A2B Adenosine Receptor Induces Protective Antihelminth Type 2 Immune Responses

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SUMMARY

The type 2 immune response evoked by intestinal nematode parasites contributes to worm expulsion and tolerance to associated tissue damage. We investigated whether this host response is affected by blocking signaling by the putative endogenous danger signal adenosine, which can be released during inflammation and host cell damage. Specific blockade of the A2B adenosine receptor (A2BAR) inhibited worm elimination and the development of innate and adaptive components of the type 2 secondary and memory response. Infected mice lacking A2BAR exhibited decreased M2 macrophage and eosinophil recruitment and reduced IL-4 and IL-13 cytokine production. Additionally, shortly after infection, upregulation of the alarmin IL-33, which drives type 2 immunity, and activation of innate lymphoid type 2 (ILC2) cells was inhibited, while exogenous IL-33 restored ILC2 cell activation and type 2 cytokine expression. Thus, adenosine acts as a danger-associated molecular pattern (DAMP) that initiates helminth-induced type 2 immune responses through A2BAR.

INTRODUCTION

Gastrointestinal parasites affect global health, inducing chronic malnutrition, morbidity, and susceptibility to infection with other infectious agents. Increasing evidence suggests that these pathogens trigger polarized type 2 immune responses (Gaze et al., 2012), similar to many experimental mouse models. Heligmosomoides polygyrus (Hp) is a strictly enteric natural murine parasite that is a widely used model for intestinal nematode parasite infection. Hp induces a polarized and potent type 2 immune response characterized by Th2 cell differentiation and the activation of multiple immune protective components that contribute to worm expulsion at both the tissue-dwelling and luminal stages of the life cycle (Herbert et al., 2009; Patel et al., 2009). Primary Hp inoculation results in chronic infection, but after clearance with anthelminthic drugs, secondary inoculation triggers a strong type 2 memory response, with worm expulsion by 2 weeks (Anthony et al., 2007; Reynolds et al., 2012).

Adenosine is an endogenous purine nucleoside, a catabolite of ATP, and can regulate multiple physiological processes by binding to and activating one or more of four transmembrane adenosine G protein-coupled cell surface receptors, denoted A1, A2A, A2B, and A3 adenosine receptors (ARs) (Jacobson and Gao, 2006). Inflammation, hypoxia, and cell damage can all result in accumulation of extracellular adenosine, which may act as an endogenous regulator of innate immunity (Haskó and Cronstein, 2004; Haskó et al., 2004; Linden, 2001). Several recent studies suggest that adenosine may promote specific components of type 2 immunity. A2BARs can activate mast cells in vitro to secrete IL-4 and dendritic cells to promote CD4 T cell IL-4 production (Ryzhov et al., 2004; Yang et al., 2010). A2BAR signaling has further been shown to promote IL-4-induced M2 macrophage activation in vitro (Csóka et al., 2012). A2BAR signaling in macrophages can also induce IL-10 gene expression in vitro (Németh et al., 2005) and enhance allergen-induced chronic pulmonary inflammation in an ovalbumin model system in vivo (Zaynagetdinov et al., 2010).

The factors that initially trigger the type 2 immune response remain uncertain. IL-33, produced by cells in barrier tissues, including epithelial cells, can initially drive both innate and adaptive components of the type 2 mucosal immune response (Mirchandani et al., 2012). But the actual signals that trigger the production and/or release of IL-33 remain unclear. Helminth excretory/secretory (ES) products may trigger type 2 immunity (Everts et al., 2009; Grainger et al., 2010; Hewitson et al., 2009; Tawill et al., 2004). Tissue damage resulting from parasite trafficking through tissues may induce release of danger-associated molecular patterns (DAMPs) (Gause et al., 2013). As the type 2 response progresses during helmith infection, its potency increases, presumably due to amplification of the immune response and associated sustained signals triggered by helminth parasites, which may include DAMPs.

As yet specific DAMPs have not been identified as playing a significant role in the development of the in vivo type 2 immune response to helminths. In these studies, we examine whether the complex type 2 immune response evoked by Hp infection...
was affected by specific blockade of adenosine signaling through the A2BAR. Surprisingly, our studies demonstrated profound impairment in the absence of A2BAR signaling of the type 2 protective immune response to Hp. They provide evidence of a DAMP essential for the development of the type 2 immune response to helminths.

RESULTS

A2BAR Receptors Contribute to Enhanced Worm Expulsion and Associated Development of the Type 2 Immune Response

Hp triggers a strong, highly polarized type 2 immune response (Anthony et al., 2007; Svetić et al., 1993). To examine whether A2BAR signaling is essential in the development of the host-protective type 2 memory immune response, which is required for Hp expulsion, A2BAR−/− and wild-type (WT) (BL/6) mice were orally inoculated with 200 Hp (HP) L3, 14 days later treated with the anthelmintic drug pyrantel pamoate to expulse parasites, and 4 weeks later given a secondary HP inoculation (Hp2). Controls included mice given only a primary inoculation (Hp1). Mice were sacrificed at 14 days after HP inoculation, and parasite and egg numbers determined (A). Serum immunoglobulins (IgE and IgG1) were measured by ELISA (B). (C–J) A2BAR−/− and wild-type mice were given an Hp1 or Hp2 inoculation as just described. On day 11 after Hp treatment, prior to worm expulsion, total parasite and egg numbers were enumerated (C). Individual parasites were isolated, and male and female parasites were recovered from corresponding treatment groups at day 11 after HP infection. Protein (D) and ATP (relative luminescence units [RLU]) (E) levels were determined for five to ten male or female worms per mouse. Small intestine Th2 cytokine gene expression was determined by qRT-PCR and expressed as treated/untreated (trt/untrt) (F). In a separate experiment after WT and A2BAR−/−/Hp2, small intestine tissue was collected and stained for periodic acid Schiff (PAS) (G). Small intestines were also stained for wheat germ agglutinin (WGA-PE, red) (H). The mucous index was measured by counting mucous-secreting cells/100 cells/villi (I). Goblet cell RELMβ and Muc2 mRNA were measured by qRT-PCR (J). All experiments were repeated two times with similar results. Mean and SE of four to five mice per treatment group are shown. **p < 0.01.
Arg1, RELMα, and Ym1 iNOS mRNA were analyzed in small intestine by real-time qRT-PCR (D), and the mean and SE from four to five mice/treatment group is shown. Experiments were performed two times with similar results. *p < 0.01.

A2B ARs contribute to enhanced worm expulsion and associated serum Ig elevations.

A2B AR−/− Mice Show Impaired Worm Damage and Compromised Peripheral Intestinal Type 2 Memory Responses

To assess whether the type 2 immune response was affected at earlier stages of luminal infection and to directly assess effects of A2B AR deficiency on luminal parasites, A2B AR−/− mice were examined at day 11 after primary and secondary Hp inoculation—a time point when cytokine elevations are readily detectable during luminal infection and parasites are still present. Total worms and egg production were increased in A2B AR−/− mice at this early time point during luminal infection (Figure 1C). Total worm ATP and protein levels were assessed to determine metabolic activity and growth (a direct measurement of parasitic impairment), as previously described (Herbert et al., 2009). Both female parasite total protein and ATP levels were significantly increased (p < 0.01) in A2B AR−/− mice compared to WT controls (Figures 1D and 1E). In intestinal tissue significant reductions in IL-4, IL-13, IL-5, and IL-9 mRNA were observed (Figure 1F). Histological analysis showed reduced mucus producing goblet cells in the small intestine of A2B AR−/− mice compared to WT mice (Figure 1G–1I). mRNA levels of RELMβ, secreted by goblet cells in response to IL-13 (Artis et al., 2004), and Mucin 2 (Muc2), a major mucin secreted by intestinal goblet cells, were also reduced significantly in the absence of A2B AR signaling (Figure 1J).

M2 macrophages contribute to the formation of a type 2 granuloma surrounding the tissue-dwelling parasite (Anthony et al., 2006; Patel et al., 2009). Swiss roll cryosections were stained for F4/80 and IL-4Rα to detect M2 cells (A); formalin-fixed and stained with H&E (B); and cryosectioned and stained for eosinophils (anti-MBP, green) (C). Samples are representative of results from four to five mice per treatment group. Arg1, RELMα, and Ym1 iNOS mRNA were analyzed in small intestine by real-time qRT-PCR (D), and the mean and SE from four to five mice/treatment group is shown. Experiments were performed two times with similar results. *p < 0.01.
Adenosine Promotes Antihelminth-Induced Immunity

A2BAR Signaling Is Required for the Polared Type 2 Response in the Mesenteric Lymph Nodes during Early Luminal Stage

The compromised immunity to parasites in the intestinal lumen after A2BAR blockade suggested that either the localized peripheral effector response at the host-parasite interface was directly impaired or, alternatively, that initial Th2 cell differentiation in the draining lymph node, required for optimal peripheral responses, was inhibited. To address this, mesenteric lymph nodes (MLNs) were collected from WT and A2BAR−/− mice at day 11 after Hp secondary inoculation and prepared for quantitative RT-PCR (qRT-PCR). As shown in Figure 3A, IL-4, IL-13, IL-5, and IL-9 mRNA were significantly reduced in A2BAR−/− mice compared to WT mice. To assess CD4+ T cell cytokines, MLN CD4+ and CD4− cells were isolated using magnetic bead cell sorting of pooled suspensions from five mice per treatment group. IFN-γ mRNA levels were increased (Figure 3B), while both IL-4 (Figure 3C) and IL-13 (Figure 3D) mRNA were reduced in CD4+ T cells in A2BAR−/− compared to WT mice. The number of IL-4- and IFN-γ-secreting MLN cells and sorted CD4+ and CD4− MLN cells were assessed by ELISPOT. Marked reductions in CD4+ IL-4-secreting cells while significant increases in IFN-γ-secreting cells were observed in A2BAR−/− mice, compared to WT mice, after Hp secondary inoculation (Figures 3E–3H). These findings indicate that CD4+ Th2 cell development is impaired in A2BAR-deficient mice during the memory response to Hp.

A2BAR Signaling Is Essential for the Host Protective Response against Tissue-Dwelling Parasites

Shortly after entering the small intestine, Hp L3 penetrate the intestinal epithelium and migrate to the submucosal region, where they reside as tissue-dwelling larvae for 7 days, when they return to the lumen as mature adults. Previous studies have suggested that immune mechanisms mediating host protection at the tissue-dwelling phase are distinct from those promoting expulsion at the luminal phase (Anthony et al., 2006; Herbert et al., 2009). Using previously described techniques (Liu et al., 2010), we next examined whether A2BAR signaling contributes to host protection at the tissue-dwelling phase. Tissue-dwelling parasites isolated at day 7 after secondary inoculation from individual granulomas were decreased in number and length compared to mice given a primary inoculation only (Figures 4A and 4B), indicating effective host protection in the tissue-dwelling phase, as...
previously described (Liu et al., 2010). However, larvae isolated from \(A_{2B}AR^{-/-}\) mice were significantly higher in number and length compared to WT controls after secondary inoculation, indicating that \(A_{2B}AR\) signaling contributes to host protection leading to impaired larval development during the tissue dwelling phase (Figures 4A and 4B). We also observed reduced \(IL-4\), \(IL-13\), and \(IL-5\) mRNA (Figure 4C) and decreased M2 macrophage markers (Arg1, RELM\(\alpha\), and Ym1) (Figure 4D) in \(A_{2B}AR^{-/-}\) mice after secondary inoculation in the small intestine. Immuno-fluorescence imaging of granulomas revealed that F4/80\(^+\) cells double stained with IL-4R\(\alpha\) were absent or reduced dramatically in \(A_{2B}AR^{-/-}\) mice (Figure 4E, upper panel). Eosinophil (MBP\(^+\)) accumulation was also blunted in \(A_{2B}AR^{-/-}\) mice (Figure 4E, lower panel). Similar to results obtained at day 11 after secondary inoculation, analysis of MLN gene expression also showed pronounced decreases in type 2 cytokines and M2 markers at day 7 after Hp inoculation of \(A_{2B}AR^{-/-}\) mice compared to WT mice (data not shown). These results indicate that \(A_{2B}AR\) deficiency markedly impairs the helminth-induced localized secondary type 2 immune response at the host:parasite interface and also significantly reduces effective protective immunity during the tissue dwelling stage.

**Type 2 Responses after Primary Inoculation Are Blocked at Tissue Dwelling Stage in \(A_{2B}AR\)-Deficient Mice**

To examine whether type 2 responses are inhibited at early stages after primary inoculation, WT and \(A_{2B}AR^{-/-}\) mice were inoculated with \(Hp\) and the peripheral intestinal type 2 cytokine
responses assessed at day 8 after inoculation, a time point at which the MLN T cell cytokine response is consistently high after primary inoculation (Lu et al., 1996; Svetić et al., 1993). Increases in type 2 cytokines (Figure 4F) and M2 markers (Figure 4G) were largely inhibited after primary Hp inoculation of A<sub>2B</sub>AR<sup>/−/−</sup> mice compared to inoculated WT mice. Previous studies have indicated that IL-33 may drive the development of both innate and adaptive type 2 immune responses (Hung et al., 2013). To examine whether exogenous IL-33 administration could rescue the impaired type 2 immune response following A<sub>2B</sub>AR blockade, A<sub>2B</sub>AR<sup>/−/−</sup> mice were exogenously administered IL-33 and inoculated with Hp. At day 8 after primary inoculation, IL-33 administration restored elevations in type 2 cytokines (Figure 4F) and M2 markers (Figure 4G). As IL-33 administration is sufficient to rescue the type 2 response, our findings suggest that adenosine interactions with other more downstream targets of A<sub>2B</sub>AR signaling, such as M2 macrophages, may not be essential if sufficient levels of IL-33 are available to support the development of the type 2 immune response.

MLN cell suspensions were also assessed for cytokine protein expression at day 8 after primary inoculation. As shown in Figure 5A, elevations in IL-4 in unsorted cell suspensions were markedly inhibited in Hp-inoculated A<sub>2B</sub>AR<sup>/−/−</sup> mice, while IFN-γ remained at low levels (Figure 5B). ELISPOT analysis of magnetic-bead-sorted CD4<sup>+</sup> and CD4<sup>+</sup> T cells from cell suspensions pooled from five mice per treatment group showed inhibition of elevations in CD4<sup>+</sup> T cell IL-4 secretion (Figure 5C). Previous studies have shown that increased B cell MHC-II expression after Hp inoculation is IL-4 dependent (Liu et al., 2002; Lu et al., 1996). B cell surface MHC-II expression was markedly higher in WT mice compared to A<sub>2B</sub>AR<sup>/−/−</sup> mice after Hp inoculation (Figures 5D and 5E), indicating that A<sub>2B</sub>AR deficiency impairs IL-4 bioactivity in vivo. We next performed flow cytometric analysis to assess CD4<sup>+</sup> T cell STAT6 phosphorylation (pSTAT6), as previously described (Perona-Wright et al., 2010). A significant reduction in the percentage of CD4<sup>+</sup> T cells expressing pSTAT6 was observed after Hp inoculation in A<sub>2B</sub>AR<sup>/−/−</sup> compared to WT mice, suggesting reduced CD4<sup>+</sup> T cell IL-4R signaling in the absence of A<sub>2B</sub>ARs (Figure 5F). IL-33 administration in the absence of Hp inoculation was not sufficient to sustain elevated IL-4 secretion in A<sub>2B</sub>AR<sup>/−/−</sup> mice (Figure 5A available online). In all these studies, exogenous administration of IL-33 substantially restored the type 2 immune response to Hp, raising the possibility that one important mechanism of A<sub>2B</sub>AR-induced type 2 immunity may be through early upregulation and release of IL-33.

A<sub>2B</sub>AR Signaling Is Essential for Initiation of the Type 2 Immune Response Shortly after Hp Inoculation

The immune response to Hp includes both innate and adaptive components that are induced shortly after infection and likely
provide an essential microenvironment supporting the development of effector and memory Th2 cells. Th2 initiating cytokines may include IL-25, TSLP, and IL-33 and can be produced by epithelial cells through as yet undetermined mechanisms of stimulation. In addition to triggering CD4⁺ Th2 cell differentiation, these cytokines also stimulate ILC2 cells (Saenz et al., 2010; Wills-Karp and Finkelman, 2011). We next examined whether initial upregulation of Th2-inducing cytokines, or possibly ILC2 cell activation, was affected by A₂BAR deficiency shortly after Hp infection. Hp larvae typically penetrate the small intestine epithelial cell barrier within 48 hr after inoculation. As shown in Figure 6A, marked increases in IL-33, but not TSLP or IL-25, were detected at 48 hr after inoculation in WT, but not in A₂BAR⁻/⁻, mice. Furthermore, elevations in IL-5 and IL-13, but not IL-4, were also markedly increased in Hp-inoculated WT controls, consistent with activation of ILC2 cells. In marked contrast, elevations in IL-5 and IL-13 were blocked at 48 hr after inoculation of A₂BAR⁻/⁻ mice (Figure 6B). To further confirm ILC2 activation in the small intestine by 48 hr after inoculation, induction of transcription factors, associated with ILC2 cell activation (Wong et al., 2012), were assessed. As shown in Figure 6C, intestinal elevations in RORγt and Id2 were decreased in A₂BAR⁻/⁻ mice compared to WT mice at 48 hr after Hp inoculation. To corroborate these findings, the specific A₂BAR antagonist, MRS1754, was administered to WT mice every 24 hr starting 3 days before Hp inoculation until thetermination of the...
experiment (day 2). As shown in Figures 6D–6F, at day 2 after inoculation, upregulation of IL-33, IL-5, IL-13, RORα, and Il2 were significantly reduced in Hp-inoculated WT mice administered MRS1754. To specifically assess ILC2 activation and cytokine expression, ILC2 cells (Lin−, IL-7Rα+, Sca-1−, c-Kit+) were electronically sorted. As shown in Figure 6G, ILC2 cells expressed IL-5 and IL-13, but not IL-4, mRNA at 48 hr after Hp inoculation of WT mice, and these increases were large inhibited in Hp inoculated A2BAR−/− mice. Similarly, increases in Il2 and RORα in ILC2 cells observed in WT mice were largely blocked in A2BAR−/− mice (Figure 6H). Flow cytometric analysis further showed that increases in ILC2 cell frequency in the small intestine after Hp inoculation were reduced in A2BAR−/− mice (Figure 6I). Taken together, these studies indicate that A2BAR signaling triggers IL-33 upregulation and the initial development of the innate type 2 response after Hp inoculation.

Exogenous Administration of IL-33 Restores the Development of Innate Type 2 Immune Response in the Absence of A2BAR Signaling

A2BAR signaling thus promotes intestinal elevations in IL-33 expression and subsequent increases in Th2 cytokines shortly after Hp inoculation. This finding raised the possibility that one mechanism through which A2BAR signaling promotes the type 2 response may be through enhanced expression of this alarm. To directly test this possibility, we next examined whether exogenous administration of IL-33 could rescue the innate type 2 immune response in Hp-inoculated A2BAR−/− mice. As shown in Figure 7A, elevations in IL-5 and IL-13 mRNA were restored in A2BAR−/− mice after Hp inoculation such that they were comparable to type 2 cytokines elevations in Hp-inoculated WT control mice. Furthermore, the ILC2-activating transcription factors, RORα and Il2, were also upregulated in small intestines of IL-33-treated A2BAR−/− mice (Figure 7B). To specifically analyze the effects of IL-33 on ILC2 cells, ILC2 cells were electronically sorted (Lin−, IL-7Rα+, Sca-1−, c-Kit+) from Hp-inoculated A2BAR−/− mice after exogenous IL-33 treatment. As shown in Figures 7C and 7D, ILC2 cells isolated from A2BAR−/− mice expressed high levels of IL-5, IL-13, RORα, and Il2 at 48 hr after Hp inoculation when they were treated with IL-33, and these increases were similar to Hp-inoculated WT mice. Collectively, these data indicate that exogenous administration of the alarm, IL-33, rescues initiation of the type 2 immune response in the absence of A2BAR signaling, including ILC2 activation and cytokine production, after Hp inoculation. ATP is a predominantly intracellular molecule; stressful and injurious events result in extracellular ATP release, and the extracellular ATP is degraded to adenosine via cell surface ectonucleotidases, including CD39 (nucleoside triphosphate diphosphohydrolase [NTPDase]) and CD73 (5′-ectonucleotidase [Ecto5′NTase]) (Yegutkin, 2008). As shown in Figure 5B, cell surface CD39 and CD73 levels were increased in the intestinal epithelial cells (IELs), but not lamina propria (LP), at 24 hr after inoculation. These findings indicate that within 24 hr after Hp inoculation, surface expression of ectonucleotidases is elevated, providing indirect evidence for increased adenosine production at this early time point.

The Immune Response to Nippostrongylus brasiliensis Is also Impaired in A2BAR−/− Mice

It was possible that the immune response to Hp preferentially required A2BAR interactions, compared to type 2 immune responses elicited by other parasites. The protective immune response to the intestinal nematode parasite Nippostrongylus brasiliensis (NB) results in parasite expulsion between 9 and
11 days after primary inoculation. To examine whether Nb egg production and parasite expulsion were influenced by A<sub>2B</sub>AR deficiency, A<sub>2B</sub>AR<sup>−/−</sup> mice and WT controls were inoculated subcutaneously (s.c.) with Nb L3. At day 9 after primary Nb inoculation, the number of intestinal parasites and the egg count were markedly elevated in A<sub>2B</sub>AR<sup>−/−</sup> mice compared to WT controls (Figure S2A). Furthermore, type 2 cytokines (Figure S2B) were significantly reduced in the small intestine of Nb-inoculated A<sub>2B</sub>AR<sup>−/−</sup> mice compared to inoculated WT controls. Taken together, these studies indicate that the protective type 2 primary immune response to the quite different intestinal nematode parasite, Nb, is impaired in A<sub>2B</sub>AR<sup>−/−</sup> mice.

**DISCUSSION**

Our findings demonstrate an essential role for adenosine interacting with A<sub>2B</sub>AR in the development of effector immune cell populations that mediate resistance against the intestinal nematode parasite Hp. They indicate that A<sub>2B</sub>AR signaling is required for the initiation of the type 2 immune response, and they further suggest that an important mechanism involves A<sub>2B</sub>AR-induced expression of the alarmin IL-33, which subsequently triggers ILC-2 and Th2 cell activation and cytokine expression. These studies identify a single DAMP required for the development of the potent and complex helmint-induced type 2 immune response, thereby indicating the importance of danger signals in inducing type 2 immunity.

The ability of Hp to trigger a potent type 2 response during the larval tissue-dwelling phase and the adult luminal phase provides a spectrum of elicited immune components, many of which contribute to worm damage and eventual expulsion. In the luminal phase, Th2 cytokine-induced increases in goblet cell secretion of mucus and Reimj impair parasite feeding and contribute to worm expulsion (Herbert et al., 2009). Our findings showed expression of these effector molecules was reduced in Hp-inoculated A<sub>2B</sub>AR<sup>−/−</sup> mice at day 11 after secondary inoculation, consistent with our findings that A<sub>2B</sub>AR signaling was required for reduced worm metabolism. The tissue-dwelling phase is characterized by a type 2granuloma, surrounding the invading larvae in the submucosa, and is composed primarily of M2 cells, neutrophils, and eosinophils (Patel et al., 2009). These studies indicate that A<sub>2B</sub>AR blockade results in an overall impairment of effector immune cell populations and associated effector molecules required for the development of a protective immune response culminating in worm expulsion. Although it remains possible that A<sub>2B</sub>AR signaling directly inhibits specific type 2 immune effector functions, the generally compromised nature of the protective response in Hp-inoculated A<sub>2B</sub>AR<sup>−/−</sup> mice raised the possibility that Th2 cytokines, required for many of these effector functions, were directly affected. A<sub>2B</sub>AR signaling in intestinal tissues has previously been shown to influence control of inflammation (Frick et al., 2009), raising the possibility that effector T cells in peripheral intestinal tissues may preferentially require adenosine and A<sub>2B</sub>AR signaling for a sustained response. It was also possible that the development of cytokine-producing Th2 cells in the draining MLN was compromised. Few studies have yet examined whether CD4<sup>+</sup> Th2 cell differentiation in vivo requires A<sub>2B</sub>AR signaling; our findings now show that MLN CD4<sup>+</sup> Th2 cell cytokine expression in vivo is reduced in Hp-inoculated A<sub>2B</sub>AR<sup>−/−</sup> mice. Furthermore, IL-4R signaling was also inhibited, as evidenced by a reduction in the frequency of MLN CD4<sup>+</sup> T cells expressing pSTAT6 (Perona-Wright et al., 2010) and also decreased B cell MHCII expression (Lu et al., 2002; Lu et al., 1996). Intriguingly, at day 11 after Hp inoculation, CD4<sup>+</sup> T cell IFN-γ mRNA and protein secretion were elevated, suggesting immune deviation toward a type 1 response—a surprising result, as the robust type 2 immune response is generally refractory to immune deviation, as observed following specific blockade of Th2 cytokines and costimulatory molecules (Gause et al., 2003). Previous in vitro studies have shown an inhibitory effect of A<sub>2B</sub>AR signaling on T cell IFN-γ production (Yang et al., 2010).

The general impairment in adaptive type 2 immunity raised the possibility that A<sub>2B</sub>AR signaling may be required for initiation of the type 2 immune response during Hp infection. Previous studies have shown that the type 2 immune response to allergenic extracts can trigger IL-33 release in the lung through ATP-binding P2 purinergic receptors (Kouzaki et al., 2011). Our studies now show that adenosine (a catabolite of ATP) signaling through the A<sub>2B</sub>AR is essential for IL-33 upregulation and associated activation of ILC2 cells following intestinal helmint infection. Hp is a large multicellular pathogen thought to potentially produce multiple factors that together trigger type 2 immunity (Gause et al., 2013). It is thus perhaps surprising that other potentially redundant parasite-associated factors cannot substitute for its absence. In future studies, A<sub>2B</sub>AR antagonists could be administered at specific time intervals to examine whether A<sub>2B</sub>AR specifically contributes to memory T cell development after primary inoculation or memory effector cell activation after secondary inoculation. Adenosine accumulates in the extracellular space following metabolic stress and cell damage (Hasko and Cronstein, 2004; Hasko et al., 2004), primarily as a result of CD39 and CD73 enzyme-mediated conversion of ATP to adenosine (Hasko et al., 2008). Adenosine is notoriously difficult to measure in vivo due to its rapid degradation by adenosine deaminase and uptake into cells through nucleoside transporters. In addition, differentiating between extracellular and intracellular adenosine in solid tissues in vivo requires employment of techniques such as microdialysis, which at this point has not been tested in a motile organ, such as the gut. Thus, CD39 and CD73 expression, which are regulated primarily by increased cell surface expression, are widely used as sentinel of adenosine production in the extracellular space (Antonioli et al., 2013). Our findings that CD39 and CD73 were elevated in worm-infected mice thus indirectly indicate increased adenosine production.

Recent studies have shown that particulate structures are sufficient to act as adjuvants promoting the development of type 2 immunity (Kuroda et al., 2011; Mishra et al., 2011), suggesting that interactions with inert structures may stress tissues in such a manner that characteristic DAMPs are released that then initiate innate and adaptive type 2 responses. Similar damage may result from migrating multicellular parasites, triggering release of characteristic DAMPs, including adenosine, which then promote type 2 immunity. Although such interactions may be necessary for the development of type 2 immunity, they may not be sufficient, and other factors released by the migrating parasite, including putative pathogen-associated molecular
patterns (Reese et al., 2007) and cytokine mimics (Grainger et al., 2010; Hewitson et al., 2009), may further influence the course of the type-2 immune response. It was also possible that the immune response to Hp was uniquely dependent on A<sub>2B</sub>AR signaling and that other parasites may trigger additional initiating signals for type-2 immunity that may substitute for this specific AR. However, our findings that the immune response and associated resistance to Nb was similarly impaired in A<sub>2B</sub>AR<sup>−/−</sup> mice indicate a general significance of adenosine interacting with this specific receptor for the development of the helminth-induced type-2 immune response. Previous studies have also suggested that trefoil factor 2 (TFF2) contributes to IL-33 upregulation and resistance to Nb (Wills-Karp et al., 2012). In future studies, it will be interesting to examine possible interactions between TFF2 and adenosine-A<sub>2B</sub>AR signaling at early stages of the type-2 immune response, as both ligand interactions appear to play a significant role in the generation of this helminth-induced response.

While adenosine has been previously shown to be involved in recruitment of immune cells to the site of injury (Haskó et al., 2008; Schnurr et al., 2004), other studies have suggested that it generally plays a protective role by dampening inflammatory responses following tissue injury, in this way promoting wound healing and homeostasis (Linden, 2001; Macedo et al., 2007). Similarly, several studies now suggest that a number of components of the type-2 immune response also promote tissue repair, thereby enhancing the capability of the host to tolerate invading multicellular parasites (Chen et al., 2012; Gause et al., 2013). It may be that adenosine functions as an essential DAMP, triggered through tissue damage, that initiates the development of a type-2 immune response during helminth infection that then contributes to tissue repair and associated homeostasis. Recent findings that Trichinella-secreted products can block nucleotide-mediated mast cell activation suggest that helminths may target this signal as an evasion mechanism (Afferson et al., 2012). Uric acid may also function as a DAMP to trigger type-2 immunity during allergic lung inflammation, and its release is associated with tissue stress and hypoxia (Kool et al., 2011). Although the mechanism through which uric acid triggers type-2 immunity is uncertain, recent studies suggest it occurs independently of purinergic receptors (Kool et al., 2011). Our studies indicate an important functional link between adenosine signaling through the A<sub>2B</sub>AR and upregulation of IL-33, an important inducer of type-2 immunity during helminth infection (Hung et al., 2013), thereby suggesting a potential mechanism for A<sub>2B</sub>AR signaling in initiating the type-2 immune response. Indeed IL-33 administration effectively restored the type-2 primary response in Hp-inoculated A<sub>2B</sub>AR<sup>−/−</sup> mice. A<sub>2B</sub>AR signaling on individual effector cells, such as M2 macrophages, may thus not be essential in the context of helminth infection. It should also be noted that in future studies it will be important to examine whether immune regulatory components of the helminth response, including immune cell expression of IL-10 and TGF-β, are also influenced by A<sub>2B</sub>AR signaling, particularly given recent studies suggesting that these regulatory cytokines may be induced independently of the canonical type-2 immune response (Hübner et al., 2012; Mishra et al., 2013).

In summary, our studies suggest that specific interactions of adenosine with the A<sub>2B</sub>AR receptor are essential for the optimal development of the potent and highly polarised type-2 immune response to the intestinal nematode parasite Hp. They raise the possibility that adenosine functions as a DAMP that plays a significant role in the development of type-2 immunity during helminth infection.

**EXPERIMENTAL PROCEDURES**

**Mice**

C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, Maine). A<sub>2B</sub>AR<sup>−/−</sup> BL/6 mice were bred and housed in a specific pathogen-free animal facility in the Comparative Medicine Resources center at New Jersey Medical School (NJMS). Four to five mice were used per treatment group, if not otherwise indicated. These studies conform to the principles for laboratory animal research outlined by the Animal Welfare Act and the Department of Health, Education, and Welfare (National Institutes of Health) guidelines for the experimental use of animals and were approved by the IACUC at New Jersey Medical School.

**Parasite Inoculation, Length, Burden, IL-33 Administration, and A<sub>2B</sub>AR Antagonist Treatment**

Mice were inoculated perorally with 200 infective Hp L3 using a rounded gavage tube, and adult worm numbers and egg production were quantitated as described previously (Anthony et al., 2006). An antihelminthic drug, pyrantel pamoate (1–2 mg), was administered orally to expulse Hp adults from the gut after primary infection and prior to secondary challenge infection. For adult parasite burdens, worms were collected from the small intestine and egg burdens determined from fecal contents, as previously described (Anthony et al., 2006). Developing larvae were also sexed and measured in the small intestine 7 days after Hp primary and secondary inoculations as previously described (Li et al., 2010). A<sub>2B</sub>AR<sup>−/−</sup> mice were injected intraperitoneally (i.p.) with 1 μg IL-33 (R&D Systems) daily starting from 1 day before Hp inoculation until the day of sacrifice. The specific A<sub>2B</sub>AR antagonist N-[4-cyanophenyl]-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl)phenoxyl]acetamide (MRS1754) (1 mg/kg body weight) was injected i.p. daily (Németh et al., 2007). In a separate experiment, mice were inoculated s.c. with infective Nb L3. Parasite egg numbers and adult worm numbers were evaluated as above.

**Total ATP Assay and Protein Measurement**

Five to ten male and female worms were isolated after Hp inoculation of WT and A<sub>2B</sub>AR<sup>−/−</sup> mice, incubated in 100 μl RPMI1640 with 100 μl Cell-Glo reagents (Promega, Madison), and ground with a motorized pestle. After centrifugation at 5,300 rpm for 5 min, 100 μl supernatant was transferred to wells of 96-well plate and luminescence measured in 1420 multilabel counter luminometer (Perkin Elmer, Waltham). Controls included the following: PBS alone, heat killed worms; worms incubated without Cell-Glo reagent. For protein measurement, worms were isolated in 100 μl RPMI1640, and the mixture ground with a motorized pestle. Parasite extract was centrifuged at 5,300 rpm for 5 min, and total proteins from parasite extracts measured by NanoDrop 2000 Spectrophotometer (Thermo scientific, Wilmington).

**Real-Time PCR, Serum Immunoglobulins, and ELISPOT**

Total RNA was prepared from tissues or sorted cells and then reverse transcribed. Real-Time PCR kits (PE Applied Biosystems, Foster City), specific for individual cytokines or ribosomal RNA, were used to quantify differences in gene expression. All data were normalized to constitutive ribosomal RNA values and expressed as mRNA fold changes relative to untreated controls, as previously described (Mishra et al., 2013). Total serum IgE and IgG1 levels were measured by ELISA, and the frequency of IL-4 and IFN-γ-producing cells was determined by ELISPOT assay as previously described (Mishra et al., 2013).

**Fluorescent Immunohistochemistry and Histology**

Cryosections were stained for macrophages (F4/80-Alexa 488, green; Caltag Laboratories, Carlsbad), IL-4Rs (mIg-4R-M1-PE, red; BD Pharmingen, San Jose), and eosinophils (anti-MBP-Alexa Fluor 488, green; Mayo Clinic, Scottsdale). Small intestine sections were formalin fixed and embedded in paraffin, and 5–6 μm sections were stained with H&E and PAS. Using previously developed computer programs, we measured the area of each section stained positive for each stain with a light microscope (Nikon, Japan) and a camera coupled to a computer (Zeiss Axios, Germany). The intensity of color was measured using Image J (NIH).
analyzed using FlowJo software (Treestar, Ashland). Cells were lysed with ACK lysing buffer to remove erythrocytes, and 0.1 × 10^7 cells were blocked with Fc Block (BD PharMingen); and stained with anti-CD4-FITC, anti-Sca-1-PE, anti-c-Kit-allophycocyanin (APC), and anti-CD127 (IL-7Rα)-PerCP-cy5.5. Cells negative for Lin and positive for IL-7Rα/C0 were sorted with FACS ARIA 2 cell sorter (BD Biosciences). Phosphorylation of STAT6 at tyrosine 641 was detected by intra-cellular staining with PE-conjugated anti-phospho-STAT6 using Flow cytometry. MDL CD4 cells were cultured and prepared from Hp1- and Hp2-inoculated WT and eBioscience), and anti-CD127 (IL-7Rα)-PECy5.5 (A7R34, BD PharMingen), anti-CD39-Alexa Fluor 647 (24DMS1, BD PharMingen), anti-c-Kit-allophycocyanin (APC) (2B8, BD PharMingen), anti-Gr1- FITC (RB6-8C5, BD PharMingen), anti-CD3- FITC (145-2c11, BD PharMingen, San Jose), anti-B220- FITC (AR3-6B2, BD PharMingen), and anti-MHCII-PE (IAb/CD14/CD10/C0, BD PharMingen; and stained with anti-CD4-FITC (RM4-5, BD PharMingen), anti-pSTAT6-PE (pY641, BD PharMingen), anti-B220-FITC (AR3-6B2, BD PharMingen), and anti-MHCII-PE (IAb, BD PharMingen). Phosphorylation of STAT6 at tyrosine 641 was detected by intra-cellular staining with PE-conjugated anti-phospho-STAT6 using Flow cytometry. MDL CD4+ and CD4- cells were isolated from small intestines, as previously described (Anthony et al., 2006). For sorting ILCs, LP cells were isolated from small intestines, as previously described (Lycke, 2001). Cells were acquired on a FACSCalibur or LSRII Flow cytometer (BD Biosciences, San Jose), and Buffer I and Perm Buffer III reagents (BD PharMingen). Phosphorylation of STAT6 at tyrosine 641 was detected by intra-cellular staining with PE-conjugated anti-phospho-STAT6 using Flow cytometry. MDL CD4+ and CD4- cells were isolated from small intestines, as previously described (Anthony et al., 2006). For sorting ILCs, LP cells were isolated from small intestines, as previously described (Lycke, 2001). Cells were acquired on a FACSCalibur or LSRII Flow cytometer (BD Biosciences, San Jose), and analyzed using FlowJo software (Treestar, Ashland).}

Sorting of CD4+ and Innate Lymphoid Cells

MLN CD4+ and CD4- cells were isolated from MLN using anti-CD4 micro beads, as previously described (Mishra et al., 2011). For sorting ILCs, LP cells were isolated from small intestines, as previously described (Lefrancois and Lycke, 2001), and stained with anti-Lin-FITC, anti-Sca-1-PE, anti-c-Kit-allophycocyanin (APC), and anti-CD127 (IL-7Rα)-PerCP-cy5.5. Cells negative for Lin and positive for IL-7Rα/C0 were sorted with FACS ARIA 2 cell sorter (BD Biosciences).

Statistical Analyses

Data were analyzed using Sigmaplot 12 (SYSTAT Software) and are reported as means ±SEM. Differences between multiple groups were assessed by one-way analysis of variance, and individual comparisons were analyzed using protected Fisher-LSD tests. Differences of p < 0.05 were considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2014.02.001.


