Differential Requirement for A2a and A3 Adenosine Receptors for the Protective Effect of Inosine In Vivo

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ABSTRACT

Inosine is an endogenous nucleoside with immunosuppressive properties that is known to inhibit the accumulation of pro-inflammatory cytokines and protect mice from endotoxin-induced inflammation and lung tissue damage. There are no known receptors specific for inosine, but A3 adenosine receptors (A3R) have been shown to bind inosine resulting in mast cell degranulation and increased vascular permeability. The present study specifically addresses the requirement for A2aR and/or A3R for the protective effect of inosine in two experimental in vivo models of inflammatory disease. The data show that A3R is essential for protection against ConA-induced fulminant hepatitis since only A3R-expressing mice were protected by inosine while wild type and A2aR-deficient mice exhibited severe liver damage even after administration of inosine. In addition, we show in a model of LPS-induced endotoxemia that inosine protected both A2aR-/- and A3R-/- mice from inflammation, but not A2aA3R-double null mice indicating that in this model both A2aR and A3R were employed by inosine. Thus, we demonstrate that A2a and A3 adenosine receptors are differentially utilized by inosine for the down-regulation of tissue damage under different inflammatory conditions in vivo.

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INTRODUCTION

Inflammatory processes are crucial for defense against pathogens, but inappropriate and/or prolonged inflammation contributes to the pathogenesis of major diseases. Therefore, identification of endogenous molecules that are capable of inhibiting activated immune cells is important in order to better understand the physiological mechanisms that regulate inflammation and to develop novel targets for anti-inflammatory drugs.

The demonstration that the extracellular adenosine A2aR participates in an endogenous immunosuppressive loop that serves to downregulate inflammation in vivo by a non-redundant mechanism heightened the interest in naturally occurring purines in the regulation of inflammation and tissue protection.

It is known that adenosine possesses anti-inflammatory properties and that adenosine analogs can protect mice from a variety of inflammatory diseases such as septic shock and rheumatoid arthritis by inhibiting the release of free radicals and pro-inflammatory cytokines from different immune cells. Relevantly, the selective A2aR agonist CGS21680 was shown to protect liver from ischemia/reperfusion injury in rats and from ConA-induce tissue damage in mice. The attractiveness of an adenosine-mediated anti-inflammatory pathway has been enhanced by the realization that widely used anti-inflammatory drugs, such as methotrexate and sulfasalazine, seem to exert their effects by inducing the release of endogenous adenosine, which then signals through adenosine receptors. Thus, the triggering a “natural” anti-inflammatory pathway by ligands of adenosine receptors represents an attractive immunomodulation strategy for the treatment of inflammatory diseases.
Inosine is an anti-inflammatory endogenous nucleoside that can be considered a natural trigger of adenosine receptors. It is formed as a product of adenosine deamination\textsuperscript{11} and is released into the extra-cellular space at times of cellular stress (e.g. systemic inflammation\textsuperscript{12} and hypoxia\textsuperscript{13}) when metabolism of adenosine is high. Indeed, while normal interstitial concentrations of inosine have been reported to be in the micromolar range, concentrations greater than 1mM in ischemic tissues and in patients with sepsis have been documented.\textsuperscript{14-18} Inosine is now known to exert wide-ranging anti-inflammatory effects that include inhibition of pro-inflammatory cytokine and chemokine production, enhancement of anti-inflammatory cytokine IL-10 production and protection from endotoxin-induced inflammation and lung tissue damage\textsuperscript{19-22} as well as skeletal muscle reperfusion injury\textsuperscript{23} in mice. In humans, inosine has shown promise as a therapeutic agent against multiple sclerosis (MS)\textsuperscript{24} and Tourette syndrome\textsuperscript{25} perhaps by indirectly increasing uric acid levels and acting as a dopamine agonist, respectively. Thus, inosine may have wide-ranging biological effects with potentially beneficial implications in humans underscoring the need to understand the molecular mechanisms of inosine action \textit{in vivo}.

Importantly, inosine has no known specific receptors(s); however, it has been shown to utilize cAMP-inhibiting Gi coupled A3Rs. In functional and radioligand binding \textit{in vitro} assays, Jin et al.\textsuperscript{26} showed that inosine in the range of 10-50µM binds to and activates A3Rs on stably transfected HEK-293 cells, RBL-2H3 rat mast-like cells, perivascular mast cells of hamster cheek pouch arterioles, and in guinea pig lung membranes. In addition, Tilley et al.\textsuperscript{27} have used mice lacking either A3Rs or mast cells
to show that A3Rs are required for the inosine-mediated potentiation of bone marrow
mast cell degranulation and promotion of plasma protein extravasation.

The question, however, remained as to whether the anti-inflammatory effect of
inosine was mediated by A3R activation alone or if inosine also signaled through other
subtypes of adenosine receptors. Indeed, while the competitive binding studies with
recombinant rat and guinea pig adenosine receptors subtypes have indicated that inosine
does not bind A1Rs or A2aRs on mast cells, it was suggested by Hasko and
colleagues that inosine-mediated suppression of TNFα production from LPS-stimulated
peritoneal murine macrophages was significantly abrogated in the presence of
pharmacological antagonists of A1 or A2a receptors. Although it was not determined in
this study if inosine acted directly or indirectly by altering adenosine levels these data
supported the possibility that inosine could trigger immunosuppressive signaling through
A1 or A2a receptors.

To determine which adenosine receptors, A2aR or A3R, were responsible for the
effects of inosine, we studied acute inflammatory damage in in vivo models of
autoimmune and viral hepatitis and endotoxin-induced sepsis using mice genetically
deficient in A2aR or A3R and A2aA3R double-deficient mice. In addition, studies of
these acute inflammation models allowed us to compare the effect of inosine on the
pathogenesis of diseases with very different initiating stimuli (polyclonal TCR activator
ConA vs Toll-like receptor activating LPS) and different types of immune cells.

In mice, ConA-induced liver damage in mice is a well-described in vivo
inflammation model of viral and autoimmune hepatitis that is mediated by T-cells, NK-T
cells, macrophages, neutrophils, and a variety of cytokines and chemokines including
tumor necrosis factor-α (TNFα), interleukin-4 (IL-4), interferon gamma (IFNγ), and macrophage inflammatory protein-2 (MIP-2) \(^{28-34}\) while endotoxin-induced shock involves mostly the activation of toll-receptors on macrophages.\(^{35-37}\) Importantly, these different cells may differ in their repertoire of expressed adenosine receptors.

The experimental data provided in this study show that A3R was essential for the inhibition of ConA-induced liver injury by inosine since the accumulation of pro-inflammatory cytokines (e.g. TNFα) and alanine transaminase was inhibited only in A3R-expressing mice, while A2aR-deficient mice had elevated levels. This was supported by histological evidence of inosine inhibiting hepatocyte apoptosis in A3R+/+ but not A3R-/- mice. In addition, the data show that LPS-induced TNFα production was inhibited by inosine in wild type, A2aR-/-, and A3R-/- mice. However, inosine was ineffective in blocking TNFα production in LPS-challenged A2aA3R-/- double-knockout mice indicating that both A2aR and A3R were employed by inosine to inhibit endotoxin-induced inflammation.

These data provide the first genetic evidence that inosine differentially utilizes A2a and A3 adenosine receptors to exert its anti-inflammatory properties, and indicate that different adenosine receptors are employed by inosine in T cell-dependent versus T cell-independent acute inflammation. Accordingly, we propose that employment of A2a and A3 adenosine receptors by the endogenous nucleoside inosine should be considered in comparative studies of mechanisms of acute inflammation with different etiology. The A2a adenosine receptors have been conclusively implicated in the down-regulation of inflammation \(\textit{in vivo}\)\(^{3}\) and the data reported here point to the need to include inosine
among those endogenous molecules that influence inflammatory responses by directly or indirectly triggering A2a and/or A3 adenosine receptors \textit{in vivo}.
Materials and Methods

Mice

Eight to twelve week old age-matched male mice were used. All mice were on a C57BL/6 background. The A2aR-/- and A3R-/- mice were backcrossed at least ten times and the A2aA3R-/- double knockout mice were backcrossed two times. A2aR and A3R gene-deficient mice are described in Chen et al.\(^ {38}\) and Salvatore et al.\(^ {39}\), respectively. A2aA3R double-deficient mice were generated by A2aR-/- x A3R-/- breeding. IL-10+/+ and IL-10-/- mice were purchased from Taconic (Germantown, NY). All mice were maintained under specific pathogen free conditions at NIH animal care facilities.

Reagents and Drugs

Inosine, LPS (from *Escherichia coli*, serotype 055:B5), and ConA were purchased from Sigma (St. Louis, MO). The A2aR agonist CGS21680 and A3R and A1R agonist 2-Cl-IB:MECA were purchased from Tocris Cookson, Inc. (Ballwin, MO).

LPS Study

Mice were injected intraperitoneally with LPS (10mgkg\(^{-1}\)) +/- inosine (100mgkg\(^{-1}\)) in sterile PBS. Inosine was injected 10min. prior to LPS. For pharmacological activation of A2aR or A3R, the agonists CGS21680 and 2-CL-IB:MECA were injected intraperitoneally at 0.5mg/kg 10min. before injection of LPS. Blood was collected from the retro-orbital vein one hour after LPS injection and TNF\(\alpha\) levels were determined from the isolated serum.
Liver Injury Study

Mice were injected intravenously (tail vein) with the indicated dose of ConA ($15\text{mgkg}^{-1}$ or $20\text{mg/kg}^{-1}$), inosine ($100\text{mgkg}^{-1}$), or a mixture of ConA plus inosine in sterile PBS. Blood was collected at 1h and 24h from the retro-orbital vein and TNF$\alpha$ and ALT levels were determined from the isolated serum. Mice were sacrificed 24h after injection and liver samples were taken for tissue histopathological evaluation.

TNF$\alpha$ and ALT Determination

TNF$\alpha$ was measured in the serum collected one hour after the indicated challenge using ELISA kits purchased from R&D Systems (Minneapolis, MN). Detection limits were <5.1 pg/ml for TNF$\alpha$. Serum alanine transaminase (ALT) was measured from serum collected 24hr after challenge with ConA, inosine, or ConA + inosine using kits purchased from Sigma (St. Louis, MO). The assays were performed according to the manufacturer’s instructions.

Histology

Samples of liver tissue from sacrificed mice were taken and fixed in formalin. In situ staining of single strand breaks in nuclear DNA to detect apoptotic cells (TdT assay) and haematoxylin and eosin (H&E) staining were done at Molecular Histology, Inc. (Montgomery Village, MD).
Results

Inosine utilizes only A3 adenosine receptors to inhibit ConA-induced liver damage in a fulminant hepatitis model in vivo

To determine whether A2a or A3 receptors or both are required for the inosine-mediated inhibition of inflammation in vivo, we compared the effect of inosine on ConA-challenged wild type, A2aR-/-, A3R-/-, and A2aA3R-/- mice. Given that inosine had been shown in published studies to inhibit inflammatory responses in vivo when administered at 100mg/kg, we chose this concentration of inosine in these and subsequent experiments. Following injection of ConA and/or inosine the levels of alanine transaminase (ALT) and of the pro-inflammatory cytokine TNFα in the serum were analyzed as a measure of intensity of the inflammatory process and of liver damage.

Many different cytokines (e.g. IFNγ, IL-4, TNFα) were shown to be involved in ConA-induced liver injury in experiments using different gene deficient and transgenic mice. As shown in Figure 1A and B, ConA challenge resulted in severe liver damage in wild type mice as assessed by high levels of ALT and TNFα in the serum. Inosine, which alone did not induce liver damage, completely inhibited ALT and TNFα accumulation in wild-type mice when co-injected with ConA. The observed inhibition of liver damage by inosine was dependent on the presence of A3 adenosine receptors as evidenced by experiments where the effect of inosine on ConA-induced liver damage and inflammation was compared between A2aR-/-, A3R-/-, and A2aA3R-/- mice. It was found that both the extent of liver damage (ALT levels) and TNFα levels were inhibited by inosine regardless of the absence of the A2aR (Figure 1C and D) indicating that A2a
receptors were not required for inhibition by inosine in this model. In contrast, mice deficient in A3R were resistant to the anti-inflammatory effect of inosine, and high levels of ALT and TNFα were detected in these mice after challenge with ConA (Figure 1E, F, G, and H), thus, indicating that A3Rs were necessary and required for inhibition of liver damage by inosine.

![Figure 1. A3 adenosine receptors are required for the inhibition of ConA-induced ALT and TNFα by inosine.](image)

C57BL/6 (A and B), A2aR-/- (C and D), A3R-/- (E and F), and A2aA3R-/- (G and H) mice were injected with ConA, inosine (100mgkg⁻¹), or both and the level of TNFα and ALT in the serum at 1 and 24 hours, respectively, after challenge was determined. Because excessive inflammation is observed in the absence of the A2aR³, A2aR-/- and A2aA3R-/- mice were injected with 15 mgkg⁻¹ while the C57BL/6 and A3R-/- mice were injected with 20mgkg⁻¹ ConA. This figure shows that inosine inhibited ConA-induced hepatitis only in mice expressing the A3R. Representative results from one of three separate experiments.

Since inosine had been shown to induce production of the anti-inflammatory cytokine IL-10¹⁹, which was reported to have protective effects over ConA-induced
hepatitis\textsuperscript{40,41}, we compared IL-10+/+ and IL-10-/\- mice for susceptibility to inhibition of liver injury by inosine. Figure 2 shows that ALT accumulation was inhibited by inosine in both IL-10+/+ and IL-10-/\- mice indicating that inhibition of liver injury by inosine in vivo was independent of IL-10. Thus, inosine inhibited ConA-induced fulminant hepatitis through an IL-10-independent but A3R- dependent mechanism.

Figure 2. Inosine inhibits ConA-induced liver injury by an IL-10 independent mechanism. IL-10+/+ (A) and IL-10-/\- (B) mice were injected with ConA (20mgkg\textsuperscript{-1}), inosine (100mgkg\textsuperscript{-1}), or both and serum ALT was determined 24 hours after injection. This figure shows that inosine inhibited ConA-induced ALT regardless of the lack of IL-10 production in IL-10-/\- mice. Representative results from one of three separate experiments.

Inosine protects liver tissue by inhibiting ConA-induced hepatocyte apoptosis.

The A3R-dependent anti inflammatory effect of inosine in the ConA model of hepatitis was further demonstrated by histopathological examination of tissue sections of liver taken from A3R+/+ and A3R-/\- mice. Figure 3 shows ConA induced severe liver damage in both A3R+/+ and A3R-/\- mice, but that only A3R+/+ mice were rescued from
liver damage by inosine as assessed by haematoxylin and eosin staining. It is shown that livers from A3R+/+ and A3R-/- mice sustained severe damage from ConA (Figure 3A and D, respectively) but no gross damage from inosine alone (Figure 3B and E, respectively). Moreover, Figure 3C and F, respectively, show that liver tissue from A3R+/+, but not A3R-/-, mice was protected from ConA–induced damage in vivo by inosine.

Figure 3. Inosine protects A3R+/+ liver tissue from ConA-induced damage in vivo. Tissue sections of liver taken from A3R+/+ (A, B, and C) and A3R-/- (D, E, F) mice 24 hours after injection with ConA (A and D), inosine (B and E), and ConA plus inosine (C and F) were stained with haemotoxylin and eosin (H&E). This figure shows that inosine protected A3R+/+, but not A3R-/-, liver from ConA-induced damage. 10X magnification.
Since the apoptosis of hepatocytes had been suggested to be involved in ConA-induced liver injury\(^{33}\) we analyzed the effect of inosine on hepatocyte apoptosis in livers from ConA-challenged A3R+/+ and A3R-/- mice by \textit{in situ} staining of single-strand breaks in nuclear DNA (TdT assay). Figure 4 demonstrates that livers of both A3R+/+ and A3R-/- mice had large numbers of apoptotic cells 24 hours after ConA challenge (Figure 4A and D), but not with inosine treatment alone. In addition, it shows that hepatocyte apoptosis was inhibited in A3R+/+ mice that had been co-injected with inosine as shown in Figure 4C. In contrast, ConA-induced hepatocyte apoptosis was not inhibited by inosine in A3R-/- mice (Figure 4F).

\textbf{Figure 4. Inosine inhibits ConA-induced hepatocyte apoptosis in vivo} Tissue sections of liver taken from A3R+/+ (A, B, and C) and A3R-/- (D, E, F) mice 24 hours
after injection with ConA (A and D), inosine (B and E), and ConA plus inosine (C and F) were assessed for apoptosis by staining single strand breaks in nuclear DNA to detect apoptotic cells (TdT assay). This figure shows that inosine protected A3R+/+, but not A3R−/−, liver from ConA-induced damage by inhibiting hepatocyte apoptosis. 10X magnification.

Taken together, these experiments show that inosine inhibited T cell-triggered inflammatory processes in liver and protected mice from tissue damage by signaling through A3 receptors to inhibit hepatocyte apoptosis. Importantly, they also show that inosine did not utilize A2a receptors in the inhibition of the pathogenesis of acute liver inflammation studied here.

**Inosine acts through both A2a and A3 adenosine receptors to inhibit endotoxin-induced inflammation in vivo.**

To determine if the mechanism by which inosine inhibited LPS-induced inflammation was also dependent only on A3R, we compared the effect of inosine on wild type, A2aR-null, A3R-null, and A2aA3R-double null mice in an *in vivo* model of LPS-induced endotoxemia. In these experiments, mice were pre-treated with inosine (100mg/kg) followed by an injection of LPS (10mg/kg). The mice were bled 1 hour after LPS injection and the concentration of TNFα in the serum was measured. As expected, LPS-stimulated TNFα was inhibited in wild-type mice as shown in Figure 5 (A and C). In addition, we found that wild type mice when injected with 60mg/kg of inosine alone had 85% mortality by 24hrs whereas 15% of mice injected with LPS plus inosine died within the same time frame (data not shown). Thus, inosine protected mice over a broad range of inflammatory insult. Importantly, the accumulation of TNFα was inhibited even in the absence of A2aR or A3R in single gene knockouts (Figs. 5B and D), suggesting that
inosine used both A2a and A3 receptors subtypes to inhibit endotoxin-stimulated TNFα production in vivo.

To test this, we developed and studied A2aA3R⁻/⁻ double-knockout mice. As predicted from the hypothesis that both A2a and A3 receptors are involved in mediating the anti-inflammatory signaling by inosine, the A2aA3R⁻/⁻ mice were unresponsive to the inhibitory effect of inosine and maintained high levels of LPS-induced TNFα production in vivo as compared to wild-type littermates (Figure 5E and F). These results thus prove that inosine utilized both A2a and A3 receptor subtypes to inhibit endotoxin-induced inflammation in vivo in this model of LPS-induced endotoxemia.
Figure 5. Inosine utilizes both A2aR and A3R to inhibit LPS-induced TNFα in vivo. Mice were injected intraperitoneally with 100mgkg\(^{-1}\) inosine followed by 10mgkg\(^{-1}\) LPS and the level of TNFα in the serum collected one hour after LPS injection was assessed. This figure shows that inosine inhibited LPS-induced TNFα production in wild type (A, C, and E), A2aR-/- (B), and A3R-/- (D) mice, but did not inhibit TNFα in A2aA3R-/- (F) mice, which lacked both receptors. Representative results from one of three separate experiments.

Pharmacological activation of A2a or A3 adenosine receptors inhibits endotoxin-induced TNFα production in vivo.

The data described above firmly implicate A2a and A3 receptors in the anti-inflammatory effects of inosine. These receptors have been suggested to be anti-inflammatory in earlier studies of the inhibitory effects of adenosine and pharmacological agonists of both A2aR and A3R on pro-inflammatory cytokine production from monocytes/macrophages and mast cells.\(^4,5,42-47\) It was important to confirm these pharmacological observations by genetic in vivo experiments to allow the conclusive implication of individual subtypes of adenosine receptors in the anti-inflammatory effect of inosine.

Therefore, to verify that it was the activation of A2a or A3 receptors that caused the inhibition of LPS-induced TNFα production in vivo, we treated wild type and A2aR-deficient or A3R-deficient mice with selective pharmacological agonists to A2aR or A3R followed by challenge with LPS. It is shown in Figure 6, that injection of the A2aR agonist CGS21680 or A3R and A1R agonist 2-Cl-IB:MECA into wild type mice resulted in decreased TNFα levels in blood serum following lethal injection of LPS (Figure 6A and C). In addition, Figure 6 shows that CGS21680 had no effect in A2aR-/- mice (Figure 6D) and, likewise, 2-Cl-IB:MECA had no effect in A3R-/- mice (Figure 6C) confirming earlier studies by Salvatore et al.\(^{39}\) Thus, the data presented here provide the
genetic proof that both A2a and A3 receptor subtypes play a role in the negative regulation of LPS-induced TNFα, and support our observation that inosine utilized both receptor subtypes to negatively regulate endotoxin-induced inflammation in vivo. These data also confirm the selectivity of tested pharmacological agonists in the assays of acute inflammation used in these experiments.

Figure 6. Pharmacological activation of A2aR or A3R inhibits LPS-induced TNFα production in vivo. A2aR+/+ (A) and A2aR -/- (B) mice were injected with 0.5mgkg⁻¹ of CGS21680, and A3R+/+ (C) and A3R -/- (D) mice were injected intraperitoneally with the 0.5mgkg⁻¹ of 2-Cl-IB:MECA. Ten minutes later the mice were injected with 10mgkg⁻¹ LPS. The mice were bled one hour after LPS injection and the level of TNFα in the serum was measured. This figure shows that LPS-induced TNFα was inhibited by CGS21680 or 2-Cl-IB:MECA in A2aR+/+ (A) and A3R -/- (C) mice, respectively. However, these adenosine receptor specific agonists did not affect A2aR -/- and A3R -/-
mice (B and D, respectively). Representative results from one of three separate experiments.
Discussion

In this report, we addressed the issue of which adenosine receptors were essential for the in vivo protective anti-inflammatory effect of the endogenous purine inosine. This was important since the biochemical and pharmacological evidence to support or refute the use of adenosine receptors other than the A3R by inosine was conflicting. Therefore, genetic in vivo testing in assays of inflammatory tissue damage was required to resolve the issue. The question as to which adenosine receptor(s) is/are utilized functionally by inosine was intriguing given that inosine was reported to induce mast cell degranulation (via A3R binding)\textsuperscript{26,27,39}, a pro-inflammatory event, yet inosine inhibited endotoxin-induced pro-inflammatory cytokine production from macrophages, an anti-inflammatory event, and protected mice from endotoxemia.\textsuperscript{19} This ability of inosine to trigger both pro- and anti-inflammatory actions suggested to us that inosine utilizes more than one type of adenosine receptor.

Our study specifically addressed the requirement for A2a and/or A3 adenosine receptors for the protective effect of inosine in two experimental in vivo murine models of disease. Our experimental data show clearly that A3R was required for the inosine-mediated inhibition of ConA-induced liver damage since wild type and A2aR-/-, but not A3R-/- or A2aA3R double knockout mice were rescued from inflammatory damage. The data also show that the requirement for adenosine receptors in the LPS model studied was different than in the ConA model. In this case, the data show that both A2aR and A3R were employed in protecting mice from LPS-induced endotoxemia since both A2aR and A3R single-knockout mice, but not A2aA3R double-knockout mice, were protected by inosine in this model. Based on these data, we concluded that both A2a and A3
adenosine receptors were utilized for inosine to protect tissue from excessive inflammatory damage \textit{in vivo}. Furthermore, we concluded that the type of inflammatory stimuli determines the overall requirement for one or both receptors.

The possibility that the observed protective effect was a consequence of an indirect action of inosine should be considered. It was possible that the anti-inflammatory effect of inosine was a consequence of indirect activation of A3 receptors leading to the release of mast cell mediators including ATP, which might be converted to adenosine and activate other adenosine receptors, and histamine, which has been shown to modulate cytokine production.\textsuperscript{48} Indeed, our data showing that only A3R-expressing mice were protected from ConA-induced liver damage might be suggestive of such an indirect effect of inosine rather than a direct activation of A2a receptors. However, if this were the case then one would expect similar results in the model of LPS-induced endotoxemia as in the ConA experiments such that only mice expressing A3R would be rescued from inflammatory damage by inosine. That is, if the observed effect of inosine is due to the release of anti-inflammatory mediators from mast cells following activation of A3 receptors by inosine then only mice expressing A3 receptors should be protected from inflammation following injection of inosine. In fact, this was not the case since A3R/- mice were rescued from inflammation in our \textit{in vivo} model of LPS-induced endotoxemia. Moreover, it was only when both A2a and A3 receptors were absent that mice were unprotected from inflammatory damage caused by LPS in this model.

It was also possible that inosine had systemic effects that influenced the observed experimental results. Inosine had been shown to have positive effects on the cardiovascular system and on neuronal growth.\textsuperscript{49,50,51} Perhaps, most interesting is the
possibility that alterations in cellular metabolic processes or of inosine itself were responsible for our observations. In a very recent article Scott, et al.\(^5^2\) reported that administration of inosine or inosinic acid suppressed clinical signs of experimental allergic encephalomyelitis (EAE) and promoted recovery from the disease in their model. Importantly, they showed that administration intraperitoneally of inosine or inosinic acid resulted in a transient, minor increase in serum levels of inosine but a strong increase in uric acid. In addition, they showed that uric acid but not inosine accumulated in the spinal cord tissue of mice with EAE fed inosine or inosinic acid. The authors concluded that the mode of action of inosine and inosinic acid on EAE was due to its metabolism to uric acid. This data suggest that metabolism of inosine might be responsible for the observations reported here. However, if the protective effect of inosine in our studies was due to an indirect consequence of its effect on a non-immune system rather than a direct activation of adenosine receptors then one might expect the same or no requirement for adenosine receptors under different inflammatory conditions. In our study, there was clearly a differential requirement for both A2a and A3 receptors in the models studied. Nevertheless, it remains to be determined if direct administration of uric acid exerts the same protective anti-inflammatory effect as inosine. If so, does uric acid have the same requirement for A2a and A3 adenosine receptors as reported here for inosine?

Although the possibility exists that activation of A2a receptors was an indirect consequence of the effect of inosine on A3 receptors, the data from the LPS model of endotoxemia clearly show the independent participation of both A2aR and A3R in response to inosine \textit{in vivo}. 
The differences in the effect of inosine on the different inflammatory models have important mechanistic implications. The obtained results are consistent with the hypothesis that different inflammatory stimuli will result in recruitment of different types of immune cells with different a repertoire of purinergic receptors. Accordingly, it is the type of immune cells and the expression of different subtypes of adenosine receptors that will determine the final outcome of regulation by extracellular purines, including inosine. For example, ConA-induced liver injury is triggered by activation of T cell receptors by ConA and the resulting hepatocyte death is mediated by the complex interplay of T cells, NK-T cells, neutrophils, Kupffer cells and cytokines such as IFN-γ, TNF-α, IL-4, IL-12, IL-18 as well as nitric oxide and other molecules. On the other hand, LPS-induced endotoxemic processes are triggered by activation of Toll-like receptors mostly on macrophages and other cells, but not T cells. It remains to be determined in detailed future studies as to why the activation of either A2a or A3 receptors was sufficient for inhibition of LPS-induced inflammation, while only A3 receptors were required the inhibition of ConA-induced liver damage. At least one explanation may be that there are differences in the time-course of up-regulation of A2aR and/or A3R on T cells versus macrophages and neutrophils during the course of ConA-induced or LPS-induced immune cells activation in the models of acute inflammation tested. It seems likely that excessive inflammatory tissue damage causes accumulation of extracellular inosine and signaling through adenosine receptors on immune cells in local tissue environment. Since the expression of purinergic receptors on immune cells is dependent on activation and differentiation, only those immune cells that express sufficient numbers of adenosine receptors will be susceptible to the protective effect of inosine. In addition, it is possible,
but has to be experimentally tested, that the time course of up-regulation of A2a receptors will differ from that of A3 receptors and, as a result, different immune cells will express different combinations of A2a versus A3 receptors at different time-points \textit{in vivo}.

Taken together, the data reported here indicate that inosine has properties that are consistent with it being an endogenous metabolic “switch” that serves to modulate inflammation \textit{in vivo}. The ability of inosine to functionally activate A2a receptors as reported here extends the anti-inflammatory effect of inosine to include negative regulation of immune cells that do not express A3R, but do express sufficient number of A2a receptors as well as enhanced inhibition of immune cells that express both A2aR and A3R. Thus, we propose that both A2a and A3 purinergic receptors are functionally utilized, directly or indirectly, by endogenous inosine with potential to protect tissue from excessive inflammatory damage, and that down-regulation of tissue damage in different inflammatory conditions is under control of the complex interplay of different types of inflammatory cells with different repertoire of adenosine receptors. Although the models employed in this study are experimental and might have limited similarities with actual disease states in humans these findings may prove important in the understanding of molecular mechanisms of the pathogenesis of acute inflammation-associated diseases and in the search for pharmacological anti-inflammatory agents for the treatment of inflammatory/autoimmune disease such as hepatitis, arthritis, and sepsis.
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Differential requirement for A2a and A3 adenosine receptors for the protective effect of inosine \textit{in vivo}

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