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Extracellular purine metabolism and signaling of CD73-derived adenosine in murine Treg and Teff cells

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Romio M, Reinbeck B, Bongardt S, Hüls S, Burghoff S, Schrader J. Extracellular purine metabolism and signaling of CD73-derived adenosine in murine Treg and Teff cells. *Am J Physiol Cell Physiol* 301: C530–C539, 2011. First published May 18, 2011; doi:10.1152/ajpcell.00385.2010.—CD73-derived adenosine acts as potent inhibitor of inflammation, and regulatory T cells (Treg) have been shown to express CD73 as a novel marker. This study explored the role of endogenously formed adenosine in modulating NF-κB activity and cytokine/chemokine release from murine Treg and effector T cells (Teff) including key enzymes/purinergic receptors of extracellular ATP catabolism. Stimulating murine splenocytes and $CD4^+$ T cells with anti-CD3/anti-CD28 significantly upregulated activated NF-κB in $CD73^{-/-}$ T cells (wild type: 4.36 ± 0.21; $CD73^{-/-}$: 6.58 ± 0.75; $n = 4$; $P = 0.029$). This was associated with an augmented release of proinflammatory cytokines IL-2, TNF-α, and IFN-γ. Similar changes were observed with the CD73 inhibitor APCP (50 μM) on NF-κB and IFN-γ in wild-type $CD4^+$ T-cells. Treatment of stimulated $CD4^+$ T-cells with adenosine (25 μM) potently reduced IFN-γ release which is mediated by adenosine A2a receptors (A2aR). AMP (50 μM) also reduced cytokine release which was not inhibited by APCP. In Teff, A2aR activation (CGS21680) potently inhibited the release of IL-1, IL-2, IL-3, IL-4, IL-12, IL-13, IFN-γ, TNF-α, granulocyte-macrophage colony-stimulating factor (GM-CSF), CCL3, and CCL4. However, in Treg, CGS21680 did not alter cytokine/chemokine release. In summary, CD73-derived adenosine tonically inhibits active NF-κB in $CD4^+$ T-cells, thereby modulating the release of a broad spectrum of proinflammatory cytokines and chemokines. Downregulation of P2X7 and upregulation of CD73 in Treg after antigenic stimulation may be an important mechanism to maintain the ability of Treg to generate immunosuppressive adenosine.

regulatory T cell; NF-κB; inflammation

THE PURINE NUCLEOSIDE ADENOSINE is involved in many physiological and pathophysiological events (35). Adenosine can be formed from AMP either intracellularly by cytosolic 5′-nucleotidase usually involving tissue hypoxia or extracellularly by ecto-5′-nucleotidase (CD73). The extracellular site of adenosine formation is generally thought to result from the sequential dephosphorylation of extracellular ATP by action of an ecto-nucleoside triphosphate diphosphohydrolase (CD39) followed by CD73 (45). While ATP primarily acts as a proinflammatory signal on purinergic P2 receptors, its degradation product adenosine signals through P1 purinergic receptors, mediating both anti- and proinflammatory effects depending on the receptor subtype. It is therefore the activity of this ecto-nucleotide cascade that determines whether P2 or P1 receptors are preferentially activated.

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Four distinct subtypes of P1 receptors have been identified: adenosine A1, A2a, A2b, and A3 receptors (R) which are G protein coupled (10). Activation of A1R is considered to be proinflammatory, whereas the A2Rs trigger potent antiinflammatory effects (3, 19). The A3R also plays an important role in immunity by inhibiting eosinophil migration (12). On murine T lymphocytes the A2aR is highly expressed (20), and after T-cell receptor (TCR) stimulation, A2aR mRNA levels increase by a factor of ~10 (16). In humans, the A2aR is more highly expressed in $CD4^+$ than in $CD8^+$ T cells (14).

The release of endogenous nucleotides represents a critical first step for the initiation of purinergic signaling. Aside from cell death, nonlytic nucleotide release has been reported for platelets (8), erythrocytes (1), neutrophils (6), monocytes/macrophages (44), and T cells (32). The mechanism of nucleotide release appears to be cell type specific and may involve membrane ion channels, ATP-binding cassette transporters, and exocytotic granule secretion (29). In addition, activation of the P2X7 receptor, present on immune cells, triggers membrane permeabilization to medium-sized molecules and thereby may permit the cellular exit of ATP (28).

The flux though the pericellular nucleotide cascade (CD39, CD73) is not precisely known. However, genetic deletion of CD73 in mice is associated with a proinflammatory phenotype and CD73-derived adenosine appears to be quantitatively sufficient to inhibit platelet activation, and leukocyte adhesion to the vascular endothelium in vivo (15) by acting through the A2aR (47). Aside from its action on the endothelium, A2aR agonists inhibit T-cell activation through increasing cAMP levels, which acts immunosuppressive (39). Genetic deletion of A2aR results in exaggerated inflammatory tissue damage (26), which in many respects is similar to the $CD73^{-/-}$ phenotype (18, 30, 47). These findings show that the adenosine-A2aR link is an important downregulatory mechanism of inflammation that acts through the inhibition of various proinflammatory cytokines. As to the signaling involved, TCR-mediated T-cell activation of mouse Th1 cells causes the release of IL-2, IFN-γ, and lymphotoxin, whereas Th2 cells secrete IL-4, IL-5, IL-6, IL-9, and IL-10 (25). This effect involves the activation of several transcription factors including NF-κB, nuclear factor of activated T cells (NFAT), Oct-1, and AP-1 (2). The activation of A2aR on effector T cells (Teff = Th1 + Th2 cells) is well known to inhibit the release of IL-2 and IFN-γ, while active NF-κB was reported to be inhibited in B cells treated with adenosine (24).

Regulatory T cells (Treg) are an immunosuppressive group of T cells that are important to prevent spontaneous autoimmune diseases and inflammation by controlling both the innate and adaptive immune system (31). Common markers of Treg cells are expression of CD25 and the forkhead transcription

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factor FoxP3. Recently, it has been shown that both CD39 and CD73 are novel marker enzymes of murine CD25$^+$ (FoxP3$^+$) Treg cells that catalyze the formation of the antiinflammatory mediator adenosine, which can dampen excessive immune reactions (4, 13). Generation and accumulation of immunosuppressive adenosine also plays a major role in Treg-mediated immunosupression in humans (23). The strategy to use adenosine in immune defense appears to be an evolutionary well-preserved mechanism, since also staphylococci and other bacterial pathogens use adenosine to escape host immune responses (42). Despite its functional importance, the relative expression of the various adenosine receptors on Treg cells is not known; there is also no information as to the Treg-associated activity of the various enzymes causing the extracellular degradation of ATP via AMP to adenosine as influenced by TCR activation.

The present study explored whether ATP released by stimulated murine CD4$^+$ T cells forms sufficient adenosine to alter active NF-kB and cytokine release. Furthermore, we studied in detail the signaling of the A2aR in Treg and Teff cells with respect to the formation of a wide array of cytokines and chemokines. Finally, the expression of all important enzymes in cellular adenosine metabolism, including purinergic receptors and nucleoside transporters, was measured in Treg cells to obtain a first comprehensive overview of the TCR-induced changes in extracellular nucleotide/adenosine metabolism.

MATERIALS AND METHODS

Mice. C57/Bl6 and CD73$^{-/-}$ mice were bred at the local animal research unit. Male mice 8 to 12 wk of age were used for this study. All experiments were performed with approval of the local government committee.

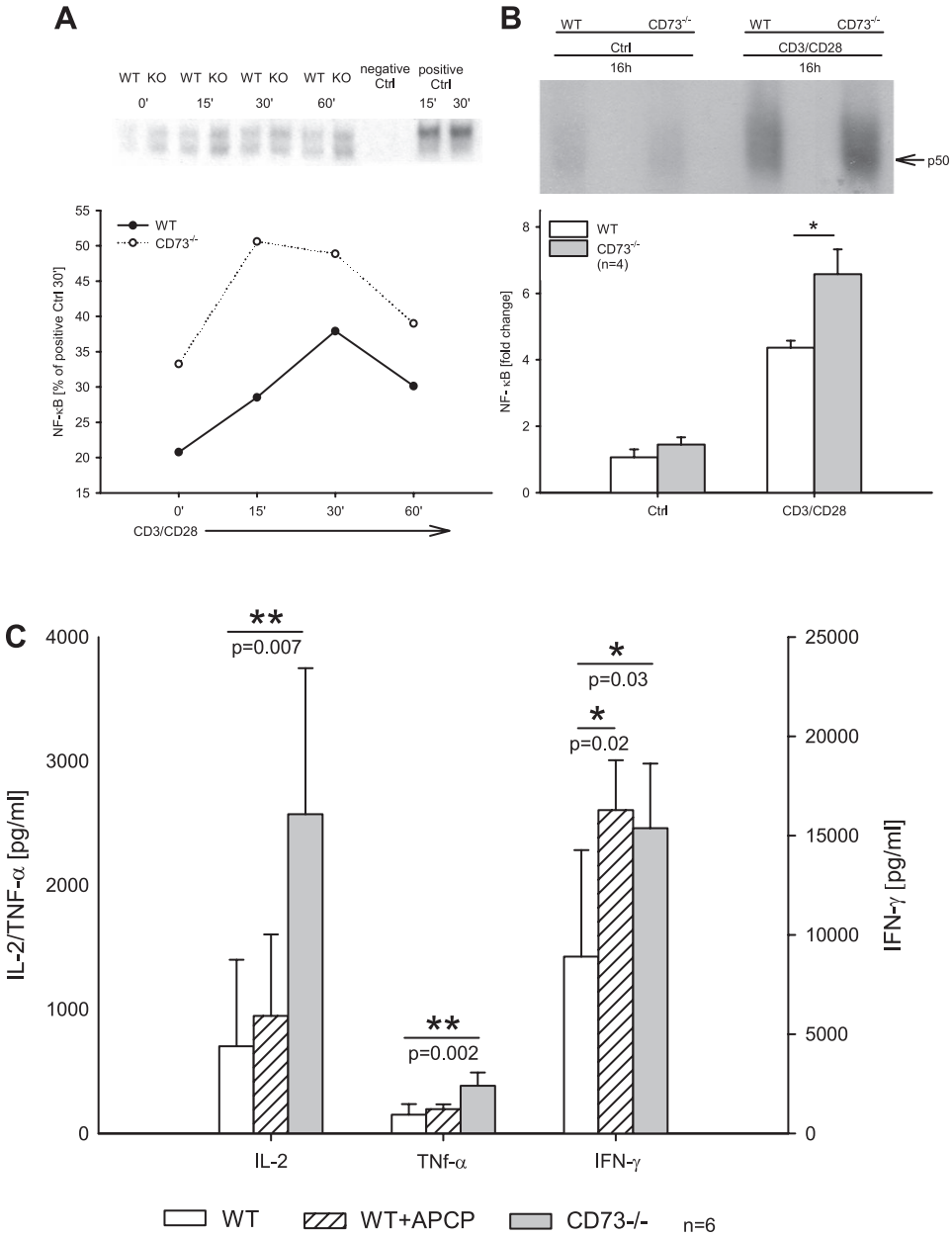

Fig. 1. Nuclear translocation of NF-κB in CD4$^+$ T cells lacking CD73 (*A* and *B*) and release of IL-2, TNF-α, and IFN-γ from wild-type (WT) CD4$^+$ T cells treated with APCP compared with CD4$^+$ T cells lacking CD73 (*C*). *A*: time course of NF-κB in splenocytes. KO, knockout; ctrl, control. *B*: NF-κB in purified CD4$^+$ T cells stimulated with anti-CD3/CD28 for 16 h. NF-κB was measured with EMSA. *C*: IL-2, TNF-α, and IFN-γ released from CD4$^+$ T cells stimulated for 18 h. Data are means ± SE. *$P \leq 0.05$. Preliminary supershift experiments (data not shown) revealed that shown bands are specific for NF-κB subunit p50.

Isolation of CD4$^+$ T cells. Splenocytes were passed through a 70-μm nylon cell strainer (BD Biosciences) and collected in PBS. CD4$^+$ T lymphocytes and CD4$^+$/CD25$^+$ Treg cells were selected by magnetic cell sorting with MicroBead isolation kits (Miltenyi). Purity of T cells for both isolation procedures was ~90%.

CD4$^+$ T-cell activation. For nuclear translocation of NF-κB and the cytokine release assays purified CD4$^+$ T cells and for quantitative real-time PCR assays purified CD4$^+$/CD25$^+$ Treg cells were resuspended in the synthetic cell culture medium Panserin 413 (RPMI medium supplemented with FCS contained nucleotidase activity that could not be heat inactivated) and cultured in flat-bottom wells precoated with 0.4 μg/ml anti-CD3 and 2.5 μg/ml anti-CD28 antibody (BD Biosciences) overnight. For the proliferation assays the T cell activation/expansion kit (Miltenyi) was used. The ratio between cells and αCD3/αCD28-coated beads was 1:1.

Nuclear protein extract preparation. CD4$^+$ T cells resuspended in 50 μl hypotonic solution [10 mM HEPES (pH 7.6), 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA] were incubated for 10 min at 4°C. Then 3.5 μl of NP-40 (10%) was added and cells were centrifuged at 14,000 *g* for 1 min. Nuclear pellet was resuspended in 30 μl high salt puffer

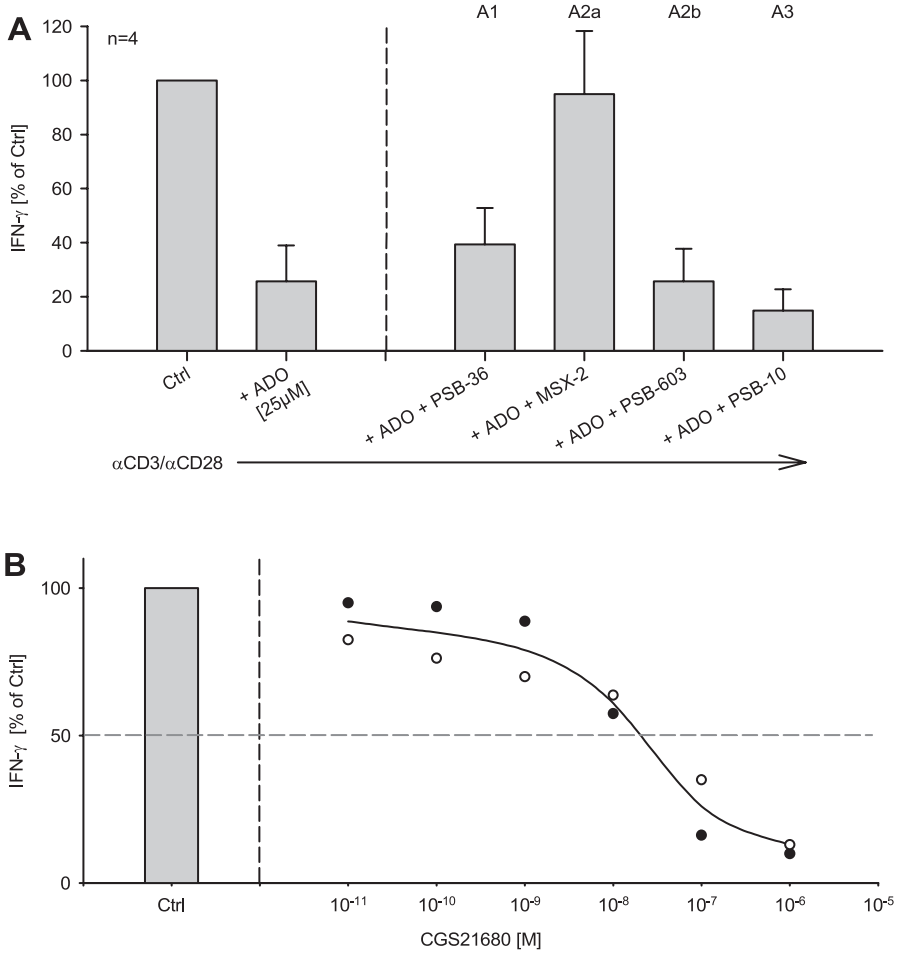

Fig. 2. Antiinflammatory effect of adenosine (ADO) is mediated by A2a adenosine receptors. CD4$^+$ T cells from WT mice were stimulated for 18 h. *A*: concentrations of adenosine A1 receptor (A1R) antagonist PSB-36, A2aR antagonist MSX-2, A2bR antagonist PSB-603, and A3R antagonist PSB-10 were 7.5 nM, 500 nM, 37.5 nM, and 50 nM, respectively. Concentrations were chosen to be 50-fold higher than the respective K_i. *B*: dose-response curve of CGS21680 on IFN-γ release. Data are from two independent experiments (dots). *C*: AMP (50 μM) inhibits IL-2, TNF-α, and IFN-γ release also in presence of APCP (50 μM). Data are means ± SE.

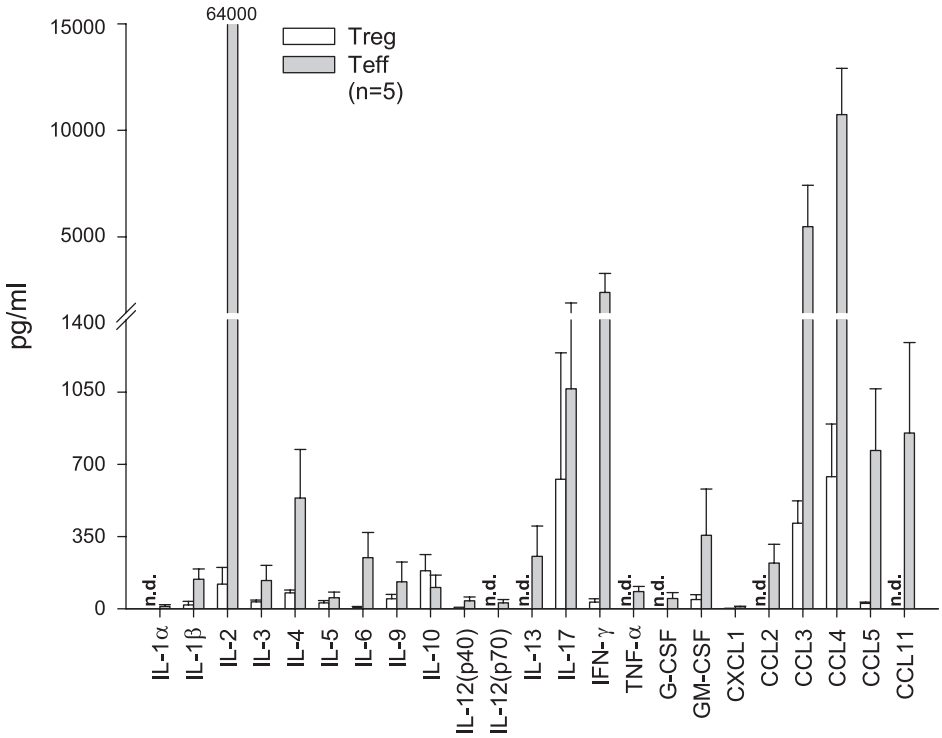

Fig. 3. Cyto-/chemokine profile (multiplex assay) of antigenic stimulated effector T cells (Teff; gray bars) and regulatory T cells (Treg cells; white bars). Anti-CD3/CD28 antibody stimulation was for 18 h. GM-CSF, granulocyte-macrophage colony-stimulating factor; n.d., not detectable. Data are means ± SD.

[50 mM HEPES (pH 7.8), 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol] and incubated for 30 min at 4°C under permanent mixing. After centrifugation at 14,000 *g* (10 min, 4°C), protein concentration of nuclear extract (supernatant) was measured and the nuclear fraction was stored at −80°C.

NF-κB activation. For measurement of NF-κB activation by electromobility shift assay, 8×10^6 purified $CD4^+$ T cells in 2 ml were stimulated in six-well plates. After 16 h incubation at 37°C and 5% CO_2, 5 μg protein of nuclear extracts were incubated at room temperature for 30 min in 20 μl containing [γ-^{32}P]-labeled double-strand oligonucleotide probe (NF-κB binding site: 5′-AGT TGA GGG GAC TTT CCC AGG G-3′). Specificity controls were done with oligonucleotides containing a mutated NF-κB binding site and supershifts. For supershifts, 1 μg/ml anti-p65 antibody or anti-p50 antibody (each from Santa Cruz) was added and incubated for 30 min on ice. Separation was on 3% acrylamide gel in TBE buffer, and visualization was done by autoradiography. Radioactivity was measured with a beta-Counter (Wallac).

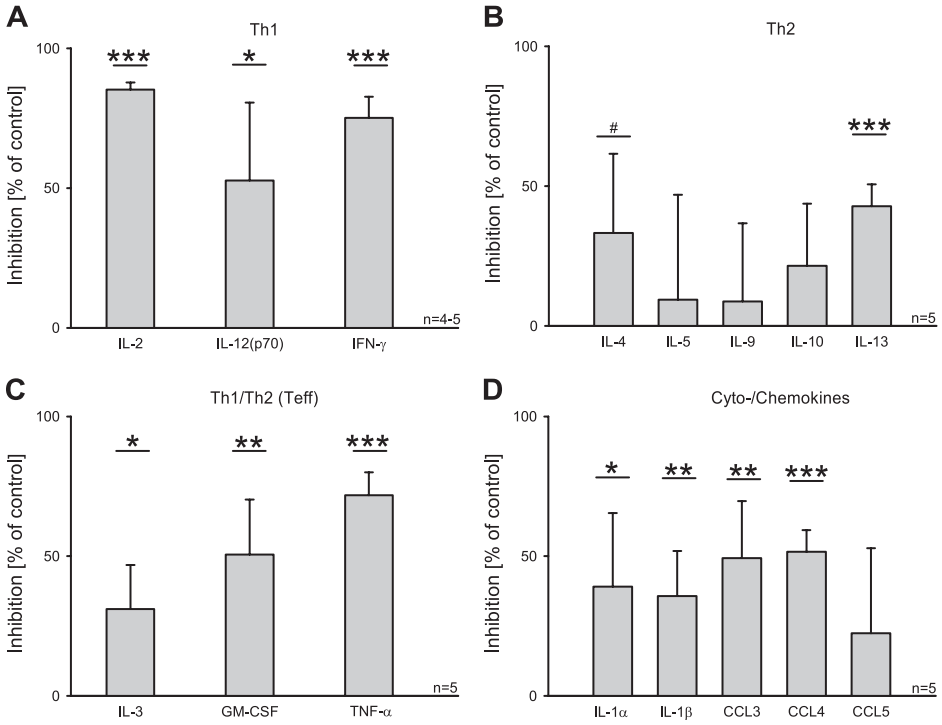

Fig. 4. *A–D*: A2aR agonist CGS21680 strongly inhibits the release of various cyto-/chemokines from antigenic stimulated Teff cells (anti-CD3/CD28 antibody stimulation was 18 h). Data are means ± SD; one sample *t*-test: ***$P < 0.001$; **$P \leq 0.01$; *$P \leq 0.05$; #$P = 0.069$.

For measurement of NF-κB by ELISA, nuclear extracts of 1×10^7 $CD4^+$ cells were isolated and stimulated in six-well plates for 18 h under the same conditions as indicated above. Nuclear extracts (12.4 μg protein) were used in conjunction with the NF-κB p50 Transcription Factor Assay Kit (Pierce) according to the manufacturers' guidelines.

IFN-γ release. Purified $CD4^+$ T cells (4×10^5 cells in 200 μl) were stimulated as indicated above in 96-well plates. After 18 h incubation at 37°C and 5% CO_2, supernatants were taken and IFN-γ was measured by ELISA (R&D Systems) in the absence and presence of adenosine (25 μM), different adenosine receptor antagonists (50-fold above the respective K_i): PSB36 (7.5 nM), MSX-2 (500 nM), PSB-603 (37.5 nM), PSB-10 (50 nM). A2a adenosine receptor agonist CGS21680 was 10 pM to 1 μM.

Carboxyfluorescein diacetate succinimidyl ester proliferation/suppression assay. $CD4^+/CD25^-$ Teff cells and $CD4^+/CD25^+$ Treg cells were isolated as indicated above. Teff cells were labeled with 3 μM carboxyfluorescein diacetate succinimidyl ester (CFSE; Sigma-Aldrich) and 1.5×10^5 of them were cocultured with 0.5×10^5 Treg cells in round-bottom 96-well plates for at least 4 days. Cells from three wells were pooled and analyzed by flow cytometry (FACSCalibur, BD). Dead cells were discriminated by light scatters and stained with ToPro-3 (Invitrogen).

Cytokine screen (multiplex assay). The release of cytokines from $CD4^+$ cells (1×10^7) after stimulation was analyzed using the Mouse Th1/Th2 6-Plex Panel and TNF-α Mouse Singleplex Bead Kit (both Invitrogen). The same kits were used to detect the inhibitory effect of AMP on released cytokines from $CD4^+$ cells (4×10^5). Purified $CD4^+/CD25^-$ Teff cells and $CD4^+/CD25^+$ Treg cells, each 4×10^5 cells, were stimulated in 96-well plates. After 18 h, supernatants were removed and analyzed for cytokine/chemokine levels with a 23-panel multiplex cytokine assay (Bio-Rad).

Quantitative real-time PCR. Total RNA was isolated either from unstimulated $CD4^+/CD25^+$ Treg cells or from $CD4^+/CD25^+$ Treg cells stimulated with precoated anti-CD3/anti-CD28 antibodies for 18 h. Total RNA was isolated with the RNeasy Mini Kit (Qiagen). The Superscript Platinum Two-Step qRT-PCR Kit (Invitrogen) was used to transcribe the obtained RNA into cDNA for real-time PCR analysis. Expression levels were related to actin.

Ecto-nucleotidase activity measurement on Treg cells. Treg isolation and stimulation were performed as described above. For measurement of CD39, CD73, and alkaline phosphatase (AP) activity, 2×10^4 cells in 200 μl were incubated (37°C, incubator) with etheno-ADP or etheno-AMP (± 50 μM APCP), respectively, at a final concentration of 50 μM for each nucleotide. Samples (15 μl) were taken after 15, 30, and 45 min and immediately denatured by 15 μl perchloric acid (1 M), neutralized with 15 μl K_3PO_4 (1 M), and centrifuged at 16,000 *g* for 5 min. Supernatant (10 μl) was loaded onto a XTerra MS C18 column (Waters, 5 μm, 4.6 × 100 mm). For the detection of etheno-AMP, a buffer consisting of 12% (vol/vol) methanol, 1.47 mM TBAP, and 73.5 mM KH_2PO_4 was used in an isocratic run for 35 min. For the detection of etheno-adenosine, two buffers were used: 6% (vol/vol) acetonitrile, 5.7 mM TBAS, and 30.5 mM KH_2PO_4 (pH 5.8) (*solution A*) and 66% (vol/vol) acetonitrile, 5.7 mM TBAS, and 30.5 mM KH_2PO_4 (pH 5.8) (*solution B*). Nucleotides and nucleosides were directly eluted with a linear gradient from 0–34% *solution B* for 5.6 min, and 34% *solution B* for 1.4 min. For fluorescence detection of etheno-adenosine and etheno-AMP, Waters Multi λ Fluorescence detector 2475 was used (λ_{EM} = 410 nm, λ_{Ex} = 280 nm). Analysis of the HPLC-data was performed using Waters Breeze software.

RESULTS

CD73-derived adenosine modulates levels of nuclear NF-κB and release of cytokines from $CD4^+$ T cells. We first investigated whether adenosine generated by CD73 present on $CD4^+$ T cells alters the nuclear translocation of NF-κB after antigenic stimulation. As depicted in Fig. 1*A*, stimulation of splenocytes from wild-type (WT) animals caused a time-dependent increase in the nuclear translocation of NF-κB and this effect was more pronounced in $CD73^{-/-}$ splenocytes. As shown in Fig. 1*B*, the TCR-triggered nuclear translocation of NF-κB was significantly increased (+30%) in purified $CD4^+$ T cells from CD73-deficient mice (WT: 4.36 ± 0.21, $CD73^{-/-}$: 6.58 ± 0.75; $n = 4$; $P = 0.029$). Supershifts revealed that the bands visible after EMSA were specific for NF-κB subunit p50 (data not shown). We have confirmed the EMSA data by independent measurements of the nuclear translocation of p50 with ELISA (WT: 66 ± 39, $n = 9$; $CD73^{-/-}$: 320 ± 365; $n = 11$; $P = 0.036$). In addition we found that APCP (50 μM) fully mimicked the effect observed in CD73-deficient cells (WT + APCP: 250 ± 243, $n = 6$; $P = 0.041$ vs. WT controls).

To investigate whether the observed changes translate into respective changes in the release of cytokines, a seven-panel

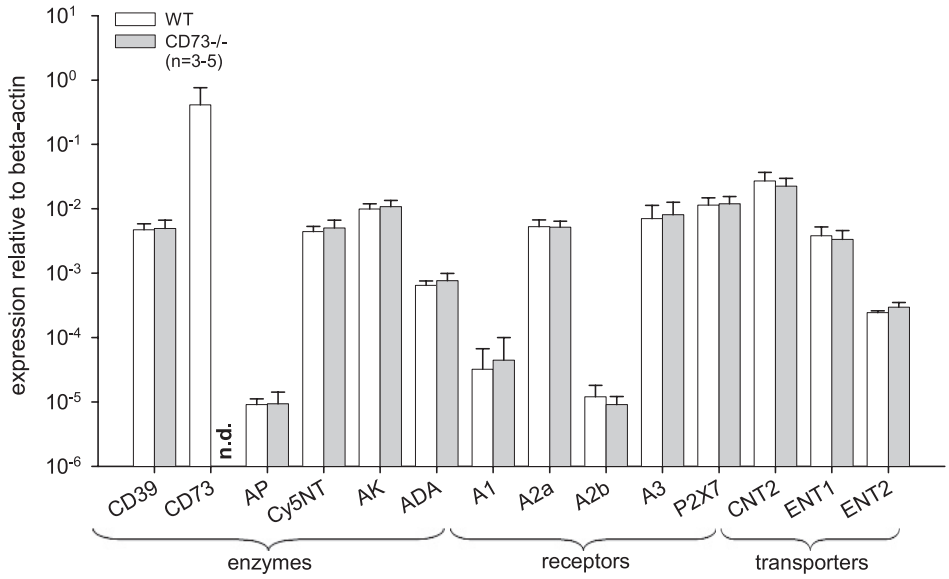

Fig. 5. Quantitative real-time PCR of various enzymes, receptors, and transporters of relevant proteins of the ecto-adenine nucleotide/nucleoside pathway in unstimulated Treg cells isolated from WT and $CD73^{-/-}$ mice. Data are means ± SD.

multiplex cytokine assay (Th1/Th2 and TNF-α) was used. As shown in Fig. 1*C*, IL-2 TNF-α, and INF-γ were significantly increased in $CD73^{-/-}$ cells, while all other cytokines/chemokines measured (IL-4, IL-5, IL-10, IL-12) remained unchanged (data not shown). Interestingly, APCP (50 μM) also stimulated the release of INF-γ but did not significantly alter IL-2 and TNF-α (Fig. 1*C*).

CD73 and $CD4^+$ T cell proliferation. To explore whether adenosine generated by CD73 on $CD4^+$ T cells alters cellular proliferation, proliferation/suppression assays with CFSE-labeled $CD4^+/CD25^-$ Teff cells and $CD4^+/CD25^+$ Treg cells were performed. Consistent with data in the literature, we found that WT Treg cells strongly inhibited proliferation of Teff cells in coculture (Supplemental Fig. S1*A*; Supplemental Material for this article is available online at the Journal website). However, there were no significant differences when experiments were performed with Treg cells lacking CD73 (Supplemental Fig. S1*B*).

Inhibition of adenosine-mediated IFN-γ release is due to A2a adenosine receptor activation. Among the specific adenosine receptor antagonists tested, only MSX-2, a specific A2aR antagonist, restored the adenosine-induced suppression of IFN-γ release (Fig. 2*A*). Similar to data in the literature (16), CGS21680, a specific A2aR agonist, potently inhibited the TCR-mediated IFN-γ release by $CD4^+$ T cells (Fig. 2*B*). We also found that AMP (50 μM) strongly inhibited the release of IL-2, TNF-α, and IFN-γ by 80%, 45%, and 84%, respectively (Fig. 2*C*). Surprisingly, however, APCP did not antagonize the effects of AMP (Fig. 2*B*) which is similar to findings of AMP on coronary vasodilatation (9).

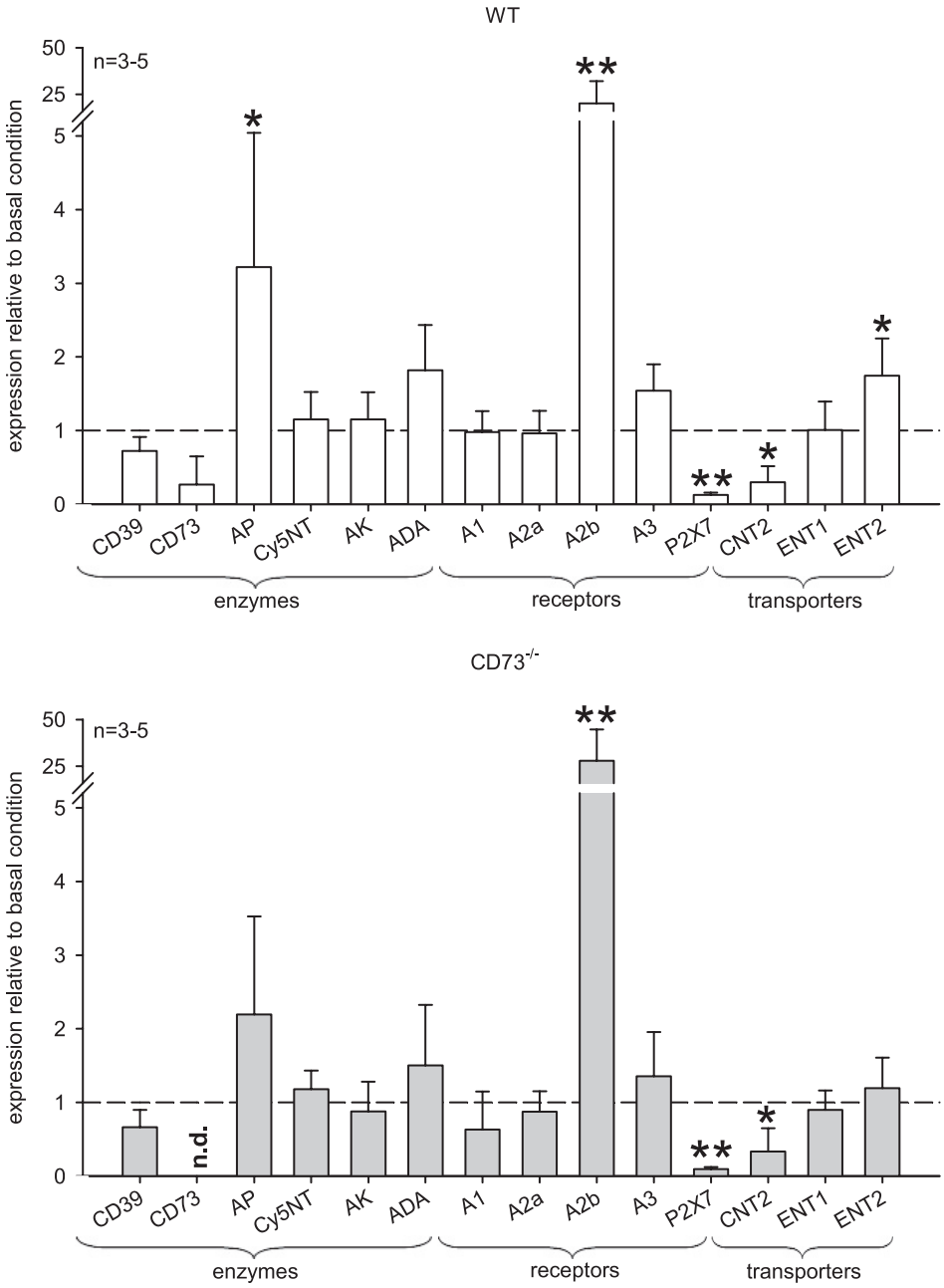

Fig. 6. Changes in gene expression of various enzymes, receptors, and transporters of the ecto-adenine nucleotide cascade after antigenic stimulation (18 h) of Treg cells isolated from WT and $CD73^{-/-}$ mice. Data are means ± SD. $*P \leq 0.05$, $**P \leq 0.01$.

Release of cytokines/chemokines from Teff and Treg cells is differently influenced by A2aR stimulation. We first measured the release of 23 cytokines (multiplex assay) from $CD4^+$/$CD25^-$ Teff $CD4^+$/$CD25^+$ Treg cells. As summarized in Fig. 3, cytokine release from Teff cells in general exceeded by far respective values obtained in Treg cells. The only exemption is IL-10, which showed a lower release in Teff cells. The cytokine/chemokines with the highest levels measured in Teff cells were IL-2, CCL3 (MIP-1α), CCL4 (MIP-1β) and IFN-γ.

Among the Th1/Th2 (Teff) cytokines (Fig. 4*C*), TNF-α showed the highest degree of inhibition (71%) when stimulated with CGS21680. Similarly, the release of the Th1 cytokines IL-2 and IFN-γ was inhibited by 85% and 75%, respectively (Fig. 4*A*). Among the Th2 cytokines, the A2aR agonist significantly inhibited the release of IL-13 (43%) and IL-4 (33%; Fig. 4*B*). Figure 4*D* shows that CGS21680 inhibited the release of IL-1α, IL-1β, CCL3, and CCL4 by 39%, 36%, 49%, and 51%, respectively. Similar experiments were carried out on the effects of A2aR stimulation on $CD4^+$/$CD25^+$ Treg cells. However, no significant differences in cytokine release could be detected (Supplemental Fig. S2).

Purine receptors and enzymes of the ecto-adeninenucleotide pathway on $CD4^+$/$CD25^+$ Treg cells. Since CD73 was reported to be expressed only on Treg cells but not on Teff cells(4, 38), we investigated gene expression of various P1 and P2 receptors (P2X7, A1, A2a, A2b, A3), adenosine transporters (CNT2, ENT1, ENT2), and adenine nucleotide/nucleoside converting enzymes [CD39, CD73, AP, cytosolic 5′-nucleotidase (Cy5NT), adenosine kinase (ADK), adenosine deaminase (ADA)] in WT and $CD73^{-/-}$ $CD4^+$/$CD25^+$ Treg cells before and after antigenic stimulation. As shown in Fig. 5 for unstimulated $CD4^+$/$CD25^+$ Treg cells, there is no difference in the expression level of all measured genes between WT and $CD73^{-/-}$ cells. CD73 showed by far the highest expression level while expression of AP was about 5 orders of magnitude lower. The difference between CD39 and CD73 was about two orders of magnitude. Among the purinergic receptors, the A2a and A3 receptors showed the highest expression levels and were similar to that of the P2X7 receptor (Fig. 5). Among the nucleoside transporters, CNT2 was the most highly expressed. There is also an active intracellular adenosine metabolism as judged by the expression of ADK, Cy5NT, and ADA. It should be noted that comparison of expression levels assumes the efficiency of amplification and the ability to convert mRNA into cDNA to be equivalent.

After antigenic stimulation (Fig. 6), we found massive upregulation of the A2bR both in WT (+2,012%) and CD73 lacking Treg cells (+2,791%), while P2X7 receptor expression was downregulated by −87% and −90%, respectively. Similarly, the expression of the nucleoside transporter CNT2 was downregulated both in WT (−70%) and CD73 lacking (−67%) Treg cells.

Since one cannot assume that changes in expression level directly translate into similar changes in enzymatic activity, we measured the activity of CD39, CD73, and AP in WT and $CD73^{-/-}$ Treg. Data are summarized in Fig. 7. As can be seen, antigenic stimulation of WT Treg significantly increased the activity of CD73, while AP remained unchanged (Fig. 7*A*). The activity of CD39 was significantly increased in $CD73^{-/-}$ Treg as compared with WT controls, while antigenic stimulation blunted this effect (Fig. 7*B*). Importantly,

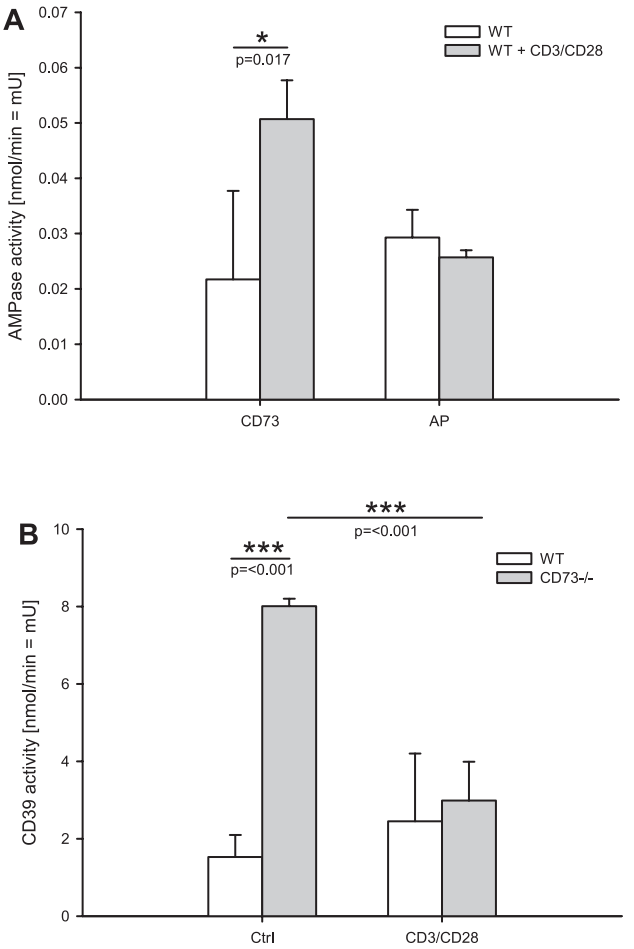

Fig. 7. Ecto-nucleotidase activity on Treg cells in absence and presence of anti-CD3/CD28 antibodies. *A*: AMPase activity of CD73 and alkaline phosphatase (AP) on WT Treg cells. *B*: CD39 activity on WT Treg cells and Treg cells lacking CD73. Anti-CD3/CD28 antibody stimulation was 18 h. Data are means ± SE.

we found that the activity of CD39 in Treg is substantially higher than CD73, which is opposite to the measured gene expression levels of the two enzymes reported in Fig. 5.

DISCUSSION

A major finding of this study was that, in stimulated $CD4^+$ T cells, CD73-derived adenosine is produced in sufficient quantities to tonically suppress nuclear translocation of NF-κB which is associated with a diminished release of proinflammatory cytokines. Furthermore, we found in Treg cells TCR-mediated downregulation of P2X7 expression which may counteract ATP-induced apoptosis of Treg cells at the site of inflammation and together with upregulated CD73 activity assures high pericellular adenosine levels. Thus, the continued formation of adenosine by CD73, a prominent nucleotide degrading enzyme on Treg cells, appears to play a crucial role in the feedback loop linking extracellular formation of adenosine to the modulation of NF-κB-dependent release of numerous proinflammatory cytokines/chemokines.

Ablation of CD73 in T-cells and pharmacological inhibition of CD73 with APCP increased NF-κB and the release of cytokines. This implies that there must have been a continuous flux of ATP to adenosine in stimulated T-cells. In fact, ATP

was recently reported to be released from activated $CD4^+$ T cells through pannexin hemichannels, which open when the cytosolic Ca^{2+} concentration is elevated (32). Released ATP than acts on purinergic P2X receptors (32) but at the same time it is degraded by the combined action of the ecto-nucleotidases CD39 and CD73 to form adenosine, stimulating P1 receptors. P2X7 is the most abundant P2XR on mouse $CD4^+$ T cells, and autocrine activation of P2X7 on T cells is thought to be required for productive T-cell activation. ATP also activates the P2X7 purinoreceptor and induces formation of large membrane pores ultimately resulting in cell death (34). Since pannexin hemichannels associate directly with P2X7, and P2X7 activation increases the opening of pannexin hemichannels, this suggests a positive feedback loop to potentiate ATP release (32). However, the threshold for activation of the P2X7 receptor by extracellular ATP is rather high ($EC_{50} \approx 0.5$ mM). More importantly, we found the expression of P2X7 to be profoundly downregulated in activated Treg cells while CD39 activity by far exceeds that of CD73. It thus appears that despite the presence of high ATP at inflammatory sites, the biological half-life of ATP may be short and P2X7-induced cell death may be substantially attenuated which together assures the continuous breakdown of ATP to adenosine. The induction of Teff cell anergy by ATP actually acting as an essential autocrine costimulatory signal (32) is therefore likely to also involve the uncompromised generation of adenosine by Treg cells and activation of A2a receptors. This pathway may be particularly relevant at sites of increased extracellular ATP such as in the vicinity of tumors (5). High ATP levels will increase the flux through the ecto-nucleotide cascade pathway to finally translate into the enhanced formation of immunosuppressive adenosine. This metabolic sequence may explain why inhibition of CD73 was recently reported to be associated with decreased tumor growth (40, 11).

ATP can be released from many cells including erythrocytes at low Po_2 (1). In an inflammatory environment with nucleotide releasing immune cells, this should result in a high concentration of extracellular ATP which translates to an augmented formation of adenosine. Aside from ATP, NAD^+ is also released from lytic cells, which induces ADP ribosylation (ART2.2) of the cytolytic P2X7 receptor, thereby alerting the immune system (33). The biological half-life of extracellular NAD^+ is determined by the activity of the CD38 NAD glycohydrolases and the CD203 nucleotide pyrophosphatases which hydrolyze NAD to nicotinamide and AMP, respectively. Similarly, extracellular ATP is hydrolyzed by membrane-bound phosphodiesterases (PDE) to ADP and/or AMP (46). Together, these findings strongly suggest that AMP can be formed by multiple enzymatic reactions, which implies that the flux through CD73 is not solely governed by the activity of CD39. Thus, the substrate flux of nucleotide-metabolizing (ecto)-enzymes is critically determined by the interconnected enzymatic activities.

We found that antigenic stimulation of mouse Treg cells significantly upregulated the activity of CD73, which, however, is still substantially lower than the activity of CD39 under these conditions. At the genomic level, in contrast, the transcription of CD39 exceeded that of CD73. This highlights the fact that the activity of these enzymes in addition to its transcription level is importantly influenced by the rate of degradation (turnover) and posttranslational modifications, as well as by possible environmental factors. One should also keep in mind that the presence of CD38, CD203, and PDE may bypass CD39 in the formation of AMP, the immediate substrate of CD73. Yet, little is known on the extracellular concentration of AMP in the immediate vicinity of T cells. Stimulation of human neutrophils was reported to be also associated with micromolar concentrations of extracellular AMP which elicited chloride secretion from epithelial cells (21) and promoted endothelial barrier function. It has also been shown that AMP is converted to adenosine by CD73 on intestinal epithelial cells (41). Under in vitro conditions we found that 50 μM

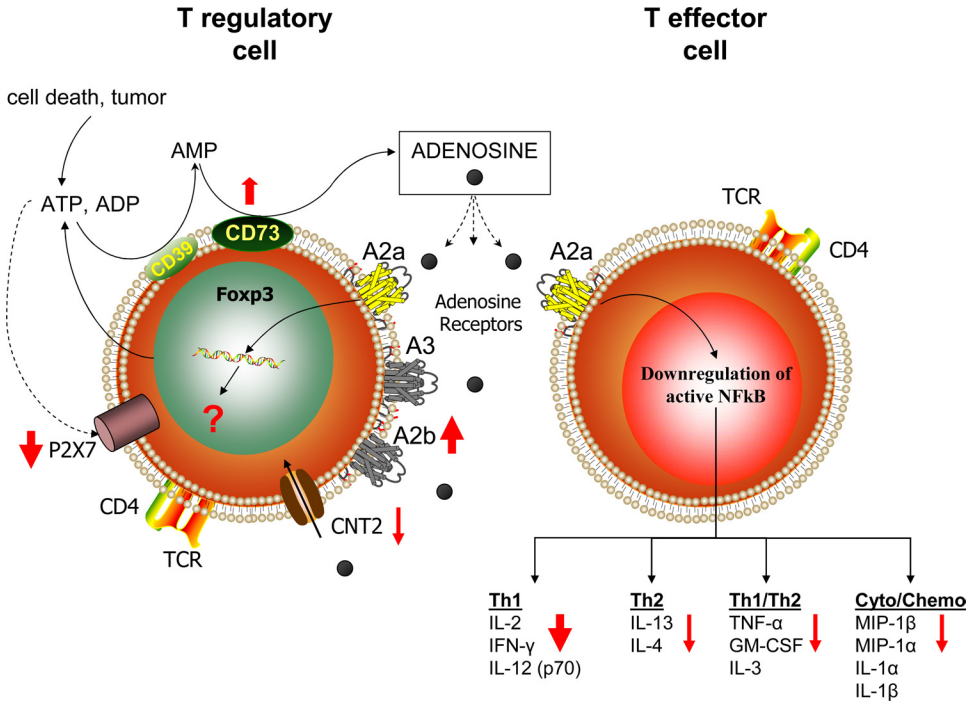

Fig. 8. Schematic overview of extracellular purine metabolism and adenosine signaling in murine Treg and Teff cells. TCR, T cell receptor.

AMP, similar as adenosine, potently inhibited the release of IL-2, TNF-α, and IFN-γ from $CD4^+$ cells. Surprisingly, the AMP effect could not be antagonized by the CD73 inhibitor APCP, suggesting at first sight that AMP acted independently of adenosine. However, T cells express alkaline phosphatase activity which is not inhibited by APCP and which is of similar magnitude as CD73 (Fig. 7). It is therefore very likely that despite effective inhibition of CD73, degradation of AMP to adenosine—at the given high concentration of AMP—was catalyzed by alkaline phosphatase. Since CD73, in contrast to alkaline phosphatase, is a low K_m enzyme, the flux though CD73 should be the dominating pathway at low extracellular nucleotide levels. It should be noted in this regard that the coronary vasodilatory action of AMP also could not be blunted by APCP (9), leading to the suggestion that the AMP effects on coronary vessels were mediated by the P1 receptor.

Expression of A2aR was reported to be high in Teff cells while that of A1R, A2bR, and A3R was barely above threshold (20). Interestingly, we found A2aR and A3R to be equally highly expressed in Treg cells. The expression level of A2bR was considerably lower; however, antigenic stimulation of Treg substantially increased its expression which did not reach that of the A2aR. Function as well as signaling mechanisms of the adenosine receptors, particularly of A2a and A3, in Treg cells are presently unknown. Since pharmacologic activation of A2aR in Treg did not significantly alter cytokine release suggests that the signaling pathway is different from that of the A2aR in Teff.

The nuclear transcription factor NF-κB is known to play an important role in the regulation of immune responses, and the NF-κB signaling pathway is a key regulator of FoxP3 expression during natural Treg cell development (17). This study shows that adenosine usually formed by CD73 present on Treg cells inhibits active NF-kB in Teff cells, which is associated with reduced formation of IL-2, TNF-α, and IFN-γ. In general, the situation in T cells appears to be similar to that in endothelial cells in which lack of CD73 was associated with upregulated active NF-κB. (20). Changes in cytokine release appear to be a direct function of NF-κB, since IL-2, IFN-γ, and TNFα are target genes of NF-κB (27). The promoter regions of IL-2 and IFN-γ show a specific binding region for the NF-κB p50 subunit (36, 37). It was therefore surprising to find that APCP stimulated TNF-γ release in WT $CD4^+$ cells but did not alter IL-2 and TNF-α, suggesting that the latter two cytokines are less stringently influenced by NF-kB.

This study gives a first comprehensive overview on the broad inhibitory action of A2aR activation on the release of various cytokines and chemokines from stimulated Treg and Teff cells. Consistent with data in the literature, we found that A2a activation potently inhibited the release of IL-2, INF-γ, and TNF-α (39). In addition, we found that A2aR activation significantly inhibited IL-1α, IL-1β, IL-3, IL-4, IL-13, and granulocyte-macrophage colony-stimulating factor (GM-CSF) release. New is the observation that activated murine $CD4^+$ T cells release significant quantities of the chemokines CCL3 and CCL4, which are of similar magnitude as the release of IFN-γ. CCL3 and CCL4 belong to the CC chemokine family, which was primarily found to be secreted from activated macrophages (43). CCL3 and CCL4 are potent chemotactic factors, inducing migration of monocytes /macrophages. They also induce the synthesis and release of other proinflammatory cytokines such as IL-1, IL-6, and TNF-α from macrophages (7). CCL4 is also known to be chemotactic for $CD4^+$ T cells whereas CCL3 is chemotactic for natural killer (NK) cells (22). Interestingly, A2aR activation inhibited CCL3 and CCL4 by ~50%. Therefore, adenosine signaling through A2aR elicits a multifaceted regulatory response on immune cells which involves quite a variety of cytokines and chemokines.

As summarized in Fig. 8, our study has shown that CD73 is upregulated on Treg cells after antigenic stimulation and CD73-derived adenosine acts through A2aRs on Teff cells to downregulate active NF-kB which is linked to the potent inhibition of various Th1 and Th2 cytokines and chemokines. The signaling cascade of A2aR on Treg cells is unknown but does not involve cytokines. Antigenic stimulation of Treg cells causes profound downregulation of the expression of P2X7R by which Treg cells may escape ATP-induced apoptosis. Consequently, one might speculate that this antiapoptotic effect indirectly preserves the activity of the adenine nucleotide cascade and together with the downregulation of the nucleoside transporter CNT2 and upregulation of CD73 activity maintains high extracellular adenosine levels. Thus, during antigenic stimulation of $CD4^+$ cells, the proinflammatory P2 response (P2X7) is shifted to a preferential antiinflammatory action (A2aR), which may be relevant when high levels of extracellular ATP are present such as in inflamed tissue and in the vicinity of tumors.

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DISCLOSURES

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