A_{2A} adenosine receptor (AR) activation inhibits pro-inflammatory cytokine production by human CD4$^+$ helper T cells and regulates *Helicobacter*-induced gastritis and bacterial persistence

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Helicobacter pylori causes a lifelong infection and provides a model of bacterial adaptation and persistent colonization. Adenosine is an anti-inflammatory mediator that limits tissue damage during inflammation. We studied the role of adenosine in the T-cell-mediated regulation of gastritis and bacterial persistence. After 4 h of activation, human T helper (Th) cells increased A_{2A} adenosine receptor (A_{2A}AR) mRNA level (sevenfold). A_{2A}AR was the predominant subtype expressed in resting and stimulated gastric or peripheral Th cells. Stimulation with ATL313, an A_{2A}AR agonist, increased cyclic AMP (cAMP) accumulation and reduced interleukin-2 (IL-2) production by 20–50%. ATL313 also attenuated tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) production, which was inhibited by an A_{2A}AR antagonist. Infection of IL-10-deficient mice with *H. pylori* is cleared spontaneously due to the marked inflammation. Administration of ATL313 during infection reduced gastritis and pro-inflammatory cytokine responses while bacterial load increased. In contrast, infection of A_{2A}AR-deficient mice enhanced gastritis. Thus, A_{2A}AR limits the pro-inflammatory effects of Th cells and favor chronic *Helicobacter* infection.

INTRODUCTION

Helicobacter pylori infects more than half of the world's population and persists for the life of its host.[1] *H. pylori* infection is associated with chronic antral gastritis, which is characterized by a mucosal infiltration of polymorphonuclear and mononuclear leukocytes.[2] Evidence for a pathogenic role of *H. pylori* infection in gastroduodenal ulcer comes from clinical investigations showing that the cure of *H. pylori* infection accelerates ulcer healing and prevents ulcer relapse.[3,4] Besides ulcer disease, *H. pylori* also causes gastric lymphoma or carcinoma.[5] Although immunization of animals can be effective, it is unclear why the immune system is unable to eliminate the infection spontaneously.

Data show that subpopulations of CD4$^+$ T lymphocytes play distinct roles in mediating and regulating *H. pylori*-induced gastritis.[6–9] For example, adoptive transfer of CD4$^+$CD45RB^{hi} effector T cells from naive donors into immunodeficient recipients causes severe gastritis in *H. pylori*-infected recipients, whereas co-transfer of CD4$^+$CD45RB^{lo} regulatory T cells (T_{reg}) protects against gastritis.[10] Several studies have suggested that CD4$^+$/CD25$^+$/Foxp3$^+$ T_{reg} control gastric inflammation and contribute to the persistence of *H. pylori* infections.[11–13] The anti-inflammatory activity mediated by these T_{reg} may reflect cell contact and/or the production of soluble anti-inflammatory mediators, including transforming growth factor-β, interleukin (IL)-10, IL-35 or adenosine.[12,14,15]

Adenosine is a purine nucleoside that accumulates in inflamed or hypoxic tissues largely because of CD39 (nucleoside triphosphate dephosphorylase) mediating the dephosphorylation of ATP to ADP then to 5′-AMP. Subsequently, CD73 (5′-ectonucleotidase) catalyzes the terminal reaction to convert 5′-AMP into adenosine.[16] The responses controlled by adenosine are

mediated by four G-protein-coupled receptors (A_1, A_{2A}, A_{2B}, and A_3). Activation of A_{2A} adenosine receptors ($A_{2A}AR$) on T cells produces a series of responses that have been categorized as anti-inflammatory.[16] For example, extracellular adenosine targets the $A_{2A}AR$ to trigger cyclic AMP (cAMP) accumulation and inhibit activation-induced CD25 expression.[17]

Adenosine analogs limit collateral damage associated with inflammation, including colitis[18] and *Clostridium difficile* toxin-induced diarrhea.[19] Lappas *et al.*[17] reported that $A_{2A}AR$ induction inhibits interferon-γ (IFN-γ) production in murine CD4$^+$ T cells. Furthermore, activation of $A_{2A}ARs$ attenuate gastric mucosal inflammation in rats.[20] Other reports suggest that T_{reg} cells exert their suppressive action through the production of[14,15,21] and/or response to adenosine.[22,23] These observations suggest that $A_{2A}AR$ play a critical role in mucosal immune regulation due to their novel effects on T-cell cytokine production.[22]

Currently, little is known about the role of $A_{2A}AR$ in human mucosal Th cell function or its role in controlling the host response to infection with *Helicobacter spp*. In this study, we examined the expression of ARs on human Th cells from the blood or gastric tissue and evaluated the role of $A_{2A}AR$ in regulating gastritis and bacterial burden.

RESULTS

Human Th cell activation induces $A_{2A}AR$ mRNA expression

Evidence suggests that the activation of mouse CD4$^+$ Th cells significantly upregulates $A_{2A}AR$ expression.[17] To translate these observations to humans, we examined the expression of ARs in human Th cells incubated on immobilized anti-CD3/CD28 monoclonal antibody (mAb) or Phorbal myristate acetate (PMA)/ionomycin for different lengths of time. The activation of Jurkat T cells or CD4$^+$ Th cells by signaling through the T cell receptor (TCR) or alternatively by PMA/ionomycin resulted in a rapid and transient increase in the expression of $A_{2A}AR$ mRNA, as determined by quantitative reverse transcriptase-PCR (RT-PCR). The induction of $A_{2A}AR$ transcripts in Jurkat T cells peaked between 2 and 4 h of incubation with PMA/ionomycin resulting in an approximate sevenfold increase in transcript compared with that in unstimulated T cells at that time point, and returned to baseline after 18 h (**Figure 1a**). When CD4$^+$ Th cells were used, the amount of $A_{2A}AR$ transcript accumulation again peaked after 2 h of activation, with a fivefold increase over resting unstimulated level. $A_{2A}AR$ mRNA levels in this cell population also returned to baseline after 24 h of activation (**Figure 2b**). To directly characterize AR expression in gastric Th cells, we isolated lamina propria lymphocytes (LPL) from human gastric biopsy specimens of uninfected subjects. LPL activation resulted in a similar augmentation of $A_{2A}AR$ transcripts (**Figure 1c**), although with different kinetics of induction. Although low levels of $A_{2B}AR$ mRNA were also detected in both peripheral Th cells and LPL T cells, no significant induction of this transcript was observed.

To show the function of $A_{2A}AR$ receptor in human Th cells, the efficacy of the selective $A_{2A}AR$ agonist, ATL313, to stimulate

cAMP accumulation was tested. We found a significant increase in cAMP level after 1,000 nm of agonist treatment in both Jurkat (data not shown) and CD4$^+$ Th cells over agonist untreated cells (**Figure 2**).

$A_{2A}AR$ activation by adenosine analogs inhibits IL-2 production

Next, we tested the effect of ATL313 treatment on IL-2 production in activated human CD4$^+$ Th cells. The activation of CD4$^+$ Th cells in the presence of various adenosine analogs resulted in a dose-dependent inhibition of TCR-mediated production of both IL-2 protein (**Figures 3** and **4a**) and mRNA (**Figure 4b**). 5′-N-Ethylcarboxamidoadenosine (NECA), a nonselective agonist, also inhibited IL-2 production (**Figures 3a** and **4a**). In addition to inhibiting activation-induced IL-2 production, treatment with 100 nm of ATL313 attenuated the TCR-induced CD25 expression (data not shown).

A selective $A_{2A}AR$ antagonist inhibits the effect of ATL313 on IL-2 production

To further confirm that adenosine acts through $A_{2A}AR$, CD4$^+$ Th cells were activated in the presence of ATL313 and 500 nм of two different $A_{2A}AR$-selective antagonists ZM241385 or SCH58261. This treatment resulted in the almost complete reversal of the suppressive activity (**Figure 4**). Treatment of ZM241385 was more effective ($P = 0.02$) reversing the suppression than that of SCH58261. ZM241385 alone did not induce any appreciable change in IL-2 production during the of time assay (data not shown). When an $A_{2B}AR$ antagonist, ATL801, was used, there was no inhibition of the agonist supporting the notion that these agonists exert an inhibitory effect predominantly through the $A_{2A}AR$ and not through $A_{2B}AR$ (**Figure 4a**).

ATL313 triggers intracellular cAMP accumulation by initiating signaling through the G_s-coupled $A_{2A}AR$ (**Figure 2**). Similarly, a type IV phosphodiesterase inhibitor, rolipram, elevates intracellular cAMP level[24] (without engaging ARs) and suppresses IL-2 production (**Figure 4a**). Human CD4$^+$ Th cells were activated in the presence of 2 μm rolipram with or without treatment of 500 nM of the selective $A_{2A}AR$ antagonist, ZM241385. $A_{2A}AR$ antagonist did not reverse the inhibition of IL-2 production by rolipram, as it did in ATL313-treated CD4$^+$ Th cells (**Figure 4a**).

### Effect of $A_{2A}AR$ agonist on other pro-inflammatory cytokines

Naganuma *et al.*[22] showed that ATL313 effectively suppresses cytokine responses in mice, including IFN-γ.[22] Here, we confirmed that an adenosine agonist inhibits anti-CD3/CD28-induced cytokine (IFN-γ, IL-4, and TNF-α) production in human CD4$^+$ T cells (**Figure 5**).

$A_{2A}AR$ agonist inhibited IL-2 and IFN-γ protein in activated gastric T cells

Although the effect of $A_{2A}AR$ agonists on murine Th cells is well known, there is no information on the sensitivity of mucosal Th cells to this stimulation. In **Figure 1c**, LPL derived from gastric
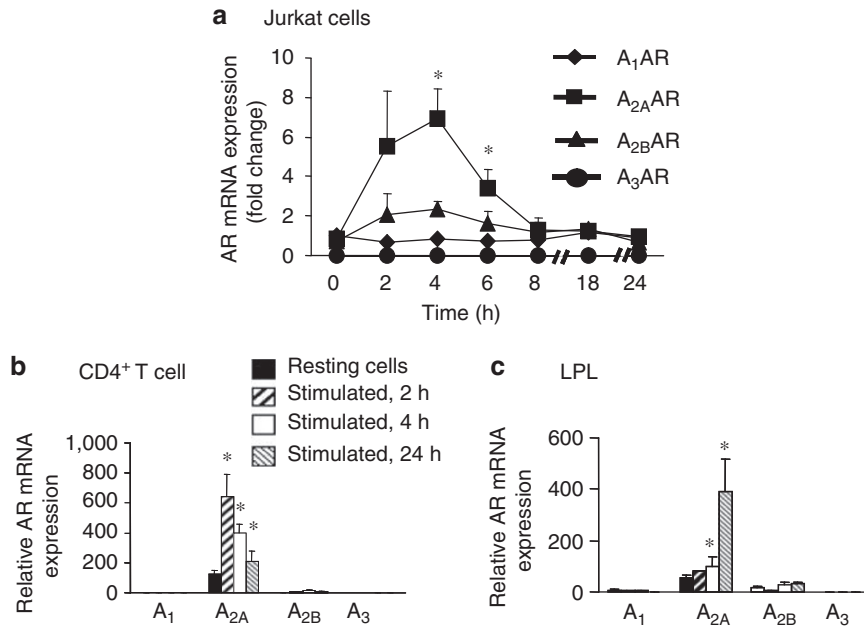
Figure 1 $A_{2A}AR$ is the predominant isoform expressed in T cells. AR expression was assessed in (**a**) Jurkat cells and reported as the fold change in mRNA in stimulated T cells relative to unstimulated T cells at the indicated time points. Subsequently, the expression of AR in (**b**) purified human CD4+ Th cells or preparations of (**c**) gastric lamina propria lymphocytes (LPL) was assessed and reported as the increase in mRNA in stimulated T cells relative to unstimulated T cells at the resting time point. In each case, T cells were activated with PMA+inomycin (50+500 ng ml^{-1}, respectively) at 37 °C for varying lengths of time, and A_1AR, $A_{2A}AR$, $A_{2B}AR$ and A_3AR mRNA expressions were measured by real-time RT-PCR. Data shown are the mean±s.e.m. from three independent experiments. *Significant difference ($P<0.05$) between unstimulated and stimulated cells.
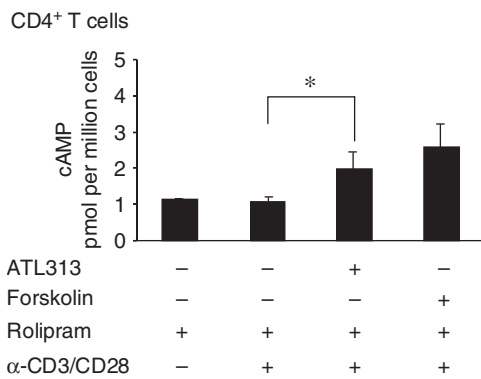

Figure 2 A selective $A_{2A}AR$ agonist, ATL313, triggers cAMP accumulation in helper T cells. Purified human CD4+ Th cells were activated for 2 h and treated for 10 min with 1 U ml^{-1} ADA in the presence of vehicle or 1 μM rolipram±1,000 nM ATL313 or forskolin, a positive control for cAMP accumulation. Intracellular cAMP accumulation was measured using a chemiluminescent immunoassay system. Data shown are the mean±s.e.m. from three independent experiments performed in triplicate. *Significant difference ($P<0.05$) between the ATL313-treated and ATL313-untreated cells.

biopsy specimens were shown to express high levels of $A_{2A}AR$. Although not entirely pure, a significant number of the cells in these preparations were CD4+ Th cells.[8,25] We found that activation of $A_{2A}AR$ with ATL313 also inhibited pro-inflammatory cytokines, including IL-2, IFN-γ, and TNF-α (**Figure 6**), in LPL, which was consistent with its effects on CD4+ Th cells from peripheral blood (**Figure 5**). These observations further

suggest that $A_{2A}AR$ play a role in controlling the pro-inflammatory cytokines in the gastric mucosa.

### $A_{2A}AR$-deficient mice have more *Helicobacter felis*-induced gastritis

H. pylori infection in humans is studied in animal models directly or using the related organism *Helicobacter felis*.[26] *H. felis* colonizes rodent gastric mucosa effectively and results in the classic sequence of histological changes seen in human infection, such as chronic gastritis, atrophy, metaplasia, dysplasia, and adenocarcinomas.[27] Infection with *H. pylori* results in a relatively mild chronic colonization and hypertrophy, but these mice do not appear to consistently develop dysplasia and carcinoma. Thus, wild-type and $A_{2A}AR$-deficient mice were infected with *H. felis* or *H. pylori*, and the effects of the mutation in the AR was compared in both species of bacteria. At 4 weeks after infection with *H. felis*, the stomachs of $A_{2A}AR$-deficient mice were significantly more inflamed compared with tissue from infected wild-type mice. **Figure 7a** show the gastric mucosa at the cardia–corpus or corpus–antral junction of a representative mouse with relatively few mucosal lymphocytes. In contrast, a marked increase in inflammation occurred in $A_{2A}AR^{-/-}$ mice 4 weeks after infection, with large numbers of mononuclear cells (MNCs) infiltrating the gastric lamina propria and submucosa. A hyperplastic thickening of the gastric wall with extensive granulocytic (myeloperoxidase, (MPO)-positive cells) infiltrates between the glands can be observed. The corpus of the stomach of the infected $A_{2A}AR^{-/-}$ mice was also significantly inflamed. **Figure 7b–d** compares the quantification of the
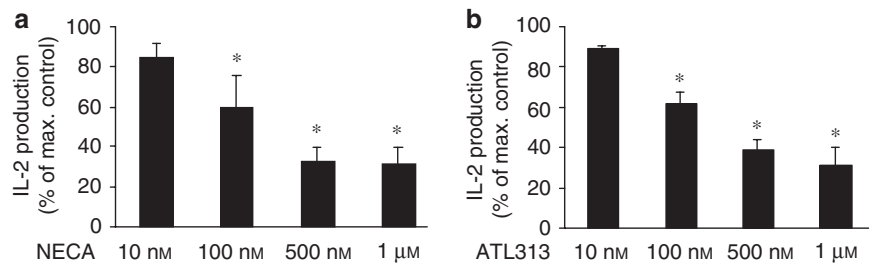
Figure 3 Suppression of IL-2 expression by CD4^{+} Th cells is mediated through the A_{2A} adenosine receptor. Purified human CD4^{+} T cells were activated in plates coated with $5\,\mu g\,ml^{-1}$ anti-CD3/CD28 mAb with $1\,U\,ml^{-1}$ ADA or after the addition of the nonselective $A_{2A}AR$ agonist, (**a**) NECA, or a selective $A_{2A}AR$ agonist, (**b**) ATL313, and IL-2 protein was measured by ELISA in supernatants collected after 24 h and expressed as the production relative to stimulated Th cells in the absence of either NECA or ATL313. Data shown are the mean±s.e.m. from three or four independent experiments performed in triplicate. *Significant difference ($P<0.05$) between the NECA- or ATL313-treated and -untreated cells.
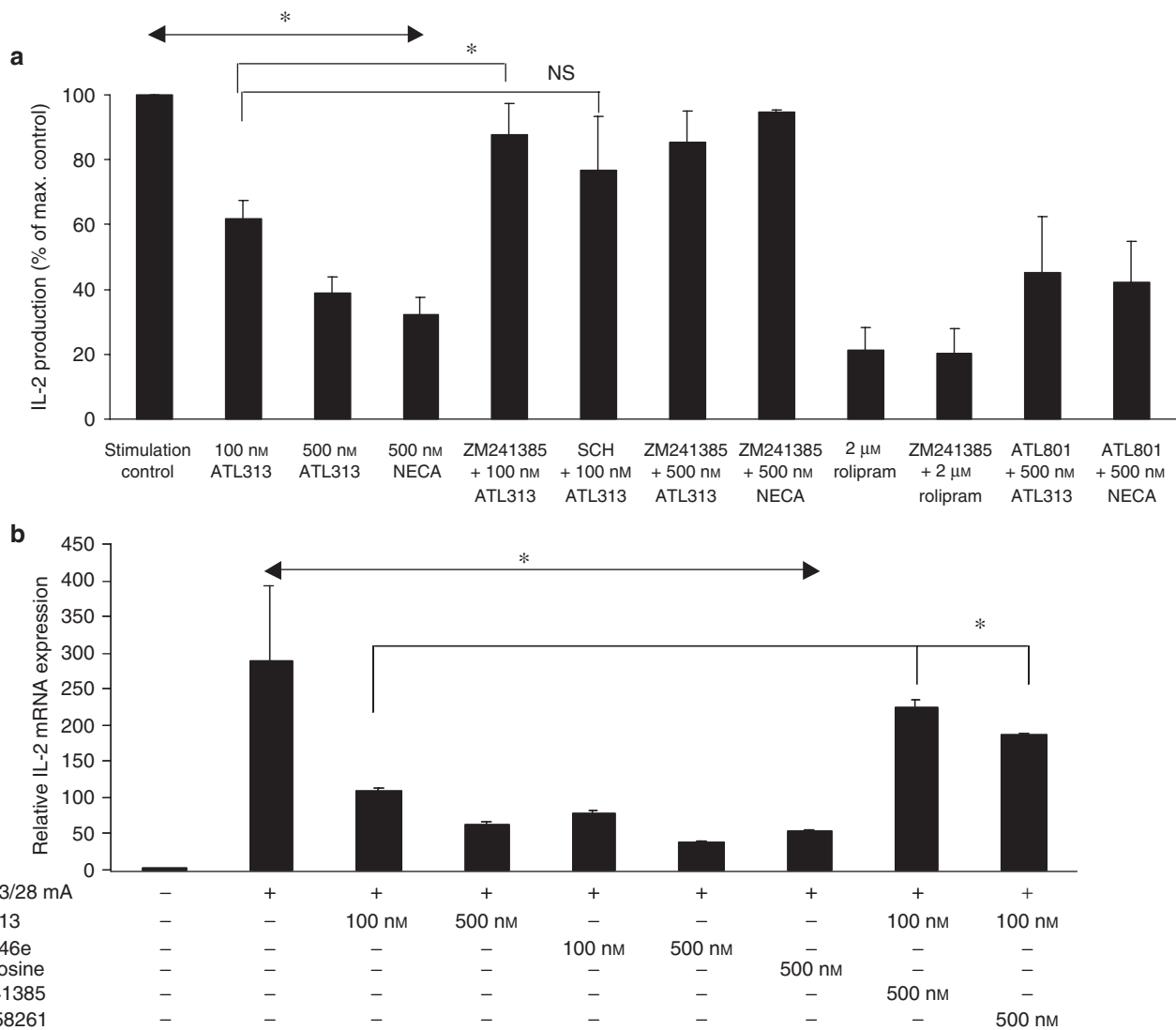
Figure 4 Inhibition of IL-2 production by ATL313. (**a**) Purified human CD4^{+} T cells were incubated on immobilized anti-CD3/CD28 mAb with $1\,U\,ml^{-1}$ ADA (Stimulation Control) or with adenosine analogs (ATL313, NECA) in the presence or absence of A_{2A} antagonists (ZM241385 or SCH58261 = SCH) or ATL801, an A_{2B} antagonist. Rolipram, a phosphodiesterase inhibitor, was used to allow cAMP to accumulate. Supernatants were collected after 24 h, and IL-2 concentrations were determined by ELISA and expressed as described in **Figure 3**. ELISA data shown are the mean±s.e.m. from three or four independent experiments performed in triplicate. (**b**) CD4^{+} T cells were cultured as described in panel a, but in this case, another A_{2A} agonist, ATL146e, was also used. Cells were collected after 24 h before IL-2 mRNA was measured by quantitative RT-PCR. Activated control refers to CD4^{+} T lymphocytes activated in the absence of any drugs. mRNA data shown are from a single experiment performed in triplicate, representative of three independent experiments. *Significant difference ($P<0.05$) of the treatment groups compared with the "stimulation control."
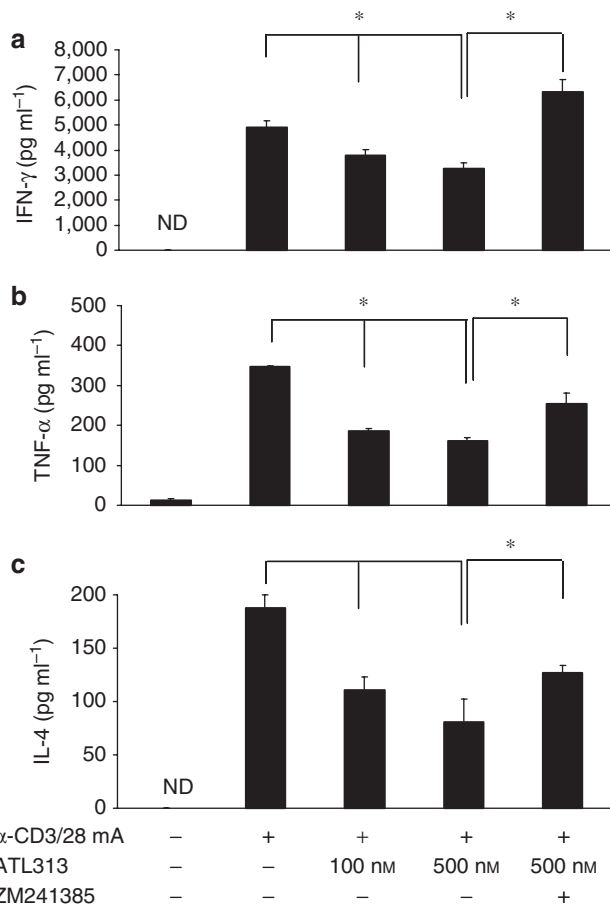
Figure 5 Suppression of pro-inflammatory cytokines by $A_{2A}AR$ agonists. $CD4^+$ T cells were stimulated and supernatants were collected after 24 h. Subsequently, (**a**) IFN-γ, (**b**) TNF-α, and (**c**) IL-4 were measured using a mutiplex bead array. The effects of the $A_{2A}AR$ agonist (ATL313) were also compared in the presence of an $A_{2A}AR$ antagonist (ZM241385). Data shown are from a single experiment performed in triplicate, representative of three independent experiments. ND = not detectable. *Significant difference ($P<0.05$) of the treatment groups compared with the "stimulation control."

gastritis and infiltrating MPO-positive cells in $A_{2A}AR^{-/-}$ mice and *H. felis*-treated control mice. In addition, gastritis was more evident in uninfected mice lacking $A_{2A}AR$. Together, these data suggest that $A_{2A}AR$ act in an anti-inflammatory role to control gastritis *in vivo*. Although these mice developed more gastritis, it did not have a significant effect on colonization (**Figure 7e**). Similar findings were also observed when $A_{2A}AR^{-/-}$ mice were infected with *H. pylori*, but overall inflammation was less (data not shown).

$A_{2A}AR$ agonists reduce inflammation, but favor *H. pylori* persistence in IL-10 deficient mice

As the inflammation induced by *H. pylori* in mice is relatively modest, investigators have challenged IL-10-deficient mice to enhance inflammation, which also leads to the spontaneous clearance of *H. pylori* infection.[28,29] To examine the effect of adenosine and its receptors on persistent infection, IL-10-deficient mice infected with *H. pylori* were fed chow contain-

ing ATL313. By 7 days after infection, both the gastritis score and the number of infiltrating MPO-positive granulocytes were lower in the adenosine agonist-treated mice (**Figure 8b** and **c**). When gastric mucosal TNF-α and IFN-γ production was tested, the adenosine agonist also significantly attenuated their production (**Figure 8d** and **e**) in the tissue site. Interestingly, suppression of inflammation by adenosine inversely affected *H. pylori* colonization in the stomach (**Figure 8f**) and resulted in significantly increased bacterial colonization. These data suggest that the inhibition of gastritis in IL-10-deficient mice favors persistent colonization with *H. pylori*.

DISCUSSION
As it is of interest to learn how to clear chronic infections that contribute to disease, it becomes essential to understand the factors that regulate the host response in a manner that protects the host from inflammation and may also favor a more commensal relationship with the persistent colonization of some organisms. This study shows that human Th cells express $A_{2A}AR$, and this receptor is induced after activation.[30] Importantly, this pattern of expression was comparable in preparations of gastric Th cells, and adenosine receptor agonists appear to attenuate the host response in a manner that may favor the commensal relationship.

Lappas *et al.*[17] reported that $A_{2A}AR$ induction inhibited IFN-γ production in murine $CD4^+$ Th cells. Similarly, Naganuma *et al.*[22] reported that $A_{2A}AR$ played a critical role in the inhibition of several pro-inflammatory cytokines that contributed to T-cell-mediated colitis. This study, using human Th cells, isolated from the blood or gastric biopsy specimens, shows that $A_{2A}AR$ could efficiently suppress IL-2 production (**Figures 3** and **4**) and other pro-inflammatory cytokines (**Figures 5** and **6**). Thus, adenosine generated in the inflamed stomach can target T cells and may attenuate the host response.

In general, T cells in the stomach are biased mostly toward the Th1 or Th17 phenotype, as marked by their ability to generate IFN-γ and IL-17,[31] but little IL-4.[7,8,32] Regardless of the evident gastritis associated with *Helicobacter* infection, infection persists for life. The failure to clear the infection may lead to a compensatory induction of Th cells with regulatory function to protect the gastric mucosa. Indeed, there are now several reports that Th cells resembling T_{reg} are present in the gastric mucosa during infection in humans and mice.[11,33–35] Moreover, Rad *et al.*[13] have provided compelling data in a mouse model that these T_{reg} may favor the development of a more commensal relationship between the host and the organism. The T_{reg} cells may produce sufficient levels of IL-10, TGF-β, or adenosine to attenuate host responses rather than to prevent gastritis totally. We observed that treatment of activated LPL with an $A_{2A}AR$ receptor agonist inhibited pro-inflammatory cytokines. In addition, using $A_{2A}AR$-deficent mice in the *H. felis* infection model, we observed increased gastric inflammation and confirmed the importance of $A_{2A}AR$ in regulating inflammatory responses *in vivo*, and thus extend this principle to mucosal tissues. In addition, treatment of *H. pylori*-infected IL-10-deficient mice with an $A_{2A}AR$ agonist resulted in a significant reduction in the gastric inflammation as well as in a significant increase in bacterial
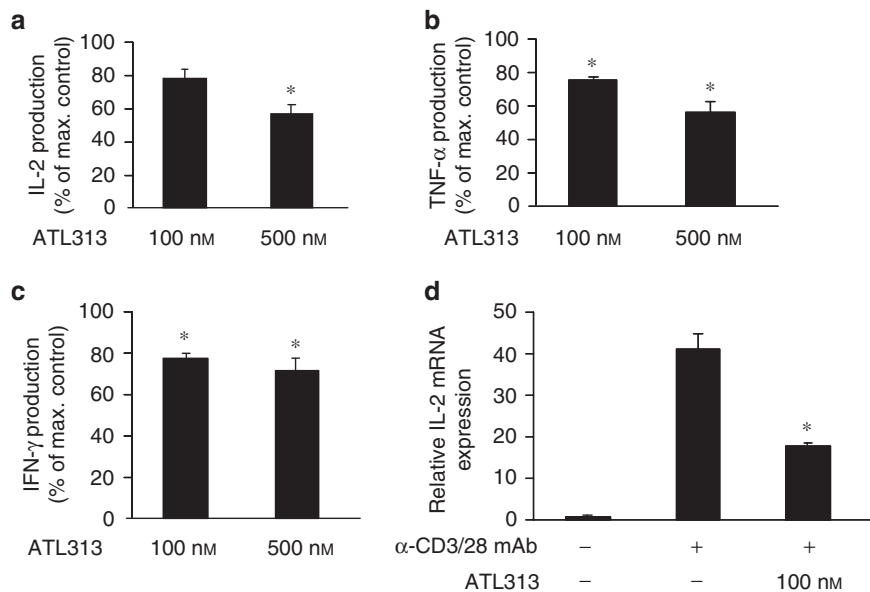
Figure 6 Suppression of pro-inflammatory cytokine production by gastric T cells is partially mediated through the A_{2A} adenosine receptor. LPL isolated from human gastric biopsy specimens were incubated on immobilized anti-CD3/CD28 mAb with $1\,U\,ml^{-1}$ ADA or in the presence of the $A_{2A}AR$ agonist, ATL313. Supernatants were collected after 24 h, and (**a–c**) IL-2, IFN-γ, and TNF-α proteins were measured using a mutiplex bead array and expressed as the production relative to stimulated Th cells in the absence of ATL313. Cytokine protein data shown are the mean±s.e.m. from three independent experiments performed in triplicate. IL-2 mRNA was extracted from T cells (from panels a–c) and assayed by real-time RT-PCR (**d**). mRNA data shown are from a single experiment performed in triplicate, representative of three independent experiments. *Significant difference ($P<0.05$) between the ATL313- treated and -untreated cells.

colonization. Although these data *in vivo* support the hypothesis, we cannot state whether the effect was mediated solely by Th cells and/or any other inflammatory targets that are known to express the $A_{2A}AR$.

A wide variety of immune cells other than Th are found to express $A_{2A}AR$, including neutrophils, monocytes, macrophages, platelets, and mast cells.[36] Other literature suggests myeloid cells, such as neutrophils, can be inhibited in the gastric mucosa as a result of $A_{2A}AR$ engagement.[20] Recently, we reported that gastric Th that express higher levels of CD39 and CD73 may act to suppress inflammatory responses during gastric infection through the production of adenosine.[22,23] Several reports now suggest that adenosine may be one anti-inflammatory mediator produced by T_{reg},[14,21,22] which could contribute to the restraint on the host response that favors persistence.

As observed in IL-10-deficient mice, marked inflammatory responses are sufficient to clear *Helicobacter spp.* infection.[29,37] Moreover, the data reported here suggest that the absence of $A_{2A}AR$ in mice led to mild gastritis before infection, inferring that endogenous adenosine cannot mediate anti-inflammatory action without its specific receptor. Furthermore, the infection of $A_{2A}AR^{-/-}$ mice led to greater inflammation when compared with that of wild-type mice. These data are consistent with the fact that activation of $A_{2A}AR$ by a specific agonist attenuates gastritis in rats.[20] It is noteworthy that the IL-10 knockout mice could decrease bacterial colonization, whereas the $A_{2A}AR$-deficient mouse had no difference. This may reflect the higher degree of gastritis in the IL-10-deficient mice or the possibility that some of the specific host responses enhanced in these mice are not found in the $A_{2A}AR$-deficient animals.

We studied the effect of *H. felis* infection in $A_{2A}AR$ KO mice for 4 weeks, and at this time point, there was an increase in inflammation with large numbers of MNCs infiltrating the gastric lamina propria and submucosa. In other models in which adenosine responses are impaired, CD73-deficient mice were shown to have a reduced *H. felis* colonization 60 days after infection. However, at this time point, there were no changes in the gastric inflammatory responses compared with infected, wild-type mice.[12] These observations suggest that the decrease in bacterial load may be associated with an attenuation in inflammation. Perhaps, the transient increase in gastritis may wane and favor clearance without any adverse effects on the development of gastric cancer. These issues remain to be studied.

In summary, activating $A_{2A}AR$ by adenosine agonists in Th cells can limit over exuberant inflammation during gastric infection with *Helicobacter spp*. The $A_{2A}AR$ provides a mechanism for limiting the pro-inflammatory effects of Th cells, thereby reducing inflammation in the gastric mucosa and impairing immunity to *Helicobacter* infection, which may favor persistence. Therefore, adenosine can be added to the list of mediators that have the potential to control gastritis in response to a local infection with *Helicobacter spp.*, and in so doing, regulate colonization.

METHODS

Reagents and antibodies. Adenosine deaminase (ADA) was purchased from Roche (Mannheim, Germany). Phorbol myristate acetate (PMA), ionomycin, NECA, adenosine, and rolipram were purchased from Sigma-Aldrich (St Louis, MO). 4-{3-[6-Amino-9-(5-cyclopropylcarbamoyl-3,4-dihydroxytetrahydrofuran-2-yl)-9*H*-purin-2-yl]
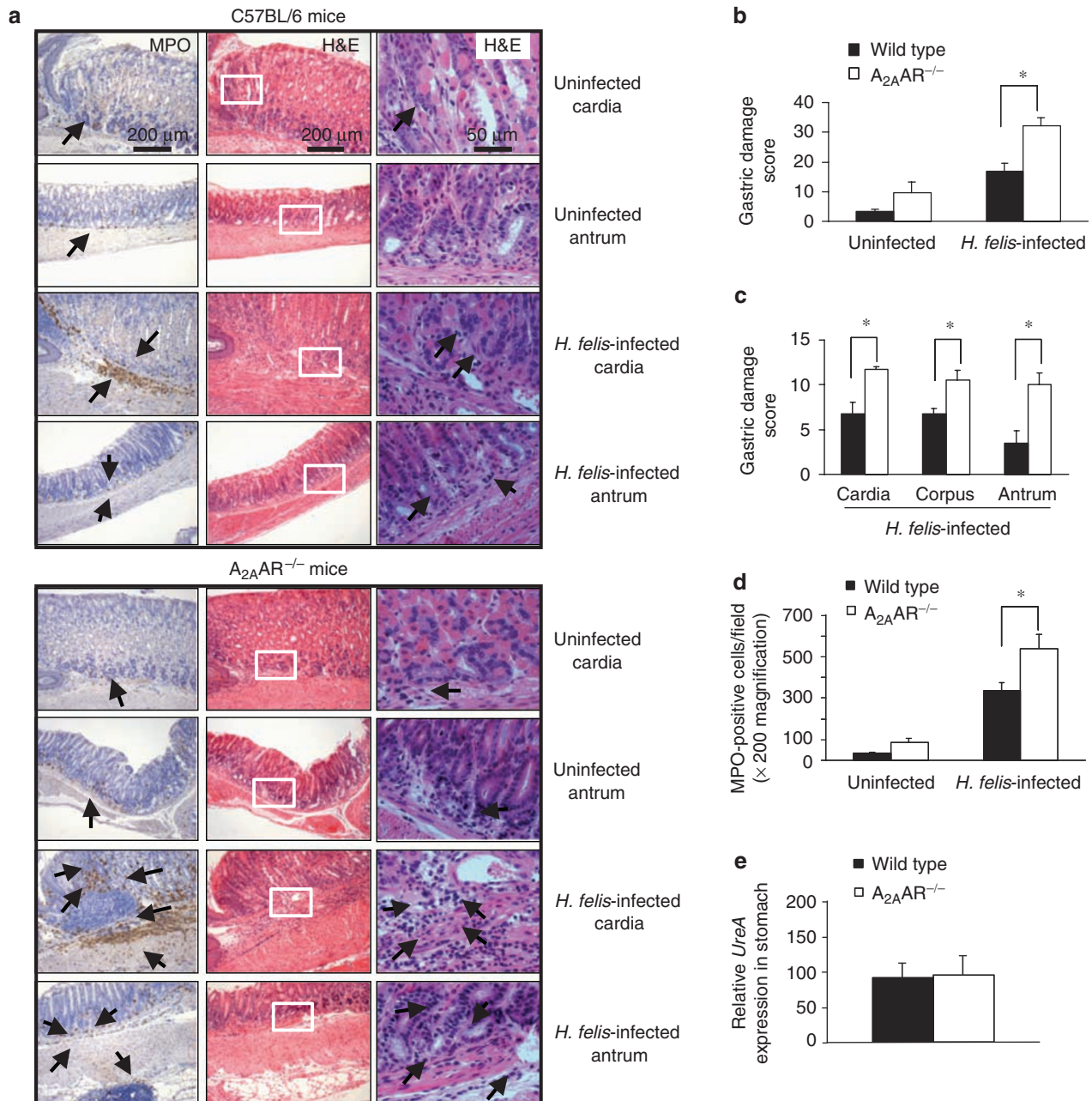
Figure 7 Gastritis is more severe in *Helicobacter felis*-infected A_{2A}AR-deficient mice. Mice were infected by gavage with 1×10^8 CFU of *H. felis* per inoculation every other day for three separate inoculations and euthanized 4 weeks later. Gastric tissue was processed for histology and *H. felis* colonization was assessed. (**a**) Myeloperoxidase (MPO) (left panel) and H&E stained (two right panels with inset from center panel shown at higher magnification in the outer right panel) of gastric sections from representative uninfected or infected C57BL/6 (upper panels), and uninfected or infected A_{2A}AR$^{-/-}$ (lower panels) mice. Arrows indicate MPO-expressing cells or MNC (original magnification of ×100). Only a few scattered MNC- and MPO-positive granulocytes can be seen in the submucosa and lamina propria with no abnormal thickening of the gastric wall of uninfected control mice. Severe gastritis with dense MNC infiltration and diffuse MPO-positive granulocytes in the submucosa and mucosa of the infected A_{2A}AR$^{-/-}$ mice in both the cardia and antral regions. MNCs aggregate between the glands are spanning the entire width of the mucosa in some cases. A concomitant thickening of the gastric wall as well as gastric of glandular atrophy can be observed. (**b**) Overall gastritis score in uninfected and infected wild-type and A_{2A}AR$^{-/-}$ mice. (**c**) Regional gastritis scores in infected wild-type and A_{2A}AR$^{-/-}$ mice. (**d**) Quantitative expression of MPO-positive cells in uninfected and infected, control, and A_{2A}AR$^{-/-}$ mice expressed per ×200 field. (**e**) *H. felis* colonization in gastric tissue analyzed by the presence of *H. felis*-specific *UreA* gene. Data are mean±s.e.m. from pooled data including two identical experiments with four mice per group. *Significant difference ($P < 0.05$) between the wild-type and A_{2A}AR$^{-/-}$ mice.

prop-2-ynyl}piperidine-1-carboxylic acid methyl ester (ATL313) was gift from Adenosine Therapeutics LLC (Charlottesville, VA). 4-(2-[7-Amino-2-[2-furyl][1,2,4]triazolo[2,3-*a*][1,3,5]triazin-5-yl-amino]ethyl)phenol (ZM241385) and 2-(2-furanyl)-7-(2-phenylethyl)-7-*H*-pyrazolo[4,3-e] [1,2,4]triazolo[1,5-c]pyrimidin-5-amine (SCH58261) were purchased from Tocris (Ellisville, MO). Anti-human CD3 mAb (clone OKT3) from eBioscience (San Diego, CA), and anti-human CD28 mAb (Clone CD28.2) was from BD Pharmingen (San Diego, CA).
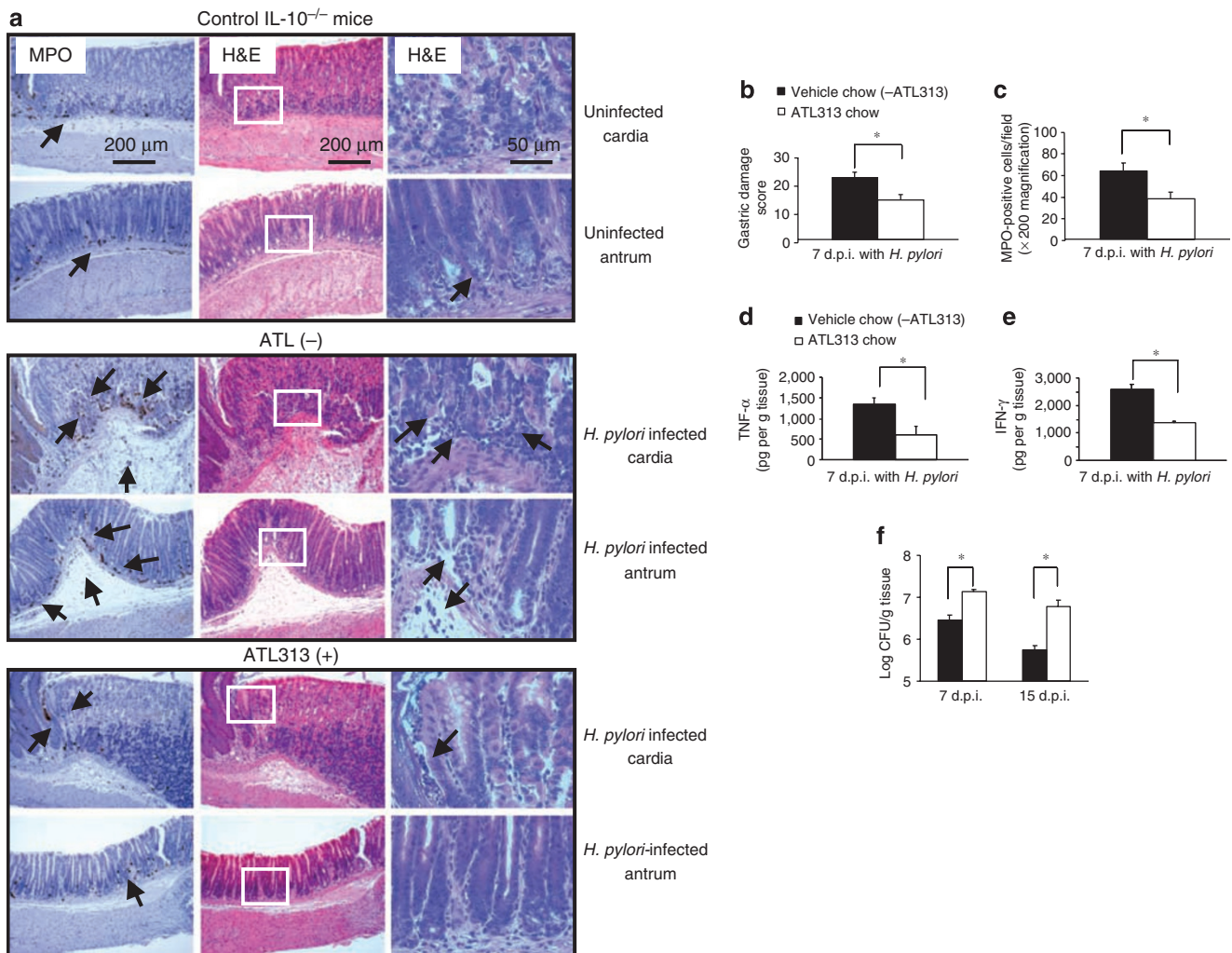
Figure 8 Oral administration of an A$_{2A}$AR agonist in *Helicobacter pylori*-infected IL-10$^{-/-}$ mice reduces gastric inflammation but increases bacterial load. IL-10-deficient mice were infected as described in **Figure 7**. All mice were given either vehicle control chow (ATL313(−), *n*=4) or ATL313 chow (ATL313(+), *n*=4) for 1 week before infection and continued until killing. Mice were killed 7 (7 d.p.i.) or 15 days (15 d.p.i.) later, and gastric tissue (each longitudinal section) was processed for histology, MPO staining, mucosal cytokine expression, and *H. pylori* colonization by colony count. (**a**) Myeloperoxidase (MPO) (left panel) and H&E stained (two right panels with inset from center panel shown at higher magnification in the outer right panel) of gastric sections from representative uninfected IL-10$^{-/-}$ (upper panels), and infected IL-10$^{-/-}$ without (middle panels) or with ATL313 chow-treated mice (lower panels). Arrows indicate MPO-expressing cells (original magnification of ×100). Some scattered mononuclear cells (MNCs) and MPO-positive granulocytes can be seen in the submucosa and lamina propria, with no abnormal thickening of the gastric wall of uninfected control IL-10-deficient mice. Severe gastritis with dense MNC infiltration and diffuse MPO-positive granulocytes in the submucosa and mucosa of the untreated infected mice in both cardia and antral regions. A concomitant hyperplasia with widespread thickening of the gastric wall as well as of gastric atrophy can be observed. ATL313-treated mice are almost normal with few inflammatory cells infiltration. (**b**) Histological scoring of gastritis, (**c**) quantitative expression of MPO-positive cells expressed per field, and (**d**) mucosal TNF-α, and (**e**), IFN-γ production in gastric tissue of in *H. pylori*-infected IL-10-deficient mice without or with ATL313 treatment. (**f**) *H. pylori* colonization in gastric tissue measured by colony count on days 7 and 15 after infection with same treatment as mentioned earlier. Data are mean±s.e.m. from one experiment with four mice per group that was carried out at least twice with similar results. *Significant difference (*P*<0.05) between the untreated and treated mice.

Cell lines. Human Jurkat T cell line (ATCC Clone E6-1, Manassas, VA) was maintained in RPMI 1640 (HyClone Laboratories, Logan, UT) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 0.1 mM nonessential amino acid solution, 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer, 100 U ml^{-1} penicillin, 100 μg of streptomycin, 1 mM sodium pyruvate, and 55 μM 2-marcaptoethanol (2-ME) at 37 °C under 5% CO_2.

Isolation of PBMC and purification of CD4$^+$ T cells. Peripheral blood mononuclear cells (PBMCs) were obtained from peripheral blood or

buffy coat preparation derived from healthy donors (Virginia Blood Bank Richmond, VA), using Ficoll/Hypaque (Amersham, Uppsala, Sweden) density centrifugation and stored in liquid nitrogen until further use. PBMCs were first depleted of monocytes using CD14 magnetic beads. CD4$^+$ T cells were then purified from CD14-negative PBMCs using CD4 magnetic-activated cell sorting beads (Miltenyi Biotec, Bergisch Gladbach, Germany).

Isolation of LPL from gastric biopsy samples. Biopsy specimens of the gastric antrum were obtained from consenting subjects undergoing

gastroesophogeal duodenoscopy for various clinical indications, as approved by our respective instructional review broads at the University of Virginia. All subjects were uninfected and were free of gastritis. Gastric T cells were isolated using a modified technique.[8,25] Briefly, the biopsy specimens were collected into sterile collection medium (calcium- and magnesium-free Hank's balanced salt solution with 5% fetal calf serum and penicillin plus streptomycin). The biopsy specimens were stored at 4 °C for up to 18 h before processing, this having been shown earlier not to alter T-cell function. The biopsy specimens were rinsed with aqueous betadine and immediately rinsed four times in collection medium containing dithiothreitol (1 mM) and EDTA (1 mM) (Sigma, St Louis, MO). The specimens were agitated for 1 h at 37 °C to detach intraepithelial lymphocytes and epithelial cells, removed, placed in complete RPMI 1640 medium (10% fetal calf serum, and penicillin/streptomycin), and washed three times with this medium. Subsequently, lamina propria T cells were liberated by treatment with collagenase (30 U ml^{-1}) in complete RPMI 1640 medium. The resulting cell suspensions were washed, and the viability of the MNCs was determined by trypan blue exclusion. The cells were not used if viability did not exceed 90%.

T-cell treatment with adenosine agonists. Purified CD4$^+$ T cells or gastric T cells (2×10^5 cells/well) were stimulated with 5 µg ml^{-1} immobilized anti-CD3 mAb and 5 µg ml^{-1} anti-CD28 mAb in the presence or absence of various concentration of ATL313, NECA, or adenosine. In some experiments, 500 nM of ZM241385 or SCH58261 (A$_{2A}$AR antagonists) or ATL801 (an A$_{2B}$AR antagonist) were added to the cultures. All co-culture assays are carried out in the presence of 1 U ml^{-1} ADA.

Measurement of intracellular cAMP. Purified CD4$^+$ T cells were suspended in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 100 U ml^{-1} penicillin, 100 µg of streptomycin. Either immediately after purification or after incubation on immobilized anti-CD3 mAb for varying lengths of time, cells were incubated at 37 °C for 10 min with 1 µM rolipram and 1 U ml^{-1} ADA in the presence or absence of ATL313. Rolipram, a type IV phosphodiesterase inhibitor, was used to stabilize cAMP production, as it inhibits the natural phosphodiesterase breakdown of cAMP to 5′-AMP.[24] cAMP accumulation in response to 1 µM of rolipram in the absence of ATL313 did not change in cells as a result of TCR activation (data not shown).

Forskolin (Sigma) was used as a positive control to induce cAMP production in cells. Cells were then lysed, and intracellular cAMP levels were measured using the chemiluminescent immunoassay system for the quantification of cAMP from mammalian cells, cAMP-Screen System, according to the manufacturer's protocol (Applied Biosystems, Foster City, CA).

Cytokines assays. Supernatants were collected for cytokine assays and cells were harvested as a source of mRNA. IL-2 concentrations in supernatants of CD4$^+$ T-cell or gastric T-cell cultures were measured by human IL-2 ELISA kit according to the manufacturer's protocol (BD Biosciences, San Diego, CA). IL-2 levels were determined using a standard curve calibrated against recombinant human IL-2 standard supplied by the manufacturer. TNF-α and IFN-γ levels were measured using a mutiplex bead array (Upstate, Temecula, CA) and analyzed with the Bio-Plex workstation and associated software (Bio-Rad, Hercules, CA). All cytokine concentrations were measured using a standard curve and expressed as picogram per milliliter and/or presented as the percentage of their activated controls, that is, percentage of maximum control. Percentile data were used to normalize the variability issues among individual human PBMCs and also antibody stimulation because of lot variation.

RNA extraction and quantitative RT-PCR. Total RNA was extracted from cells using the QIAamp Blood Mini Kit (Qiagen, Valencia, CA), and was reverse transcribed to yield cDNA using the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA). Real-time RT-PCR

was performed in a SmartCycler (Cepheid, Sunnyvale, CA), using primer and dual-labeled probe (Applied Biosystem) for IL-2 and adenosine receptors (A$_1$, A$_{2A}$, A$_{2B}$, and A$_3$). Real-time PCR reactions were carried out in a total volume of 25 µl containing 1 µl of cDNA, 12.5 µl of Universal PCR Master Mix (Applied Biosystems), 300–900 nM of each forward and reverse primer, and 200 nM of each probe. Duplicate PCRs were performed for each sample, and the average threshold cycle number was determined using the Opticon software. Normalized levels of each mRNA were determined using the formula $2^{(Rt-Et)}$, where Rt is the threshold cycle for the reference gene (18S rRNA) and Et the threshold cycle for the experimental gene ($\Delta\Delta C_T$ method).[38] Data are expressed as arbitrary units relative to an appropriate unstimulated control.

Mice. C57BL/6 mice and IL-10-deficient (IL-10$^{-/-}$) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) whereas A$_{2A}$AR-deficient mice (A$_{2A}$AR$^{-/-}$) mice inbred onto the C57BL/6 background[17] were maintained in a conventional animal care facility at the University of Virginia (Charlottesville, VA). All procedures were approved by the Animal Care and Use Committee at the University of Virginia.

***Helicobacter* growth and challenge, and A$_{2A}$AR agonist treatment of mice.** *H. pylori* is the pathogen of humans, but *H. felis* infection of mice more closely mimics the histological sequences of *H. pylori* infection in humans. Therefore, we have compared the effects of adenosine receptor stimulation using both microbes in two different models. *H. pylori* Sydney strain (SS2000) or *H. felis* strain (ATCC 51211) were cultured on trypticase soy agar (TSA) II agar plates containing 5% sheep blood (BBL, BD, Becton, Dickinson Laboratory, Sparks, MD) in a microaerophilic condition as described earlier. The bacteria were then harvested and inoculated into *Brucella* broth (BD Biosciences, Sparks, MD) supplemented with 10% heat-inactivated fetal calf serum (Hyclone, Logan, UT) as described elsewhere.[39,40] To establish a primary *H. pylori* infection, mice were inoculated intragastrically through a 20-G feeding needle with a 200-µl suspension of *H. pylori* containing 1×10^8 CFU of the bacteria on 3 consecutive days and were killed different weeks after inoculation. In some experiments, mice receiving *H. pylori* or *H. felis* were fed chow containing 1.875 mg kg^{-1} of ATL313 or vehicle chow containing dimethylsulfoxide control 1 week before their infection. ATL313 chow was continued until the experiments are completed, and all mice were fed with fresh chow, which was changed every 4–5 days interval to maintain its drug effect. At 4–6 weeks after challenge, mice were killed, and gastric tissue was processed for *H. pylori* culture, histology, and immunohistochemistry as described below.

Assessment of bacterial colonization. For a quantitative measurement of *H. pylori* bacteria, longitudinal segments of gastric tissue were homogenized in 0.5 ml of *Brucella* broth supplemented with 10% fetal calf serum, and replicate serial 10-fold dilutions were plated on *Helicobacter*-selective blood agar plates to determine bacterial colonization as described earlier.[41] The plates were incubated at 37 °C under microaerobic conditions (10% CO_2, 5% O_2, and 85% N_2), quantified 5–7 days later, and reported as the number of CFU per gram.

Gastric tissue DNA extraction and assessment of *H. felis* colonization. Stomach DNA from infected wild-type mice was prepared using Maxwell 16 Tissue DNA Purification Kit (Promega, Madison, WI) exactly as per the manufacturer's instructions. Gastric colonization of *H. felis* was assessed by quantifying a *H. felis*-specific *UreA* gene relative to host tissue housekeeping gene, as described earlier.[12,13] The amount of *H. felis* *UreA* DNA was expressed relative to mouse gastric GAPDH (glyceraldehyde-3-phosphate dehydrogenase), as determined by the comparative $\Delta\Delta C_T$ method mentioned above. Primer sequences for *H. felis UreA*, 5′-TCGATCGCGCAAAAGCTT-3′ and 5′-CGCACCGTTCCAGAT-3′. Primers sequences for GAPDH, 5′-GCTAAGCAGTTGGTGCA-3′ and 5′-TCACCACCATGGAGAAGGC-3′.

Preparation of gastric homogenates for TNF-α and IFN-γ measurement. One longitudinal strip of stomach mucosa (40–50 mg) was homogenized for 30 s with a polytron homogenizer (PT 1200, Kinematica AG, Littau, Switzerland) in 0.5 ml of ice-cold phosphate buffer (pH 7.4) with Complete, Mini (Protease Inhibitor Cocktail Tablets; Roche). After homogenization, all tissues weight was expressed as 25 mg ml^{-1} concentration and sonicated. Aliquots of homogenate supernatants were obtained by centrifugation and kept at $-70\,°C$ until use. Total protein was measured by Bradford's method. Concentration of TNF-α and IFN-γ cytokine in the supernatant of mucosal homogenates was determined by ELISA (BD Biosciences, CA) as mentioned above.

Gastric tissue analyses and histological evaluation. For histopathology, longitudinal segments, including the antrum and corpus plus proximal duodenum, were fixed in Bouin's fixative solution (Ricca Chemical, Arlington, TX) for 24 h, washed twice with 70% ethanol and embedded in paraffin, cut into 3- to 5-μm sections, and stained with hematoxylin and eosin. For immunohistochemistry, similar 3-μm gastric sections were stained with polyclonal anti-MPO antibody (Novus Biochemicals, Littleton, CO), and tissue-bound peroxidase activity was visualized with DAB (3,3′-diaminobenzidine). Hematoxylin was used for nuclear counter staining. The number of MPO-positive cells were counted in 10 different fields at an original magnification of ×200 and expressed per field. Gastric inflammation was assessed using a modified scoring system, as described earlier by Ismail *et al.*[42] Briefly, two sections were collected from each stomach, and each region of the stomach (forestomach or cardia, corpus, and antrum) was assessed individually for three parameters: (1) thickening, (2) infiltration of polymorphonuclear cells and (3) infiltration of MNCs. Severity was graded based on the absence (0) or presence (1) of each parameter, with polymorphonuclear infiltration further examined (absence or presence) for focal, diffuse, or abscess involvement. Similarly, MNC infiltration was examined for focal, diffuse, or aggregate involvement in the lamina propria. A total score was calculated by summing the score values for each region of the stomach for one section. Results are reported as total damage scores±s.e.m.

Statistical analysis

Results are expressed as mean±s.e.m. Data were compared by Student's *t*-test (unpaired), and results were considered significant if *P*-values were <0.05.

DISCLOSURE

Drs Figler, Linden, and Rieger hold shares in Adenosine Therapeutics LLC.

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