

# **ATP inhibits regulatory T cell function and generation through activation of P2X receptors**

## **One sentence summary**

Activation of purinergic P2X7 receptor by ATP mediates lineage instability of immunosuppressive regulatory T cells (T<sub>regs</sub>) and their conversion to pro-inflammatory interleukin-17 secreting T<sub>H</sub>17 cells, thus promoting autoimmunity.

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**Extracellular nucleotides are pleiotropic regulators of mammalian cell function. In CD4<sup>+</sup> T helper cells adenosine triphosphate (ATP) released upon T cell receptor (TCR) stimulation contributes to mitogen activated protein kinase (MAPK) activation through purinergic P2X receptors. High expression of P2X7 is part of the transcriptional signature of immunosuppressive CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells (T<sub>regs</sub>). Here we show that P2X7 activation by ATP inhibits T<sub>regs</sub> suppressive potential and lineage stability. The inflammatory cytokine IL-6 increased ATP synthesis and P2X7 mediated signaling, which in vivo determined T<sub>regs</sub> lineage instability and conversion to IL-17 secreting T helper (T<sub>H</sub>17) effector cells. Moreover, pharmacological P2X antagonism promoted cell autonomous conversion of naïve CD4<sup>+</sup> T cells into T<sub>regs</sub> upon TCR stimulation. Thus, ATP integrates extracellular cues and cellular energetics to tune the development and immunosuppressive program of the T cell as an external soluble factor.**

## **Introduction**

Naive T cell stimulation by cognate antigen determines a functional response, the nature of which depends on concomitant stimuli provided by accessory molecules and cytokine milieu. Distinct metabolic phenotypes characterize T cell activation; early increase of mitochondrial oxidative phosphorylation is followed by conversion to an anabolic metabolism to increase nutrient uptake as well as protein and lipid synthesis (1-3). Cellular metabolism has recently emerged as an element contributing to shaping a T cell response (reviewed in ref. 4). For example, inhibition of mammalian target of rapamycin (mTOR), a cellular nutrient and energy sensor, was shown to promote differentiation of CD8<sup>+</sup> memory

T cells (5, 6) as well as generation of CD4<sup>+</sup>CD25<sup>+</sup> adaptive T<sub>regs</sub> expressing the transcription factor forkhead box p3 (Foxp3) (7, 8). Along the same line a metabolic switch to mitochondrial fatty acid oxidation was shown to be required for differentiation of CD8<sup>+</sup> memory T cells (9).

Increased oxidative synthesis of ATP in CD4<sup>+</sup> T cells by TCR triggering is followed by release of ATP from stimulated cells through pannexin hemichannels (10). ATP binds to two classes of purinergic P2 receptors in the plasma membrane of eukaryotic cells: P2X receptors, which open to ion channels, and G protein coupled P2Y receptors. Stimulation of P2 receptors by extracellular ATP contributes in many cells in setting their activation state and regulates various signal transduction pathways (11, 12). The impact of ATP and purinergic signaling in regulating innate immune system responses has been intensely investigated (13-17); in contrast, after pioneering studies on the role of extracellular ATP in regulating lymphocyte activation (18, 19), it remains unclear how ATP synthesis and release shape T-cell functions. Autocrine purinergic stimulation by ATP through P2X receptors was shown to play a crucial role in protracting TCR-initiated mitogen activated protein kinase (MAPK) activity (10) and IL-2 secretion (10, 20), thus determining productive T cell activation. Importantly, P2X antagonism concomitant with TCR stimulation blunted MAPK activation without affecting nuclear factor of activated T cells (NFAT) nuclear translocation, resulting in T cell unresponsiveness to subsequent stimulation (anergy) (10). These results suggested that ATP synthesis and release could tune T cell activation in a cell autonomous fashion.

Immunosuppressive T<sub>regs</sub> expressing Foxp3 play an indispensable role in maintaining tolerance to self antigens (21). We have previously shown that T<sub>regs</sub> produce significantly

lower amounts of ATP than conventional CD4<sup>+</sup> T (T<sub>conv</sub>) cells following TCR stimulation (10). In addition, they are characterized by the combined expression of CD39 and CD73 ectonucleotidases, which rapidly degrade extracellular ATP to adenosine (22-24). It was therefore puzzling that *p2x7* was comprised in the T<sub>regs</sub> signature genes (25, 26). The P2X7 receptor was hypothesized to sensitize T<sub>regs</sub> to ATP mediated apoptosis (27, 28). Whether extracellular ATP also affected other aspects of T<sub>regs</sub> physiology such as suppressive function, lineage differentiation and/or stability has not been addressed to date.

Recently, several reports have shown that T<sub>regs</sub> may lose Foxp3 expression and convert to effector cells both in non-inflammatory and inflammatory conditions (29-31). Our study shows that P2X7 stimulation inhibits the tissue-specific immunosuppressive potential of T<sub>regs</sub> and facilitates their conversion to T<sub>H</sub>17 cells during chronic inflammation. Notably, pharmacological P2X antagonism or P2X7 deficient T<sub>regs</sub> ameliorated tissue inflammation by preserving T<sub>regs</sub> functional profile. Furthermore, in vitro and in vivo activation of naïve CD4<sup>+</sup> T cells in the presence of the P2X receptor antagonist periodate-oxidized 2',3'-dialdehyde ATP (oATP) inhibited their differentiation to effector cells and induced a developmental program leading to generation of adaptive T<sub>regs</sub>. Therefore, endogenous ATP released upon activation shapes T cell function as a pro-inflammatory soluble factor.

## **Results**

### **P2X7-mediated inhibition of T<sub>regs</sub> suppressive function**

Experiments defining T<sub>regs</sub> transcriptional signature showed that *p2x7* is highly expressed in T<sub>regs</sub> (25, 26). This result was confirmed by analysis of *p2x* genes expression by real-time (RT)-PCR of cDNA from sorted thymic CD4 single positive, peripheral naïve CD4<sup>+</sup> and CD4<sup>+</sup>CD25<sup>high</sup> (>98% Foxp3<sup>+</sup>) cells, which confirmed robust expression of *p2x7* in T<sub>regs</sub>

(Fig. 1A). To investigate whether P2X7 signaling might modulate  $T_{\text{regs}}$  function, we assessed the suppressive potential of purified C57BL/6 wild-type (WT) and  $p2x7^{-/-}$   $T_{\text{regs}}$  in an in vitro suppression assay.  $p2x7^{-/-}$   $T_{\text{regs}}$  were significantly more potent suppressors than their WT counterpart (fig. S1). To understand whether the higher suppression derived from better survival or enhanced suppressive function of  $p2x7^{-/-}$   $T_{\text{regs}}$ , we performed suppression assays with carboxyfluorescein diacetate succinimyl ester (CFSE) labeled  $CD45.1^{+}$  naïve responder and  $CD45.2^{+} CD4^{+} CD25^{\text{high}}$   $T_{\text{regs}}$  sorted from WT or  $p2x7^{-/-}$  animals. After 72h we analyzed the CFSE dilution in  $CD45.1^{+} T_{\text{conv}}$  responder cells as well as the number of surviving  $CD45.2^{+} T_{\text{regs}}$  ( $CD4^{+} CD25^{+} Foxp3^{+}$ ) by flow cytometry. The overall survival of  $CD45.2^{+}$  cells was comparable between WT and  $p2x7^{-/-}$  T cells (Fig. 1B). However, we found a significant decrease of Foxp3 expressing  $CD45.2^{+}$  cells in WT versus  $p2x7^{-/-}$  cells at  $T_{\text{regs}}/T_{\text{conv}}$  ratios ranging from 1:8 to 1:32 (Fig. 1C). At  $T_{\text{regs}}/T_{\text{conv}}$  ratios below 1:64 no significant differences in the total number of  $CD45.2^{+} Foxp3^{+}$  T cells were observed, however  $p2x7^{-/-}$   $T_{\text{regs}}$  displayed a dramatically enhanced suppressive potential (Fig. 1D). These data suggest that P2X7 stimulation in the course of  $T_{\text{regs}}$  activation diminishes their suppressive potential possibly through downregulation of Foxp3 expression. Accordingly, the addition of the P2X antagonist,  $\alpha$ ATP, increased the level of Foxp3 expression in sorted  $T_{\text{regs}}$  from WT mice stimulated with CD3 monoclonal antibody (mab) in the presence of irradiated splenocytes and IL-2 (Fig. 1E).

Recently, a conserved non-coding DNA element (CNS) in the Foxp3 gene, namely CNS2, was shown to be required for Foxp3 expression in the progeny of dividing  $T_{\text{regs}}$ .

Demethylation-dependent binding of Foxp3 together with Cbf- $\beta$  and Runx1 transcription

factors to this “cellular memory module” is required for heritable maintenance of Foxp3 and lineage stability in dividing T<sub>regs</sub> (32). We addressed whether P2X7 signaling in T<sub>regs</sub> affected Foxp3 expression upon cell division. Sorted T<sub>regs</sub> from WT and *p2x7<sup>-/-</sup>* mice were labeled with CFSE and Foxp3 expression analysed after 6 days of stimulation with CD3 mab in the presence of irradiated splenocytes from syngenic *cd3ε<sup>-/-</sup>* mice. A minor loss of Foxp3 expression was observed in proliferating *p2x7<sup>-/-</sup>* with respect to WT T<sub>regs</sub> (Fig. 1F). Accordingly, stimulation of WT T<sub>regs</sub> in the presence of the P2X antagonist oATP or Rottlerin, which uncouples mitochondrial respiration from oxidative phosphorylation and reduces intracellular ATP (33), determined significantly increased maintenance of Foxp3 expression in dividing T<sub>regs</sub> (Fig. 1G). Importantly, T<sub>regs</sub> stimulation in the presence of the prototypic P2X7 agonist 2'(3')-O-(4-Benzoylbenzoyl)ATP (BzATP) resulted in a dramatic loss of Foxp3 expression in WT but not *p2x7<sup>-/-</sup>* T<sub>regs</sub> (Fig. 1F and G), thereby showing that P2X7 signaling affects T<sub>regs</sub> lineage stability during cell division through downregulation of Foxp3.

### **P2X7-mediated ATP synthesis and extracellular signal-regulated kinase (ERK)**

#### **phosphorylation in T<sub>regs</sub>**

Intramitochondrial Ca<sup>2+</sup> is a key factor in driving ATP synthesis (34). P2X7 expression was shown to increase intramitochondrial Ca<sup>2+</sup>, which boosted oxidative phosphorylation and ATP synthesis resulting in increased intracellular ATP content; moreover, ATP release was enhanced and supported cell growth through autocrine/paracrine P2X7 activation (35). In T<sub>conv</sub> cells, activation-induced ATP synthesis depends on mitochondrial respiration (10, 36). Ex vivo T<sub>regs</sub> from WT and *p2x7<sup>-/-</sup>* mice displayed similar intracellular ATP concentrations

(WT  $T_{\text{regs}}$ :  $1.8 \pm 0.2 \times 10^{-3}$  pMol/cell;  $p2x7^{-/-}$   $T_{\text{regs}}$ :  $1.7 \pm 0.09 \times 10^{-3}$  pMol/cell), however in WT  $T_{\text{regs}}$  CD3 stimulation resulted in protracted increase in intracellular ATP whereas in  $p2x7^{-/-}$   $T_{\text{regs}}$  intracellular ATP progressively decreased (Fig. 2A), indicating that P2X7 expression in  $T_{\text{regs}}$  exerts a trophic effect on mitochondrial oxidative phosphorylation.

In  $T_{\text{conv}}$  cells activation induced ATP synthesis and release contributes to protracted ERK activation (10). Interestingly, activated  $T_{\text{regs}}$  showed lower ERK activation with respect to  $T_{\text{conv}}$  cells (37) and ERK inhibition was shown to enhance  $T_{\text{regs}}$  suppressive potential (38). We therefore investigated whether impaired ATP synthesis in  $p2x7^{-/-}$   $T_{\text{regs}}$  affected ERK phosphorylation upon TCR stimulation. Intracellular ATP concentration expressed as fold increase at 90 min after stimulation correlated with mean fluorescence intensity of phospho-ERK detected in flow cytometry (Pearson correlation coefficient  $r = 0.917$  for C57BL/6 and  $r = 0.9291$  for  $p2x7^{-/-}$   $T_{\text{regs}}$ ) (fig. S2), which was dramatically reduced in  $p2x7^{-/-}$   $T_{\text{regs}}$  (Fig. 2B and S3). In contrast to ERK, no significant difference in p38 MAPK and c-jun phosphorylation were observed in stimulated  $p2x7^{-/-}$   $T_{\text{regs}}$  with respect to WT  $T_{\text{regs}}$  (Fig. 2C). These results suggest that P2X7 might inhibit  $T_{\text{regs}}$  function through ERK activation. Accordingly, pharmacological inhibition of ERK with the MEK1 inhibitor PD 98059 in stimulated WT  $T_{\text{regs}}$  resulted in significantly increased maintenance of Foxp3 expression in dividing  $T_{\text{regs}}$  (Fig. 2D), thus suggesting that P2X7 activation inhibits  $T_{\text{regs}}$  stability and function through ERK activation.

The proinflammatory cytokine interleukin-6 (IL-6) was shown to downregulate Foxp3 expression and promote conversion of  $T_{\text{regs}}$  to Th17 cells (30). In WT  $T_{\text{regs}}$ , CD3 stimulation in the presence of IL-6 resulted in increased ATP synthesis with respect to CD3 stimulated cells. However, in  $p2x7^{-/-}$   $T_{\text{regs}}$  the addition of IL-6 resulted in the progressive decrease in

intracellular ATP content, analogously to CD3 stimulated cells (Fig. 2A). Accordingly, IL-6 increased ERK phosphorylation in WT but not  $p2x7^{-/-}$   $T_{\text{regs}}$ . (Fig. 2B). Stimulation of WT  $T_{\text{regs}}$  in the presence of IL-6 resulted in diminished Foxp3 expression compared to cells stimulated without IL-6. Notably, no decrease in Foxp3 expression was observed in  $p2x7^{-/-}$   $T_{\text{regs}}$  stimulated in the presence of IL-6 (Fig. 2E). Addition of the ectonucleotidase inhibitor ARL, which prolongs the half-life of extracellular ATP, had a similar effect as IL-6 in reducing Foxp3 expression in WT but not  $p2x7^{-/-}$  cells (Fig. 2E). Altogether, our results indicate that in  $T_{\text{regs}}$  P2X7 determines an increase in the intracellular ATP content, contributes to activation of the ERK pathway and decreases Foxp3 expression in a cell autonomous fashion, thereby modulating their immunosuppressive potential.

#### **ATP-mediated $T_{\text{regs}}$ instability and enhanced conversion to $T_{\text{H}}17$ cells in vitro**

To investigate whether the ATP mediated decrease in Foxp3 expression might sensitize  $T_{\text{regs}}$  for conversion to  $T_{\text{H}}17$  effector cells, we analysed by RT-PCR the expression of retinoic acid receptor (RAR)-related orphan receptor gamma t (ROR $\gamma$ t) as  $T_{\text{H}}17$  lineage marker at regular intervals after stimulation of  $T_{\text{regs}}$ . In WT  $T_{\text{regs}}$  we observed a gradual increase of ROR $\gamma$ t mRNA. In contrast, ROR $\gamma$ t expression was significantly reduced in  $p2x7^{-/-}$   $T_{\text{regs}}$ . This result could not be attributed to the nature of co-cultured splenocytes, since activation of WT cells in co-culture with either  $p2x7^{-/-}$  or WT splenocytes led to identical results (Fig. 3A). In WT  $T_{\text{regs}}$  both intracellular ATP and ROR $\gamma$ t transcript levels displayed progressive increases with increasing strength of TCR stimulation (fig. S4). Since release of ATP and autocrine P2X stimulation are positively correlated to TCR signal strength (10), these results suggest that increased signal strength might destabilize  $T_{\text{regs}}$  with

the contribution of released ATP. In support of this hypothesis TCR stimulation in the presence of IL-6 or IL-6 together with arylreceptor agonist FICZ promoted ROR $\gamma$ t (Fig. 3B) and IL-17 (fig. S5) expression, consistent with the T<sub>H</sub>17 differentiation programme (39) in WT but not *p2x7*<sup>-/-</sup> T<sub>regs</sub>. Finally, T<sub>regs</sub> stimulation for 16 h in the presence of BzATP resulted in diminished Foxp3 and increased ROR $\gamma$ t expression in WT but not *p2x7*<sup>-/-</sup> T<sub>regs</sub> (Fig. 3C). These observations indicate that ATP destabilizes cell autonomously T<sub>regs</sub> signature and promotes T<sub>regs</sub> differentiation to pro-inflammatory T<sub>H</sub>17 effector cells through P2X7.

### **Lack of conversion of *p2x7*<sup>-/-</sup> T<sub>regs</sub> to T<sub>H</sub>17 cells and protection from inflammatory bowel disease (IBD)**

Lymphopenia and inflammation stimulate the conversion of T<sub>regs</sub> into effector cells in vivo (29, 31, 40, 41). We investigated the role of ATP and P2X7 signaling in T<sub>regs</sub> conversion to effector cells in a model of IBD. Lymphopenic *cd3 $\epsilon$* <sup>-/-</sup> mice were injected with naïve CD45.1<sup>+</sup> CD4<sup>+</sup> T cells together with a number of CD45.2<sup>+</sup> T<sub>regs</sub> insufficient to protect them from the development of colitis. Four weeks after transfer mice receiving WT T<sub>regs</sub> displayed inflammation of colonic mucosa, splenomegaly and increase in mesenteric lymph nodes (LN) size. In contrast, *p2x7*<sup>-/-</sup> T<sub>regs</sub> prevented the disease completely as shown by the normal size of lymphoid organs and absence of inflammation in the colon (Fig. 4A,G); expansion of CD45.1 T<sub>conv</sub> cells was significantly reduced in the presence of *p2x7*<sup>-/-</sup> T<sub>regs</sub> (Fig. 4B). Total CD45.2<sup>+</sup> cell recoveries from mesenteric LN were not significantly different in the two groups of animals (mean cell number $\pm$ SD: 1.39 $\pm$ 0.67 x 10<sup>6</sup> WT T<sub>regs</sub> group vs 0.75 $\pm$ 0.12 x 10<sup>6</sup> *p2x7*<sup>-/-</sup> T<sub>regs</sub> group; n=5, *P*=0.11), however the percentage of cells

maintaining Foxp3 expression decreased by 70.3% in WT and 47.8% in  $p2x7^{-/-}$  CD45.2<sup>+</sup> cells in mesenteric LN (Fig. 4D). Comparable decreases of Foxp3 expressing cells were observed in WT and  $p2x7^{-/-}$  CD45.2<sup>+</sup> cells isolated from non draining LN or the spleen (Fig. 4D), in agreement with the described loss of Foxp3 expression during T<sub>regs</sub> homeostatic expansion in lymphopenic environments (29, 31, 40, 41). Notably, in mesenteric LN the percentage of T<sub>regs</sub>, which converted to IL-17 secreting effector cells was significantly higher in the WT group whereas no difference in the percentage of IFN- $\gamma$  secreting CD45.2<sup>+</sup> cells was observed (Fig. 4E,F). Nevertheless, the absolute number of IFN- $\gamma$  secreting CD45.2<sup>+</sup> cells in mesenteric LN was reduced in mice injected with  $p2x7^{-/-}$  T<sub>regs</sub> (Fig. 4F). These data confirmed the higher suppressive potential of  $p2x7^{-/-}$  T<sub>regs</sub> and point to a role for P2X7 in the differentiation of T<sub>regs</sub> to T<sub>H</sub>17 cells under inflammatory conditions; in contrast, P2X7 would not significantly affect lymphopenia stimulated acquisition of the Th1 IFN- $\gamma$  secreting phenotype.

### **Enhanced generation of adaptive T<sub>regs</sub> by P2X antagonism**

Although the inclination of CD4 single positive cells to differentiate to T<sub>regs</sub> decreases with maturation (42) adaptive T<sub>regs</sub> may be generated from peripheral T<sub>conv</sub> cells upon encounter of self antigens (reviewed in ref 43). We investigated whether P2X signaling might influence T<sub>regs</sub> conversion of CD4 naïve T cells. Stimulation of naïve T<sub>conv</sub> cells with anti-CD3 antibodies, IL-2 and irradiated splenocytes led to poor differentiation of Foxp3<sup>+</sup> cells (Fig. 5A) and transcriptional upregulation of Tbet, consistent with polarization toward T<sub>H</sub>1 fate (Fig. 5C).  $P2x7^{-/-}$  T<sub>conv</sub> cells did not differ significantly from WT cells in the transcriptional upregulation of Tbet. Since T<sub>conv</sub> cells express lower levels of P2X7 than T<sub>regs</sub>

and P2X4 is expressed at comparable levels (Fig. 1A), we hypothesized that in  $T_{conv}$  cells P2X7 might not play an exclusive role in shaping T cell function as observed in  $T_{regs}$ . We therefore used oATP as a pharmacological antagonist of P2X receptors in order to investigate whether ATP might influence  $T_{conv}$  cells polarization. Addition of oATP during the first 48h of T cell activation determined a dramatic increase in the percentage of Foxp3<sup>+</sup> cells (Fig. 5A) and transcriptional upregulation of Foxp3 with undetectable Tbet and ROR $\gamma$ T transcripts (Fig. 5C). Analogous increase in Foxp3 expressing cells was observed when a combination of selective pharmacological antagonists for P2X4 and P2X7 was added during T cell activation (Fig. S6), indicating the non-exclusive role of P2X7 in promoting conversion of  $T_{conv}$  cells toward  $T_{regs}$ .

oATP mediated  $T_{regs}$  conversion was not caused indirectly by inhibition of P2X receptors expressed in cocultured splenocytes since we obtained identical results when  $T_{conv}$  cells were stimulated with beads coated with anti-CD3 and CD28 antibodies (fig. S7A). The conversion of  $T_{conv}$  cells to  $T_{regs}$  upon TCR stimulation in the presence of oATP was presumably caused by diminished ERK activation (10) since addition of PMA abrogated oATP induced  $T_{regs}$  generation (fig. S7C). In accordance with the indispensable role of TGF- $\beta$  in adaptive  $T_{regs}$  generation (43), we found that oATP induced  $T_{regs}$  conversion was almost completely abolished when TGF- $\beta$  neutralizing antibodies were added to the cultures (Fig. S7C). The combination of oATP and TGF- $\beta$  had an additive effect on  $T_{regs}$  conversion (Fig. 5B,D). Moreover, oATP inhibited the moderate  $T_H17$  differentiation induced by TGF- $\beta$  alone (Fig. 5B and S7B). To get insights in the gene expression profile of  $T_{conv}$  cells stimulated in the presence of oATP we performed microarray analysis of T

cells activated for 48h in vitro. Figure 5E shows in a fold change plot how the combination of oATP with TGF- $\beta$  increased the expression of several T<sub>regs</sub> signature genes with respect to cells stimulated with TGF- $\beta$  alone. The same tendency is evident in a color plot from the same experiment (Fig. 5D). Notably, hierarchical clustering (Fig. S8) and TGF- $\beta$  network analysis (Fig. 6) showed similar patterns of expression of TGF- $\beta$  signaling components in T<sub>conv</sub> cells treated with TGF- $\beta$ , oATP or TGF- $\beta$  + oATP. Thus, inhibition of P2X receptors during T cell activation in the periphery enhances the TGF- $\beta$  signaling network and favors a developmental program leading to generation of T<sub>regs</sub>.

To test whether naïve T cells might be converted to immunosuppressive T<sub>regs</sub> by oATP in vivo, we induced IBD in *cd3 $\epsilon$ <sup>-/-</sup>* mice by transfer of T<sub>conv</sub> cells. These animals develop IBD due to the absence of T<sub>regs</sub> and insufficient T<sub>regs</sub> conversion in vivo. We started oATP treatment contemporaneously with the transfer of T<sub>conv</sub> cells, and administered the drug daily for 2 weeks. All mice injected with T<sub>conv</sub> cells developed severe disease four weeks after adoptive cell transfer; in contrast, 13 out of 20 oATP treated mice were disease free and 7 displayed only moderate signs of inflammation; mesenteric LN as well as spleen were of normal size (Fig. 7A). oATP treated animals displayed significantly higher percentages of T<sub>regs</sub> in mesenteric LN but not in non draining LN or spleen (Fig. 7B). Moreover, whereas lamina propria and intraepithelial T<sub>regs</sub> were barely detectable in untreated mice, they were equally represented in oATP treated animals and mice injected with protective numbers of T<sub>regs</sub> (Fig. 7C). These data suggest that P2X antagonism may induce tissue specific adaptive T<sub>regs</sub>, which may efficiently avoid inflammatory tissue destruction (44).

## Discussion

The release of ATP by eukaryotic cells influences cell physiology in autocrine/paracrine fashion. The response of P2 receptors to ATP regulates the “set point” of second messengers by affecting cytosolic  $\text{Ca}^{2+}$  concentration, cAMP levels and protein kinases activation. ATP released by  $\text{CD4}^+$  T cells upon TCR stimulation activates the MAPK pathway through P2X signaling (10, 20). *P2x7* is prominently expressed in  $\text{T}_{\text{regs}}$  and is part of the  $\text{T}_{\text{regs}}$  transcriptional signature. Herein, ATP released by activated  $\text{T}_{\text{regs}}$  mediated downregulation of Foxp3 and rendered them susceptible of conversion to  $\text{T}_{\text{H}}17$  cells through P2X7 activation, thereby indicating a role for autocrine ATP in modulating  $\text{T}_{\text{regs}}$ -mediated immunosuppression and lineage stability. Notably, the proinflammatory cytokine IL-6 increased ATP synthesis and ERK phosphorylation in  $\text{T}_{\text{regs}}$  through autocrine P2X7 activation. Therefore, enhancement of ATP synthesis by protracted/increased TCR stimulation combined with proinflammatory mediators may result in impaired suppressive function and lineage stability of  $\text{T}_{\text{regs}}$ .

In addition to influencing  $\text{T}_{\text{regs}}$  suppressive function in an autocrine fashion, ATP might also derive from other cells in an inflammatory environment and tune  $\text{T}_{\text{regs}}$  function in a paracrine fashion. We have previously shown that calreticulin-deficient  $\text{T}_{\text{conv}}$  cells, which release increased amounts of ATP (10), are less susceptible to suppression by  $\text{T}_{\text{regs}}$  (45). A similar T cell phenotype was also described in the NOD mouse model of type I diabetes (46).  $\text{T}_{\text{regs}}$  express high levels of the ectonucleotidase CD39, which enables them to rapidly degrade extracellular ATP to ADP and AMP. Further conversion of AMP to adenosine by CD73 was proposed as one mechanism by which  $\text{T}_{\text{regs}}$  inhibit the late phase of effector cell expansion (23, 24). However, in the initial phase of  $\text{T}_{\text{regs}}$  activation (first 48 hours) CD39

hydrolyzes ATP with low efficiency (22), which would allow accumulation of extracellular ATP and inhibition of T<sub>regs</sub> suppressive function while stimulating the expansion of effector T cells. Lowering P2X costimulation in T cells diminishes ERK phosphorylation without affecting TCR mediated NFAT nuclear translocation (10). In T<sub>regs</sub> this mechanism would favor the stability of T<sub>regs</sub> transcriptional program through the stabilization of nuclear NFAT/Foxp3 complexes (47).

ATP was shown to determine Th17 effector cell differentiation from naïve T cells in the lamina propria of the colon via activation of CD70<sup>high</sup>CD11c<sup>low</sup> cells (48). In contrast, we have shown that ATP directly influences T<sub>regs</sub> conversion to Th17 cells in a cell autonomous fashion. Recently, several reports demonstrated that T<sub>regs</sub> do not represent a terminally differentiated T cell lineage. Indeed, they can differentiate to effector cells in vivo under particular circumstances such as lymphopenia or inflammation (29, 31, 40, 41). Since natural T<sub>regs</sub> display increased self reactivity (49) their conversion to effector cells might lead to loss of tolerance to self. On the other hand, controlled T<sub>regs</sub> conversion to effector cells may contribute to a more efficient clearance of particular pathogens (50).

Another important finding of the present study is the generation of adaptive T<sub>regs</sub> by conversion of naïve T cells by P2X pharmacological antagonism. This conversion was reversed by PMA, thereby supporting the role of ERK activation in counteracting T<sub>regs</sub> conversion. Accordingly, activation of naïve T cells in the presence of an ERK inhibitor was shown to induce T<sub>regs</sub> conversion (51). Signaling by endogenous ATP might help in shaping the T cell response in a context dependent manner by integrating extracellular cues with cellular energetics. Our study further emphasizes the role of ATP as an adjuvant in immune function (52); the increase in extracellular ATP at an inflammatory site would

determine effector T cell activation as well as P2X7-mediated inhibition of T<sub>regs</sub> function and stability. Altogether these results underscore the potential value of the P2X signaling pathway as a pharmacological target for adaptive immune regulation.

## **Materials and Methods**

### **Mice**

C57Bl/6 mice were from Charles River Germany, C57Bl/6 *cd3ε*<sup>-/-</sup> mice (53) and P2X7<sup>-/-</sup> mice (54) were from the Jackson Laboratory. The animals were bred and treated in accordance with the Swiss Federal Veterinary Office guidelines. Experiments were approved by the “Dipartimento della Sanità e della Socialità”. Individual experiments with at least 4 mice/group are shown. All experiments were repeated at least three times. IBD was induced by adoptive transfer of 200,000 naïve CD4<sup>+</sup> T cells purified from C57Bl/6 mice into *cd3ε*<sup>-/-</sup> animals. Mice that received 100,000 CD4<sup>+</sup>CD25<sup>+</sup> T<sub>regs</sub> together with naïve CD4<sup>+</sup> T cells served as healthy controls, whereas cotransfer of 40,000 T<sub>regs</sub> was used to induce partial protection from disease. For in vivo induction of T<sub>regs</sub>, 200,000 naïve CD4<sup>+</sup> T cells were resuspended in 100 µl PBS containing 3 mM oATP (Medestea) and injected. Then, mice were daily treated with 100 µl PBS containing 3 mM oATP i.v. for two weeks. After four weeks, the animals were sacrificed and LN, spleen, and colon lamina propria cells were analyzed by flow cytometry. For histological analysis, the large intestine (from the ileo-ceco-colic junction to the ano-rectal junction) was removed, fixed in 10% buffered formalin solution, and routinely processed for histological examination. Sections were stained with eosin-hematoxin or Alcian-Periodic-Acid-Schiff (PAS).

### **Antibodies and cells**

For flow cytometric analyses, mabs conjugated with either fluorescein isothiocyanate (FITC), phycoerythrin (PE), CyChrome (Cy), allophycocyanin (APC), PE-Cy7 or APC-Cy7 (eBioscience) were used. Cytokine-producing T cells were detected by intracellular staining after incubation with 100 nM PMA (Sigma-Aldrich) and 1  $\mu$ M ionomycin (Sigma-Aldrich) for 4h. Ten  $\mu$ g/ml brefeldin A (Sigma-Aldrich) were added for the last 2h of incubation to prevent cytokine secretion. Cytokines were detected with APC-conjugated antibodies to IFN- $\gamma$ , and IL-17 (eBioscience) after cell fixation and permeabilization with Cytofix/Cytoperm (BD Pharmingen). To detect T<sub>regs</sub>, cells were fixed, permeabilized according to the manufactures instructions and stained for the transcription factor Foxp3 with an APC conjugated antibody (eBioscience). For analysis of ERK, p38 MAPK and c-jun phosphorylation, sorted T<sub>regs</sub> were stimulated for 90 min with the indicated stimuli in the presence of irradiated splenocytes from *cd3 $\epsilon$ <sup>-/-</sup>* mice, permeabilized and stained with the following rabbit mabs: anti-phospho ERK (Thr202/Tyr204) D13.14.4E, anti-phospho p38 MAPK (Thr180/Tyr182) 3D7 and anti-phospho c-jun (Ser63) 54B3 (Cell Signaling Technology). Samples were analyzed with a FACScalibur or FACSCanto (Becton Dickinson). Viable cells were electronically gated by exclusion of propidium iodide.

For differentiation/proliferation assays, T cells were isolated from peripheral LN and spleens by positive selection with anti-CD4 immunomagnetic beads (Miltenyi Biotech). CD4<sup>+</sup> naïve (CD4<sup>+</sup>CD25<sup>-</sup>CD44<sup>-</sup>CD62L<sup>+</sup>) and regulatory (CD4<sup>+</sup>CD25<sup>high</sup>) T cell subsets were sorted with a FACS Aria (Becton Dickinson) from pooled LN and spleens. We grew 100,000 naïve or T<sub>regs</sub>/well (96 well flat bottom plate) in RPMI supplemented with 5% FCS and Penicillin/Streptomycin with 0.5  $\mu$ g/ml anti-CD3 mab, 50 U/ml IL-2 and 250,000 T

cell depleted splenocytes, which were previously  $\gamma$ -irradiated with 2000 rad. Where indicated 5 ng/ml TGF- $\beta$  (R&D Systems), 20 ng/ml IL-6 (R&D Systems), 100  $\mu$ M oATP, 2 nM PMA or 200 nM FICZ (Biomol) were added. Unless otherwise stated, pharmacological treatments were removed from naïve T cells after 48 h culture and cells were placed in fresh medium containing 50 U IL-2 and analysed at day 7.

For suppression assays 50,000 naïve T cells were labelled with 1  $\mu$ M 5,6-CFSE (Molecular Probes) and stimulated with 0.5  $\mu$ g/ml of anti-CD3 mab and 125,000 T cell depleted irradiated splenocytes together with serial dilutions of T<sub>regs</sub>. CFSE dilution in naïve T cells and survival of T<sub>regs</sub> were analyzed after 72 h of culture. FACS acquisitions were standardized by fixed numbers of calibration beads (BD Pharmingen). For experiments using <sup>3</sup>H-thymidine incorporation, <sup>3</sup>H thymidine (2  $\mu$ Ci/well) was added for the last 12 hours of culture. For measurement of cellular ATP, cells were lysed in 1% Triton X-100 and frozen on dry ice until analysed with the ATP determination kit (Molecular Probes). Statistical analysis was performed by using a Student's *t* test, unless otherwise indicated. Data are reported as mean  $\pm$  SE or SD. Values of *P* < 0.05 were considered significant.

\*: *P* < 0.05; \*\*: *P* < 0.01; \*\*\*: *P* < 0.001

### **Quantification of mRNA levels**

Total RNA was precipitated in Trizol (Invitrogen) and then reverse transcribed to cDNA using Random hexamer primers and an M-MLV reverse-transcriptase kit (Invitrogen). For quantification of transcripts, mRNA samples were treated with 2 U/sample of DNase (Applied Biosystems). Transcripts were quantified by real-time quantitative PCR on an ABI PRISM 7700 Sequence Detector with predesigned TaqMan Gene Expression Assays

and reagents according to manufacturer's instructions (Perkin-Elmer Applied Biosystems).

Probes with the following Applied Biosystems assay identification numbers were used:

Foxp3: Mm00475162\_m1

ROR $\gamma$ t: Mm01261022\_m1

Tbet: Mm00450960\_m1

IL-17: Mm00439618\_m1

P2X1: Mm00435460\_m1

P2X2: Mm01202369\_g1

P2X3: Mm00523699\_m1

P2X4: Mm00501787\_m1

P2X5: Mm00473677\_m1

P2X7: Mm00440582\_m1

18S: EUK 18S rRNA (DQ) Mix

For each sample, mRNA abundance was normalized to the amount of 18S rRNA and is presented in arbitrary units.

### **Microarray**

Naïve CD4<sup>+</sup> T cells were stimulated with anti-CD3 mab, 50 U/ml IL-2 and irradiated splenocytes, as described above, either without any further addition or with TGF- $\beta$ , oATP or a combination of both. After 48 h, CD4<sup>+</sup> cells were sorted at FACS Aria and total RNA was isolated using the RNAeasy kit (Qiagen). Quality and integrity of total RNA isolated from sorted cells was assessed by running all samples on an Agilent Technologies 2100 Bioanalyser (Agilent Technologies). 12.5  $\mu$ g of each biotinylated cRNA preparation was

fragmented and placed in a hybridization cocktail containing four biotinylated hybridization controls (BioB, BioC, BioD, and Cre) as recommended by the manufacturer. Samples were hybridized to an identical lot of Affymetrix MOE430 2.0 for 16 hours. After hybridization the GeneChips were washed, stained with SA-PE and read using an Affymetrix GeneChip fluidic station and scanner. Analysis was done with gene expression software GCOS 1.2 (Affymetrix), Genesis 1.6 and GenePattern software package.

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## Figure legends

**Figure 1. P2X7 mediated inhibition of T<sub>regs</sub> function.** **A.** RT-PCR of P2X transcripts in CD4 single positive thymocytes, CD4 peripheral naïve T cells and T<sub>regs</sub>. **B-D.** Suppression assay with CFSE-labeled CD45.1<sup>+</sup> responder and CD45.2<sup>+</sup> T<sub>regs</sub>. Means±SE are shown (n=3; \*: *P* <0.05, \*\*: *P* <0.01). Results are representative of three independent experiments. **E.** Flow cytometry analysis at 7 days of sorted CD4<sup>+</sup>CD25<sup>high</sup> T cells stimulated with CD3 mab either untreated or treated with oATP. Percentage of cells in the displayed quadrant and mean fluorescence intensity (MFI) for Foxp3 are indicated. Results are representative of five independent experiments. **F.** Foxp3 staining of CFSE labeled WT or *p2x7*<sup>-/-</sup> T<sub>regs</sub> stimulated with CD3 mab, irradiated *cd3ε*<sup>-/-</sup> splenocytes, IL-2 at 50 U/ml and BzATP where indicated for 48 h, then maintained in IL-2 at 50 U/ml for additional 4 days. Numbers refer to percentages of cells in the respective quadrant. Results are representative of three independent experiments. **G.** Statistical analyses of Foxp3<sup>+</sup>CFSE<sup>low</sup> cells (corresponding to upper quadrant in F) of WT T<sub>regs</sub> stimulated as indicated above in the presence of oATP, rottlerin or BzATP for the first 48 h (mean±SD, n=3, \*\*: *P* <0.01).

**Figure 2. P2X7-mediated ATP synthesis, ERK phosphorylation and Foxp3**

**downregulation.** **A.** Intracellular ATP variations in WT (black lines) and *p2x7*<sup>-/-</sup> (red lines) T<sub>regs</sub> upon stimulation with CD3 mab (left panel) and CD3 mab together with IL-6 (right panel). *P* values calculated by analysis of variance (ANOVA) for each set of data are indicated. Results are representative of three independent experiments. **B.** MFI with phospho-ERK mab in flow cytometry of WT (black bars) and *p2x7*<sup>-/-</sup> (red bars) T<sub>regs</sub> stimulated for 90 min in the presence of *cd3ε*<sup>-/-</sup> irradiated splenocytes with the indicated stimuli (mean±SD, n=3, \*: *P* <0.05). **C.** Statistical analyses of MFI with the indicated anti-phospho protein mab

in flow cytometry of WT (black bars) and  $p2x7^{-/-}$  (red bars)  $T_{\text{regs}}$  stimulated with CD3 mab as above (mean $\pm$ SD, n=4, \*\*\*:  $P < 0.001$ ). **D.** Foxp3 staining of CFSE labeled WT  $T_{\text{regs}}$  stimulated in the presence of PD 98059 for the first 48 h where indicated. Numbers refer to percentages of cells in the respective quadrant. Histograms represent the statistical analysis of the experiment (n=3, \*:  $P < 0.05$ ). Results are representative of two independent experiments. **E.** RT-PCR of Foxp3 mRNA from  $T_{\text{regs}}$  stimulated for 16 h in the presence of the indicated treatments. Means $\pm$ SE of triplicates from single cultures are shown. Data are representative of three independent experiments. Black bars, WT cells; red bars,  $p2x7^{-/-}$  cells. AU: arbitrary unit.

**Figure 3. P2X7 mediated expression of ROR $\gamma$ t in  $T_{\text{regs}}$ .** **A.** RT-PCR of ROR $\gamma$ t mRNA from WT (black lines) and  $p2x7^{-/-}$  (red lines)  $T_{\text{regs}}$  stimulated for the indicated times in cocultures with splenocytes of WT and  $p2x7^{-/-}$  origin, as indicated. Means $\pm$ SE are displayed (n=3), asterisks refer to both data sets (\*,  $P < 0.05$ ). Results are representative of three independent experiments. **B.** RT-PCR of ROR $\gamma$ t mRNA from WT (black bars) and  $p2x7^{-/-}$  (red bars)  $T_{\text{regs}}$  stimulated for 6 days in the presence of IL-6 and IL-6 + FICZ. Means $\pm$ SE of triplicates from single cultures are shown. Results are representative of three independent experiments. **C.** RT-PCR of Foxp3 and ROR $\gamma$ t mRNA from WT (black bars) and  $p2x7^{-/-}$  (red bars)  $T_{\text{regs}}$  stimulated for 16 h in the presence of BzATP where indicated. Means $\pm$ SD of duplicates from single cultures are shown. Results are representative of two independent experiments. AU: arbitrary unit.

**Figure 4. Increased in vivo suppressive potential of  $p2x7^{-/-}$   $T_{\text{regs}}$ .** **A.** Large intestine, spleen and mesenteric LN from  $cd3\epsilon^{-/-}$  mice transferred with CD45.1  $T_{\text{conv}}$  cells and nonprotective

numbers of either WT or  $p2x7^{-/-}$  CD45.2  $T_{\text{regs}}$ . Bar=1 cm. **B.** Cell recoveries of CD45.1<sup>+</sup>  $T_{\text{conv}}$  cells from mesenteric LN and spleens. **C.** Cell recoveries of CD45.2<sup>+</sup>  $T_{\text{regs}}$  from mesenteric LN and spleens. **D.** Percentages of CD45.2<sup>+</sup>  $T_{\text{regs}}$  from mesenteric LN and spleens. **E.** Percentages (left) and absolute numbers (right) of IL-17 secreting CD45.2<sup>+</sup> CD4<sup>+</sup> T cells from mesenteric LN and spleens. **F.** Percentages (left) and absolute numbers (right) of IFN- $\gamma$  secreting CD45.2<sup>+</sup> CD4<sup>+</sup> T cells from mesenteric LN and spleens. Black bars, mice receiving WT CD45.2<sup>+</sup>  $T_{\text{regs}}$ ; Red bars, mice receiving  $p2x7^{-/-}$  CD45.2<sup>+</sup>  $T_{\text{regs}}$ . Means  $\pm$  SE are displayed (n=5; \*:  $P < 0.05$ , \*\*\*:  $P < 0.001$ ). **G.** Hematoxilin/eosin (three left panels) and Alcian/PAS (right panel) staining of colon sections. Colons from mice transferred with both naïve CD4<sup>+</sup> cells and  $T_{\text{regs}}$  from wild-type mice ( $T_{\text{regs}}$  WT) display inflammatory cells infiltrates (arrows) and goblet cells depletion. In colons from mice reconstituted with wild-type naïve CD4<sup>+</sup> cells and  $T_{\text{regs}}$  from  $p2x7^{-/-}$  mice ( $T_{\text{regs}}$   $p2x7^{-/-}$ ) no inflammatory changes are evident and large number of goblet cells with Alcian-PAS-positive droplets lines the colonic crypts. Results are representative of two independent experiments.

**Figure 5.  $T_{\text{regs}}$  conversion by P2X antagonism.** **A.** Flow cytometry for CD25 and Foxp3 expression by  $T_{\text{conv}}$  cells at day 7 after stimulation for 48 h with CD3 mab (control) or CD3 mab with oATP (oATP). **B.** Flow cytometry of  $T_{\text{conv}}$  cells stimulated with CD3 mab in the presence of TGF- $\beta$  (left) or TGF- $\beta$  and oATP (right) at day 7 after stimulation; CD25/Foxp3 and CD4/IL-17 staining, as indicated. Numbers refer to percentages of cells in the respective quadrant. **C.** RT-PCR of Foxp3, ROR $\gamma$ t and Tbet mRNAs extracted from  $T_{\text{conv}}$  cells stimulated for the indicated times. Black traces, control; red traces, oATP treatment. Results are representative of two experiments. **D.** Color plot of fold change gene

expression at 48 hours in CD3 stimulated  $T_{conv}$  cells treated with TGF- $\beta$ , oATP and TGF- $\beta$  + oATP. **E.** Fold change plot of gene expression at 48 hours in CD3 stimulated  $T_{conv}$  cells treated with TGF- $\beta$  alone versus TGF- $\beta$  + oATP. Some spots corresponding to  $T_{regs}$  signature genes are identified. AU: arbitrary unit.

**Figure 6. TGF- $\beta$  network analysis.** Network was constructed based on the interaction of TGF- $\beta$  receptor pathway molecules as described by NetPath (<http://www.netpath.org>).

Molecules (genes) shown as purple, red and blue circles represent non-regulated, up-regulated and down-regulated members of the TGF- $\beta$  signaling network, respectively.

**Figure 7. Protection from IBD by oATP. A.** Large intestines, spleen, mesenteric LN (left), hematoxylin/eosin (middle) and Alcian/PAS (right) staining of colon sections of  $cd3\epsilon^{-/-}$  mice transferred with  $T_{conv}$  cells (upper panels),  $T_{conv}$  cells together with oATP (middle panels) and  $T_{conv}$  cells with natural  $T_{regs}$  (lower panels). In mice reconstituted with  $T_{conv}$  cells, colonic crypts are severely dysplastic with large inflammatory infiltrates and almost complete loss of goblet cells. Colons from mice reconstituted with  $T_{conv}$  cells together with oATP lack signs of inflammation analogously to colons from mice reconstituted with  $T_{conv}$  cells and natural  $T_{regs}$ . Bar=1 cm **B.** Percentages of recovered  $T_{regs}$  in non draining (black bars), mesenteric (white bars) LN and spleen (grey bars) from  $cd3\epsilon^{-/-}$  mice reconstituted as indicated (n=15, CD4 group; n=20, CD4+oATP and CD4+ $T_{regs}$  groups) **C.** Percentages of lamina propria (black bars) and intraepithelial (grey bars)  $T_{regs}$  recovered from mice reconstituted as indicated (n=4). Mean values  $\pm$  SE are displayed; ns: non significant; \*\*\*:  $P < 0.001$ .