Generation and Accumulation of Immunosuppressive Adenosine by Human CD4+CD25high FOXP3+ Regulatory T Cells*

Naturally occurring regulatory T cells (nTreg) are crucial for maintaining tolerance to self and thus preventing autoimmune diseases and allograft rejections. In cancer, Treg down-regulate anti-tumor responses by several distinct mechanisms. This study analyzes the role the adenosinergic pathway plays in suppressive activities of human nTreg. Human CD4+CD25high FOXP3+ Treg overexpress CD39 and CD73, ectonucleotidases sequentially converting ATP into AMP and adenosine, which then binds to A2a receptors on effector T cells, suppressing their functions. CD4+CD39+ and CD4+CD25high T cells express low levels of adenosine deaminase (ADA), the enzyme responsible for adenosine breakdown, and of CD26/ADA but express low levels of CD39 and CD73. Inhibitors of ectonucleotidase activity (e.g. ARL67156) and antagonists of the A2a receptor (e.g. ZM241385) blocked Treg-mediated immunosuppression. The inhibition of ADA activity on effector T cells enhanced Treg-mediated immunosuppression. Thus, human nTreg characterized by the presence of CD39 and the low expression of CD26/ADA are responsible for the generation of adenosine, which plays a major role in Treg-mediated immunosuppression. The data suggest that the adenosinergic pathway represents a potential therapeutic target for regulation of immunosuppression in a broad variety of human diseases.

Treg2 are a subpopulation of CD4+ T cells, which are central to the acquisition and maintenance of immunological self-tolerance as well as tolerance to tissue grafts and prevention of autoimmune diseases (1). Treg demonstrate considerable diversity. To date, two major types of Treg have been described in humans: (a) thymus-derived, naturally occurring Treg (nTreg) and (b) inducible Treg. Phenotypically defined as CD4+CD25high FOXP3+, nTreg modulate immune responses by suppressing functions of other T cells, mainly by cell-to-cell contact-dependent and antigen-independent mechanisms. Suppression may be mediated by membrane-bound transforming growth factor-β and/or other surface-associated molecules such as CD95, perforin, or granzyme B (1–5). In contrast, inducible Treg, including T regulatory type 1 cells, arise in the periphery upon antigen contact and express CD132 rather than CD25 and perhaps FOXP3high (6). They mediate suppression by cell-to-cell contact-independent secretion of cytokines such as IL-10 and transforming growth factor-β (5, 7, 8). Suppressive functions of Treg are regulated by the forkhead transcription factor (FOXP3), currently considered to be a marker for Treg. While FOXP3 is exclusively expressed on murine Treg, in humans, low levels of FOXP3 are also detectable in effector T cells as well as a variety of malignant cells (9, 10). FOXP3 regulates expression and function of several membrane proteins operationally important for Treg, including IL-2, CD25, CTLA-4, and GITR.

Although the dynamics of Treg/responder cell (RC) interactions have been under intense investigation in recent years, considerable controversy exists as to the mechanisms utilized by Treg to mediate immunosuppression. In cancer, elevated frequencies and suppressor activity of Treg at the tumor site and in the peripheral blood are, at least in part, responsible for tumor immune escape (11). Attenuation of Treg functions in patients with cancer is thus desirable (12). In autoimmune disorders, the Treg number and their capacity to inhibit autoreactive T cells are decreased (13), suggesting that a restoration of Treg numbers and function could be beneficial. Currently, Treg-based therapies are being clinically evaluated in patients with cancer, graft-versus-host disease or autoimmune diseases (14–16). However, the existence of phenotypically and functionally distinct subsets of human Treg, which can mediate suppression via distinct mechanisms, from secretion of IL-10, transforming growth factor-β, cAMP, granzyme B, perforin, and IL-35 to expression of LAG3 and GITR or CTLA4/B7 interactions (17–20), suggests that the use of Treg in the clinic requires further studies.

Recently, it has been reported that CD39 is expressed on human and murine Treg, whereas CD73 is found only on the

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The abbreviations used are: Treg, regulatory T cell; nTreg, naturally occurring Treg; ADA, adenosine deaminase; NC, normal control; S, suppressor cell; RC, responder cell; A2aR, A2a receptor; GFTR, glucocorticoid-induced tumor necrosis factor receptor; PBMC, peripheral blood mononuclear cell; MFI, mean fluorescence intensity; FITC, fluorescein isothiocyanate; FOXP3, forkhead transcription factor; Ab, antibody.

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A study on the production of adenosine by human regulatory T cells, with a focus on the role of CD39, CD73, and CD26 in adenosine generation and its regulatory effects on T-cell function.

**Surface of murine Treg**

CD39, a member of the ectonucleotidase triphosphate diphosphohydrolase family, is the rate-limiting enzyme that hydrolyzes ATP and ADP into AMP, while CD73, an ecto-5’-nucleotidase, exists in a soluble or membrane-bound form and catalyzes the dephosphorylation of AMP to adenosine. CD39 and CD73 in combination with adenosine deaminase (ADA), the enzyme degrading adenosine to inosine, work together in regulating pericellular adenosine concentrations. Cellular expression of CD39 and CD73 is distinct: CD39, initially identified as an activation marker for B cells, is also expressed on the surface of activated T cells and other hematopoietic cells, including natural killer cells, monocytes/macrophages, and dendritic cell; CD73 is expressed by CD4⁺CD25⁺FOXP3⁺ Treg cells as well as CD4⁺CD25⁻uncommitted precursor helper T cells. There may also be differences in expression of these markers between humans and mice. For example, in humans, ADA is associated with the extracellular domain of CD26, a widely distributed cell membrane-bound 110-kDa glycoprotein with intrinsic dipeptidyl peptidase IV activity, whereas this complex does not form in mice. The presence of CD26-ADA complex in human T cells provides an opportunity to assess the expression and function of ADA in Treg. In the immune system, CD26 promotes T-cell activation as well as interactions of T cells with antigen-presenting cells.

Recent studies indicate that adenosine produced by Treg may be in part responsible for their suppressive functions. Adenosine is a signaling molecule that binds to adenosine receptors (A₁, A₂a, A₂b, and A₃) present on many cell types, and it regulates multiple physiological responses, including anti-inflammatory effects. The ligation of adenosine to the A₂a receptor on immune cells activates an immunoinhibitory loop through the elevation of cytoplasmic cAMP. Specifically, adenosine receptor signaling interferes with functions of immune cells, inhibiting proliferation and cytokine production.

Whereas tissue-derived adenosine acting via adenosine A₂a receptor has been reported to be an important regulator of T-cell functions, the role of adenosine plays in the suppressive activity of human Treg has not been investigated. Therefore, in this study we evaluated contributions of the adenosinergic pathway to the functional activity of human Treg. We determined that the CD39 and CD73 expression as well as the low expression level CD26 and ADA in Treg seem to be characteristic features of this very special subset of human T cells. In contrast, human CD4⁺CD25⁻effector T cells express low levels of CD39 but abundant CD26 and ADA. The ability of Treg to hydrolyze ATP and generate high pericellular concentrations of adenosine is in part responsible for their suppressive activity.

**Experimental Procedures**

**Healthy Volunteers**—Peripheral venous blood samples for phenotypic and functional analyses of lymphocyte subsets were obtained from 15 normal controls. All subjects signed an informed consent approved by the Institutional Review Board of the University of Pittsburgh. The normal control group included 4 males and 11 females with a mean age of 39 years (range, 24–58 years).

**Collection of PBMCs**—Blood samples (20–30 ml) were drawn into heparinized tubes and centrifuged on Ficoll-Hypaque gradients. PBMCs were recovered, washed in AIM-V medium (Invitrogen), counted in a trypan blue dye, and immediately used for experiments evaluating the phenotype.

**Separation of Treg**—CD4⁺CD25⁺ as well as CD4⁺CD25⁻ T cells were freshly isolated fromuffy coats using the Regulatory T cell Separation Kit and AutoMACS (Miltenyi Biotech). After the cells were washed twice, the CD4⁺ T cells were negatively selected first from the total PBMCs and, afterward, positive selection was performed on anti-CD25 magnetic beads, separating CD4⁺CD25⁺ and CD4⁺CD25⁻ cells. The purity of each cell fraction was >97%. CD4⁺CD25³high cells (i.e., those with an MFI of >120) were single cell-sorted following staining with an anti-CD25 antibody and using a Beckman Coulter cell sorter.

**Antibodies**—The following anti-human monoclonal antibodies were used for flow cytometry: anti-CD3-EC, anti-CD4-EC, anti-CD4-PC5, anti-CD8-PC5, anti-CD25-PC5, anti-GITR-FITC, anti-FOXO3-FITC, anti-CD39-FTTC, anti-CD39-PE, anti-CD26-PE, anti-CD73-PE, unconjugated anti-CD73, and anti-CTLA4-PE. Antibodies and their respective isotypes, which served as a negative control for surface as well as intracellular staining, were purchased from eBioscience; anti-CD26-PE and anti-CD73-PE were purchased from BD Pharmingen; anti-GITR-FITC and anti-CTLA4-PE were purchased from R&D Systems; unconjugated anti-CD73 (clone: 10f1) was purchased from Santa Cruz Biotechnology; and FITC-conjugated AffiniPure Goat anti-mouse secondary antibody was purchased from Jackson ImmunoResearch. Before use, all antibodies were titrated using resting as well activated PBMCs obtained from normal controls to determine the optimal staining dilution for each antibody.

**Surface and Intracellular Staining**—Freshly isolated cells or activated cells were stained for flow cytometry as previously described. Briefly, cells were incubated with the antibodies for surface markers for 30 min at 4 °C in the dark and then fixed with 2% (w/v) paraformaldehyde in PBS for 15 min. Afterward, cells were permeabilized with 0.1% (w/v) saponin and stained with antibodies specific for intracellular markers for 30 min at 4 °C in the dark. Cells were washed twice with 0.1% saponin in PBS, resuspended in a flow solution and immediately analyzed by flow cytometry. Appropriate isotype controls were included for each sample.

**Flow Cytometry**—Flow cytometry was performed using an EPICS® XL-MCL flow cytometer equipped with Expo32 software (Beckman Coulter). The acquisition and analysis gates were restricted to the lymphocyte gate based on characteristic properties of the cells in the forward (FSC) and side scatter (SSC). Forward and side scatter were set in a linear scale, and 10⁵ cells were acquired for analysis, which was performed using the Coulter EXPO 32v2.2 analysis program. For additional analyses, gates were restricted to the CD3⁺CD4⁺, CD3⁺CD8⁺, or different CD4⁺CD25⁺ T-cell subsets.
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Suppression Assay—Single cell-sorted CD4+CD25\textsuperscript{high} and CD4+CD25\textsuperscript{neg} cells were cytocentrifuged onto glass slides and stained using a standard immunoperoxidase method. Cells were first fixed using a 1:1 methanol/acetic acid solution and then dried at room temperature for 4 h. Afterward, cells were treated with a serum-free protein block (Dako) for 1 h at room temperature, followed by washing with PBS and an overnight incubation at 4 °C in the dark with the primary Ab. The following Abs were used: un conjugated anti-human CD73 antibody (1:100 dilution, Santa Cruz Biotechnology); rabbit anti-human CD26 Ab; mouse anti-human ADA or appropriate isotype controls. Slides were then washed and, for CD73 staining, incubated with a secondary peroxidase-conjugated goat anti-mouse antibody for 1 h (Dako). Finally, peroxidase activity was developed using a liquid diaminobenzidine chromogen system (DAB+, Dako). Sections were washed and counterstained with hematoxylin (Dako). Slides were mounted with Glycergel Mounting Medium (DakoCytomation) and analyzed under a light microscope. For CD26 and ADA staining, slides were incubated with the following secondary antibodies: donkey anti-rabbit-FITC (1:200, Santa Cruz Biotechnology) and donkey anti-mouse-Cy3 (1:500, Jackson ImmunoResearch). Afterward slides were washed, fixed, and evaluated in an inverted Olympus FluoView 1000 laser scanning confocal microscope under an oil immersion objective (Center for Biology Imaging Core Facility, University of Pittsburgh). For digital image analysis, the software Adobe Photoshop version 7.0 was used.

Mass Spectrometric Analysis of Adenosine Production—CD4+CD25\textsuperscript{neg} or CD4+CD25\textsuperscript{+} T cells (25,000 cells/well) were incubated with 10 \mu M exogenous ATP in wells of 96-well flat bottom plates. To some wells ARL67156 (250 \mu M) or \alpha,\beta-methylene-ADP (100 \mu M) was added 30 min prior to the assay. Cell supernatants were collected after various periods of incubation time. Samples were boiled for 2 min and stored on dry ice until analysis. Adenosine was measured on a ThermoFinnigan LCQ Duo mass spectrometer equipped with electrospray ionization. The samples were separated using a C18 column (Eclipse XDB-C18, 4.6 × 150 mm, 5 \mu m). A mobile phase consisted of 0.1% formic acid water and methanol solution. The flow rate of a mobile phase was 0.6 ml/min. The analytes were monitored with single-ion monitoring in the positive-ion mode; for adenosine the mass-to-charge ratio was 268; for 10–13 C-adenosine (internal standard) the mass-to-charge ratio was 278. The internal standard (10–13 C-adenosine) in supernatants is 10 pg/\mu l. The average concentration of adenosine was determined in duplicate wells.

Western Blots—Whole cell protein extracts were prepared by CD4+CD25\textsuperscript{neg} as well as CD4+CD25\textsuperscript{high} T cells, which were freshly isolated fromuffy coats as described above. Afterward, cells were washed twice and lysed at 4 °C in a lysis buffer containing 0.5% Nonidet P-40, 150 mM NaCl, and 50 mM Tris base (Sigma). Laemmli loading buffer (4% SDS, 10% \beta-mercaptoethanol 20% glycerol, 0.004% bromphenol blue, 0.125 M Tris-HCl) was added to the cell lyses at a 1:1 ratio (v/v), and the lyses were then boiled for 5 min. Protein extracts were subjected to electrophoresis on 4–15% Tris-HCl gradient gels (Bio-Rad) and were subsequently transferred to polyvinylidene fluoride membranes (Millipore). The membranes were treated with polyclonal primary antibodies specific for human CD39 (Santa Cruz Biotechnology, rabbit 1:200), CD73 (Abcam, mouse, 1:200), CD26 (Santa Cruz Biotechnology, rabbit, 1:200), and ADA (Santa Cruz Biotechnology, mouse, 1:100) followed by goat anti-rabbit or goat anti-mouse secondary antibody conjugated to horseradish peroxidase (Pierce, goat, 1:40,000). Supersignal West Femto Maximum Sensitivity Substrate (Pierce) and Kodak BioMax MR Film were used to visualize the target proteins.

Statistical Analysis—All data were presented as the means of at least three experiments ± 1 S.D. The data were analyzed using the Student’s t test, and the p values of <0.05 were con-
Correlations were calculated using the Spearman rank correlation test.

RESULTS

Expression of CD39, CD73, CD26, and ADA on Treg versus CD4$^+$ Effector Cells—Lymphocytes in PBMCs were identified based on their forward scatter (FS) and sideward scatter (SS) characteristics (Fig. 1A). Next, the frequency of CD4$^+$ CD25$^{high}$ and CD4$^+$ CD25$^{neg}$ cells in the total CD3$^+$ CD4$^+$ cell population was determined. Gating strategies are shown in Fig. 1B. CD4$^+$ cells with CD25 expression \( \geq 120 \) MFI were considered as CD4$^+$ CD25$^{high}$ cells; those with a MFI \(< 120\) as CD25$^{intermediate/low}$ (1).

FIGURE 1. Gating strategy for CD4$^+$ CD25$^{high}$ T cells and expression of CD39 or CD26 in human CD4$^+$ CD25$^{high}$ cells and CD4$^+$ CD25$^{neg}$ cells. A, lymphocytes were identified based on their characteristic properties shown in the forward scatter (FS) and sideward scatter (SS). B, the subset of CD4$^+$ CD25$^{high}$ T cells was identified based on the MFI \( > 120 \) for CD25 expression on CD4$^+$ T cells. C, distribution of CD39 and CD26 in CD3$^+$ CD4$^+$ cells. The dot plot shows data for one representative healthy individual. D, CD4$^+$ CD25$^{high}$ and CD4$^+$ CD25$^{neg}$ T-cell subsets were stained for CD39 and CD26. Histograms show the levels of expression of these markers (bold line) in the gated populations compared with the appropriate isotype control (thin line). Data for one representative donor of 15 tested are shown. E, cells of 15 donors were stained for the surface expression of CD39 and CD26 as described under “Experimental Procedures.” The mean \% \pm S.D. values of T cells positive for these markers within each subset are shown.

considered to be significant.
The majority of the CD4<sup>+</sup>CD25<sup>high</sup> cells were positive for FOXP3 (mean of 75.6%) and mediated strong suppression, as previously reported by us (1). Double staining revealed that the majority of CD3<sup>+</sup>CD4<sup>+</sup> T cells were CD39<sup>neg</sup>CD26<sup>neg</sup> and 7.5% were CD39<sup>neg</sup>CD26<sup>high</sup>. Only a very small portion of cells (0.2%) were CD39<sup>high</sup>CD26<sup>neg</sup> (Fig. 1C). These data shown in Fig. 1C confirm that CD39 and CD26 are expressed on different subsets of CD4<sup>+</sup> T cells.

**FIGURE 2.** Expression of CD73, CD39, and CD26 on different T-cell subsets. A, CD4<sup>+</sup>CD25<sup>high</sup> and CD4<sup>+</sup>CD25<sup>neg</sup> subsets of T cells were tested for permeabilization for expression of CD73 on the cell surface or in the cytoplasm. Flow cytometry data show percentages of positive cells. The data are mean values ± S.D. obtained with cells of 15 normal controls. B, lymphocytes were surface or intracellularly stained for CD73. Histograms show level of expression in the gated populations compared with appropriate isotype controls. Data for one representative individual of 15 tested are shown. C, Western blot analysis of CD39, CD73, CD26, and ADA expression in single cell-sorted CD4<sup>+</sup>CD25<sup>high</sup> and CD4<sup>+</sup>CD25<sup>neg</sup> cells. Blots show data of one representative individual from at least three experiments performed with cells of different donors. D, multicolor confocal microscopy of single cell-sorted CD4<sup>+</sup>CD25<sup>high</sup> and CD4<sup>+</sup>CD25<sup>neg</sup> cells shows the simultaneous expression of CD26 and ADA on the surface of CD4<sup>+</sup>CD25<sup>neg</sup> T cells. Relatively little expression of these markers is seen in CD4<sup>+</sup>CD25<sup>high</sup> Treg.
FIGURE 3. Multiparameter flow cytometry analyses of CD4⁺CD25⁺, CD4⁺CD39⁺, and CD4⁺CD26⁻ Treg subsets. A, phenotypic characteristics of circulating CD4⁺CD25⁺ T cells. B, phenotypic characteristics of circulating CD4⁺CD39⁺ T cells. C, phenotypic characteristics of circulating CD4⁺CD26⁻ T cells. The data are mean % ± S.D. from experiments performed with cells of 15 donors. D, correlation of CD25⁺ and CD26 expression of CD3⁺CD4⁺ T cells based on the MFI as determined by flow cytometry. E, correlation of the CD39 and CD26 expression (MFI) in CD4⁺CD25⁺ T cells. F, correlation of FOXP3 and CD39 expression based on the MFI in CD3⁺CD4⁺CD25⁺ T cells.
Next, CD39 expression on human CD4⁺CD25(high) and CD4⁺CD25(neg) cells was examined by flow cytometry (Fig. 1, D and E). As shown in Fig. 1E, only 8 ± 4% of cells within the CD3⁺CD4⁺ cell subset were CD39⁺. The CD4⁺CD25(high) subset was highly enriched in CD39⁺ cells (79 ± 15%, p < 0.001) relative to CD4⁺CD25(neg) cells (5 ± 2%). The CD4⁺CD25(int/low) cell subset contained T cells with low FOXP3 (11 ± 2%) and CD39 (9 ± 3%) expression but a relatively high CD26 surface expression (76 ± 7%). Neither the CD4⁺CD25(low) subset (data not shown) nor the CD3⁺CD8⁺ subset (Fig. 1E) expressed CD39. These results indicate that CD39 expression is largely restricted to CD4⁺CD25high Treg. In contrast, CD26 was broadly expressed on >80% of all CD3⁺CD4⁺ cells (Fig. 1E). Gating on CD4⁺CD25(high) and CD4⁺CD25(neg) T cell subsets, we observed a significantly higher CD26 expression on CD4⁺CD25(neg) subset relative to CD4⁺CD25high Treg (Fig. 1, D and E), suggesting that CD26 was preferentially expressed on the conventional CD4⁺ T cells. Its expression increased significantly upon T-cell activation (data not shown).

CD73 surface expression was very low in the CD4⁺CD25high T-cell subset, ranging from below 1 up to 7% positive cells (Fig. 2A). This is in contrast to murine Treg, which were reported to express high levels of cell membrane-associated CD73 (21). Although no difference in the level of CD7 expression on the cell surface was seen between CD4⁺CD25(high) versus CD4⁺CD25(neg) T cell subsets, intracellular staining using permeabilized Treg followed by flow cytometry showed that most CD4⁺CD25high Treg (71 ± 5%) were positive for CD73 (Fig. 2A). In addition, mean fluorescence intensity (MFI) measurements of CD73 expression levels in permeabilized T cells showed that the CD4⁺CD25high cells had higher levels of CD73 expression (p < 0.001) than CD4⁺CD25(neg) T cells (Fig. 2B).

Given the observed differential expression of ectonucleotidases and CD26 in the T cell subsets, we next performed Western blot analyses using freshly isolated and single cell-sorted CD4⁺CD25high and CD4⁺CD25(neg) cells. Because CD26 expression was previously reported to be associated with ADA

(25), we also determined the levels of ADA in both these cell subsets. As expected, CD39 and CD73 were expressed in CD4⁺CD25high Treg but not in CD4⁺CD25(neg) conventional CD4⁺ T cells (Fig. 2C). In contrast, CD26 and ADA were predominantly expressed in CD4⁺CD25(neg) T cells and only weakly in Treg (Fig. 2C). To further confirm that both CD26 and ADA expression levels were high in CD4⁺CD25(neg) conventional T cells and low in Treg, confocal microscopy was performed using single cell-sorted cells. Fig. 2D shows high levels of both markers on the surface of CD25(neg) conventional CD4⁺ T cells and low expression levels in CD4⁺CD25high Treg. It also convincingly demonstrates co-expression of CD26 and ADA on the surface of conventional CD4⁺ T cells (Fig. 2D).

Phenotypic Characterization of the CD4⁺CD39⁺ and CD4⁺CD26neg T-cell Fractions—Our data showed that CD39 and CD26 potentially define distinct subsets of CD4⁺ T cells. To further characterize the Treg subpopulations that expressed CD39 versus conventional CD4⁺ T cells with high CD26 expression, additional multiparameter flow cytometry analyses were performed. Gating on CD4⁺CD25high cells, we again observed enrichment in CD39⁺ cells and low percentages of CD26⁺ and GITR⁺ cells (Fig. 3A). When gates were set on CD39⁺ or CD26neg subsets of CD4⁺ cells, a similar marker profile was observed (Fig. 3, B and C), suggesting a reciprocal relationship between these subsets. As shown in Fig. 3D, CD26high expression levels correlated negatively with those for CD26 on CD4⁺ T cells (r = 0.56). Within the CD4⁺CD25high Treg subset, levels of CD39 expression correlated negatively with those for CD26 (r = 0.836) (Fig. 3E), whereas a positive correlation was seen for the levels of FOXP3 and CD39 expression in CD4⁺CD25high cells (r = 0.90) (Fig. 3F). These results confirm that CD39 is a biomarker of CD4⁺FOXP3⁺ and CD4⁺CD25high Treg.

Suppressor Function of CD4⁺CD39⁺ and CD4⁺CD26neg T-cell Subsets—To analyze suppressor activity of CD4⁺CD39⁺ and CD4⁺CD26neg cells, these cell subsets were single cell sorted from freshly isolated PBMCs. The sorted cells serving as suppressor(s) were co-incubated with autologous CD4⁺CD25(neg) RC at different S/RC ratios. After a 5-day culture, the mean suppressor activity of CD4⁺CD39⁺ cells at the 1:5RC ratio was 43 ± 3%, and the suppression of proliferation linearly decreased upon further dilution of S (Fig. 4A). The mean suppressor activity of CD4⁺CD26neg cells at the 1:5RC ratio was 24 ± 1%, and it also significantly decreased upon S cell dilution (Fig. 4B). CD4⁺CD39neg as well as CD4⁺CD26⁺ cells did not suppress proliferation of RCs. These results indicate that both CD39⁺ and CD26neg subsets of Treg mediate suppressive activity in conventional CFSE-based proliferation assays with autologous RC. The somewhat lower suppressive
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Effects of CD25neg cells relative to CD39+ cells can be explained by the fact that not all CD26neg cells expressed CD39 (Fig. 3, B and C).

Hydrolysis of ATP by CD4+ CD25+ versus CD4+ CD25neg Cells—Because CD39 is an ectonucleotidase hydrolyzing ATP, it was important to determine whether the isolated Treg expressing this marker also had enzymatic activity. Single cell-sorted CD4+ CD25+ cells and CD4+ CD25neg cells were stimulated with OKT-3 and anti-CD28 Ab for 3 days and afterward incubated with various concentrations of exogenous ATP for 30 min. In these and subsequent analyses of T-cell functions, we used CD4+ CD25+ and not CD4+ CD25low T cells because of a requirement for relatively high numbers of Treg in these functional assays. Compared with autologous CD4+ CD25neg cells, CD4+ CD25+ Treg hydrolyzed significantly more ATP (Fig. 5A; p < 0.02 to 0.001). Upon experimental design, we incubated both cell types with 1 μM exogenous adenosine and harvested the cell supernatant after various time points. In Treg cultures, the amount of unmetabolized adenosine was four times higher than in CD4+ CD25neg cell cultures (data not shown).

Adenosine Generation by CD4+ CD25+ Cells versus CD4+ CD25neg Cells—To further analyze the activity of the ectonucleotidases CD39 and CD73 in Treg, we determined the ability of activated CD4+ CD25+ and CD4+ CD25neg cells to produce adenosine following the addition of 10 μM exogenous ATP. Adenosine levels were measured in the cell supernatants collected at various time points after ATP addition. As shown in Fig. 5C, CD4+ CD25+ cells produced significantly more adenosine than CD4+ CD25neg cells. Upon co-incubation of CD4+ CD25+ cells with ARL67156, adenosine production was almost completely blocked (p < 0.09). Also, we observed a complete inhibition of adenosine production by CD4+ CD25+ as cells when α,β-methylene ADP, a specific CD73 inhibitor, was added to selected wells (Fig. 5D). Both inhibitors did not have any effects on CD4+ CD25neg cells (data not shown). Next, using the same pretreatment with ARL67156, a selective inhibitor of ecto-ATPases, the capability of CD4+ CD25+ cells to hydrolyze ATP was decreased at different ATP concentrations (Fig. 5B). Whereas the inhibitors did not show any effect on CD4+ CD25neg cells (data not shown). We also noted that unstimulated, freshly isolated CD4+ CD25+ cells hydrolyzed the same amount of exogenous ATP as stimulated CD4+ CD25+ cells, but needed more time for hydrolysis (data not shown).

FIGURE 5. Enzymatic activity of the ectonucleotidase CD39 and adenosine production in CD4+ CD25+ and CD4+ CD25neg cells. A, MACS-sorted CD4+ CD25+ and CD4+ CD25neg cells were plated in 96-well plates in serum-free medium with either 10 or 50 μM of exogenous ATP for 30 min, and unhydrolyzed ATP was measured. “Standard” represents exogenous ATP in serum-free medium incubated without cells plus endogenous ATP released by lysed cells, which were cultured alone in serum-free medium without the addition of exogenous ATP. Data are mean values ± S.D. obtained in three independent experiments. B, measurement of remaining ATP after CD4+ CD25+ cells were incubated with different concentrations of exogenous ATP with or without the addition of ARL67156 (selective ecto-ATPase inhibitor). Data are from one representative experiment of three performed with cells of three different donors. C, CD4+ CD25+ or CD4+ CD25neg were incubated for various periods of time in the presence of 10 μM ATP. The levels of adenosine were determined in the cell supernatant using mass spectrometry. Data are from one representative experiment of three performed with cells of different donors. D, CD4+ CD25+ cells/well were incubated with or without ARL67156 (25 μg/ml, an ectonucleotidase inhibitor) or α,β-methylene ADP (100 μg/ml, selective CD73 inhibitor). Adenosine production by CD4+ CD25+ cells in the absence or presence of the ectonucleotidase inhibitors were assessed using mass spectrometry. Data are from one representative experiment of three performed with cells of different donors.
used these cells isolated by AutoMACs to confirm that adenosine produced during ATP cleavage by the associated ectonucleotidases is responsible for suppression of RCs. We showed that ARL67156, a structural analogue of ATP and an ectonucleotidase inhibitor, added to co-cultures of CD4+CD39+ cells serving as S cells with autologous RCs significantly decreased suppression levels (p < 0.02) compared with cultures without the inhibitor (38 ± 9% versus 19 ± 1%) (Fig. 6B). In the same type of co-culture, the addition of α,β-methylene-ADP, an inhibitor of CD73, also significantly reduced (p < 0.03) Treg-mediated suppression (Fig. 6C). In the presence of erythro-9-(2-hydroxy-3-nonyl)adenine, an inhibitor of ADA, suppression mediated by Treg was significantly increased (p < 0.02) compared with cultures without the inhibitor (36 ± 1 versus 52 ± 4%) (Fig. 6C). Thus, blocking of ADA activity in the co-cultures resulted in a higher level of adenosine produced by Treg and greater Treg-mediated suppression of RC proliferation.

**DISCUSSION**

Treg-mediated immunosuppression appears to involve multiple molecular mechanisms and signal transduction pathways. In this study, we conclusively showed that in humans, adenosine is a byproduct of Treg activity, which is used by these cells to suppress immune responses. Treg-derived adenosine is a hydrolysis product of ATP cleaved in tandem by two Treg-associated ectonucleotidases, CD39 and CD79. Upregulated expression of CD39 and CD73 by Treg was previously shown in mice by two independent groups (21, 22). Our data concerning the abundant surface expression of CD39 in human Treg are consistent with their data. Nearly all CD4+CD25highFOXP3+ human Treg were positive for CD39 and negative for CD26. This feature distinguishes human Treg from all other T-cell subsets. Surface expression of CD73, described as a characteristic surface marker of murine Treg, was only observed in a minor proportion of human Treg, although close to 50% of CD8+ T cells expressed it on the cell surface as well. Intracellular expression of CD73 was observed in over 70% of CD4+CD25high Treg and in only 20% of CD4+CD25low T cells. These data suggest that CD73 is readily shed from the surface of human lymphocytes as previously suggested (38, 39). Although CD73 surface expression is stable in

**Effects of an Adenosine Receptor Antagonist on Suppression Mediated by CD4+CD39+ Cells**—The immunosuppressive effects of adenosine are mediated via the A<sub>2a</sub>R, which is expressed on effector T cells (1). We hypothesized that, if adenosine suppresses proliferation of RCs, then blocking of the A<sub>2a</sub>R with a specific antagonist should inhibit Treg-mediated suppression. To test this hypothesis, we added ZM241385, a selective A<sub>2a</sub> and A<sub>2b</sub> receptor antagonist, to the above described S/RC co-cultures. The addition of ZM241385 almost completely blocked the suppression mediated by CD4+CD39+ cells at the 1S:1RC ratio (9 ± 1% versus 36 ± 1%; p < 0.001) (Fig. 6D). The addition of dipropylcyclopentylxanthine, a selective A1 receptor antagonist, or MRS1191, a selective A<sub>3</sub> receptor antagonist, or MRS1706, a selective A<sub>2b</sub> receptor antagonist, did not show any effect on CD39+ T cell-mediated suppression (data not shown). All antagonists incubated with RCs in the absence of S cells did not influence the proliferation of RCs.
endothelial cells (39), in lymphocytes it is not, and antibody binding to CD73 could promote its removal from the surface of T cells. Thus, in contrast to CD39, CD73 protein is mainly localized in the cytosol of human Treg. More importantly, increased levels of adenosine in cultures of Treg imply that CD73 is present in its active form in these cells, and that it is made available to cleave AMP at the cell surface. The metabolic blockade of CD73 reduced Treg-mediated suppression of RC proliferation in our hands.

Functionally, the two ectonucleotidases expressed in Treg are important for extracellular removal of ATP. This anti-inflammatory process allows Treg to infiltrate sites of chronic inflammation, including tumor tissue, synovial fluids in patients with rheumatoid arthritis, as well as the target organs in other autoimmune diseases, such as multiple sclerosis, autoimmune vasculitis, ankylosing spondylitis, or progressive systemic sclerosis. Because extracellular concentration of ATP present in injured tissues is high, Treg accumulate at sites of inflammation because they are needed to hydrolyze ATP to adenosine. The absence of Treg or their dysfunction in autoimmunity can result in exacerbated local inflammation.

Adenosine, a well studied anti-inflammatory molecule, has a number of biological effects. It reduces the production of pro-inflammatory cytokines, is anti-proliferative, abrogates autoimmune diseases and tissue destruction, promotes T-cell anergy, and protects tumors from immune-mediated destruction (27, 30, 40). This could be a rationale for accumulation of Treg at the tumor site in patients with cancer (42). Adenosine mediates a negative feedback loop by enabling cells to cope with high inflammatory stimuli and, therefore, prevents further tissue damage. Adenosine, the end product of ATP hydrolysis by Treg, enables these cells to suppress functions of effector T cells via the engagement of the A2aR. Further, adenosine-mediated suppression is favored by the up-regulation of the A2aR on effector T cells during activation (35). It not only limits or suppresses activities of effector T cells and other inflammatory cells but also interferes with dendritic cell maturation and function (41). The depletion of extracellular ATP may also contribute to Treg survival, because high levels of ATP have been shown to induce cell death acting via the purinergic P2X7 receptor (26, 42). Recently, it has been shown that tissue-derived adenosine also induces the generation of FOXP3+ and LAG-3+ Treg in mice and induces peripheral T-cell tolerance (35).

Although ectonucleotidases, CD39 and CD73, are present in most human Treg and could be considered as potential markers for this T-cell subset, CD26 is absent/low on the surface of these cells. CD26 is, however, highly expressed on all other CD4+ T-cell subsets, where it could serve as an anchor for ADA, which is capable of reducing the pericellular adenosine concentration. Because ADA is associated with the extracellular domain of surface-localized CD26, forming a cell-membrane complex and because of the lack of anti-ADA Ab suitable for flow cytometry, CD26 analysis could serve as a surrogate marker for ADA expression in T cells. The characteristic molecular profile of human Treg (CD39+CD73−CD26low/ADA low) has a functional significance, because it confers to Treg the capability to produce and sustain high levels of pericellular adenosine. Thus, Treg are the only type of T cells that possesses the full enzymatic machinery necessary to not only generate adenosine, but to sustain it at relatively high concentrations due to low ADA expression and the reduced capacity to activate ADA. The CD26-ADA complex seems to be ubiquitously expressed on effector T cells, which are thus capable of enzymatically degrading adenosine to inosine. This enzymatic activity may be important for protecting T effector cells from suppressive effects of adenosine and for regulating suppression mediated by Treg. The Treg/T effector cell ratios in the tissue microenvironment might be critical for this regulation.

To the best of our knowledge, ours is the most comprehensive study to date describing the role of adenosine in the functional activity of human Treg, and introducing CD39 as a new phenotypic and functional marker of human Treg. Because this marker is expressed on the cell surface, it is applicable to the isolation of Treg as we recently reported (43). Furthermore, the paucity of CD26-ADA expression in Treg, described in this study for the first time, provides important insights into the utilization of adenosine by Treg, which not only can produce it from ATP but accumulate it pericellularly for presentation to effector T cells via their A2a receptors.

REFERENCES

1. Strauss, L., Bergmann, C., Gooding, W., Johnson, J. T., and Whiteside, T. L. (2007) Clin. Cancer Res. 13, 6301–6311
2. Cao, X., Cai, S. F., Fehniger, T. A., Sonj, J., Collins, L. I., Piwnica-Worms, D. R., and Ley, T. J. (2007) Immunity 27, 635–646
3. Jonuleit, H., Schmitt, E., Stassen, M., Tuettenberg, A., Knop, J., and Enk, A. H. (2001) J. Exp. Med. 193, 1285–1294
4. Levings, M. K., Sangregorio, R., and Roncarolo, M. G. (2001) J. Exp. Med. 193, 1295–1302
5. Dieckmann, D., Plotter, H., Berchtold, S., Berger, T., and Schuler, G. (2001) J. Exp. Med. 193, 1303–1310
6. Bergmann, C., Strauss, L., Zeidler, R., Lang, S., and Whiteside, T. L. (2007) Cancer Immunol. Immunother. 56, 1429–1442
7. Groux, H., O’Garra, A., Bigler, M., Rouloeu, M., Antenkon, S., de Vries, J. E., and Roncarolo, M. G. (1997) Nature 389, 737–742
8. Roncarolo, M. G., Bacchetta, R., Bordignon, C., Narula, S., and Levings, M. K. (2001) Immunol. Rev. 182, 68–79
9. Ziegler, S. F. (2006) Annu. Rev. Immunol. 24, 209–226
10. Karanikas, V., Speletas, M., Zamanakou, M., Kalafa, F., Loules, G., Kerenidi, T., Barda, A. K., Gourgoulians, K. L., and Germenis, A. E. (2008) J. Transl. Med. 6, 19
11. Curiel, T. J., Coukos, G., Zou, L., Alvarez, X., Cheng, P., Mottram, P., Edvemon-Hogan, M., Conejo-Garcia, J. R., Zhang, L., Burow, M., Zhu, Y., Wei, S., Kryczek, I., Daniel, B., Gordon, A., Myers, L., Lackner, A., Disis, M. L., Knutson, K. L., Chen, L., and Zou, W. (2004) Nature Med. 10, 942–949
12. Curiel, T. J. (2007) J. Clin. Invest. 117, 1167–1174
13. Baecher-Allan, C., and Hafler, D. A. (2006) Immunol. Rev. 212, 203–216
14. Beyer, M., and Schultze, J. L. (2008) Annu. Rev. Immunol. 26, 563–596
15. Verbsky, J. W. (2007) Annu. Rev. Immunol. 25, 541–568
16. Roncarolo, M. G., Bacchetta, R., Bordignon, C., Narula, S., and Levings, M. K. (2001) Immunol. Rev. 182, 68–79
17. Ziegler, S. F. (2006) Annu. Rev. Immunol. 24, 209–226
18. Karanikas, V., Speletas, M., Zamanakou, M., Kalafa, F., Loules, G., Kerenidi, T., Barda, A. K., Gourgoulians, K. L., and Germenis, A. E. (2008) J. Transl. Med. 6, 19
19. Curiel, T. J., Coukos, G., Zou, L., Alvarez, X., Cheng, P., Mottram, P., Edvemon-Hogan, M., Conejo-Garcia, J. R., Zhang, L., Burow, M., Zhu, Y., Wei, S., Kryczek, I., Daniel, B., Gordon, A., Myers, L., Lackner, A., Disis, M. L., Knutson, K. L., Chen, L., and Zou, W. (2004) Nature Med. 10, 942–949
20. Collison, L. W., Workman, C. J., Kuo, T. T., Boyd, K., Wang, Y., Vignali, K. M., Cross, R., Sehy, D., Blumberg, R. S., and Vignali, D. A. (2007) Nature 450, 566–569
21. Huang, C. T., Workman, C. J., Flies, D., Pan, X., Marson, A. L., Zhou, G., Hipkiss, E. L., Ravi, S., Kowalski, J., Levitsky, H. I., Powell, J. D., Pardoll, D. M., Drake, C. G., and Vignali, D. A. (2004) Immunity 21, 503–513
22. Paust, S., Lu, L., McCarty, N., and Cantor, H. (2004) Proc. Natl. Acad. Sci. U.S.A. 101, 10398–10403
23. Bodor, J., Fehervari, Z., Diamond, B., and Sakaguchi, S. (2007) Eur. J. Im-
Adenosine Production by Human Regulatory T Cells

munol. 37, 884–895
21. Deaglio, S., Dwyer, K. M., Gao, W., Friedman, D., Usheva, A., Erat, A., Chen, J. F., Enjoji, K., Linden, J., Oukka, M., Kuchroo, V. K., Strom, T. B., and Robson, S. C. (2007) J. Exp. Med. 204, 1257–1265
22. Borsellino, G., Kleinewietfeld, M., Di Mitri, D., Stertnak, A., Diamantini, A., Giometto, R., Hörner, S., Centonze, D., Bernardi, G., Dell’Acqua, M. L., Rossini, P. M., Battistini, L., Rötzschke, O., and Falk, K. (2007) Blood 110, 1225–1232
23. Resta, R., Yamashita, Y., and Thompson, L. F. (1998) Immunol. Rev. 161, 95–109
24. Kobie, J. J., Shah, P. R., Yang, L., Rebhahn, J. A., Fowell, D. J., and Mosmann, T. R. (2006) J. Immunol. 177, 6780–6786
25. Schrader, W. P., West, C. A., Miczek, A. D., and Norton, E. K. (1990) J. Biol. Chem. 265, 19312–19318
26. Dong, R. P., Kameoka, J., Hegen, M., Tanaka, T., Xu, Y., Schlossman, S. F., and Morimoto, C. (1996) J. Immunol. 156, 1349–1355
27. Burnstock, G. (1997) Neuropharmacology 36, 1127–1139
28. Sitkovsky, M. V. (2003) Biochem. Pharmacol. 65, 493–501
29. Klinger, M., Freissmuth, M., and Nanoff, C. (2002) Cell Signal. 14, 99–108
30. Ohta, A., and Sitkovsky, M. (2001) Nature 414, 916–920
31. Panther, E., Corinti, S., Idzko, M., Herouy, Y., Napp, M., la Sala, A., Girolomoni, G., and Norgauer, J. (2003) Blood 101, 3985–3990
32. Raskovalova, T., Huang, X., Sitkovsky, M., Zacharia, L. C., Jackson, E. K., and Gorelik, E. (2005) J. Immunol. 175, 4383–4391
33. Sitkovsky, M. V., and Ohta, A. (2005) Trends Immunol. 26, 299–304
34. Sitkovsky, M. V., Lukashev, D., Apasov, S., Kojima, H., Koshiba, M., Caldwell, C., Ohta, A., and Thiel, M. (2004) Annu. Rev. Immunol. 22, 657–682
35. Zarek, P. E., Huang, C. T., Lutz, E. R., Kowalski, J., Horton, M. R., Linden, J., Drake, C. G., and Powell, J. D. (2008) Blood 111, 251–259
36. Raskovalova, T., Lokshin, A., Huang, X., Su, Y., Mandic, M., Zarour, H. M., Jackson, E. K., and Gorelik, E. (2007) Cancer Res. 67, 5949–5956
37. Strauss, L., Whiteside, T. L., Knights, A., Bergmann, C., Knuth, A., and Zippelius, A. (2007) J. Immunol. 178, 320–329
38. Thomson, L. F., Ruedi, J. M., Glass, A., Moldenhauer, G., Moller, P., Low, M. G., Klemens, M. R., Massaia, M., and Lucas, A. H. (1990) Tissue Antigens 35, 9–19
39. Airas, L., Niemelä, J., Salmi, M., Puurunen, T., Smith, D. J., and Jalkanen, S. (1997) J. Cell Biol. 136, 421–431
40. Ohta, A., Gorelik, E., Prasad, S. J., Ronchese, F., Lukashev, D., Wong, M. K., Huang, X., Caldwell, S., Liu, K., Smith, P., Chen, J. F., Jackson, E. K., Apasov, S., Abrams, S., and Sitkovsky, M. (2006) Proc. Natl. Acad. Sci. U.S.A. 103, 13132–13137
41. la Sala, A., Ferrari, D., Corinti, S., Cavani, A., Di Virgilio, F., and Girolomoni, G. (2001) J. Immunol. 166, 1611–1617
42. Aswad, F., Kawamura, H., and Dennert, G. (2005) J. Immunol. 175, 3075–3083
43. Mandapathil, M., Lang, S., Gorelik, E., and Whiteside, T. L. (2009) J. Immunol. Meth. 346, 55–63