling with the violet live/dead stain (VIVID, Invitrogen), the following antibodies were used for surface staining: CD3-PE-Texas Red, CD14- and CD19-Pacific Blue (all Invitrogen), CD4-PeCy7, CD8-HorizonV500, CD73-PerCPCy5.5 (all BD Biosciences, Eerembodegem, Belgium), and CD39-PE (Biolegend, London, UK).

For intracellular staining, we used Intrastain reagents (Dako-Cytomation, Heverlee,
Belgium) according to the instructions of the manufacturer and the following antibodies: CCL4-FITC (R&D Systems, Abingdon, UK), Foxp3-Alexa Fluor 700 (eBioscience, Hatfield, UK), LAG-3-atto 647N (ENZO Life Sciences, Antwerp, Belgium), CD25-allophycocyanin-H7 (BD Biosciences), and IL-10-allophycocyanin (Miltenyi Biotec, Teterow, Germany). Samples were acquired on a BD LSRSpectra using FACSDiva software (version 6.2, BD Biosciences) and analyzed using FlowJo software (version 9.5.3, Treestar, Ashland, OR, USA).

Cell sorting. CD8+ cells were enriched by positive selection using magnetic beads (MACS, Miltenyi Biotec). Cells were fluorescence-activated cell sorted (FACS) by BD FACSAriaIII cell sorter using CD39-PE (Biolegend). Purity of all cell sorts was ≥ 97% as assessed by flow cytometry.

Suppression assays. Cell lines were tested for their capacity to inhibit proliferation of a Th1 responder clone (Rp15 1-1) and its cognate M. tuberculosis hsp65 p3–13 peptide, presented by HLA-DR3 positive, irradiated (20 Gy) PBMCs as APCs in a co-culture assay that has been previously reported [8;32]. Proliferation was measured after 3 days of co-culture by addition of 0.5 μCi/well and (3H)thymidine incorporation was assessed after 18 h. Values represent means from triplicate wells.

For the CFSE-labeling assay, the Rp15 1-1 Th1-responder clone was labeled with 0.005 μM of CFSE and the irrelevant, isogenic T-cell clone (R2F10), with different peptide specificity and HLA-DR2 restriction, with 0.5 μM of CFSE, similar in design to previously described [13]. After 16h of co-culture with 5x10^4 CD8+CD39+ T-cells, the p3-13 peptide (50 ng/mL) and HLA-DR3 positive APCs, cells were harvested and stained for CD3, CD4, and CD8. CFSE intensity was measured on a BD LSRSpectra using FACSDiva software and analyzed using FlowJo software.

Blocking experiments. ARL 67156 trisodium salt hydrate (Sigma-Aldrich) was added to the well in 150 μM and daily during the 3 days of co-culture. Anti-CD39 monoclonal antibody BY40/OREG-103 (Orega Biotech, Ecully, France) was added to the well at the first day of co-culture at a final concentration of 10 μg/mL, as was the IgG1 isotype control (R&D Systems). Values represent mean ± SE from triplicate wells.
Suppressive capacity of CD8^+CD39^+ T-cells was independent of original proliferation of the Th1 clone, as tested by reducing the cognate peptide concentration in the co-culture assays. Reversal of suppression was calculated in proportion to original clone proliferation in the absence of Treg cells, since ARL and anti-CD39 monoclonal antibody interfered directly with Th1 clone proliferation signals in the CD39 pathway, as demonstrated by reduced (3H)thymidine incorporation after 3 days. Percentage blocking was calculated after natural logarithmic transformation, and inhibition of proliferation in the presence and absence of blocking agents was calculated and expressed as percentage [8]. Raw data can be provided per request.

Statistical analyses. Mann-Whitney tests and Wilcoxon signed-rank tests were performed using GraphPad Prism (version 5, GraphPad Software, San Diego, CA, USA) and SPSS statistical software (version 20, SPSS IBM, Armonk, NY, USA).

Results

Live BCG activates CD39 expression on CD4^+ and CD8^+ T-cells
We isolated PBMCs from healthy human donors and stimulated these PBMCs with live BCG [8]. Flow cytometric analysis was performed after 6 days (the full gating strategy is shown in Supplementary figure S1, in compliance with the most recent MIATA guidelines [33]). CD39 was expressed on T-cells of donors that responded to purified protein derivative (PPD) in vitro, but not on T-cells from PPD non-responsive donors or on unstimulated cell lines (figure 1). CD39 and CD25 were co-expressed on both CD4^+ and CD8^+ T-cells from PPD-responsive donors after stimulation with live BCG (figure 1).
Figure 1. Live *Mycobacterium bovis* BCG activates CD4⁺CD39⁺ and CD8⁺CD39⁺ T-cells. Flow cytometric analysis of CD39 and CD25 expression on CD8⁺ and CD4⁺ T-cells of *in vitro* PPD-responders and non-responders 6 days after live *M. bovis* BCG stimulation. Gating was performed as shown in Supplementary figure S1. Data are representative of five PPD-responders and five non-responders.

**CD8⁺CD39⁺ T-cells specifically co-express Treg-cell markers**

CD8⁺CD39⁺ T-cells co-expressed the Treg-cell markers CD25, LAG-3, CCL4, and Foxp3 (figure 2A). There was no co-expression of CD39 with CD73, consistent with other studies on human Treg cells [34] (data not shown). Gating CD8⁺ T-cells on Foxp3 and LAG-3 [8] demonstrated that the majority of these cells also expressed CD39 as well as CD25 (figure 2B). Boolean gating was used to analyze expression of multiple markers on single cells (figure 2C). A significantly higher percentage of CD3⁺CD8⁺CD4⁺ T-cells from PPD-responders expressed CD39 as compared with non-responders (p = 0.03; Mann-Whitney test). The CD8⁺CD39⁺ T-cells from responders significantly co-expressed CD25, LAG-3, CCL4, and/or Foxp3 as compared with non-responders and this difference was highly significant for the CD8⁺ T-cells that were CD39⁺CD25⁺LAG-3⁺CCL4⁺Foxp3⁺ (p = 0.02 - 0.03 and p = 0.0079, respectively; Mann-Whitney test). The majority of the CD3⁺CD8⁺CD4⁺ T-cells co-expressed CD25, LAG-3, CCL4, and/or Foxp3 in combination with CD39, such that CD39 appears to be a preferential marker of CD8⁺ Treg cells expressing multiple Treg-associated markers (p = 0.0625; Wilcoxon signed-rank test).
**Figure 2 (A and B).** CD39 expression is associated with Treg-cell markers on CD8$^+$ T-cells. A: Flow cytometric analysis of CD8$^+$ T-cells for a selection of Treg markers in a PPD-responder 6 days after live *Mycobacterium bovis* BCG infection. Gating was performed as shown in Supplementary figure S1. CD8$^+$CD4$^-$ gate consisted of at least 10 000 cells. Data are representative of five donors and four experiments performed. B: CD8$^+$ T-cells that express Foxp3 and LAG-3 (left) also express CD25; the majority of these also express CD39 (right).
Figure 2 (continued). CD39 expression is associated with Treg-cell markers on CD8+ T-cells. C: Combined analysis of individual Treg markers. Expression of individual markers was combined using Boolean gating and data are expressed as the percentage of CD3+CD8+CD4- T-cells that express these markers. Data are shown of five PPD-responders in gray boxes versus five non-responders in open boxes, 6 days after live *M. bovis* BCG infection and are representative of four experiments. Boxes: 25th-75th percentiles; line at median; whiskers: minimum to maximum (*p < 0.05; **p < 0.01; Mann-Whitney test).

**CD8+CD39+ T-cells suppress CD4+ Th1 responses**

To determine the possible suppressive function of CD39+ T-cells, CD39-positive and negative T-cell populations were FACS-sorted and tested for their capacity to inhibit the activity of an unrelated CD4+ Th1 responder clone, recognizing a cognate peptide presented in the context of HLA-DR3 [8;32]. CD8+CD39+ T-cells, purified to ≥ 97% purity, indeed suppressed the proliferative response of (cloned) CD4+ Th1 cells in response to peptide in the context of HLA-class II. This suppressive activity was strongly enriched in the CD8+CD39+ T-cell population as compared with CD8+CD39− T-cells and unsorted CD8+ T-cells (figure 3A). Flow cytometric analysis of sorted T-cell lines demonstrated enrichment for LAG-3, CD25, Foxp3, and CCL4 in the CD8+CD39+ compared with the CD8+CD39− T-cells (figure 3B).
Figure 3. *Mycobacterium bovis* BCG-induced CD8⁺CD39⁺ T-cells suppress antigen-specific proliferation of (cloned) CD4⁺ Th1 cells. A: CD8⁺ T-cells of PPD-responders were enriched after BCG stimulation by positive selection using magnetic beads and FACS-sorted on CD39 expression. Their potential suppressive capacity was tested in a co-culture assay by titrating CD8⁺CD39⁺ T-cell lines, CD8⁺CD39⁻ T-cell lines, or unsorted bulk CD8⁺ T-cell lines (containing on average 75% CD8⁺CD39⁺ T-cells) onto a Th1 reporter clone that was stimulated with its cognate peptide. Proliferation was measured by (3H)TdR (where TdR is thymidine) incorporation after 3 days. Proliferation was divided by Th1 reporter clone proliferation in the absence of Treg cells to obtain relative proliferation (Wilcoxon signed-rank test, *p* < 0.001). Dashed lines represent background proliferation of the different CD8⁺ T-cell subsets (at the indicated CD8⁺ T-cell concentrations) relative to Th1 reporter clone proliferation. Data are depicted as mean ± SE of three cell lines. B: Flow cytometric analysis demonstrating enrichment for Treg-cell markers in CD39-sorted CD8⁺ T-cell lines, compared with CD39⁺CD8⁺ T-cell lines. (A, B) Data are shown from one representative experiment out of three performed.
Blocking CD39 results in partial reversal of suppression by *M. bovis* BCG-stimulated CD8⁺CD39⁺ T-cells

CD8⁺CD39⁺ T-cells preserved their expression of CD39 (≥99%), as well as of other Treg-cell markers, including CD25, Foxp3, and CCL4 (Supplementary figure S2) following further *in vitro* expansion. We next tested the ability of ARL 67156 trisodium salt hydrate (ARL) and the anti-CD39 monoclonal antibody BY40/OREG-103 to reverse the suppressive activity of CD8⁺CD39⁺ T-cells. ARL is an ATP analog that can bind to, but is not hydrolysable by, CD39 [35], and has been used to inhibit the suppressive activity of CD4⁺CD25⁺CD39⁺ cells [27]. Here, ARL partially reversed the capacity of CD8⁺CD39⁺ T-cells, with previously demonstrated T-cell inhibitory capacity, to suppress the proliferation of CD4⁺CD25⁺ T-cell lines in a dose-dependent manner (Figure 4). ARL was added daily in 150 μM and proliferation was measured as (3H)TdR (where TdR is thymidine) incorporation after three days. The response was calculated by dividing cognate-peptide-induced proliferation with unstimulated values after natural logarithmic transformation. To obtain a relative response, the response for each Treg concentration was normalized for proliferation in the absence of Treg cells (100%). Reversal of suppression was calculated by dividing relative responses in the presence or absence of ARL (14 - 47% reversal of suppression; *p* = 0.023; Wilcoxon signed-rank test) as previously described [8]. Data represent mean ± SE for three independent CD8⁺CD39⁺ T-cell lines tested in two experiments on the same standardized Th1 reporter clone.
cells to suppress the proliferative responses of the Th1 responder clone (14 - 47% reversal of suppression; in three cell lines; \( p = 0.023 \); Wilcoxon signed-rank test) (figure 4).

Suppression by the CD8\(^+\)CD39\(^+\) T-cells was also (partially) reversed by the anti-CD39 blocking monoclonal antibody BY40/OREG-103 \([36;37]\) (0 - 35% reversal of suppression; in four experiments; \( p = 0.005 \); Wilcoxon signed-rank test) (figure 5); further supporting the direct functional involvement of CD39 in suppression mediated by CD8\(^+\)CD39\(^+\) Treg cells.

![Figure 5. Suppression mediated by CD8\(^+\)CD39\(^+\) T-cell lines is partly reversed by an anti-CD39 monoclonal antibody BY40/OREG-103.](image_url)

**Figure 5.** Suppression mediated by CD8\(^+\)CD39\(^+\) T-cell lines is partly reversed by an anti-CD39 monoclonal antibody BY40/OREG-103. CD8\(^+\)CD39\(^+\) T-cells were expanded using \( \alpha \)CD3/CD28 beads and suppression assays were performed by titrating these CD8\(^+\)CD39\(^+\) T-cell lines onto a Th1 reporter clone stimulated with its cognate peptide. Proliferation values were divided, after natural logarithmic transformation, by Th1 reporter clone proliferation values obtained in the absence of Treg cells, in order to obtain relative proliferation. Inhibition of proliferation in the presence and absence of anti-CD39 monoclonal antibody was calculated and expressed as percentage (0 - 35% reversal of suppression; \( p = 0.005 \); Wilcoxon signed-rank test). Data represent mean ± SE for four cell lines in four independent experiments.
To exclude that suppressive activity by CD8⁺CD39⁺ T-cell lines was due to lysis rather than active suppression of the CD4⁺ Th1 responder clone, the Th1 responder clone and an equal number of cells of an irrelevant T-cell clone were labeled with low and high doses CFSE, respectively, and were added in equal numbers to the co-culture assay, identical to previously described [13]. After 16h, before division of the Th1 responder clone occurs, the percentages of responder and irrelevant T-cell clone without CD8⁺CD39⁺ T-cells were similar to the percentages of responder and irrelevant T-cell clone with the addition of CD8⁺CD39⁺ T-cells (Supplementary figure S3), indicating that in these co-culture assays, inhibition of responder cell proliferation by CD8⁺CD39⁺ T-cells is not the result of cytotoxicity.

Discussion

In this study, we describe for the first time the expression of, and a functional role for, CD39 on human pathogen activated CD8⁺ Treg cells. CD8⁺CD39⁺ T-cells from PPD-responsive individuals specifically co-expressed the known classical Treg-cell markers CD25, Foxp3, LAG-3, and CCL4. To assess if CD39 expression was merely a marker of CD8⁺ Treg cells or was directly involved in the CD8⁺CD39⁺ T-cells’ suppressive activity, we purified CD8⁺CD39⁺ T-cells, and showed that they were strongly enriched for suppressive activity and the expression of Treg markers, and that both the chemical CD39 antagonist, ARL, as well as a blocking anti-CD39 antibody were able to partly inhibit the suppressive activity of CD8⁺CD39⁺ T-cells. Altogether these data indicate that CD39 is a marker for regulatory CD8⁺ T-cells and that CD39 contributes functionally to the suppression mediated by human CD8⁺CD39⁺ T-cells. Both ARL as well as the blocking anti-CD39 antibody only partly inhibited suppressive activity, indicating that also other mechanisms may contribute to suppression. We previously demonstrated the expression of LAG-3 and the functional involvement of CCL4 in immune regulation by BCG-activated CD8⁺ Treg cells. In the current study, ≥ 43% of CD8⁺CD39⁺ T-cells also expressed CCL4, while we did not find any expression of IL-10 on these T-cells.
CD8+ Treg cells have been described in human *Mycobacterium*-infected LNs [8] and lepromatous lesions [9;10], demonstrating that CD8+ Treg cells are present at the site of disease and suggesting a potential role for these cells in disease pathogenesis. In line with our previous studies showing that BCG activated CD8+ Treg cells in PPD-responsive individuals, but not in donors that did not recognize PPD *in vitro* [8], also in the current study CD8+CD39+ Treg cells were confined to PPD-responders, suggesting that these cells originated from preexistent antigen-specific memory T-cells. We have previously hypothesized that Treg cells could contribute to the relative failure of BCG-vaccination in conferring protection against pulmonary TB in adults [6].

In TB, recent results have suggested a role for Th17 cells both in protection and pathology. IL-17 producing CD4+ T-cells in the lung, induced by BCG-vaccination, were associated with protective immunity to TB in mice [2;38]; interestingly, in human tuberculous pleural effusions, the number of CD4+CD39+ Treg cells was inversely related to the number of Th17 cells, and CD39+ Treg cells suppressed the differentiation of naive CD4+ cells into Th17 cells [39]. Frequencies of CD4+CD39+ T-cells correlated negatively with IL17A responses in stimulated PBMCs after MVA85A vaccination [40]. Stimulation of PBMCs in the presence of the CD39 chemical antagonist ARL 67156 increased coproduction of IL-17 and IFNγ by CD4+ T-cells, 1 and 2 weeks after MVA85A vaccination [27]. Whether CD8+CD39+ T-cells are associated with IL-17 responses and/or protection needs further investigation.

In this article, we describe for the first time a functional role for CD39 on human BCG-activated CD8+CD39+ Treg cells. We show that CD39 expression marks a CD8+ Treg-cell subset, which co-expresses LAG-3, CD25, Foxp3, and CCL4, and that CD39 may play a direct role in exerting CD8+ Treg-dependent suppression. CD8+CD39+ Treg cells represent a new player in balancing immunity and inflammation in host defense against mycobacteria, and possibly contribute to (lack of) vaccine-mediated protection.
Acknowledgments

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Conflict of interest statement

Jérémy Bastid is chief operating officer at OREGA BIOTECH and provided the anti-CD39 monoclonal antibody BY40/OREG-103. Dr. Bastid was not involved in design and execution of experiments or in data analysis.
References


Supplementary figures

Supplementary figure S1. Gating strategy. Studies were conducted in a laboratory guided by exploratory research principles with established Standard Operating Procedures (SOP): cells were isolated and cryopreserved according to SOP. Cells were thawed (78% median viable cell yield) and stimulated with live BCG for six days in medium supplemented with pooled human serum (pretested in standardized protocols; only sera were pooled that had no inhibitory activity in standard mixed allogeneic lymphocyte cultures). Demonstrated is the gating strategy used for flowcytometric analysis at day 6: cells were gated on single cells, violet-live/dead-CD14-CD19-negative, CD3⁺ and CD8⁺CD4⁻ vs. CD4⁺CD8⁻.


Supplementary figure S2. Expression of regulatory T-cell markers in restimulated CD8⁺CD39⁺ T-cell lines. CD8⁺CD39⁺ T-cells were expanded using αCD3/CD28 beads and analyzed for expression of regulatory T-cell markers by flowcytometry. Gating was similar for both plots and performed as shown in Supplementary figure S1. 97% Of the restimulated CD8⁺CD39⁺ T-cells expressed CD25 (left) and 43% of restimulated CD8⁺CD39⁺ T-cells co-expressed CCL4 and Foxp3 (right). These cells did not express IL-10. Data are representative for three CD8⁺CD39⁺ T-cell lines.
Supplementary figure S3. Inhibition of Th1-responder cell proliferation is not the result of lysis by CD8+ T-cells. The Th1-responder clone Rp15 1-1 was labeled with 0.005 μM of CFSE, and another, isogenic T-cell clone, with a different peptide specificity and HLA-DR restriction, with 0.5 μM of CFSE [13]. Both cells were then co-cultured in equal numbers with the CD8+CD39+ T-cell lines in the presence of the peptide (50 ng/ml) recognized by the responder clone Rp15 1-1 in the context of HLA-DR3+ APCs. After 16 hours CFSE intensity was measured by flowcytometry. The percentages of responder and irrelevant T-cells without CD8+CD39+ T-cells were similar to the percentages of responder and irrelevant T-cells with the addition of 5 x 10^4 CD8+CD39+ T-cells, the highest concentration used in our co-culture experiments; thus indicating that the responder clone is not lysed by the BCG-activated CD8+CD39+ T-cells.