DOI: 10.1002/eji.201142213 Eur. J. Immunol. 2012. 42: 3310–3321

European Journal of Immunology

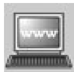
ATP conditions intestinal epithelial cells to an inflammatory state that promotes components of DC maturation

Yu Yao[1], Megan K. Levings[2] and Theodore S. Steiner[1]

[1] Department of Medicine, University of British Columbia and Vancouver Coastal Health Research Institute, Vancouver, B.C., Canada
[2] Department of Surgery, University of British Columbia and Child and Family Research Institute, Vancouver, B.C., Canada

Intestinal epithelial cells (IECs) normally promote the development of gut resident tolerogenic dendritic cells (DCs) and regulatory T cells, but how this process is altered in inflammatory bowel disease is not well characterized. Recently, we published that the cell injury signal ATP modulates IEC chemokine responses to the TLR5 ligand flagellin and exacerbates colitis in the presence of flagellin. We hypothesized that ATP switches these IECs from tolerogenic to proinflammatory, enhancing DC activation and immune responses to commensal antigens. Here, we report that ATP enhanced murine IEC production of KC, IL-6, TGF-β, and thymic stromal lymphopoietin in response to TLR1/2 stimulation by Pam_3CSK_4 (PAM). Moreover, supernatants from IECs stimulated with ATP+PAM enhanced expression of CD80 on bone marrow derived dendritic cells, and increased their production of IL-12, IL-6, IL-23, TGF-β, and aldh1a2, suggesting a Th1/Th17 polarizing environment. DCs conditioned by stressed IECs stimulated an enhanced recall response to flagellin and supported the expansion of IFN-γ^+ and IL-17^+ memory T cells. Lastly, colonic administration of nonhydrolysable ATP increased production of IL-6 and Cxcl1 (KC) by IECs. These findings indicate that ATP influences the response of IECs to TLR ligands and biases the maturation of DCs to become inflammatory.

Keywords: CD4 T cells · Dendritic cells · Immune regulation · Intestinal immunity · Toll-like receptors


Supporting Information available online

Introduction

Intestinal epithelial cells (IECs) coordinate the dynamic interactions between luminal microbes and local immune cells and represent the front line of enteric defense [1]. In addition to acting as a physical barrier to prevent passage of luminal contents, IECs are crucial for maintaining intestinal homeostasis by sampling the luminal microenvironment, integrating signals received from pattern recognition receptors (PRRs) and local immune cells, and secreting factors that regulate adaptive immunity by priming intestinal dendritic cells (DCs) [2]. Under steady state conditions, IECs maintain a hyporesponsive state to commensal flora by secreting factors such as thymic stromal lymphopoietin (TSLP), TGF-β, and retinoic acid (RA), which together facilitate the development of tolerogenic DCs and regulatory T (Treg) cells [3, 4]. During enteric infection, however, TLR-mediated activation of IECs can

Correspondence: Dr. Theodore S. Steiner
e-mail: flagellin@gmail.com

© 2012 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

initiate robust inflammatory responses. How IECs discriminate between TLR ligands presented by invasive pathogens versus commensal microbes remains largely unknown [5].

One way that IECs may respond to pathogens is by recognizing specific danger signals such as extracellular ATP [6, 7]. Recently, several groups reported that ATP contributes to the pathogenesis of a variety of inflammatory disorders, such as asthma, graft versus host disease, and inflammatory bowel disease (IBD), suggesting it is an endogenous danger signal that promotes inflammation [8–12]. Importantly, low expression of CD39, an enzyme that hydrolyzes ATP, is associated with increased risk for Crohn's disease (CD) [12]. Consistent with this finding, CD39-deficient mice have increased susceptibility to dextran sulfate sodium (DSS) induced colitis [12]. The association of ATP with enhanced inflammation in the gut has also lead to the recent identification of commensal bacteria that actively produce ATP in the lumen [13, 14], providing another important source of ATP in addition to epithelial injuries as a result of inflammation [14, 15]. In the context of IBD, where patients have weaker intestinal barrier function and bacterial leakage across the mucus layer, IECs represent the important first responders to cellular stress and microbial ligands. Therefore, it is essential to understand how ATP regulates inflammatory responses to TLR activation in IECs.

Previous work in our laboratory showed that ATP regulates the inflammatory response to flagellin-TLR5 ligation in human IECs and that rectal administration of ATP enhanced flagellin-mediated inflammation during DSS-colitis [16]. In this study, we extend our findings to murine IECs and utilize an in vitro coculture model with supernatants from IECs and bone marrow derived dendritic cells (BMDCs) to test how stressed IECs regulate DC maturation. Furthermore, we explored in vivo whether colorectal administration of ATP alone, in the absence of DSS colitis, is able to modulate the how IECs respond to commensal microbes. We hypothesized that simultaneous exposure to ATP and TLR ligands would modulate how IECs respond to TLR stimulation, facilitating the maturation of DCs and ultimately determining how T cells respond to commensal antigens.

Results

ATP modulates the production of cytokines and chemokines by TLR1/2-activated IECs

During cellular damage and inflammation, ATP can be released immediately into the extracellular space, attaining local concentrations in the millimolar range. At that concentration, it acts as an important danger signal by activating P2 purinergic receptors, alerting immune cells to the presence of tissue damage and mobilizing them to the site of injuries to fight pathogens and clear cellular debris [17, 18]. We previously showed that ATP alters TLR5 signaling in Caco-2 human IECs, and sought to determine whether this phenomenon applied to murine IECs as well. To test this, Mode-K IECs were stimulated with different concentrations of ATP and TLR1/2 agonist Pam3CSK4 (PAM). We focused on pro-inflammatory mediators and measured secretion of the neutrophil chemoattractant KC (Cxcl1) and IL-6 by ELISA. As shown in Figure 1A, 1 mM ATP significantly augmented TLR1/2 activation-induced production of KC (from 29.9 ± 1.41 to 73.3 ± 2.67 ng/mL), while ATP alone did not induce KC expression. A similar increase in IL-6 production was observed with ATP at a lower concentration (from 156.4 ± 20.04 to 259.2 ± 9.49 pg/mL, Fig. 1B). Furthermore, the ATP-induced KC secretion was completely blocked by treatment with apyrase, which degrades ATP into ADP and AMP (from 73.3 ± 2.67 to 32.3 ± 1.97 ng/mL, Fig. 1A), indicating that this effect requires ATP and not its hydrolytic products.

Since IECs are known to produce tolerogenic factors that maintain immune tolerance to commensal microbes in the intestinal tract [3, 4], we also asked whether ATP modulated the expression of anti-inflammatory mediators. We thus measured expression of TGF-β, TSLP, and aldh1a2, a rate-limiting enzyme in the synthesis of RA. ATP increased TLR1/2-mediated expression of mRNA encoding for each of these proteins (Fig. 1C–E), which would suggest induction of a tolerant state. However, these tolerogenic factors can promote inflammation in the appropriate milieu [19–21], such as in the presence of large amounts of IL-6 or IL-15. Hence they may act to reduce tolerance in the presence of cellular injuries.

ATP acts primarily through the P2X7 receptor on IECs

Because most of the biological effects of extracellular ATP are mediated through P2 purinergic receptors [22], we explored which P2 receptors mediated the pro-inflammatory effect on the IECs. To study this, we first tested two different ATP analogues, UTP (uridine 5′-triphosphate), and BzATP (2′(3′)-O-(4-benzoylbenzoyl)adenosine 5′-triphosphate), both of which have preferential binding to different P2 receptors. As shown in Figure 2A, BzATP, a preferential P2X7 receptor agonist [23], potently enhanced KC production (BzATP 3.4 ± 0.51 fold compared with ATP 3.1 ± 0.57 fold). In contrast, UTP, which is a relatively selective P2Y receptor agonist, only minimally increased KC production (Fig. 2A, 1.36 ± 1.58 fold). In keeping with these results, KN-62, a noncompetitive antagonist for P2X7, completely blocked the enhancement of KC secretion by ATP (Fig. 2B). These data indicate that the effects of ATP on IECs are largely mediated through the P2X7 receptor and not P2Y receptors.

Since the P2X7 receptor is known for the important downstream effect of inflammasome activation and IL-1β processing [24], we then asked whether paracrine release of IL-1β is responsible for the inflammatory effect of ATP in Mode-K cells, as has been reported in T84 human IECs [25]. We did not detect any production of IL-1β at the protein level as a result of ATP and PAM treatment (data not shown). Moreover, when we treated Mode-K cells with IL-1ra in concentrations sufficient to block IL-1 receptor signaling (Fig. 2C), we did not inhibit KC production by Mode-Ks (Fig. 2D), suggesting that neither extracellular IL-1α nor IL-1β is responsible for the reported Mode-K phenotype that we observed.

© 2012 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

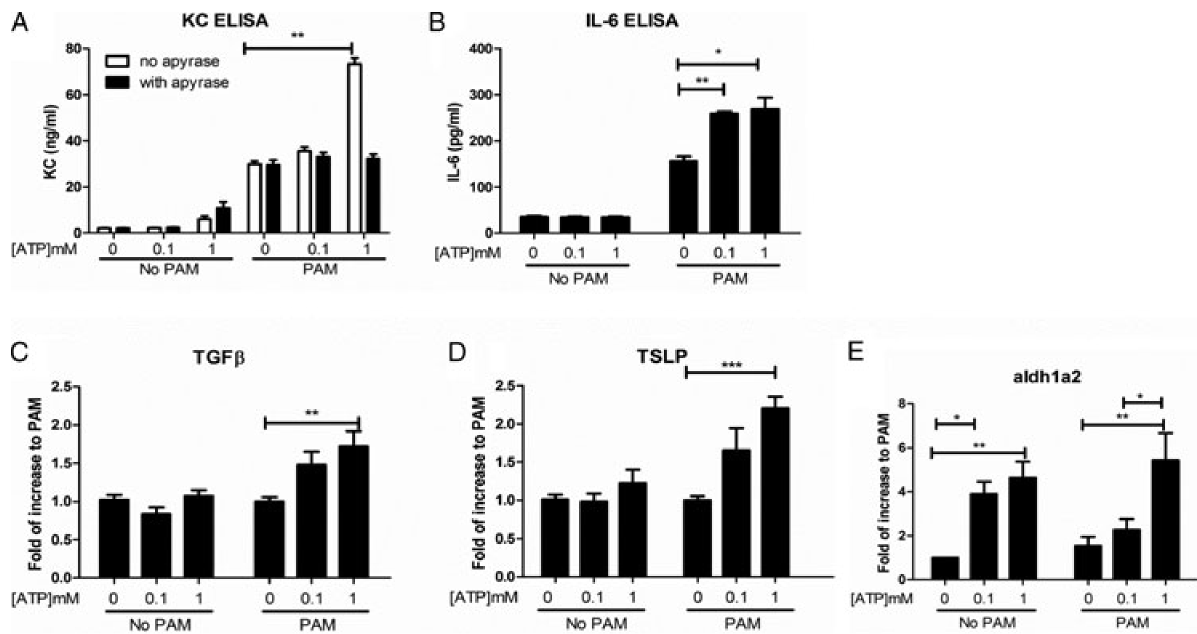


Figure 1. ATP enhances IEC cytokine/chemokine production. Mode-K cells were pretreated with ATP at the indicated concentrations with/without apyrase for 20 minutes followed by 100 ng/mL Pam_3CSK_4 (PAM) for (A, B) 24 hours or (C-E) 6 hours. (A) KC and (B) IL-6 concentrations in supernatants were measured by ELISA. (C) TGF-β, (D) TSLP, and (E) aldh1a2 mRNA were measured by RT-PCR and expressed as the fold change compared with the mRNA level of cells treated with PAM alone in each experiment. The results are expressed as mean ± SEM of 10–14 replicates/samples compiled from at least five different experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; one-way ANOVA followed by Tukey's multiple comparison test.

Supernatants from stressed IECs enhance expression of CD80 on DCs

In order to define the immunological consequence of stressed IECs on DC maturation, we exposed BMDCs to supernatants from IECs that were treated with PAM in the absence or presence of ATP for 24 h, and then analyzed expression of CD80 and MHC class II (MHC II) on CD11c^{+} cells by flow cytometry. Because the ATP+PAM-treated IEC supernatants contained residual ATP and PAM after overnight culture, we neutralized the remaining ATP with apyrase (Supporting Information Fig. 1A). Next, we determined the residual TLR1/2-stimulatory activity in ATP+PAM-treated IEC supernatants using an IL-8 release bioassay in TLR2-transfected HEK-293T cells. The TLR1/2 agonist activity present in

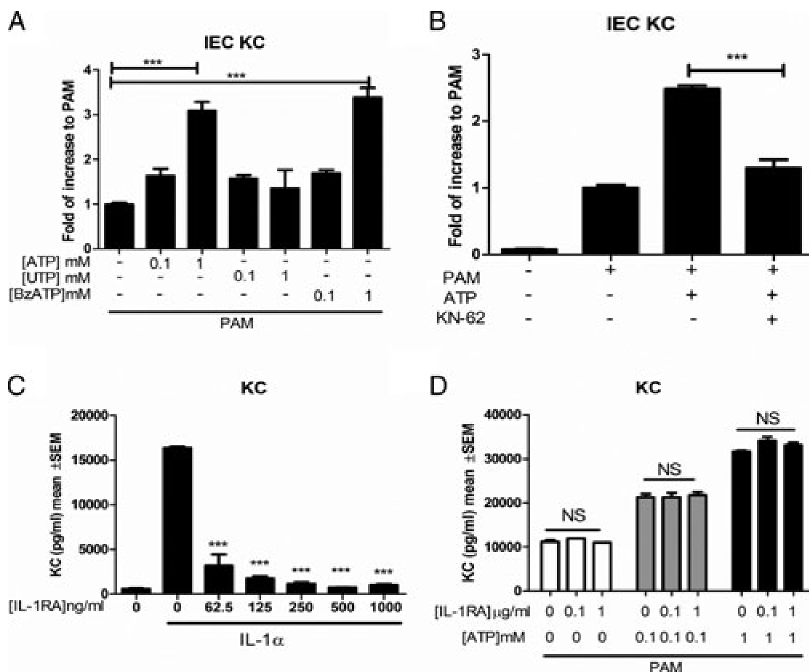


Figure 2. The inflammatory effect of ATP is mediated primarily through the P2X7 receptor and the effect is independent of IL-1. Mode-K cells were preincubated with KN-62 (K) for 30 min, followed by ATP, UTP, or BzATP for 20 min and PAM. (A, B) Supernatants were collected after 24 h of stimulation and KC concentration was measured by ELISA and expressed as fold of increase to PAM-treated cells. (A) BzATP and UTP differentially influence KC production. (B) KN-62 treatment affects KC production by ATP. (C) Dose-response study of inhibition of IL-1α signaling by IL-1RA (where RA is retinoic acid) in Mode-K cells. (D) IL-RA affects the production of KC by ATP in Mode-K cells. The data are shown as mean ± SEM of two replicates compiled from three separate experiments. $^{***}p < 0.001$ compared with IL-1α-treated Mode-K cells or as shown, one-way ANOVA followed by Tukey's multiple comparison test.

© 2012 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

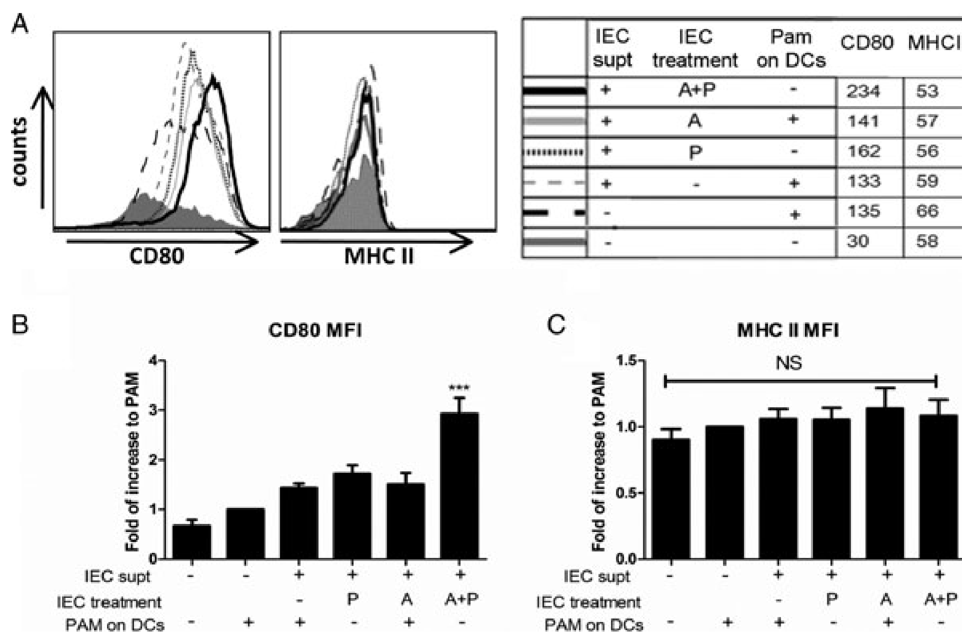


Figure 3. ATP-treated IECs enhance BMDC CD80 expression. Mode-K cells were stimulated with the following: ATP 1 mM, Pam_3CSK_4 100 ng/mL, or both (A+P) for 24 h. IEC supernatants were collected and placed on BMDCs for 24 h. Apyrase was added to ATP-treated Mode-K supernatants to remove residual ATP. To match the residual PAM concentration in the ATP+PAM-treated IEC supernatants, 15 ng/mL of PAM was added onto the BMDCs that were treated with IEC supernatants that did not contain PAM. (A–C) The live DCs were gated on 7-Amino-actinomycin D (7-AAD)$^-$ and CD11c$^+$ population and mean fluorescence intensities of MHCII (I-A^k) and CD80 were calculated. (A) Representative CD80 and MHC II histograms (left) and MFIs (right) from one of four experiments. (B) CD80 MFIs are shown. ***$p < 0.001$ compared with all other groups, one-way analysis of variance (ANOVA) and Dunnett's posttest. (C) MHC II MFI. NS: not significant. (B, C) Data are expressed as mean ± SEM from four independent experiments normalized to the MFI of PAM-matured DCs in regular media in each experiment (one-way ANOVA and Dunnett's posttest).

the conditioned supernatants was equivalent to 15 ± 5 ng/mL of PAM (Supporting Information Fig. 1C). Therefore, when we stimulated BMDCs, we added 15 ng/mL of PAM to each control supernatant if there was no PAM in it. DCs treated with PAM alone in the absence of IEC supernatants served as our matured DC control.

As shown in Figure 3A and B, ATP+PAM-treated IEC supernatants enhanced CD80 expression compared with supernatants from untreated IECs or IECs treated with either PAM or ATP alone. Only simultaneous ATP+PAM-treated IEC supernatants were able to induce a significant increase of CD80, as opposed to single stimulus-treated IECs. In contrast to CD80, we did not observe any effect on the expression of MHC II molecules (Fig. 3A and C). To exclude the possibility that ATP metabolites (such as AMP or adenosine) stimulate CD80 expression, we compared apyrase-treated ATP with unstimulated DCs in the presence or absence of PAM and did not find any significant induction (Supporting Information Fig. 1B). Overall, these data suggest that conditioned media from stressed IECs cause a significant upregulation of CD80 expression on BMDCs.

We next measured CD80 expression in response to the UTP or BzATP stimulated Mode-K cell supernatants. We observed a similar effect of BzATP to ATP but not UTP in modulating CD80 expression on BMDCs (Fig. 4A), while KN-62 completely blocked the downstream effect of ATP on BMDC CD80 expression (Fig. 4B). Furthermore, IL-1RA did not inhibit CD80 induction on BMDCs by stressed IEC supernatants (Fig. 4C), confirming the effect is independent of extracellular IL-1.

We next tested the effects of stressed IEC supernatants on other DC populations. We found that ATP/PAM-stressed Mode-K cells enhanced CD80 expression on C57Bl/6 BMDCs (data not shown) that are not deficient in TLR4 signaling. We found a similar trend in CD80 expression in C3H/HeJ splenic DCs (Supporting Information Fig. 2A), and C3H/HeJ BMDCs derived in the presence of RA (Supporting Information Fig. 2B), the latter of which are reported to exhibit tolerogenic properties resembling ex vivo intestinal DCs [26]. These data suggest that multiple subsets of DCs can upregulate their CD80 expression upon exposure to soluble factors released by stressed epithelial cells, and this effect depends on P2X7 receptor expression on IECs but is independent of IL-1β.

ATP+PAM-treated IEC supernatants promote production of pro-inflammatory cytokines from DCs

To further characterize how stressed IECs affect DCs, we examined cytokine production from these cells. We found that DCs cultured in supernatants from IECs exposed to PAM and ATP had a significant increase in expression of IL-12p35, IL-12p40, IL-6, IL-23p19, and TGF-β, compared with that of controls (Fig. 5). There was also a trend toward increased IL-10 and IL-15 although it was not statistically significant. Similar to what we observed in Mode-K cells, there was also increased expression of aldh1a2 in DCs (Fig. 5F), suggesting a likely increase in RA production. Such a cytokine

© 2012 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

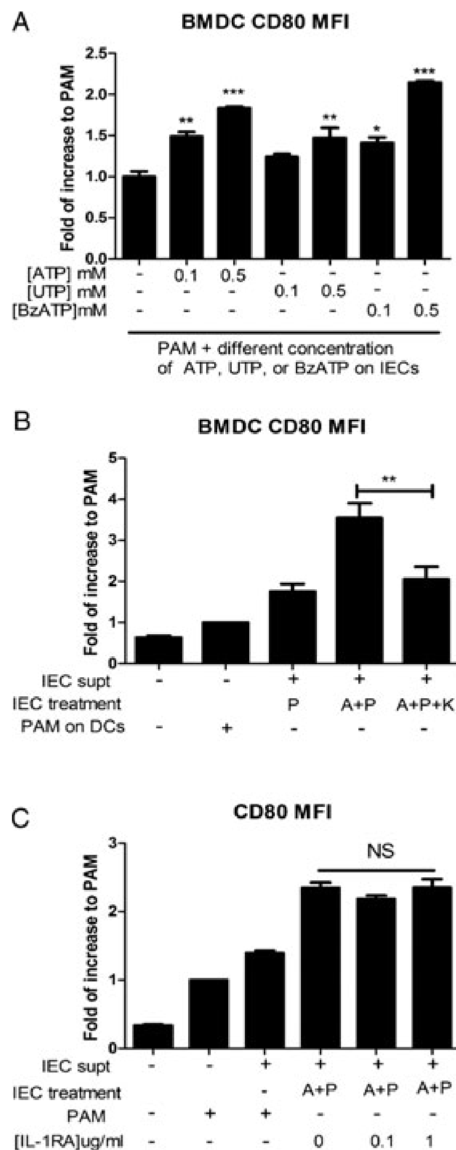


Figure 4. The ability of ATP-treated Mode-K cells to induce CD80 on BMDCs depends primarily on the P2X7 receptor and is independent of IL-1β secretion. Stimulated Mode-K supernatants were treated with apyrase and put onto DCs. CD80 MFI was calculated as described previously. (A) Mode-K cells were stimulated with different concentrations of ATP, UTP, or BzATP for 20 min followed by PAM. (B) Mode-K cells were preincubated with/without KN-62 (K) for 30 min, followed by ATP for 20 min and PAM. (C) Different concentrations of IL-1ra were added to the supernatants prior to culturing with DCs. Data are expressed as mean ± SEM of two samples pooled from three independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. NS, not significant; one-way ANOVA followed by Tukey's multiple comparison test.

environment would be predicted to favor the expansion of Th1 and Th17 cells.

Stressed IEC supernatants support flagellin-specific T-cell proliferation and Th1/17 cell expansion

We next assessed the ability of BMDCs exposed to stressed-IEC supernatants to stimulate a recall T-cell response to the model intestinal antigen, flagellin. First, to generate memory T cells that react to flagellin, we immunized C3H/HeJ mice with the *Escherichia coli* H18 flagellin (FliC) and isolated $CD4^+$ T cells from the spleen. C3H/HeJ BMDCs were incubated with FliC in the presence of different conditioned supernatants overnight prior to coculture with these $CD4^+$ T cells. As shown in Figure 6C, DCs conditioned with supernatants from IECs exposed to ATP and PAM stimulated a significant increase in FliC-specific T-cell proliferation compared with conditions without ATP. As predicted from the cytokine production profile from DCs, ATP+PAM IEC supernatant-treated DCs supported the expansion of IFN-γ^+ $CD4^+$ T cells (Fig. 6A and D), and IL-17^+ $CD4^+$ T cells (Fig. 6B and E) compared with conditions without ATP. Using a fixable viability dye, we confirmed that all the supernatants induce similar levels of cell death therefore excluding the possibility that this is simply due to selective cell survival (data not shown). These data support our hypothesis that exposure of IECs to both ATP and PAM on IECs drives the proliferation of proinflammatory T cells and facilitates the expansion of Th1 and Th17 cells.

To determine whether stressed IEC-conditioned DCs would affect primary responses in naïve T cells, we performed a mixed lymphocyte reaction (MLR), incubating conditioned C3H/HeJ DCs with splenic T cells from Foxp3-eGFP reporter mice. We found that DCs conditioned with ATP+PAM-treated IEC supernatants increased T-cell proliferation with a greater percentage of proliferating cells producing IFN-γ (Supporting Information Fig. 3). We did not observe any significant modulation in the percentage of total $Foxp3^+CD4^+$ T cells (Supporting Information Fig. 3C and F). Together, these results suggest that stressed IECs can condition DCs to enhance both primary and memory T-cell responses.

Colorectal injection of ATP enhances proinflammaotry cytokine production by IECs in vivo

To investigate whether ATP alters the IEC responses to endogenous TLR ligands presented by commensal microbes, we injected ATPγS, a nonhydrolysable form of ATP intrarectally, and harvested colons 18 h later. We found that ATPγS was able to significantly increase the expression of KC and IL-6 mRNA in IECs (Fig. 7A and B). To identify whether this treatment induced CD80 expression in intestinal DCs, we analyzed the $CD11c^+$ DC population in lamina propria (LP-DCs) and mesenteric lymph nodes (MLN-DCs). We found a trend toward an increased CD80 in LPL-DCs after 18 h (Fig. 7C) and MLN-DCs after 48 h (Fig. 7D), although the difference was not statistically significant.

Discussion

As an important extracellular signal of stress or injury, ATP on its own can direct the maturation of DCs and facilitate the differentiation of naive T cells [27–30], while little is known about its effect on IECs. Our previously published work suggests that the ability to sense ATP is one way that IECs can distinguish between homeostatic and dangerous conditions. In this work, we sought

© 2012 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

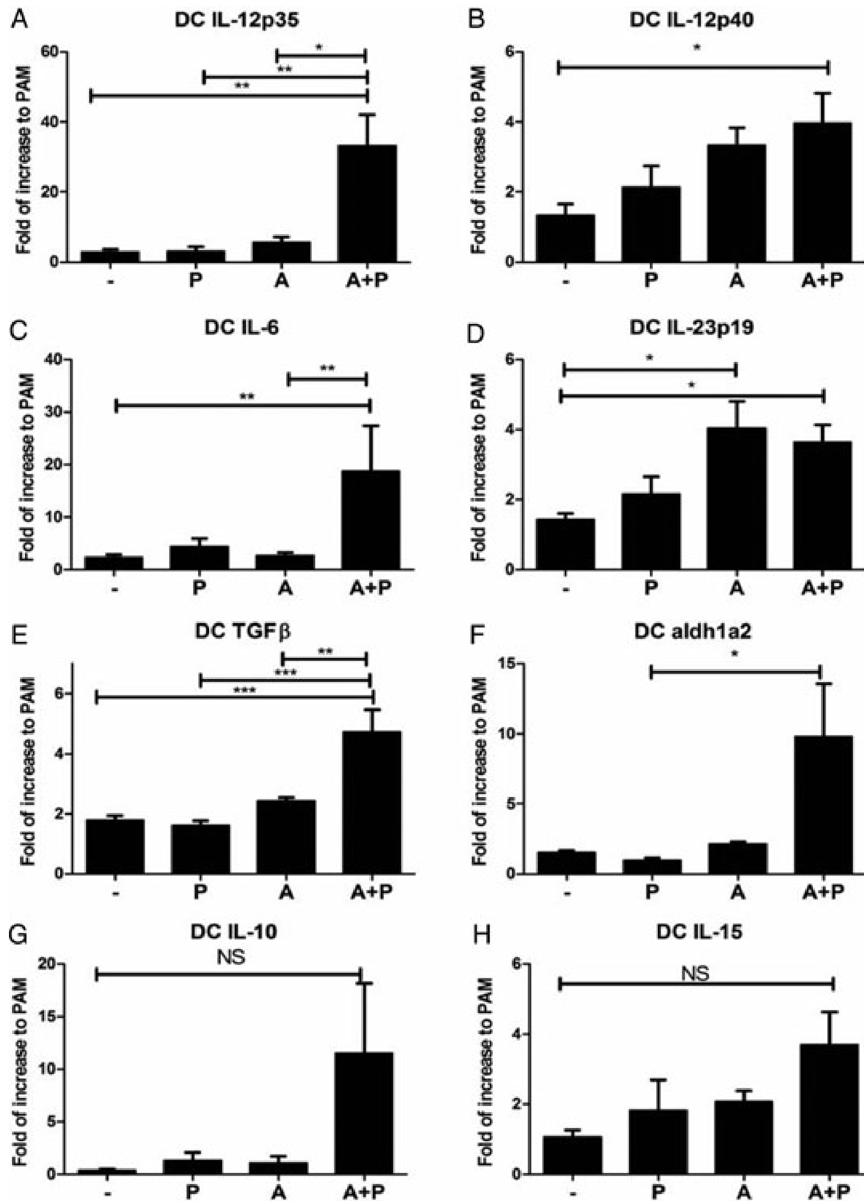


Figure 5. Stressed IEC supernatants modulate BMDC cytokine production. Supernatants from Mode-K cells treated with 1 mM ATP, 100 ng/mL PAM, or both (A+P) were removed and treated with apyrase prior to culture with BMDCs. A total of 15 ng/mL PAM was added to supernatants from the nontreated Mode-K cells or ATP alone treated Mode-K cells during the coculture, to match the residual PAM in the PAM-treated Mode-K conditions. BMDC RNA was isolated after 24 h, reverse-transcribed and quantified with real-time PCR to determine levels of (A) IL-12p35, (B) IL-12p40, (C) IL-6, (D) IL-23p19, (E) TGF-β, (F) aldh1a2, (G) IL-10, and (H) IL-15 gene expression. Data are expressed as means ± SEM from two replicates pooled from five independent experiments normalized to the PAM-treated DCs in each experiment. X-axis labeled as different treatments on Mode-K cells. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. NS, not significant; one-way ANOVA followed by Tukey's multiple comparison test.

to prove that ATP not only affects IEC chemokine production, but also switches them from being tolerogenic to being proinflammatory. Herein, we demonstrated that ATP and TLR1/2 stimulation together promote an inflammatory response in IECs, leading to secretion of soluble factors that enhance expression of CD80 and pro-inflammatory cytokines by DCs. The resulting DCs cause an enhanced stimulation of IFN-γ- and IL-17-expressing $CD4^{+}$ T cells specific for the important intestinal antigen flagellin. Furthermore, we showed that ATP enemas can lead to increased IL-6 and KC production in colonic IECs in the absence of exogenous TLR stimulation, suggesting that ATP is able to change how IECs respond to endogenous TLR ligands. Together our data support the notion that sensing extracellular ATP is one mechanism IECs use to become more inflammatory cells.

The increased cytokine/chemokine production in Mode-K IECs upon TLR1/2 ligation is similar to what we have observed in Caco-2 cells with TLR5 stimulation [16] and the effects were largely mediated by the P2X7 receptor. This suggests that the ATP effect does not depend on specific TLR stimulation and it is likely to be a more universal effect. We selected Pam3CSK4 for these experiments because Mode-K cells, derived from C3H/HeJ mice, lack functional TLR4 and show minimal responses to TLR5. The involvement of the P2X7 receptor on IECs is not surprising as it has been implicated in a variety of chronic inflammatory diseases [31], where P2X7 activation is tightly linked to inflammasome assembly and IL-1β production [8, 10, 11, 30, 32]. In contrast to these studies, we did not observe any IL-1β production by Mode-K IECs and blocking IL-1 receptor signaling by IL-1ra did not inhibit the enhanced cytokine production by IECs, suggesting this effect

© 2012 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

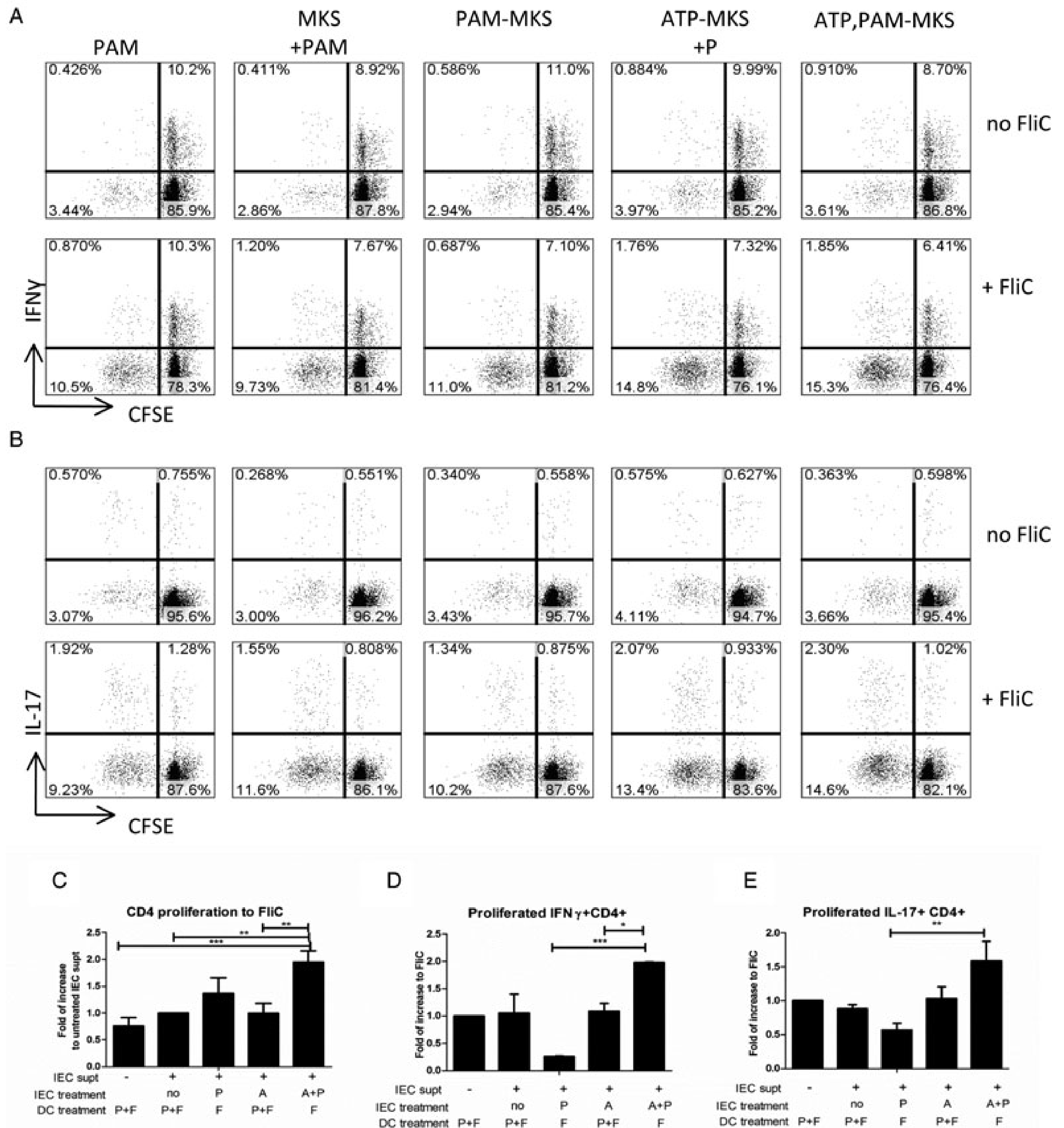


Figure 6. Stressed IEC supernatants increase T-cell recall response to flagellin. (A–E) BMDCs were pulsed with or without *Escherichia coli* flagellin (FliC) 10 μg/mL for 24 h in media alone, untreated Mode-K supernatants, or ATP and/or PAM-treated Mode-K supernatants. The DCs were then washed and cocultured with CFSE-stained splenic $CD4^+$ T cells isolated from FliC-immunized C3H mice. DCs and T cells were cocultured for 72 h and analyzed for T-cell proliferation and intracellular cytokine production. The percentage of proliferated T cells that respond to FliC was analyzed based on the difference between FliC-treated and -untreated DCs in the same Mode-K conditioned supernatants. From that the percentage of T cells that proliferated in response to flagellin in each conditioned media was determined. Each condition was normalized to the unstimulated IEC supernatant to account for experimental variance. (A, D) $CD4^+$ T cells were analyzed by intracellular cytokine staining with IFN-γ in the presence of different Mode-K supernatants. (A) Representative flow cytometry plots for flagellin-stimulated DCs in different supernatants. (D) Fold of increase of FliC-specific $CFSE^-IFN\text{-}\gamma^+$ cells, expressed as mean ± SEM of two replicates pooled from five independent experiments. (B, E) $CD4^+$ T cells were analyzed for IL-17 expression. (B) Representative flow cytometry plots for flagellin-stimulated DCs in different supernatants. (E) Fold of increase of FliC-specific $CFSE^-$ $IL\text{-}17^+$ cells, expressed as mean + SEM of two replicates pooled from three independent experiments. (C) Fold increase in total $CD4^+$ T-cell proliferation in each condition, expressed as mean + SEM of two replicates pooled from three independent experiments. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; one-way ANOVA followed by Tukey's multiple comparison test.

© 2012 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

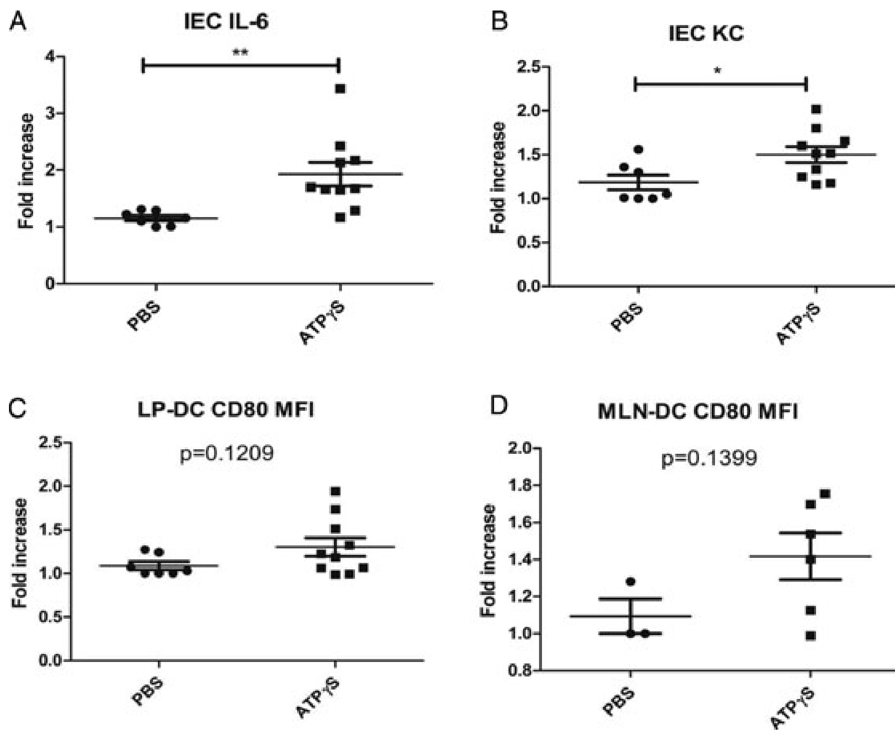


Figure 7. Colorectal administration of ATPγS increases IEC cytokine production but not DC maturation. Mice were given either ATPγS or PBS enemas and euthanized 18 h later. The epithelial cells were isolated from the mouse colon, and RNA was extracted from each sample and analyzed by RT-qPCR for (A) IL-6 and (B) KC mRNA expression. $^{*}p < 0.05$; $^{**}p < 0.01$; Student's t-test. (C) Lamina propria DCs were isolated and the expression of CD80 was analyzed in the 7-AAD^{-}CD11c^{+} population, and expressed as fold increase to normalize the difference between experiments. $p = 0.1209$; Student's t-test. (D) Mice were given enemas daily for 2 days, and euthanized on day 3. Mesenteric lymph nodes were collected and cells were analyzed by flow cytometry. The expression of CD80 was analyzed in the 7-AAD^{-}CD11c^{+} population and expressed as fold increase compared with one PBS-treated mouse. $p = 0.1399$; Student's t-test.

is unlikely to be mediated through paracrine release of either IL-1α or IL-1β. Most recently, the P2X7 receptor has been found to positively regulate MyD88-dependent NF-κB activation [33, 34], which offers a possible mechanistic explanation for the heightened TLR response in IECs. However, it should be noted that the specificity for BzATP and KN-62 for P2X7 is not perfect, and other P2X receptors (particularly P2X4) may also be affected by these compounds. While there are no specific P2X4 agonists or antagonists available, we reported that P2X4 and P2X7 can both augment TLR5 signaling in HEK-293T cells, suggesting that both receptors could be operational here as well [16].

To investigate the biological relevance of the ATP effect on IECs, we characterized the phenotypic changes of BMDCs conditioned with IEC supernatants. We showed that only when Mode-K cells were treated with a TLR1/2 ligand plus ATP they were able to induce a significant increase in CD80 on BMDCs, splenic DCs, and RA-conditioned DCs. In contrast, we did not observe any induction of MHCII, likely because the GM-CSF that is present in the culture media stimulates maximum expression of MHCII [35]. This is supported by our observation that IFN-γ-treated BMDCs expressed similar levels of MHCII (data not shown).

Further analysis of the BMDCs conditioned with ATP+PAM-IEC supernatants showed a large increase in both Th1 and Th17 polarizing cytokine transcripts, namely IL-12p35, IL-12p40, IL-23p19, IL-6, and TGF-β. In contrast when DCs are cocultured with healthy IEC supernatants, they demonstrate decreased expression of IL-12 and IL-23 and increased IL-10 [3, 4].

The increase in aldh1a2 and TGF-β expression in stressed IEC-conditioned DCs compared with that of the controls was somewhat surprising since RA is generally believed to promote tolerance rather than inflammation. However, Depaolo et al. have recently shown that RA can further enhance Th1/17 development in the presence of IL-15 via DC-dependent mechanisms [21]. Notably, IL-15 expression is increased in inflamed mucosa of IBD patients [36–38] and it is able to induce IFN-γ and TNF-α production in lamina propria T cells isolated from IBD patients but not in healthy controls [36, 37]. In our coculture system, we confirmed IL-15 transcript expression both by Mode-K cells (data not shown) and BMDCs as shown by previous studies [39, 40]. Together with the enhanced costimulatory molecule CD80 expression, the increase in Th1 and Th17 cytokine production suggests a more mature phenotype of these DCs as a result of epithelial cell stress.

In light of the phenotypic changes on BMDCs resulting from epithelial cells stress, we hypothesized that these DCs would have increased capacity to activate T cells. We tested this hypothesis using our model antigen flagellin since anti-flagellin responses have important implications in the pathogenesis of IBD [41–43]. In addition to its well-characterized role in TLR5 activation, flagellin has been identified as a dominant antigen in patients with CD [41, 44, 45], with about half of patients producing serum antibodies against flagellin [44, 45]. The increased flagellin antibodies in these patients suggest activation of flagellin-specific T cells. Supporting evidence from a murine model has shown that a flagellin-specific CD4^{+} T-cell clone can induce colitis when adoptively transferred into immune deficient mice [46]. The antigenic properties of flagellin have been further explored in TLR5$^{-/-}$ mice, and we have shown that flagellin enemas exacerbate DSS induced colitis independent of TLR5 [47]. In our coculture model, we tested whether ATP stimulation of IECs can modulate anti-flagellin responses. Our data suggest that TLR

© 2012 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

stimulation of stressed IECs leads to further enhancement of inflammation, resulting in increased flagellin presentation by DCs as shown by increased antigen-induced $CD4^+$ T-cell proliferation. Furthermore, this enhanced T-cell proliferation was also observed in a MLR with naive $CD4^+$ T cells, an effect that could be due to the increased production of IL-6 and IL-12 by the DCs.

Our in vitro findings also indicated that ATP stimulated IECs can facilitate the expansion of Th1 and Th17 cells. The increased Th17 response was consistent with observations by Atarashi et al. [48], who showed that colonic administration of ATP exacerbated colitis in a T-cell transfer model and that this was mediated through increased Th17 polarization and activation of a distinct subset of lamina propria DCs expressing $CD11c^{low}CD70^{high}$. Increased Th1 and Th17 responses have also been reported in CD39 null mice, which lack efficient ATP degradation [49, 50]. Despite seeing an increased percentage of Th1 and Th17 cells, we failed to observe any significant population of $Foxp3^+$ cells in the proliferated cells due to the low sensitivity of the flagellin-specific antigen response in this nontransgenic system. To further investigate this question, we used an MLR system with responder Foxp3-eGFP T cells, and found that the proportion of $Foxp3^+$ Treg cells did not change in the presence of stressed IEC supernatant. These data suggest that that ATP causes a skewing toward proinflammatory cells without a commensurate increase in regulatory cells.

In our in vivo model, we found that ATPγS increased production of IL-6 and KC mRNA from primary IECs, confirming the in vitro data. We also found a trend toward increased expression of CD80 expression in the lamina propria DCs and later in MLN DCs. The inconsistent ability of ATP alone to induce a significant increase in CD80 might be due to the insufficient diffusion of ATP across the mucosal barrier when the mice are not colitic, or not enough TLR ligand under the noninflamed state to reach the epithelial cells to initiate a strong IEC response as seen in an in vitro system. Indeed, we found that IL-6 was more highly upregulated than KC, consistent with our in vitro data showing IL-6 expression is facilitated at lower concentrations of ATP than KC. Furthermore, the lamina propria $CD11c^+$ cell population has recently been shown to contain gut resident macrophages that do not behave the same as conventional DCs [51]. These macrophages may have masked the ability to observe an effect of ATP on intestinal DCs specifically. Nonetheless, our results suggest that ATP alone is able to modulate how IECs respond to endogenous TLR ligands, which may in turn affect the maturation status and inflammatory activity of DCs.

In conclusion, our studies provide evidence that cellular stress signals are able to activate epithelial cells, leading to enhanced DC CD80 expression and intensified T-cell responses. Since IBD is driven partly by overreactive T-cell responses to commensal antigens [2, 52], and heightened Th1/17 responses are pathogenic in CD [46, 53], our results constitute a significant advance in understanding how the chronic cycle of intestinal inflammation in IBD is maintained.

Materials and methods

Mice

Foxp3-eGFP reporter (generation F11) [54], C3H/HeJ and C57Bl/6 mice were purchased from Jackson Laboratories (Bar Harbor, Maine, USA). All mice were bred in-house and maintained under specific pathogen-free conditions at the animal facility at the Jack Bell Research Center. The experiments described in this study were approved by the University of British Columbia (UBC) Animal Care and Use Committee.

Cell culture

Mode-K cells were kindly provided by Dr. Karen Madsen (University of Alberta), and cultured from passages 20 to 30 in HyQ DMEM/High glucose with 5% heat-inactivated FBS, nonessential amino acids, penicillin, streptomycin (both at 100 μg/mL and from Sigma, St. Louis. MO, USA). For stimulation of cells, Mode-Ks were seeded at 2×10^5/mL in 24-well plates and used for experiments after 24 h when the cells were 70% confluent.

BMDCs were generated from C3H/HeJ mice, cultured using a protocol developed by Lutz et al. [55]. Briefly, BM cells were flushed out of the femur and tibia, and cultured at a density of 2.5×10^5/mL in RPMI-1640 media containing mouse recombinant GM-CSF (a gift from Dr. Alice Mui, University of British Columbia), 10% heat-inactivated FBS, 10 mM HEPES (StemCell), 2 mM L-glutamine, 50 μM 2-ME (Sigma), penicillin, and streptomycin. The cells were cultured for 7 days with half of the media changed on day 3 and day 6. To stimulate BMDCs with RA, 1 μM RA was added to the BMDCs on day 3.

Splenic DCs and $CD4^+$ T cells were isolated using a mouse anti-CD11c-enrichment kit and anti-CD4-enrichment kit, respectively, according to manufacturer's instructions (StemCell Technologies, Vancouver, BC, Canada) achieving over 90% purity of cells. All cell culture reagents, except noted, were purchased from Fisher (HyClone, CA, USA).

Stimulation of Mode-K cells and treatment of Mode-K supernatants

Mode-K cells were stimulated with freshly prepared solutions of ATP, UTP, or BzATP (2′ (3′)-O-(4-benzoylbenzoyl)adenosine 5′-triphosphate) (all from Sigma) for 20 min followed by 100 ng/mL Pam_3CSK_4 (PAM; InvivoGen, San Diego, CA, USA). In some experiments, cells were incubated with KN-62 (Sigma) for 30 min prior to addition of ATP. Supernatants were collected after 24 h of stimulation and analyzed for KC and IL-6 by ELISA (OptEIA, BD Biosciences, San Jose, CA, USA for IL-6 and Duo-set, R&D, Minneapolis, MN, USA for KC) according to the manufacturers' instructions. Results are expressed as fold increase in cytokine concentration

© 2012 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

compared with PAM alone in each experiment. Total mRNA was isolated after 6 h of stimulation, and mRNA for TGF-β, TSLP, aldh1a2, and IL-15 were quantified with RT-PCR as described below.

For DC experiments, supernatants from Mode-K cells stimulated as above were incubated with 20 U/mL apyrase (Sigma) for 30 min at 37°C to neutralize any residual ATP. ATP concentrations in Mode-K supernatants before and after apyrase treatment were measured using a luminescent ATP assay kit (SUNY, Buffalo, NY, USA) according to the manufacturer's instructions.

HEK 293T cells were maintained and transfected as described [16] with the following conditions, per well: pEGFP-N1 (Clontech) 1 ng, pEF6-hTLR2 or pEF6-hTLR5 5 ng, and salmon-sperm DNA to total 100 ng. The TLR5 construct was a gift from Alan Aderem (University of Washington) and the TLR2 construct was generated as described previously [56].

BMDC conditioning and activation

BMDCs were incubated for 24 h with medium alone or conditioned cell supernatants, with or without 15 ng/mL of PAM. After 24 h, cells were harvested for FACS analysis using the following antibodies: CD80-PE (ebioscience), I-A^k-FITC (Santa Cruz), and CD11c-APC (ebioscience). Cell viability was confirmed using 7-AAD. To measure the cytokine responses in BMDCs, total RNA was isolated after conditioning with Mode-K supernatants for 24 h. In some experiments, DCs were pulsed with *E. coli* H18 flagellin (FliC) [57] in the presence of differentially treated Mode-K supernatants for 24 h prior to coculture with T cells isolated from FliC-immunized C3H/HeJ mice.

BMDC and T-cell coculture

To expand flagellin-specific T cells, we injected 10 μg of FliC intraperitoneally into C3H/HeJ mice, followed by two booster immunizations with 1 μg of FliC at 2 week intervals. CD4$^+$ splenocytes were isolated from these immunized mice and stained with carboxyfluorescein succinimidyl ester (CFSE), or cell proliferation dye eFluor® 760 for MLR experiments (eBioscience) prior to coculture. C3H/HeJ BMDCs were pulsed with or without 10 μg/mL of FliC for 24 h in the presence of different conditioned supernatants as described. DCs were then washed and cultured with isolated T cells at a ratio of 1:5 (DC/T cell). After 72 h of coculture, cells were stimulated with 10 ng/mL PMA and 500 ng/mL ionomycin for 5 h, with 10 mg/mL brefeldin A (all from Sigma-Aldrich) added 1 h after PMA/ionomycin addition. After surface staining for CD4 and fixable viability dye eFluor® 780 (eBioscience), the cells were fixed with 2% formaldehyde and permeabilized with 0.5% Saponin (Sigma). The antibodies used for cytokine stains were IFN-γ-PE-Cy7 and IL-17-allophycocyanin (eBioscience). The cells were then analyzed on a FACS Canto (BD Biosciences) to measure T-cell proliferation and cytokine expression.

RNA isolation and quantitative reverse-transcription polymerase chain reaction (RT-PCR)

RNA isolation, cDNA synthesis, and quantification were performed as described previously [16]. Primers used are shown in Supporting Information Table 1. Each reaction was performed in duplicate. The mRNA levels of β-actin for each sample were used for normalization and the fold induction for each cytokine compared with unstimulated control cells was calculated based on the $2^{-\Delta\Delta Ct}$ method. All reagents, except as noted, were obtained from Fermentas (Burlington, ON, Canada).

Intrarectal delivery of ATP

Six- to eight-week-old mice were used for the experiments. One hundred microliters volumes were administered to isofluorane-anesthetised mice as described previously [16]. ATPγS enemas contained 100 μL of 10 mM ATPγS in 50mM Tris-HCl, pH 7.4, adjusted to 1 mL with PBS. Animals were euthanized after 18 h and colons (excluding cecum) and MLNs collected. Colonic epithelial cells and lamina propria cells were collected as described [58]. Isolated IECs were put into TriZol followed by RNA analysis. Isolated lamina propria cells and lymphocytes from MLNs were stained for 7-AAD, CD11c-PE-Cy7, CD80-APC, and MHC II (I-A^k)-FITC followed by FACS analysis.

Statistical analysis

Statistical analyses were performed in GraphPad. Groups were analyzed by one-way ANOVA followed by Tukey's multiple comparison test, except noted. Significant differences were set at p less than 0.05. Results are expressed as mean ± SEM.

Acknowledgments: This work was supported by a New Emerging Team grant in Autoimmunity from the CIHR (III 84037 93793). Y.Y. holds CIHR Doctoral Research Award, CIHR Transplant Training Award, and UBC Affiliated Scholarship. M.K.L. holds a Canada Research Chair in Transplantation.

Conflict of interest: The authors declare no financial or commercial conflict of interest.

References

1 **Maloy, K. J. and Powrie, F.**, Intestinal homeostasis and its breakdown in inflammatory bowel disease. *Nature* 2011. **474**: 298–306.

2 **Artis, D.**, Epithelial-cell recognition of commensal bacteria and maintenance of immune homeostasis in the gut. *Nat. Rev. Immunol.* 2008. **8**: 411–420.

© 2012 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

3 **Iliev, I. D., Mileti, E., Matteoli, G., Chieppa, M. and Rescigno, M.,** Intestinal epithelial cells promote colitis-protective regulatory T-cell differentiation through dendritic cell conditioning. *Mucosal Immunol.* 2009. **2**: 340–350.

4 **Zeuthen, L. H., Fink, L. N. and Frokiaer, H.,** Epithelial cells prime the immune response to an array of gut-derived commensals towards a tolerogenic phenotype through distinct actions of thymic stromal lymphopoietin and transforming growth factor-beta. *Immunology* 2008. **123**: 197–208.

5 **Medzhitov, R.,** Recognition of microorganisms and activation of the immune response. *Nature* 2007. **449**: 819–826.

6 **Matzinger, P.,** The danger model: a renewed sense of self. *Science* 2002. **296**: 301–305.

7 **Kono, H. and Rock, K. L.,** How dying cells alert the immune system to danger. *Nat. Rev. Immunol.* 2008. **8**: 279–289.

8 **Lister, M., Sharkey, J., Sawatzky, D., Hodgkiss, J., Davidson, D., Rossi, A. and Finlayson, K.,** The role of the purinergic P2X7 receptor in inflammation. *J. Inflamm.* 2007. **4**: 5.

9 **Chen, L. and Brosnan, C. F.,** Exacerbation of experimental autoimmune encephalomyelitis in P2X7R-/- mice: evidence for loss of apoptotic activity in lymphocytes. *J. Immunol.* 2006. **176**: 3115–3126.

10 **Riteau, N., Gasse, P., Fauconnier, L., Gombault, A., Couegnat, M., Fick, L., Kanellopoulos, J. et al.,** Extracellular ATP is a danger signal activating P2X7 receptor in lung inflammation and fibrosis. *Am. J. Respir. Crit. Care Med.* 2010. **182**: 774–783.

11 **Wilhelm, K., Ganesan, J., Muller, T., Durr, C., Grimm, M., Beilhack, A., Krempl, C. D. et al.,** Graft-versus-host disease is enhanced by extracellular ATP activating P2X7R. *Nat. Med.* 2010. **16**: 1434–1438.

12 **Friedman, D. J., Künzli, B. M., A-Rahim, Y. I., Sevigny, J., Berberat, P. O., Enjyoji, K., Csizmadia, E. et al.,** CD39 deletion exacerbates experimental murine colitis and human polymorphisms increase susceptibility to inflammatory bowel disease. *Proc. Natl. Acad. Sci.* 2009. **106**: 16788–16793.

13 **Ivanova, E. P., Alexeeva, Y. V., Pham, D. K., Wright, J. P. and Nicolau, D. V.,** ATP level variations in heterotrophic bacteria during attachment on hydrophilic and hydrophobic surfaces. *Int. Microbiol.* 2006. **9**: 37–46.

14 **Iwase, T., Shinji, H., Tajima, A., Sato, F., Tamura, T., Iwamoto, T., Yoneda, M. et al.,** Isolation and identification of ATP-secreting bacteria from mice and humans. *J. Clin. Microbiol.* 2010. **48**: 1949–1951.

15 **Yin, J., Xu, K., Zhang, J., Kumar, A. and Yu, F. S.,** Wound-induced ATP release and EGF receptor activation in epithelial cells. *J. Cell. Sci.* 2007. **120**: 815–825.

16 **Ivison, S. M., Himmel, M. E., Mayer, M., Yao, Y., Kifayet, A., Levings, M. K. and Steiner, T. S.,** The stress signal extracellular ATP modulates antiflagellin immune responses in intestinal epithelial cells. *Inflamm. Bowel Dis.* 2011. **17**: 319–333.

17 **Di Virgilio, F.,** Purinergic mechanism in the immune system: a signal of danger for dendritic cells. *Purinergic Signal* 2005. **1**: 205–209.

18 **Rock, K. L., Lai, J. J. and Kono, H.,** Innate and adaptive immune responses to cell death. *Immunol. Rev.* 2011. **243**: 191–205.

19 **Aggarwal, S., Ghilardi, N., Xie, M. H., de Sauvage, F. J. and Gurney, A. L.,** Interleukin-23 promotes a distinct CD4 T-cell activation state characterized by the production of interleukin-17. *J. Biol. Chem.* 2003. **278**: 1910–1914.

20 **Langrish, C. L., Chen, Y., Blumenschein, W. M., Mattson, J., Basham, B., Sedgwick, J. D., McClanahan, T. et al.,** IL-23 drives a pathogenic T-cell population that induces autoimmune inflammation. *J. Exp. Med.* 2005. **201**: 233–240.

21 **Depaolo, R. W., Abadie, V., Tang, F., Fehlner-Peach, H., Hall, J. A., Wang, W., Marietta, E. V. et al.,** Co-adjuvant effects of retinoic acid and IL-15 induce inflammatory immunity to dietary antigens. *Nature* 2011. **471:** 220–224.

22 **Bours, M. J., Dagnelie, P. C., Giuliani, A. L., Wesselius, A. and Di Virgilio, F.,** P2 receptors and extracellular ATP: a novel homeostatic pathway in inflammation. *Front. Biosci. (Schol Ed)* 2011. **3**: 1443–1456.

23 **North, R. and Surprenant, A.,** Pharmacology of cloned P2X receptors. *Annu. Rev. Pharmacol. Toxicol.* 2000. **40**: 563–580.

24 **Ferrari, D., Pizzirani, C., Adinolfi, E., Lemoli, R. M., Curti, A., Idzko, M., Panther, E. et al.,** The P2X7 receptor: a key player in IL-1 processing and release. *J. Immunol.* 2006. **176**: 3877–3883.

25 **Cesaro, A., Brest, P., Hofman, V., Hebuterne, X., Wildman, S., Ferrua, B., Marchetti, S. et al.,** Amplification loop of the inflammatory process is induced by P2X7R activation in intestinal epithelial cells in response to neutrophil transepithelial migration. *Am. J. Physiol. Gastrointest. Liver Physiol.* 2010. **299**: G32–G42.

26 **Feng, T., Cong, Y., Qin, H., Benveniste, E. N. and Elson, C. O.,** Generation of mucosal dendritic cells from bone marrow reveals a critical role of retinoic acid. *J. Immunol.* 2010. **185**: 5915–5925.

27 **la Sala, A., Ferrari, D., Corinti, S., Cavani, A., Di Virgilio, F. and Girolomoni, G.,** Extracellular ATP induces a distorted maturation of dendritic cells and inhibits their capacity to initiate Th1 responses. *J. Immunol.* 2001. **166**: 1611–1617.

28 **Bles, N., Horckmans, M., Lefort, A., Libert, F., Macours, P., El Housni, H., Marteau, F. et al.,** Gene expression profiling defines ATP as a key regulator of human dendritic cell functions. *J. Immunol.* 2007. **179**: 3550–3558.

29 **Schnurr, M., Then, F., Galambos, P., Scholz, C., Siegmund, B., Endres, S. and Eigler, A.,** Extracellular ATP and TNF-alpha synergize in the activation and maturation of human dendritic cells. *J. Immunol.* 2000. **165**: 4704–4709.

30 **Yip, L., Woehrle, T., Corriden, R., Hirsh, M., Chen, Y., Inoue, Y., Ferrari, V. et al.,** Autocrine regulation of T-cell activation by ATP release and P2X7 receptors. *FASEB J.* 2009. **23**: 1685–1693.

31 **Keating, C., Pelegrin, P., Martínez, C. M. and Grundy, D.,** P2X7 receptor-dependent intestinal afferent hypersensitivity in a mouse model of postinfectious irritable bowel syndrome. *J. Immunol.* 2011. **187**: 1467–1474.

32 **Muller, T., Paula Vieira, R., Grimm, M., Durk, T., Cicko, S., Zeiser, R., Jakob, T. et al.,** A potential role for P2X7R in allergic airway inflammation in mice and humans. *Am. J. Respir. Cell Mol. Biol.* 2010 **44:** 456–464.

33 **Liu, Y., Xiao, Y. and Li, Z.,** P2X7 receptor positively regulates MyD88-dependent NF-kappaB activation. *Cytokine* 2011 **55:** 229–236.

34 **Théâtre, E., Bours, V. and Oury, C.,** A P2X ion channel–triggered NF-κB pathway enhances TNF-α–induced IL-8 expression in airway epithelial cells. *Am. J. Resp. Cell Mol. Biol.* 2009. **41**: 705–713.

35 **Labeur, M. S., Roters, B., Pers, B., Mehling, A., Luger, T. A., Schwarz, T. and Grabbe, S.,** Generation of tumor immunity by bone marrow-derived dendritic cells correlates with dendritic cell maturation stage. *J. Immunol.* 1999. **162**: 168–175.

36 **Liu, Z., Geboes, K., Colpaert, S., D'Haens, G. R., Rutgeerts, P. and Ceuppens, J. L.,** IL-15 is highly expressed in inflammatory bowel disease and regulates local T cell-dependent cytokine production. *J. Immunol.* 2000. **164**: 3608–3615.

37 **Vainer, B., Nielsen, O. H., Hendel, J., Horn, T. and Kirman, I.,** Colonic expression and synthesis of interleukin 13 and interleukin 15 in inflammatory bowel disease. *Cytokine* 2000. **12**: 1531–1536.

© 2012 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

38 **Kirman, I. and Nielsen, O. H.,** Increased numbers of interleukin-15-expressing cells in active ulcerative colitis. *Am. J. Gastroenterol.* 1996. **91**: 1789–1794.

39 **Bas, A., Swamy, M., Abeler-Dorner, L., Williams, G., Pang, D. J., Barbee, S. D. and Hayday, A. C.,** Butyrophilin-like 1 encodes an enterocyte protein that selectively regulates functional interactions with T lymphocytes. *Proc. Natl. Acad. Sci. USA* 2011. **108**: 4376–4381.

40 **Dubois, S. P., Waldmann, T. A. and Muller, J. R.,** Survival adjustment of mature dendritic cells by IL-15. *Proc. Natl. Acad. Sci. USA* 2005. **102**: 8662–8667.

41 **Lodes, M. J., Cong, Y., Elson, C. O., Mohamath, R., Landers, C. J., Targan, S. R., Fort, M. et al.,** Bacterial flagellin is a dominant antigen in crohn disease. *J. Clin. Invest.* 2004. **113**: 1296–1306.

42 **Lunardi, C., Bason, C., Dolcino, M., Navone, R., Simone, R., Saverino, D., Frulloni, L. et al.,** Antiflagellin antibodies recognize the autoantigens toll-like receptor 5 and pals 1-associated tight junction protein and induce monocytes activation and increased intestinal permeability in crohn's disease. *J. Intern. Med.* 2009. **265**: 250–265.

43 **Vijay-Kumar, M. and Gewirtz, A. T.,** Flagellin: key target of mucosal innate immunity. *Mucosal Immunol.* 2009. **2**: 197–205.

44 **Sitaraman, S. V., Klapproth, J. M., Moore, D. A., 3rd, Landers, C., Targan, S., Williams, I. R. and Gewirtz, A. T.,** Elevated flagellin-specific immunoglobulins in Crohn's disease. *Am. J. Physiol. Gastrointest. Liver Physiol.* 2005. **288**: G403–G406.

45 **Targan, S. R., Landers, C. J., Yang, H., Lodes, M. J., Cong, Y., Papadakis, K. A., Vasiliauskas, E. et al.,** Antibodies to CBir1 flagellin define a unique response that is associated independently with complicated Crohn's disease. *Gastroenterology* 2005. **128**: 2020–2028.

46 **Elson, C. O., Cong, Y., Weaver, C. T., Schoeb, T. R., McClanahan, T. K., Fick, R. B. and Kastelein, R. A.,** Monoclonal anti-interleukin 23 reverses active colitis in a T cell-mediated model in mice. *Gastroenterology* 2007. **132**: 2359–2370.

47 **Ivison, S. M., Himmel, M. E., Hardenberg, G., Wark, P. A., Kifayet, A., Levings, M. K. and Steiner, T. S.,** TLR5 is not required for flagellin-mediated exacerbation of DSS colitis. *Inflamm. Bowel Dis.* 2009. **16**: 401–409.

48 **Atarashi, K., Nishimura, J., Shima, T., Umesaki, Y., Yamamoto, M., Onoue, M., Yagita, H. et al.,** ATP drives lamina propria T(H)17 cell differentiation. *Nature* 2008. **455**: 808–812.

49 **Kunzli, B. M., Nuhn, P., Enjyoji, K., Banz, Y., Smith, R. N., Csizmadia, E., Schuppan, D. et al.,** Disordered pancreatic inflammatory responses and inhibition of fibrosis in CD39-null mice. *Gastroenterology* 2008. **134**: 292–305.

50 **Deaglio, S., Dwyer, K. M., Gao, W., Friedman, D., Usheva, A., Erat, A., Chen, J. F., et al,** Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *J. Exp. Med.* 2007. **204**: 1257–1265.

51 **Rivollier, A., He, J., Kole, A., Valatas, V. and Kelsall, B. L.,** Inflammation switches the differentiation program of Ly6Chi monocytes from antiinflammatory macrophages to inflammatory dendritic cells in the colon. *J. Exp. Med.* 2012. **209**: 139–155.

52 **Niess, J. H.,** Role of mucosal dendritic cells in inflammatory bowel disease. *World J. Gastroenterol.* 2008. **14**: 5138–5148.

53 **Bouma, G. and Strober, W.,** The immunological and genetic basis of inflammatory bowel disease. *Nat. Rev. Immunol.* 2003. **3**: 521–533.

54 **Haribhai, D., Lin, W., Relland, L. M., Truong, N., Williams, C. B. and Chatila, T. A.,** Regulatory T cells dynamically control the primary immune response to foreign antigen. *J. Immunol.* 2007. **178**: 2961–2972.

55 **Lutz, M. B., Kukutsch, N., Ogilvie, A. L. J., Rößner, S., Koch, F., Romani, N. and Schuler, G.,** An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J. Immunol. Methods* 1999. **223**: 77–92.

56 **Ivison, S. M., Graham, N. R., Bernales, C. Q., Kifayet, A., Ng, N., Shobab, L. A. and Steiner, T. S.,** Protein kinase D interaction with TLR5 is required for inflammatory signaling in response to bacterial flagellin. *J. Immunol.* 2007. **178**: 5735–5743.

57 **Donnelly, M. A. and Steiner, T. S.,** Two nonadjacent regions in enteroaggregative *Escherichia coli* flagellin are required for activation of toll-like receptor 5. *J. Biol. Chem.* 2002. **277**: 40456–40461.

58 **Hardenberg, G., Yao, Y., Piccirillo, C. A., Levings, M. K. and Steiner, T. S.,** Toll-like receptor 5 deficiency protects from wasting disease in a T-cell transfer colitis model in T-cell receptor-beta-deficient mice. *Inflamm. Bowel Dis.* 2012. **18**: 85–93.

Abbreviations: **IEC**: intestinal epithelial cell · **RA**: retinoic acid · **TSLP**: thymic stromal lymphopoietin

Full correspondence: Dr. Theodore S. Steiner, Rm. D452 HP East, VGH, Vancouver, BC V5Z 3J5, Canada
Fax: +1-604-875-4013
e-mail: flagellin@gmail.com

Received: 31/10/2011
Revised: 2/8/2012
Accepted: 7/9/2012
Accepted article online: 14/9/2012

© 2012 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim