Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression

Silvia Deaglio,[1] Karen M. Dwyer,[1] Wenda Gao,[1] David Friedman,[1] Anny Usheva,[1] Anna Erat,[1] Jiang-Fan Chen,[3] Keiichii Enjyoji,[1] Joel Linden,[4] Mohamed Oukka,[5] Vijay K. Kuchroo,[5] Terry B. Strom,[1,2] and Simon C. Robson[1]

[1]Department of Medicine and [2]Department of Surgery, Harvard Medical School, Transplantation Research Center, Beth Israel Deaconess Medical Center, Boston, MA 02215
[3]Boston University Medical Center, Boston, MA 02118
[4]Department of Medicine, University of Virginia, Charlottesville, VA 22908
[5]Department of Neurology, Center for Neurological Diseases, Brigham and Women's Hospital, Boston, MA 02115

The study of T regulatory cells (T reg cells) has been limited by the lack of specific surface markers and an inability to define mechanisms of suppression. We show that the expression of CD39/ENTPD1 in concert with CD73/ecto-5′-nucleotidase distinguishes $CD4^+/CD25^+/Foxp3^+$ T reg cells from other T cells. These ectoenzymes generate pericellular adenosine from extracellular nucleotides. The coordinated expression of CD39/CD73 on T reg cells and the adenosine A2A receptor on activated T effector cells generates immunosuppressive loops, indicating roles in the inhibitory function of T reg cells. Consequently, T reg cells from *Cd39*-null mice show impaired suppressive properties in vitro and fail to block allograft rejection in vivo. We conclude that CD39 and CD73 are surface markers of T reg cells that impart a specific biochemical signature characterized by adenosine generation that has functional relevance for cellular immunoregulation.

CORRESPONDENCE
Terry B. Strom:
tstrom@bidmc.harvard.edu
OR
Simon C. Robson:
srobson@bidmc.harvard.edu

$CD4^+/CD25^+$ regulatory T cells (T reg cells) are important in the induction of immunological tolerance (1). The elimination of this population enhances immune responses to alloantigens, favoring the rejection of tissue grafts (2), spontaneous appearance of autoimmune diseases, and inflammation (3). T reg cells also suppress natural immune responses to parasites (4) and viruses (5) and inhibit or attenuate antitumor immunity induced by cancer vaccines. The X chromosome–encoded forkhead transcription factor Foxp3 is uniquely expressed by T reg cells, serves as a lineage specification factor, and confers suppressive function to these cells (6–8). However, the intracellular location of Foxp3 limits its usefulness in studying and purifying this subpopulation.

S. Deaglio, K.M. Dwyer, and W. Gao contributed equally to this paper.
T.B. Strom and S.C. Robson contributed equally to this paper.
S. Deaglio's present address is Dept. of Genetics, Biology, and Biochemistry and CeRMS, University of Torino Medical School, 10126 Torino, Italy.
This work was presented, in part, at the Keystone Symposium on Regulatory T cells in Vancouver, Canada in February 2007.

Cell surface expression of CD25 expression is often used as a marker for $CD4^+$ T reg cells, but CD25 is somewhat nonspecific, as it is also up-regulated on other $CD4^+$ T cell populations after activation.

Putative mechanisms of suppression by T reg cells remain ill defined but include cell–cell contacts considered predominant in vitro and the release of soluble mediators predominant in vivo. Surface molecules linked to T reg cell suppression include CTLA-4 (9, 10), chemokine receptors (CCR4 and CCR8; reference 11), selectins (CD62L; reference 12), integrins (CD103; reference 13), and CD127 (14). In vivo, putative mechanisms include the release of soluble factors IL-10 and TGF-β (15, 16). Recently, other T reg cell Foxp3-dependent suppressor functions have been suggested by gene clustering experiments (8). Moreover, the deletion of Foxp3 results in the reduced expression of such putative suppressor genes (17).

Extra- and/or immediate pericellular accumulation of adenosine elicits immunosuppressive cellular responses that are mediated through

The Journal of Experimental Medicine

several type 1 purinergic (adenosine) receptors, including A2A (18). Seminal, important genetic data indicate that adenosine, which is operative via the A2A adenosine receptor, plays critical, nonredundant, autonomous, and autochthonous roles in inhibiting effector functions of activated T cells (19, 20). Modulation of inflammation by adenosinergic mechanisms has been rigorously tested in models of T cell–dependent autoimmune and viral hepatitis (21) and in antitumor T cell immunity (22). In addition, the stimulation of A2A-mediated responses modulates the control of T cell–mediated colitis in experimental mouse models by suppressing the expression of proinflammatory cytokines in a manner independent of both IL-10 and TGF-β (23).

We have proposed that the regulation of extracellular nucleotide catabolism by T reg cells might be responsible for the coordination of such adenosinergic effects. Consequently, we have investigated the regulation of extracellular nucleotide catabolism by T reg cells that ultimately leads to the generation of pericellular adenosine. The rate-limiting step of this ectonucleotidase cascade is determined by CD39 (ENTPD1 [ectonucleoside triphosphate diphosphohydrolase-1]; EC 3.6.1.5), an ectoenzyme that hydrolyzes ATP/UTP and

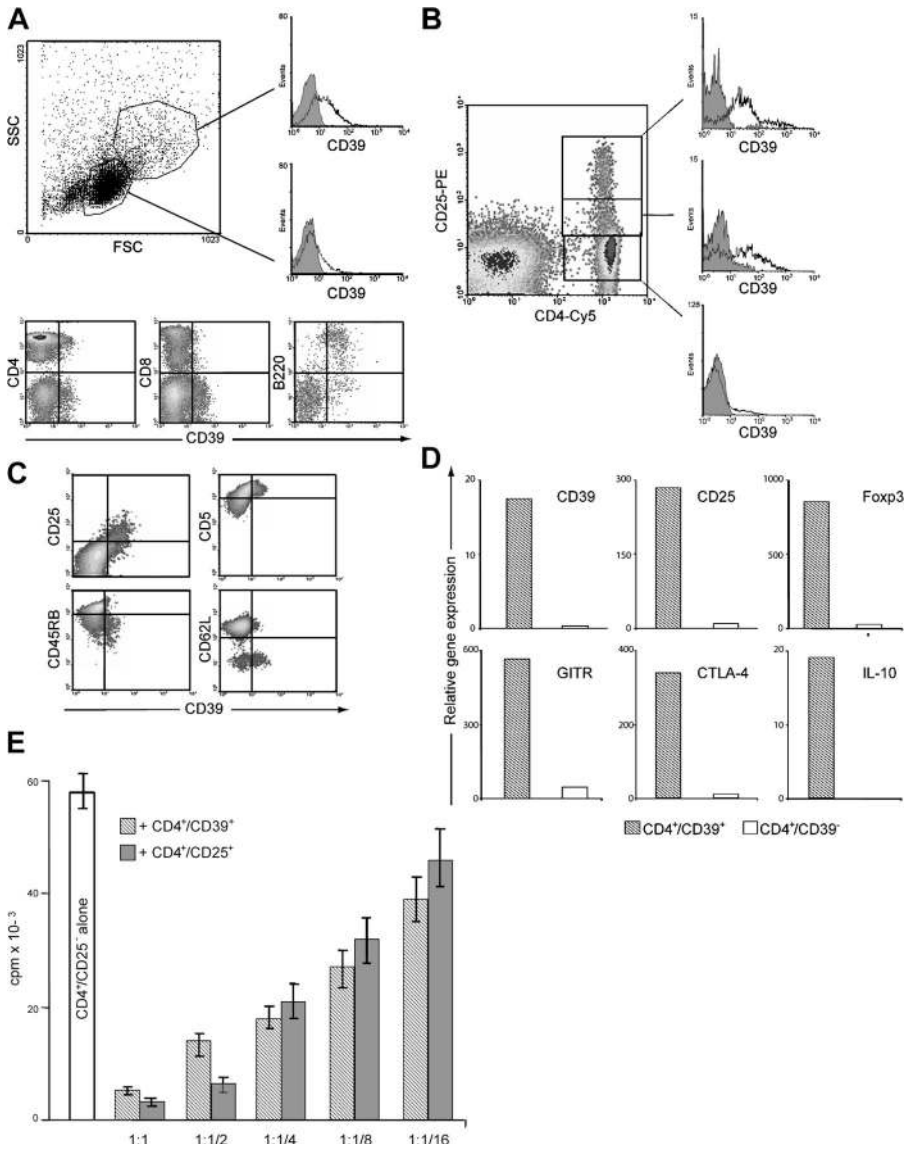

Figure 1. CD4⁺/CD25⁺ cells express CD39. (A) The expression of CD39 (open profiles) on mouse lymph node cell suspensions is shown in the histograms on the right, contrasted against control IgG (gray). The expression of CD39 on $CD4^+$, $CD8^+$, and $B220^+$ subsets is shown in the density plots. (B) Lymph node cells were stained with CD4-Cy5 and CD25-PE. CD39 expression in gated populations is shown in the histograms (open) with irrelevant control (gray). (C) $CD4^+$ cells were stained for CD39 (horizontal axis) and the indicated markers. (D) Real-time PCR analysis in $CD4^+/CD39^+$ and $CD4^+/CD39^-$ cells. (E) 5×10^4 $CD4^+/CD25^-$ T cells were mixed with $CD4^+/CD39^+$ or $CD4^+/CD25^+$ cells. $[^3H]$Thymidine incorporation was measured after 3 d. Data are means with SEM (error bars) of three independent experiments.

ADP/UDP to the respective nucleosides such as AMP. Extracellular nucleoside monophosphates are, in turn, rapidly degraded to nucleosides, (e.g., adenosine) by soluble or membrane-bound ecto-5′-nucleotidases (e.g., CD73 [EC 3.1.3.5]; reference 24). Ectonucleotidase biochemical activity has already been shown to be relevant in immune responses. Specifically, CD39 expressed by Langerhans cells is critically involved in the suppression of inflammation (25). More recently, CD73 has been shown to be expressed by $CD25^+FoxP3^+$ T reg cells and $CD25^-$ uncommitted primed precursor T helper cells that, through the generation of adenosine, dampen inflammatory responses (26).

RESULTS AND DISCUSSION

This study reports on the identification of CD39 together with CD73 as novel cell surface markers of $CD4^+$ T reg cells. In addition to the expression of both ectonucleotidases by T reg cells, the robust generation of adenosine controlled by these ectoenzymes functionally distinguishes classic T reg cells from other $CD4^+$ T cell populations. Our observations concerning extracellular adenosine production by T reg cells have been further developed by applying models conceived by Sitkovsky and others (19–22) that show that adenosine receptors on targeted T effector cells mediate suppressive functions. Gavin et al. (8) predict from gene clustering models that there are at least three potential T reg cell mechanisms inclusive of "suppressive soluble factors, generation of extracellular adenosine and release of reactive oxygen." We demonstrate in this study that alterations in pericellular levels of adenosine brought about by CD39 (and CD73) associated with T reg cells have a major, albeit not total, suppressive effect on cellular responses in vitro. We also show that CD39 plays a nonredundant role in the suppressive capabilities of T reg cells in vivo, as demonstrated by the relative failure of *Cd39*-null T reg cells to block skin allograft rejection. We propose that cell-associated CD39 initiates and regulates the generation of adenosine and comprises an important component of the suppressive machinery of T reg cells.

T reg cells express CD39

Rabbit anti–mouse CD39 polyclonal antibody was raised previously by cDNA immunization (27) and was used to stain mononuclear cells purified from lymph nodes (Fig. 1 A) and spleen (not depicted) of naive WT mice. CD39 is expressed by the majority of monocytes and by a subset of lymphocytes (Fig. 1 A) in C57BL/6 mice, as inferred by gating on forward scatter and side scatter. Among lymphocytes, the majority of $CD39^+$ cells are $B220^+$ B cells. The remaining $CD39^+$ cells reside within the $CD4^+$ subset, where they consistently range from 8 to 12% of all $CD4^+$ cells in lymph nodes and spleen (Fig. 1 A). Importantly, no cells within the $CD8^+$ T cell subset express CD39.

Further characterization of resting C57BL/6 lymphocytes reveals that CD39 is predominantly expressed on those $CD4^+$ cells that concurrently express CD25 (Fig. 1 B). CD39 is consistently and abundantly expressed by $CD4^+/CD25^{high}$ T cells, whereas the $CD4^+/CD25^{dim}$ population shows a dichotomic pattern with respect to CD39 expression, with ∼50% of the cells being positive. Less than 1% of $CD39^+$ cells are found within the $CD4^+/CD25^-$ compartment (Fig. 1 B). Predominant expression of CD39 by $CD4^+/CD25^+$ T cells was also observed in other strains of mice such as BALB/c and, additionally, in primates and humans (unpublished data). Nearly all $CD4^+/CD39^+$ cells are also $CD5^{high}$, $CD45RB^{low}$ and are mostly $CD62L^{low}$ (Fig. 1 C). $CD4^+/CD8^-/CD25^+$ thymocytes express CD39 at equivalent amounts to peripheral $CD4^+/CD25^+$ T cells (unpublished data).

RT-PCR analyses of sorted mouse cell populations confirm that $CD4^+$ cells express the highest levels of CD39 mRNA among lymphocytes (unpublished data). Gene expression profiling of $CD4^+$ T cells, which was sorted on the basis of CD39 cell surface expression, indicates that $CD4^+/CD39^+$ but not $CD4^+/CD39^-$ T cells robustly express Foxp3, CD25, glucocorticoid-induced tumor necrosis factor receptor (GITR), and CTLA-4 transcripts, which is in keeping with the recognized T reg cell phenotype (Fig. 1 D).

When functional properties are tested, $CD4^+/CD39^+$ cells are found to suppress $CD4^+/CD25^-$ proliferation with an efficacy similar to that observed with classic naive $CD4^+/CD25^+$ T reg cells (Fig. 1 E). In concordance with the T reg cell phenotype, $CD4^+CD39^+$ cells are nonresponsive to TCR stimulation in the presence of irradiated T-depleted accessory cells and in the absence of IL-2 (unpublished data).

The incomplete overlap between $CD4^+/CD39^+$ and $CD4^+/CD25^+$ subsets is interesting given recent data demonstrating that Foxp3 is expressed in a significant percentage of $CD4^+$ cells that is potentially independent of CD25 expression (7). Further work suggests that a combination of surface markers, including CD127, provides a more accurate definition of T reg cells than CD25 alone (14). Lastly, CD39 is one of 67 genes, as shown by gene profiling, to be highly associated with Foxp3 expression, even more so linked than CD25 (7). Indeed, Foxp3 amplifies and stabilizes the expression of genes encoding cell surface or secreted molecules (e.g., Fgl2, CD73, CD39, TRAIL, or CTLA4; reference 8).

To discern the relationship between CD39 expression, CD73, and the T reg cell phenotype-function relationships, we studied purified T cells from mice with the GFP reporter gene introduced into the endogenous Foxp3 locus (designated as $Foxp3^+(GFP^+)$ knock-in cells; reference 28). The great majority (∼80%) of $Foxp3^+(GFP^+)$ cells from spleen and lymph nodes strongly express CD39 (Fig. 2 A). CD39 is a more consistent and reliable marker for T reg cells than CD25; importantly, only ∼50% of $Foxp3^+(GFP^+)$ knock-in cells also express CD25 (Fig. 2 A). However, a limited fraction of $Foxp3^-(GFP^-)$ cells are also $CD39^+$ (Fig. 2 A). $Foxp3^+/CD39^-$ cells are rarely seen and have been excluded from further analyses.

Therefore, three substantive populations can be defined on the basis of differential CD39 and Foxp3 expression: $Foxp3^+/CD39^+$, $Foxp3^-/CD39^+$, and $Foxp3^-/CD39^-$. These populations were sorted, and gene expression profiles together

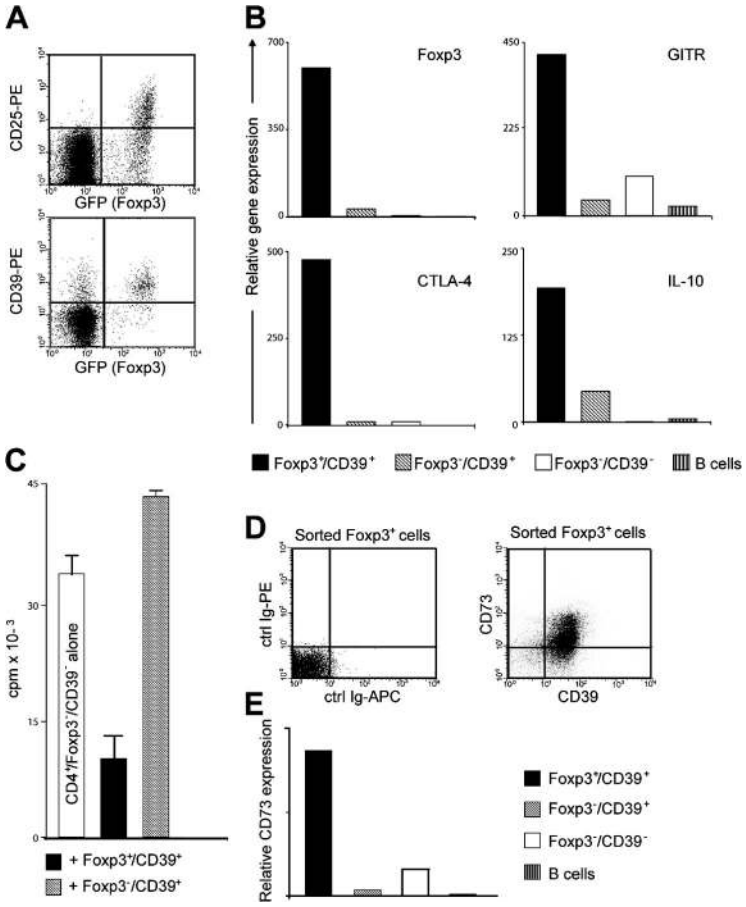

Figure 2. Foxp3$^+$ cells express CD39. (A) CD4$^+$ T cells from Foxp3$^+$ (GFP$^+$) knock-in mice stained with CD25-PE or CD39-PE. (B) Real-time PCR analysis of Foxp3$^+$/CD39$^+$ (black bars), Foxp3$^-$/CD39$^+$ (shaded bars), and Foxp3$^-$/CD39$^-$ (white bars) cells. (C) Foxp3$^+$/CD39$^+$ suppresses T effector (Foxp3$^-$/CD39$^-$ CD4$^+$) proliferation. [^{3}H]Thymidine incorporation was measured after 72 h. Data are representative of two independent experiments, with error bars representing the SD of triplicate wells. (D) Foxp3$^+$(GFP$^+$) cells stained for CD39-APC and CD73-PE. (E) CD73 mRNA expression in Foxp3$^+$/CD39$^+$ (black bars), Foxp3$^-$/CD39$^+$ (shaded bars), Foxp3$^-$/CD39$^-$ (white bars), and B lymphocytes (striped bars).

with functional suppressive properties were determined. The Foxp3$^+$/CD39$^+$ fraction was shown to mirror the recognized genetic profile of T reg cells, as defined by the presence of Foxp3, GITR, CTLA-4, and IL-10 transcripts (Fig. 2 B). These Foxp3$^+$/CD39$^+$ cells also efficiently suppress CD4$^+$/CD25$^-$ proliferation in vitro (Fig. 2 C). Conversely, the Foxp3$^-$/CD39$^+$ subset contains T lymphocytes that appear to be associated with the memory compartment, pointing to a comparable derivation of T reg cells and other activated memory cells (unpublished data). As expected, the Foxp3$^-$/CD39$^-$ population expresses neither phenotypic nor functional features of T reg cells (Fig. 2, B and C).

We next sought to determine the exact functional relationship between the capacity for adenosine generation and Foxp3 expression within recognized mouse CD4$^+$ T cell populations. Using Foxp3 knock-in mice, Foxp3$^+$(GFP$^+$) T reg cells were found to coexpress both ectonucleotidases CD39 and CD73. This is a unique situation among T lymphocytes and provides precise phenotypic markers and designates biochemical functions for T reg cells (Fig. 2 D). Consistent with the phenotypic data, RT-PCR analysis confirms that Foxp3$^+$/CD39$^+$ cells exhibit high levels of the gene expression of both CD39 and CD73 (Fig. 2 D). Moreover, CD4$^+$/CD39$^+$/CD73$^+$ cells have comparable suppressive properties to classic CD4$^+$/CD25$^+$ cells in vitro (not depicted and Fig. 2 E).

T reg cells generate extracellular adenosine

CD39 and CD73 coexpression are associated with Foxp3$^+$ T cells (Fig. 2 D). However, CD39 and CD73 are also typically expressed by monocytes and endothelial cells, in which altered expression can be linked to disordered circulatory homeostasis, thrombosis, and platelet activation (29). Moreover, CD39 expression encompasses different cellular lineages, including B lymphocytes and Langerhans cells, where it may play a role in modulating immune cell–cell contacts by catalyzing local changes in extracellular nucleotide-triggered responses (25). CD73 is more generally expressed on the vasculature by select T and B cell subsets, on follicular dendritic cells, and on thymic medullary reticular fibroblasts together with various epithelial cells (24).

The finding that a population of $CD4^+$ lymphocytes endowed with suppressive functions selectively coexpresses CD39 and CD73 illustrates potential functional roles of extracellular nucleotide-metabolizing enzymes within the T cell pool. In the biochemical systems that we propose for T cell regulation, a degree of specificity could be dictated by three essential modulatory components: titrated and various nucleotide release into the extra- or pericellular milieu, specific receptors for these mediators, and, finally, a varied repertoire of ectonucleotidases upon regulatory and effector cells.

CD39 biochemical activity is the rate-limiting component of the ectoenzymatic chain that metabolizes extracellular nucleoside di- and triphosphates, ultimately to the respective nucleosides, such as adenosine. Nucleoside triphosphate diphosphohydrolase (NTPDase) biochemical activity levels of intact WT $CD4^+/CD25^+$ T cells are 8–10 times greater than that of the $CD4^+/CD25^-$ counterparts, constitutively not expressing CD39 (Fig. 3 A). Furthermore, using ^{14}C-radiolabeled nucleotides, we demonstrate that WT $CD4^+$ cells rapidly hydrolyze exogenous ADP to efficiently generate adenosine in vitro. *Cd39*-null $CD4^+$ cells show markedly diminished rates of ADP phosphohydrolysis with no production of adenosine (Fig. 3 B). These data confirm that functional, classic $Foxp3^+$/T reg cells constitutively express CD39 and CD73 and that the biochemical pathway leading to the synthesis of adenosine is operative.

In contrast, the two other $CD4^+$ subpopulations, namely $Foxp3^-/CD39^+$ and $Foxp3^-/CD39^-$, fail to generate adenosine from ADP (Fig. 3 C), which is in keeping with low CD73 expression levels. $CD8^+$ T cells included as a $CD39^-/CD73^+$ control do not generate adenosine. Therefore, within the T cell compartment, only the $Foxp3^+(GFP^+)$ T reg cell possesses the intact ectonucleotidase catalytic machinery, and these properties are maintained during cellular activation (unpublished data). Such cells might dictate the generation of pericellular adenosine (at extravascular sites) to potentially mediate immunoregulation at a cellular level.

Adenosine suppresses T cell responses

The hypothesis that adenosine may comprise, at least in part, a component of the suppressive machinery of T reg cells is supported by the independent demonstration of CD73 expression by T reg cells (26). Adenosine exerts its effects by binding to adenosine type 1 purinergic G protein–coupled cell surface receptors, which are termed A1, A2a, A2B, and A3. Adenosine is a potent inhibitor of T cell responses (19), and the A2A receptor has been identified as the major anti-inflammatory adenosine receptor associated with T cells (21).

The expression patterns of the A2A receptor were evaluated in T reg cells and $CD4^+/CD25^-$ cell populations. Under resting conditions, the highest A2A mRNA levels are present in T reg cells, which, in the presence of adenosine generated from CD39, may account for the anergic state typical of this population. Minimal A2A mRNA expression could be detected in $CD4^+/CD25^-$ cells (Fig. 4 A), but effector T cells acquire A2A receptors during activation, starting at 4 d after stimulation and with expression peaking at day 6 (Fig. 4 A).

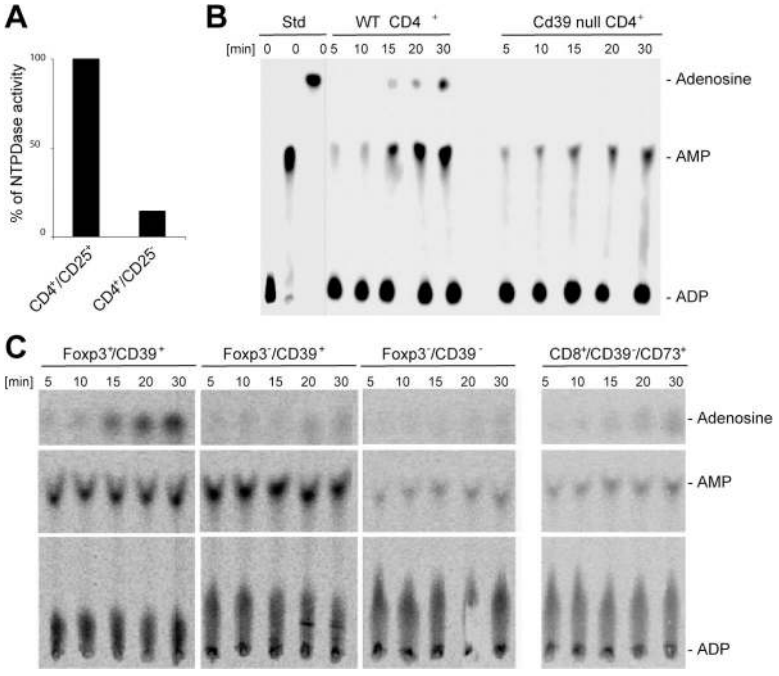

Figure 3. Concomitant expression of CD39/CD73 and generation of adenosine distinguishes T reg cells from other T cells. (A) CD39/NTPDase activity in $CD4^+/CD25^+$ and $CD4^+/CD25^-$ cells. (B) Hydrolysis of extracellular ^{14}C-radiolabeled ADP to adenosine is catalyzed by WT $CD4^+$ (lanes 4–8) but not *Cd39*-null $CD4^+$ (lanes 9–13) mouse T cells. The radiolabeled standards are designated as ADP, AMP, and Adenosine (lanes 1–3). (C) Hydrolysis of extracellular ADP by $Foxp3^+/CD39^+$ (first to fifth lanes), $Foxp3^-/CD39^+$ (6th to 10th lanes), and $Foxp3^-/CD39^-$ (11th to 15th lanes) cells.

Little or no expression of A2B receptors is identified in T cells (unpublished data). To determine whether signaling through the A2A receptor has functional consequences in $CD4^+/CD25^-$ cells, a panel of adenosine agonists with defined selectivity toward the full array of adenosine receptors was tested. The proliferation of $CD4^+/CD25^-$ cells stimulated by allogeneic cells in culture was substantially inhibited in a dose-dependent fashion in the presence of A2A-specific agonists (ATL146e), confirming a potent pharmacological effect (Fig. 4 B). Much weaker effects were obtained using compounds with somewhat nonselective A1 and A3 receptor agonist activity (Fig. 4 C).

The central role of adenosine generation in the control of $CD4^+/CD25^-$ proliferation was then tested by using lymphocytes from mutant mice null for A2A receptors. We noted that A2A-null $CD4^+/CD25^-$ proliferation rates in response to TCR stimulation in the presence of irradiated accessory cells are markedly increased by day 6 (Fig. 4 D). These observations are in agreement with the known kinetics of A2A receptor up-regulation and validate further previous observations regarding A2A receptor properties in T effector cells (21). The timing of up-regulation of the A2A receptor during T cell activation suggests that this novel immunosuppressive loop is functional during the late phases of T effector cell activation and proliferation, likely coinciding with maximal nucleotide leak from increasingly hypoxic or activated cells (30).

Effects of CD39 upon adenosinergic loops that comprise a component of the suppressive machinery of T reg cells

CD39 critically regulates levels of ADP (Fig. 3 B), a crucial feed-forward inhibitor of CD73 (31). Therefore, we further studied *Cd39*-null mice to determine how disruption of the ectonucleotidase cascade impacts T reg cell functions. *Cd39*-null mice express $CD4^+/CD25^+$ T cells in the expected numbers in peripheral blood and lymphoid organs. These cells express traditional markers of T reg cells, such as Foxp3 and GITR. However, T reg cells isolated from *Cd39*-null animals display features consistent with an activated state, such as enhanced mRNA expression of CD25 and CTLA-4 (Fig. 5 A). These features possibly reflect aberrant purinergic signaling, resulting in the loss of any putative inhibitory adenosinergic autocrine loop. Moreover, *Cd39*-null T reg cells are markedly dysfunctional in other ways: specifically, they are not anergic and proliferate excessively in response to anti-CD3 and anti-CD28 triggering and to alloantigens without exogenous IL-2 (Fig. 5 B).

Concordant with data derived from A2A-null $CD4^+/CD25^-$ (Fig. 4 D), proliferation responses of $CD4^+/CD25^-$ T cells obtained from *Cd39*-null mice are exaggerated after 6 d of stimulation, which is coincident with the up-regulation of adenosine receptor A2A in $CD4^+/CD25^-$ cells (Fig. 5 C). As a further confirmation of direct connections between CD39 expression and adenosine generation, effects of the

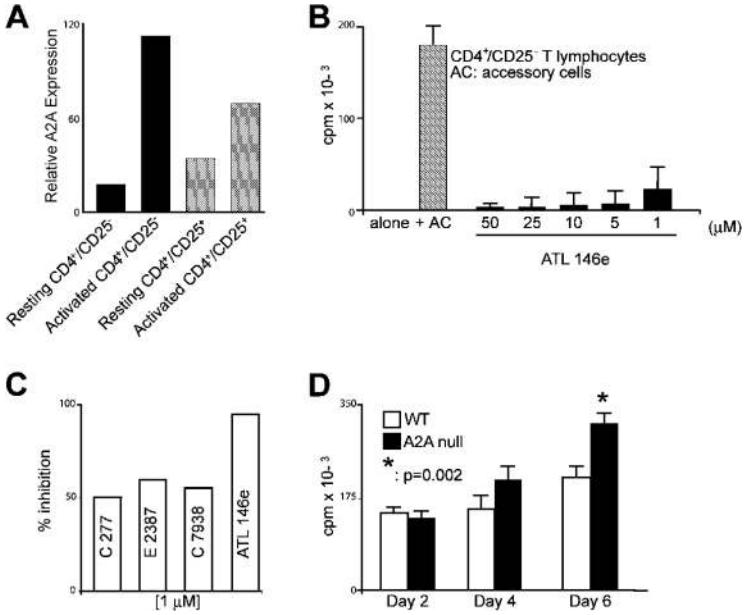

Figure 4. Adenosine generated by CD39 and CD73 suppresses T cell proliferation. (A) mRNA expression of adenosine A2A receptor. Cells were activated for 5 d in vitro. Data are means of three independent experiments. (B) ATL146e inhibits T cell proliferation in response to alloantigens. 5×10^4 $CD4^+/CD25^-$ T cells were cultured alone or in the presence of irradiated allogeneic splenocytes (shaded bar) with ATL146e at indicated concentrations. Data are means with SEM (error bars) of more than five independent experiments ($P < 0.05$ vs. $CD4^+/CD25^-$ cells alone). (C) Inhibition of T cell proliferation by adenosine receptor agonists. C277, E2387, C7938, and ATL146e (1 μM final) were added at the beginning of the cultures. [^{3}H]Thymidine incorporation was assayed at day 5. Data are representative of five independent experiments. (D) Proliferation of $CD4^+/CD25^-$ T cells purified from A2A-null (black bars) or WT (white bars) mice and stimulated by 2.5 μg/ml of plate-bound anti-CD3 and 2.5 μg/ml of soluble anti-CD28. [^{3}H]Thymidine was added for the last 8 h of culture. Representative data are from three experiments. *, $P < 0.002$ between WT and A2A null at 6 d of culture.

addition of soluble exogenous NTPDases (apyrase grade VII) to *Cd39*-null T cell cultures were tested. This reconstitution effectively suppresses the abnormal proliferation of $CD4^+/CD25^-$ cells (Fig. 5 C) and restores anergy within the T reg cell pool (not depicted).

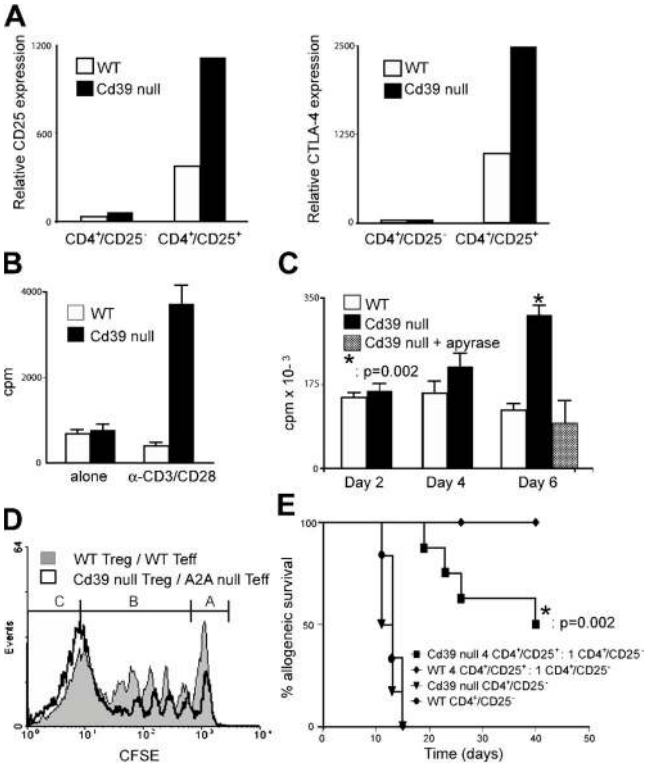

Figure 5. T reg cells from *Cd39*-null mice are constitutively activated and fail to suppress $CD4^+/CD25^-$ cell proliferation. (A) CD25 and CTLA-4 mRNA expression in $CD4^+/CD25^-$ and $CD4^+/CD25^+$ cells isolated from *Cd39*-null (black bars) or WT mice (white bars). (B) Proliferation assay of $CD4^+/CD25^+$ T cells purified from *Cd39*-null (black bars) or WT (white bars) mice. Cells were cultured in the presence or absence of 2.5 μg/ml of plate-bound anti-CD3 and 2.5 μg/ml of soluble anti-CD28 for 3 d. (C) Proliferation of $CD4^+/CD25^-$ T cells purified from *Cd39*-null (black bars) or WT (white bars) mice cultured in the presence of irradiated allogeneic splenocytes. Apyrase reconstitution effects are noted at day 6 (shaded bars). Error bars represent the SEM of three independent experiments. (D) T reg cell function. *Cd39*-null T reg cell effects of the stimulation of A2A-null Teff (open) and WT T reg cells on WT Teff (closed; 1:1 ratio) are compared at day 5. A represents the nonproliferating cell populations, B represents $CD4^+/CD25^-$ cells that have entered into the cell cycle, and C represents unlabeled T reg cells. The percentage of cells present in A or B was quantified using FlowJo software. Data are representative of three independent experiments. (E) Skin allograft survival. C57BL/6 Rag 1–deficient mice received allogeneic skin grafts 24 h after the passive transfer of $CD4^+/CD25^+$ and/or $CD4^+/CD25^-$ cells from WT or *Cd39*-null mice in a ratio of 4:1 (32, 33). Mice receiving WT (circles) or *Cd39*-null (triangles) $CD4^+/CD25^-$ cells rejected the skin graft at a median of 13 d ($n = 6$). Mice receiving WT $CD4^+/CD25^+$ cells transferred in excess (diamonds; 4:1) with WT $CD4^+/CD25^-$ cells showed long-term graft survival ($n = 9$). Four out of eight mice receiving *Cd39*-null $CD4^+/CD25^+$ cells transferred in excess (squares; 4:1) with Cd39-null $CD4^+/CD25^-$ cells rejected the skin allograft at a median of 24.5 d ($P = 0.02$).

We next examined the suppressive ability of WT and *Cd39*-null T reg cells to inhibit the proliferation of WT or *Cd39*-null effector cells, respectively. An in vitro system was chosen whereby effector cells were activated with anti-CD3 and anti-CD28. This system was chosen to eliminate the expression of CD39 on stimulating cells, which confounds analyses. We found that *Cd39*-null T reg cells were 50–60% less effective at inhibiting *Cd39*-null effector cell proliferation compared with WT counterparts at all ratios tested (unpublished data).

To further determine the importance of the CD39-adenosinergic axis upon T cell function, we examined the ability of T reg cells from WT and *Cd39*-null animals to suppress the proliferation of $CD4^+/CD25^-$ cells from WT and A2A-null mice. Although the elimination of *Cd39* substantially retards adenosine generation, it does not account for local adenosine release. Therefore, we generated an in vitro system in which the cellular machinery that generates adenosine or the dominant receptors that bind adenosine are intact or disrupted, respectively. Given the kinetics of A2A receptor up-regulation (Fig. 4, A and D), this system was analyzed at day 5, a time point when, in the absence of T reg cells, almost all effector T cells have entered into proliferation. Under such conditions, *Cd39*-null T reg cells are ~50% less effective at suppressing A2A-null T cell proliferation when compared with the ability of WT T reg cells to suppress WT $CD4^+/CD25^-$ proliferation when mixed at a ratio of 1:1 (Fig. 5 D). This effect is shown by the increased numbers of A2A-null $CD4^+/CD25^-$ T cells that have entered into proliferation. The disparity between the suppressive capacities of WT and *Cd39*-null T reg cells holds at decreased numbers relative to effectors, with greater numbers of effector T cells entering into proliferation at each reduced ratio (unpublished data). Interestingly, analysis of cultures at earlier time points failed to demonstrate a difference in the suppressive ability between WT and *Cd39*-null T reg cells (unpublished data), once again indicating that *Cd39* and the subsequent generation of adenosine are effective late in the proliferation of T cells.

These data further imply that adenosine generated from the hydrolysis of nucleotides exerts substantive inhibitory effects primarily through the A2A receptor in vitro, which is additive to any other cell–cell contact putative mechanisms that dictate T reg cell function. $CD4^+/CD25^+$ T cells isolated from *Cd39*-null animals also exhibit attenuated suppressive capabilities in vivo, as assessed in a transplant rejection model in immune reconstituted mice. Here, WT and mutant T reg cells are transferred in excess with $CD4^+/CD25^-$ T cells, and the capacity to prevent the rejection of skin allografts was examined. As expected (32, 33), adoptive transfer of WT T reg cells with WT $CD4^+/CD25^-$ cells at the well-defined 4:1 ratio results in long-term allograft survival (>40 d). In contrast, when *Cd39*-null T reg cells are transferred together with *Cd39*-null T effectors at the same cellular ratios, four out of eight grafts are rejected at a median of 24.5 d ($P = 0.02$). Transfer of $CD4^+/CD25^+$ T cells alone from either WT or

Cd39-null animals does not induce rejection (not depicted); no statistically significant differences are observed in the rejection times induced by the transfer of WT or *Cd39*-null $CD4^+/CD25^-$ cells alone (Fig. 5 E). These results are the first demonstration that the CD39-adenosinergic axis plays a nonredundant role in $CD4^+/CD25^-$ cell suppression mechanisms in vivo, giving credence to our phenotypic studies and other expression analyses (8). Our data strongly suggest that adenosine generation is an important component of the T reg cell armamentarium and is essential for full suppressive function.

CD39 and CD73 are components of a larger family of ectoenzymes that degrade nucleotides and include molecules for which a clear-cut role in lymphocyte activation and migration has been extensively proven, such as CD26 (34) and CD38 (35). All of these enzymes evolved from highly conserved ancestral proteins, usually acquiring membrane anchorage and developing parallel, albeit independent, functions. The acquisition of receptor functions such as signal transduction, localization in membrane lipid microdomains, and physical and functional association with partners specialized in signal transduction are particularly relevant. Similar properties also have been shown, albeit in a more limited manner for CD39 (36).

Conclusion

Our data demonstrate that CD39 is a novel cell surface marker of Foxp3 T reg cells. Functionally, the coexpression of CD39 and CD73 with the pericellular generation of adenosine dictates a substantial component of the suppressive capabilities of T reg cells. CD39 might regulate immune T cell suppression by the downstream production of adenosine acting via the A2A receptor. Advances in the understanding of how CD39 impacts T reg cell suppressive functions may help develop novel therapeutic avenues to target common inflammatory and immune disorders.

MATERIALS AND METHODS

Mice. Mice used in this study are as follows: *Cd39* null (29), FoxP3 knock-in (28); DBA/2, C57BL/6 Rag 1–deficient mice (Jackson ImmunoResearch Laboratories); A2A null (Boston University Medical Center), and littermate controls. Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee (Animal Ethics Committee) approval was obtained for all experimental work.

Cell preparations. Cells were positively selected using MACS (Miltenyi Biotec) or MoFlow cell sorter (BD Biosciences) or were purified using the mouse $CD4^+/CD25^+$ isolation kit (Miltenyi Biotec). Cells from Foxp3 knock-in animals were sorted on the basis of GFP fluorescence.

Antibodies and reagents. The following antibodies were used: rabbit α–mouse CD39 polyclonal antibody (27), FITC- and PE-conjugated goat α–rabbit Ig (Jackson ImmunoResearch Laboratories), α–mouse CD4, CD8, B220, CD25, CD5, CD73, CD62L, and CD45RB (eBioscience), and α–mouse CD3 and CD28 (BD Biosciences). Adenosine receptor agonists used in this study were CGS-21680, NECA, C7938, C277 (Sigma-Aldrich), and ATL146e (Adenosine Therapeutics).

Quantitative TaqMan real-time PCR. A sequence detection system (ABI PRISM 7900HT; Applied Biosystems) was used for real-time PCR analysis. Primer-probe sets and TaqMan Universal PCR Master Mix were purchased from Applied Biosystems. Gene expression was analyzed against mouse GAPDH.

ATPase and ADPase assays. 5×10^4 $CD4^+/CD25^+$ or $CD4^+/CD25^-$ cells were isolated and washed three times in cold phosphate-free buffer. Cells were warmed in incubation buffer (10 mM glucose, 20 mM Hepes, pH 7.5, 5 mM KCl, 120 mM NaCl, 2 mM $CaCl_2$, and 5 mM tetramisole) to 37°C for 10 min. Cells were then incubated in the same buffer with 2 mM ATP for 10 min. Reactions were stopped with the addition of trichloroacetic acid to a final concentration of 5% and immediately put on ice. Phosphate concentration was measured after the addition of Malachite green/polyvinyl alcohol/ammonium molybdate solution for 20 min by a spectrophotometer (ELx808 Ultra Microplate Reader; Bio-Tek Instruments, Inc.) at 610 nm and compared against a standard curve.

TLC. 2 mCi/ml [^{14}C]ADP (Ge Healthcare) was added to cell cultures; aliquots were removed and analyzed for the presence of [^{14}C]ADP hydrolysis products by TLC (three different cell culture preparations).

Functional assays. T cells (5×10^4/well) were cultured with irradiated DBA2 splenic leukocytes (2×10^5/well) or 2.5 μg/ml plate-bound α-CD3 and 2.5 μg/ml soluble α-CD28. $CD4^+/CD25^+$ or $CD4^+/CD39^+$ T cells were mixed with 5×10^4 $CD4^+/CD25^-$, $CD4^+/CD39^-$, or $CD4^+/CD25^-/CD39^-$ T cells in the presence of irradiated syngeneic splenocytes depleted of $CD3^+$ cells and supplemented with 5 μg/ml α-CD3. Data are expressed as mean counts per minute in triplicate wells. WT or *Cd39*-null T reg cells were mixed with WT or A2A-null $CD4^+/CD25^-$ that was previously labeled with 5 μM CFSE (Invitrogen), stimulated with α-CD3 and α-CD28, and analyzed by FACS.

Adoptive transfer experiments. T cells from *Cd39*-null or WT mice were transferred into C57BL/6 Rag 1–deficient mice. The next day, mice received an allogeneic skin graft from BALB/c mice. Grafts were considered to be rejected when ~60% of the graft was destroyed.

We thank Jean Sevigny for generating anti–mouse CD39 polyclonal antibodies and Christina Dore for help with the quantitative real-time PCR studies.

This project was funded through grants from the National Institutes of Health (to S.C. Robson and T.B. Strom) and the Juvenile Diabetes Research Foundation (to T.B. Strom). K.M. Dwyer is the recipient of a CJ Martin fellowship from the National Health and Medical Research Council of Australia.

The authors have no conflicting financial interests.

Submitted: 30 November 2006
Accepted: 19 April 2007

REFERENCES

1. Sakaguchi, S., N. Sakaguchi, M. Asano, M. Itoh, and M. Toda. 1995. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J. Immunol.* 155: 1151–1164.
2. Zheng, X.X., A. Sanchez-Fueyo, M. Sho, C. Domenig, M.H. Sayegh, and T.B. Strom. 2003. Favorably tipping the balance between cytopathic and regulatory T cells to create transplantation tolerance. *Immunity.* 19:503–514.
3. Sakaguchi, S. 2000. Regulatory T cells: key controllers of immunologic self-tolerance. *Cell.* 101:455–458.
4. Belkaid, Y., and B.T. Rouse. 2005. Natural regulatory T cells in infectious disease. *Nat. Immunol.* 6:353–360.
5. Rouse, B.T., and S. Suvas. 2004. Regulatory cells and infectious agents: detentes cordiale and contraire. *J. Immunol.* 173:2211–2215.
6. Hori, S., T. Nomura, and S. Sakaguchi. 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science.* 299:1057–1061.
7. Fontenot, J.D., J.P. Rasmussen, L.M. Williams, J.L. Dooley, A.G. Farr, and A.Y. Rudensky. 2005. Regulatory T cell lineage specification by the forkhead transcription factor foxp3. *Immunity.* 22:329–341.

8. Gavin, M.A., J.P. Rasmussen, J.D. Fontenot, V. Vasta, V.C. Manganiello, J.A. Beavo, and A.Y. Rudensky. 2007. Foxp3-dependent programme of regulatory T-cell differentiation. *Nature*. 445:771–775.
9. Paust, S., L. Lu, N. McCarty, and H. Cantor. 2004. Engagement of B7 on effector T cells by regulatory T cells prevents autoimmune disease. *Proc. Natl. Acad. Sci. USA*. 101:10398–10403.
10. Mellor, A.L., and D.H. Munn. 2004. IDO expression by dendritic cells: tolerance and tryptophan catabolism. *Nat. Rev. Immunol.* 4:762–774.
11. Iellem, A., M. Mariani, R. Lang, H. Recalde, P. Panina-Bordignon, F. Sinigaglia, and D. D'Ambrosio. 2001. Unique chemotactic response profile and specific expression of chemokine receptors CCR4 and CCR8 by CD4(+)CD25(+) regulatory T cells. *J. Exp. Med*. 194:847–853.
12. Salomon, B., D.J. Lenschow, L. Rhee, N. Ashourian, B. Singh, A. Sharpe, and J.A. Bluestone. 2000. B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes. *Immunity*. 12:431–440.
13. Lehmann, J., J. Huehn, M. de la Rosa, F. Maszyna, U. Kretschmer, V. Krenn, M. Brunner, A. Scheffold, and A. Hamann. 2002. Expression of the integrin alpha Ebeta 7 identifies unique subsets of CD25+ as well as CD25- regulatory T cells. *Proc. Natl. Acad. Sci. USA*. 99:13031–13036.
14. Liu, W., A.L. Putnam, Z. Xu-Yu, G.L. Szot, M.R. Lee, S. Zhu, P.A. Gottlieb, P. Kapranov, T.R. Gingeras, B. Fazekas de St Groth, et al. 2006. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. *J. Exp. Med*. 203:1701–1711.
15. Asseman, C., S. Mauze, M.W. Leach, R.L. Coffman, and F. Powrie. 1999. An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. *J. Exp. Med*. 190:995–1004.
16. Chen, M.L., M.J. Pittet, L. Gorelik, R.A. Flavell, R. Weissleder, H. von Boehmer, and K. Khazaie. 2005. Regulatory T cells suppress tumor-specific CD8 T cell cytotoxicity through TGF-beta signals in vivo. *Proc. Natl. Acad. Sci. USA*. 102:419–424.
17. Williams, L.M., and A.Y. Rudensky. 2007. Maintenance of the Foxp3-dependent developmental program in mature regulatory T cells requires continued expression of Foxp3. *Nat. Immunol*. 8:277–284.
18. Sitkovsky, M.V., and A. Ohta. 2005. The 'danger' sensors that STOP the immune response: the A2 adenosine receptors? *Trends Immunol*. 26:299–304.
19. Huang, S., S. Apasov, M. Koshiba, and M. Sitkovsky. 1997. Role of A2a extracellular adenosine receptor-mediated signaling in adenosine-mediated inhibition of T-cell activation and expansion. *Blood*. 90: 1600–1610.
20. Armstrong, J.M., J.F. Chen, M.A. Schwarzschild, S. Apasov, P.T. Smith, C. Caldwell, P. Chen, H. Figler, G. Sullivan, S. Fink, et al. 2001. Gene dose effect reveals no Gs-coupled A2A adenosine receptor reserve in murine T-lymphocytes: studies of cells from A2A-receptor-gene-deficient mice. *Biochem. J.* 354:123–130.
21. Ohta, A., and M. Sitkovsky. 2001. Role of G-protein-coupled adenosine receptors in downregulation of inflammation and protection from tissue damage. *Nature*. 414:916–920.
22. Ohta, A., E. Gorelik, S.J. Prasad, F. Ronchese, D. Lukashev, M.K. Wong, X. Huang, S. Caldwell, K. Liu, P. Smith, et al. 2006. A2A adenosine receptor protects tumors from antitumor T cells. *Proc. Natl. Acad. Sci. USA*. 103:13132–13137.
23. Naganuma, M., E.B. Wiznerowicz, C.M. Lappas, J. Linden, M.T. Worthington, and P.B. Ernst. 2006. Cutting edge: critical role for A2A adenosine receptors in the T cell-mediated regulation of colitis. *J. Immunol*. 177:2765–2769.
24. Resta, R., Y. Yamashita, and L.F. Thompson. 1998. Ecto-enzyme and signaling functions of lymphocyte CD73. *Immunol. Rev*. 161:95–109.
25. Mizumoto, N., T. Kumamoto, S.C. Robson, J. Sevigny, H. Matsue, K. Enjyoji, and A. Takashima. 2002. CD39 is the dominant Langerhans cell-associated ecto-NTPDase: modulatory roles in inflammation and immune responsiveness. *Nat. Med*. 8:358–365.
26. Kobie, J.J., P.R. Shah, L. Yang, J.A. Rebhahn, D.J. Fowell, and T.R. Mosmann. 2006. T regulatory and primed uncommitted CD4 T cells express CD73, which suppresses effector CD4 T cells by converting 5′-adenosine monophosphate to adenosine. *J. Immunol*. 177:6780–6786.
27. Sevigny, J., C. Sundberg, N. Braun, O. Guckelberger, E. Csizmadia, I. Qawi, M. Imai, H. Zimmermann, and S.C. Robson. 2002. Differential catalytic properties and vascular topography of murine nucleoside triphosphate diphosphohydrolase 1 (NTPDase1) and NTPDase2 have implications for thromboregulation. *Blood*. 99:2801–2809.
28. Bettelli, E., Y. Carrier, W. Gao, T. Korn, T.B. Strom, M. Oukka, H.L. Weiner, and V.K. Kuchroo. 2006. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature*. 441:235–238.
29. Enjyoji, K., J. Sevigny, Y. Lin, P.S. Frenette, P.D. Christie, J.S. Esch II, M. Imai, J.M. Edelberg, H. Rayburn, M. Lech, et al. 1999. Targeted disruption of cd39/ATP diphosphohydrolase results in disordered hemostasis and thromboregulation. *Nat. Med*. 5:1010–1017.
30. Erdmann, A.A., Z.G. Gao, U. Jung, J. Foley, T. Borenstein, K.A. Jacobson, and D.H. Fowler. 2005. Activation of Th1 and Tc1 cell adenosine A2A receptors directly inhibits IL-2 secretion in vitro and IL-2-driven expansion in vivo. *Blood*. 105:4707–4714.
31. Slakey, L.L., K. Cosimini, J.P. Earls, C. Thomas, and E.L. Gordon. 1986. Simulation of extracellular nucleotide hydrolysis and determination of kinetic constants for the ectonucleotidases. *J. Biol. Chem*. 261: 15505–15507.
32. Zelenika, D., E. Adams, S. Humm, C.Y. Lin, H. Waldmann, and S.P. Cobbold. 2001. The role of CD4+ T-cell subsets in determining transplantation rejection or tolerance. *Immunol. Rev*. 182:164–179.
33. Chai, J.G., S.A. Xue, D. Coe, C. Addey, I. Bartok, D. Scott, E. Simpson, H.J. Stauss, S. Hori, S. Sakaguchi, and J. Dyson. 2005. Regulatory T cells, derived from naive CD4+CD25- T cells by in vitro Foxp3 gene transfer, can induce transplantation tolerance. *Transplantation*. 79:1310–1316.
34. De Meester, I., S. Korom, J. Van Damme, and S. Scharpe. 1999. CD26, let it cut or cut it down. *Immunol. Today*. 20:367–375.
35. Deaglio, S., T. Vaisitti, S. Aydin, E. Ferrero, and F. Malavasi. 2006. In-tandem insight from basic science combined with clinical research: CD38 as both marker and key component of the pathogenetic network underlying chronic lymphocytic leukemia. *Blood*. 108:1135–1144.
36. Koziak, K., E. Kaczmarek, A. Kittel, J. Sevigny, J.K. Blusztajn, J. Schulte Am Esch II, M. Imai, O. Guckelberger, C. Goepfert, I. Qawi, and S.C. Robson. 2000. Palmitoylation targets CD39/endothelial ATP diphosphohydrolase to caveolae. *J. Biol. Chem*. 275:2057–2062.