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Adenosine is required for sustained inflammasome activation via the A_{2A} receptor and the HIF-1α pathway

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Abstract

Inflammasome pathways are important in chronic diseases, but it is not known how the signalling is sustained after initiation. Inflammasome activation is dependent on stimuli such as LPS and ATP that provide two distinct signals resulting in rapid production of IL-1β, with lack of response to repeat stimulation. Here we report that adenosine is a key regulator of inflammasome activity, increasing the duration of the inflammatory response via the A_{2A} receptor. Adenosine does not replace signals provided by stimuli such as LPS or ATP, but sustains inflammasome activity via a cAMP/PKA/CREB/HIF-1α pathway. In the setting of lack of IL-1β responses after previous exposure to LPS, adenosine can supersede this tolerogenic state and drive IL-1β production. These data reveal that inflammasome activity is sustained, after initial activation, by A_{2A} receptor-mediated signalling.

INTRODUCTION

The production of IL-1β is a central step in a wide range of acute and chronic inflammatory and fibrotic responses. The identification of the molecules which form the inflammasome has been a significant advance in our understanding of the required cytosolic machinery, and up-stream signals for IL-1β production[1]. Two distinct pathways are known to be required for initial inflammasome activation and IL-1β production. The signal 1 pathway is typically

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considered to be activated via Toll-like receptors resulting in NFkβ mediated up-regulation of the Pro-ILβ gene, as well as genes for inflammasome components[2]. A second pathway (signal 2) is required for activation of the inflammasome machinery. Signal 2 is delivered by a wide range of stimuli, which range from pathogen derived molecules such as flagellin and cytosolic DNA, and non-pathogen derived particulates such as uric acid crystals.

The above two pathways appear to provide the minimum requirements for inflammasome activation, however their activation is associated with an acute production of IL-1β which is significantly resolved within 24 hrs[3]. Inflammasome activation is, however, also firmly established to have an important role in a number of chronic inflammatory and fibrotic diseases. Sustained production of IL-1β could theoretically occur within the framework of the above pathways by a greater number, concentration or duration of exposure to ligands which initiate signal 1 and 2 pathways. However, it is well known that persistent exposure to PAMPs results in the development of a tolerogenic state, and signal 2 pathways such as ATP induce cell death[4–7]. We speculated that there are additional regulatory signals, which are independent of the ligands which provide signal 1 and 2. The additional advantage of such signals is that they may provide distinct functional information. With this question in mind we tested the role of adenosine in the regulation of inflammasome activation. Adenosine biology is well suited to regulate inflammasome activity because extracellular adenosine concentrations are elevated in response to tissue damage, and adenosine is rapidly removed from tissues by cellular uptake and adenosine deaminase-mediated metabolism[8]. This provides for a rapidly responsive mechanism which signals local tissue ischemia and injury. Adenosine, however, has not been considered as a DAMP because it co-ordinates the adaptive responses to tissue injury in many ways in addition to inflammation, and more importantly because most of the immunological effects have been to reduce cytokine production, with the notable exception of IL-1β[9–11].

In this study we demonstrate that adenosine acting via the A_{2A} receptor is a key regulator of inflammasome activity. Concentrations of adenosine found during tissue injury increase the maximal amplitude and duration of the inflammasome response. Inflammasome regulation by adenosine does not replace either signal 1 or 2, but regulates inflammasome activity initiated by a wide range of PAMPs and DAMPs. A cAMP/PKA/CREB/HIF-1α signalling pathway downstream A_{2A} receptor is activated, and results in up-regulation of Pro-IL1β and NLRP3, and greater caspase-1 activation. In addition to regulation of inflammasome activity by pathological concentrations of adenosine, there is a requirement for physiological levels of adenosine for maximal IL-1β production. Finally, after macrophages have received signals 1 and 2, adenosine can regulate further IL-1β production, without the need for either initiating signal. This demonstrates that such cells are not simply tolerant or un-responsive to further signals, but are in a post-activation state where they have switched from an initial DAMP-driven phenotype, to a subsequent adenosine, cAMP driven phenotype.

RESULTS

Adenosine stimulates IL-1β in an inflammasome-dependent manner

Initially we tested if adenosine can increase IL-1β production above that produced by LPS and ATP, both of which activate signals 1 and 2 respectively. The combination of LPS and

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ATP resulted in a high level of IL-1β production as assayed 5 hrs after ATP, and this was significantly increased by adenosine (Fig. 1a). To confirm that metabolites such as inosine produced via degradation of adenosine were not responsible for the elevated IL-1β the metabolism of adenosine from cellular sources was inhibited by using the adenosine deaminase inhibitor EHNA (erythro-9-(2-hydroxy-3-nonyl) adenine, and this also resulted in a significant increase in IL-1β production (Fig. 1a)[12]. Of note adenosine and EHNA in the absence of either LPS or ATP did not result in any detectable IL-1β. To determine the effect of experimentally reducing adenosine concentration, adenosine deaminase was added and resulted in a significant reduction in IL-1β release (Fig. 1a).

A time course was performed, and demonstrated a sustained increase in IL-1β production by inhibition of adenosine deaminase (Fig. 1b). To further confirm that adenosine metabolites were not responsible for the increase in IL-1β via some other pathway, the non-degradable pan-adenosine receptor agonist NECA (5′-N-ethylcarboxamidoadenosine) was used, and also stimulated an increase in IL-1β production in LPS and ATP, and time-dependent manner (Fig. 1c and d). The IL-1 receptor uses the same Myd88 adaptor protein used as most TLRs and can increase IL-1β production via an autocrine loop[13]. To rule out that NECA was enhancing this autocrine pathway we demonstrated that NECA can increase IL-1β production in macrophages from wild-type and IL-1 receptor deficient mice to a similar degree (Fig. 1e)[13]. We next tested if this phenomenon had applicability to other types of macrophages. LPS and ATP induced stimulation of bone marrow derived macrophages and Kupffer cells resulting in production of IL-1β, and this was significantly increased by NECA (Supplementary Fig. S1). Cell death was examined by the assay of LDH (lactate dehydrogenase) release, and did not show correlation with the secretion of IL-1β in the presence or absence of EHNA, indicating that differences in macrophage survival are not the reason for the increased IL-1β production (Fig. 1f).

The effect of the adenosine pathway on the production of other cytokines was tested and inhibition of adenosine deaminase resulted in a reduction in the production of TNF-α, no effect on IL-10 and an increase in IFN-γ (Fig. 1g, h and i). We next tested if the observed increase in IL-1β production was dependent on caspase-1, NLRP3, ASC and P2x7 receptor. In the absence of any of these molecules stimulation of the adenosine signal by adding the pan-adenosine receptor agonist (NECA) or inhibiting adenosine deaminase (EHNA) did not result in significant production of IL-1β by LPS and ATP (Fig. 1j). NECA also increased IL-1β production by the combination of LPS and mono-sodium urate (MSU) crystals (Supplementary Fig. S2a). This was supported by data from different manipulations with inhibition of adenosine metabolism resulting in an increase in CpG-B and ATP induced increase in IL-1β (Supplementary Fig. S2b). The reverse question of the contribution of A_{2A} receptor signaling to IL-1β production was tested by using the A_{2A} receptor antagonist ZM241385 in inflammasome activation by a variety of stimuli (CpG-B and ATP, Pam3 and ATP, LPS and beads, LPS and MSU and liver cell lysate) (Supplementary Fig. S2c–g). Finally the contribution of A_{2A} receptor signaling *in vivo* was tested in the inflammasome dependent model of MSU induced intraperitoneal sterile inflammation which demonstrated a significant reduction in the presence of A_{2A} receptor antagonism (Supplementary Fig. S2h). These data show that activation of an adenosine pathway enhances the amount and

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duration of LPS and ATP induced IL-1β production in a NLRP3 inflammasome dependent manner, and this is not due to an IL-1β autocrine loop.

A_{2A} receptor activation amplifies signal 1 and 2 pathways

There are four identified adenosine receptors (A_1, A_{2A}, A_{2B}, and A_3). These receptors are widely distributed and are coupled to stimulatory (A_{2A}, A_{2B}) or inhibitory (A_1 and A_3) adenylate cyclases[9,11]. NECA increased IL-1β production, and this was inhibited by A_{2A}R antagonist (ZM241395), but not A_1R (DPCPX), A_{2B}R (MRS1706) and A_3R (MRS1523) specific receptor antagonists (Fig. 2a). This result was replicated by examining the ability an A_{2A} receptor agonist (CGS21680) to increase IL-1β production to a comparable degree to NECA and EHNA. All of these stimuli were inhibited by ZM241395 (Fig. 2b). LDH assay indicates that the inhibitory function of ZM241395 on LPS and ATP induced IL-1β secretion was not due to its cell toxicity (Fig. 2c). To confirm the role of the A_{2A} receptor, the ability of stimulation of the adenosine pathway to increase IL-1β production was tested in macrophages from A_{2A} receptor deficient mice (*Adora2a*$^{-/-}$). In the absence of A_{2A} receptor there was virtually no production of IL-1β by LPS and ATP, and this was not increased by stimulation of several components related to broad activation of adenosine pathways (Fig. 2d). To identify if the increased production of IL-1β was due to adenosine induced stimulation of signal 1 and signal 2, we initially examined up-regulation of *Il1b* gene in peritoneal macrophages from wild-type and A_{2A} deficient mice in response to CGS21680 (Fig. 2e, f). The requirement for A_{2A} receptor was confirmed by a conventional strain of *Adora2a* knockout mice and *Adora2a*$^{flox/flox}$ / Lysozyme M-(LysM)-Cre (*A_{2A}R-cKO*) mice which had significant reduction in A_{2A} receptor expression in macrophages (Supplementary Fig. S3), the macrophage from both strains had significantly less up-regulation of *Il1b* mRNA in response to LPS (Fig. 2e, f). To examine the role of A_{2A} receptor activation on signal 2 activated inflammasome pathway we examined the effect of activation of A_{2A} receptor on the formation of active caspase-1. The A_{2A} receptor agonist CGS21680 alone did not result in detectable active caspase-1 (Fig. 2g). In response to LPS/ATP, active caspase-1 was detected, and this was decreased by blocking A_{2A} receptor with ZM241385 and increased by activating A_{2A} receptor with CGS21680. The increase in active caspase-1 by CGS21680 was also inhibited by ZM241385. In addition, the clear reduction of active caspase-1 was also detected in *A_{2A}R-cKO* macrophages in response to LPS/ATP (Fig. 2h). Collectively, these data show that adenosine induced increase in LPS and ATP stimulated IL-1β production is via A_{2A} receptor and is due to an increase in both signal 1 and signal 2 pathways.

Adenosine supersedes LPS tolerance via a CAMP-PKA pathway

The demonstration that adenosine signaling can increase not just the amplitude but also the duration of IL-1β production directly impacts the well characterized phenomenon of LPS tolerance[5,6]. In this phenomenon exposure to LPS results in hypo-responsiveness to subsequent stimulation by LPS and other TLR agonists, and occurs in-part by a lack of up-regulation in pro-IL-1β[5]. We tested the ability of activation of the adenosine pathway to regulate Pro-IL-1β induction from PECs with previous exposure to LPS. As expected 3 hrs after initial stimulation of PECs with LPS (Stim) there is up-regulation of *Il1b* mRNA expression (Fig. 3a). PECs pretreated with LPS (Pri) for 16 hrs had low levels of pro-IL-1β,

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and did not respond to a repeat stimulation with LPS (Fig. 3a). In sharp contrast to a lack of response of LPS pretreated PECs to repeat LPS stimulation, there was a dramatic increase in *Il1b* mRNA expression in response to either NECA or CGS21680 (Fig. 3a). These results demonstrate that after an initial signal 1, when PECs are un-responsive to further signal 1 ligands, they become highly responsive to adenosine by up-regulating *Il1b*. We next tested if this ability of adenosine to supersede LPS tolerance was true for other cytokines. Over 6 and 24 hours CGS21680 and NECA were able to increase the expression levels of *Il1b, ll6*, *Il4* but not *tnfa* (Supplementary Fig S4 a–d). As sustained inflammasome activation has also been shown to be important in chronic tissue fibrosis and remodeling we examined expression of relevant genes and found a consistent ability of CGS21680 and NECA to supersede LPS tolerance for tissue inhibitor of metalloproteinases-1 (TIMP-1), vascular endothelial growth factor (VEGF), and Glucose transporter 1 (GLUT-1) (Supplementary Fig. S4e–h). This is consistent with previous data showing that adenosine increases TIMP-1 in IL-4 or IL-13 activated macrophages[14]. In contrast, other antimicrobial gene like macrophage receptor Marco was up-regulated by adenosine signal activation, and this was found to be dependent on A_{2A} and IL-1 receptor signaling (Supplementary Fig. S5a–f).

The A_{2A} receptor is coupled to a stimulatory adenylate cyclase, which results in up-regulation of cAMP and activation of protein kinase A (PKA)[8]. To test this pathway we used the adenylate cyclase activator forskolin, which induced the induction of *Il1b* gene in a dose dependent manner (Fig. 3b). This was confirmed by directly using a stable analogue of cAMP (db-cAMP), which along with NECA and CGS induced up-regulation of Pro-IL-1β (Fig. 3c). To test the requirement for the adenlate cyclase/cAMP/PKA pathway on A_{2A} receptor signal induced IL-1β production, an adenylate cyclase inhibitor (SQ22536) was tested and was able to block the increase in IL-1β induced by CGS21680 and EHNA (Fig. 3d)[15]. We next tested the requirement of PKA downstream of cAMP activation using the specific inhibitors of H-89 and PKI 14-22 amide (PKi). PKA inhibition significantly reduced the IL-1β production in response to NECA and CGS21680 (Fig. 3d). This was confirmed for pro-IL-1β protein level by western blot (Fig. 3e). We next confirmed that the lack of response in cells from A_{2A} receptor deficient mice (Fig. 2e) was not due to developmental effects, by testing the ability of db-cAMP to increase pro-IL1β protein levels in *A_{2A}R-cKO* mice (Fig. 3f).

To determine if the greater amounts of Pro-IL-1β transcripts after A_{2A} receptor activation were due to increased transcript stability, we used actinomycin D to inhibit transcription, and examined the effect of CGS21680 on the loss of Pro-IL-1β transcript. The rate of loss of *Il1b* mRNA expression was identical showing that CGS did not affect transcript stability (Fig. 3g). To confirm the role of A_{2A} receptor in increasing TLR induced Pro-IL-1β up-regulation, the response of macrophages from A_{2A} receptor deficient mice was tested. In the absence of A_{2A} receptor there was minimal increase in LPS induced up-regulation of *Il1b* mRNA expression (Fig. 3h). Finally we tested the role of adenosine signaling and A_{2A} receptor in the changes associated with a number of cytokine and inflammasome related transcripts (Fig. 3h). In contrast to decreased *Il1b* and *Il6* mRNA in A_{2A} receptor deficient cells, there was a higher level of *tnfa*. Of interest there were also lower levels of *Nlrp3* and *Txnip* transcripts in A_{2A} receptor deficient cells. This promted us to test if the adenylate

cyclase/cAMP/PKA pathway was also required for the A_{2A} receptor induced increase in active caspase-1. This was the case, with the CGS21680 induced up-regulation in LPS/ATP induced active caspase-1 being reduced by SQ22536 and H-89 (Fig. 3i). Interestingly, in contrast with these findings, under non-LPS tolerogenic condition, adenosine-cAMP signaling did not show increase in IL1β secretion by inflammasome activation indicating distinct role of adenosine in deriving IL-1β production (Supplementary Fig. S6a–d). Collectively, these data show that adenosine signaling can supersede LPS tolerance of signal 1 via the A_{2A} receptor and does so via an adenlate cyclase/cAMP/PKA mediated pathway.

Adenosine induces pro-IL-1β via CREB and HIF-1α

To understand the link between PKA activation and Pro-IL1β up-regulation, we and others identified HIF-1α response elements in the human and mouse *Il1b* promoter (Fig. 4a)[16]. This led us consider that HIF-1α activation may be a central step in upregulating Pro-IL-1β. We assayed if A_{2A} receptor activation resulted in up-regulation of *Hif1a* mRNA, and found this to be the case (Fig. 4b). The HIF-1α inhibitor CAY10585 was able to significantly decrease adenosine agonist induced *Il1b expression* and IL-1β production (Supplementary Fig. S7a, b). To specifically test the role of HIF-1α in A_{2A} receptor activation induced up-regulation of *Il1b* mRNA, *HIF-1α$^{flox/flox}$*/Lysozyme M (LysM)-Cre mice (*HIF-1α-cKO*) were generated, and high deletion efficiency was demonstrated in bone marrow derived macrophages (Supplementary Fig. S3b). LPS primed *HIF-1α-cKO* macrophages had significantly less A_{2A} receptor stimulation induced up-regulation of *Il1b* than wild-type macrophages (Fig. 4c). We further demonstrated that LPS primed *HIF-1α-cKO* macrophages had significantly less A_{2A} receptor stimulation induced up-regulation of *Nlrp3* and *Txnip* mRNA than wild-type macrophages (Supplementary Fig. S7c, d).

A direct link between PKA activation and HIF-1α upregulation is provided by cAMP response element-binding protein (CREB) which enhances two C-terminus transactivating domains in HIF-1α[17]. The requirement for CREB in adenosine induced up-regulation of HIF-1α was tested by transfection of THP-1 human monocyte cell line with the HRE-promoter luciferase construct and β-galactosidase plasmid in the presence or absence of CREB dominant negative plasmid (CREB) for 24 hrs, and then primed with LPS/PMA for 16 hrs followed by NECA treatment over 8 hrs. As predicted an adenosine stimulus resulted in HRE reporter luciferase activity, and this was inhibited by the presence of the dominant negative CREB plasmid (Fig 4d)[18,19]. In an analogous manner transfection of the IL-1β promoter luciferase construct and β-galactosidase plasmid in the presence or absence of CREB dominant negative form demonstrated a requirement for CREB signaling for the production of IL-1β (Fig. 4e)[20]. Transcriptional up-regulation of Pro-IL-1β on initial LPS stimulation is via activation of the NF-kB pathway. We tested if the subsequent transcriptional up-regulation of Pro-IL-1β also uses this pathway, and found that adenosine agonists do not increase activity of the NF-kB pathway above that already induced by LPS (Fig. 4f). This is in agreement with previous studies[21,22]. We finally confirmed that in the absence of HIF-1α there was a significant reduction at the protein level in pro-caspase-1, activated caspase-1, pro-IL-1β, and ultimately active IL-1β (Fig. 4g and h). These data show that adenosine induced up-regulation of IL-1β is dependent on a CREB/HIF-1α pathway

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which is distinct from the NF-kB pathway used for initial production of IL-1β in response to LPS.

Liver injury is dependent on A_{2A} receptor in macrophages

The above data shows a requirement for adenosine signaling via the A_{2A} receptor for maximal production of IL-1β by macrophages *in vitro*. With tissue inflammation and injury extracellular adenosine levels are known to increase by a variety of means from the basal levels below 1 μM to levels up to 100 mM[23,24]. We tested if A_{2A} receptor driven inflammasome activity was relevant *in vivo* and using two models of liver injury, one acute LPS driven model where liver injury was assessed in 6 hrs after LPS, and a second model of sterile injury by a toxic metabolic insult to the liver by thioacetamide (TAA) in which injury was examined at days 1, 2 and 7. We used *A_{2A}R-cKO* mice that specifically delete *Adora2a* gene in macrophages as described above. Liver immune populations were intact in these mice (Supplementary Fig S8). Six hours after LPS and D-galactosamine induced liver injury there was significantly less hemorrhage and necrosis in the livers from *A_{2A}R-cKO* mice and this was confirmed by lower serum ALT values (Fig. 5a). Reduced activation of the inflammasome was confirmed by demonstrating that whole liver had lower levels of *Il1b* mRNA, less active caspase-1, and that there were lower levels of serum IL-1β (Fig. 5b, c, d). To test the effect of increasing adenosine signaling *in vivo* wild-type mice were treated with LPS in the presence or absence of the adenosine agonist NECA. The presence of NECA resulted in a significant elevation in serum ALT, whole liver transcripts of *Il1b*, *Il6*, *Hif1a* and *Nlrp3*, and a reduction in the transcript for *tnfa* (Supplementary Fig. S9).

Toxic injury by TAA demonstrated maximal hemorrhage and necrosis at day 1. There was less liver hemorrhage and necrosis, and lower levels of liver *Il1b* mRNA and serum ALT in *A_{2A}R-cKO* mice (Fig. 5e, f, g, h). In addition to inflammation, inflammasome activity has been shown to be required for the development of the fibrotic response in many organs[25–27]. To assess if fibrosis was also affected in the absence of A_{2A} receptor on tissue macrophages, liver tissues from 7 days after TAA were stained for collagen by Sirius Red. There was significantly less fibrosis in livers from *A_{2A}R-cKO* mice compared to wild-type (Fig. 5g). A sustained IL-1β production was also seen in liver tissue and serum after a course of repeated TAA injection in wild-type control, but not *A_{2A}R-cKO* mice (Supplementary Fig. S10a–c). To further test the effect of increased adenosine signaling on fibrosis wild-type and caspase-1 deficient mice were injected with TAA with and without co-injection of the adenosine agonist CGS21680. After one week liver tissues were stained for collagen with Sirus Red. CGS21680 increased TAA induced liver fibrosis and *Timp1* expression in wild-type but not caspase-1 deficient mice (Supplementary Fig. S11a, b). Collectively these data confirm that adenosine induced signaling via the A_{2A} receptor is important for *in vivo* activation of the inflammasome in acute and chronic injury. Original blots for the data shown are in Supplementary Fig. S12.

DISCUSSION

The current model of inflammasome activation in macrophages explains the initial steps in acute inflammation, but is inadequate to explain how activity is sustained in chronic

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inflammation, repair and fibrosis[1,2]. The issue is not only the self limited inflammatory response induced by signals 1 and 2, but also the unresponsiveness of macrophages to similar subsequent signals[5,6]. As macrophages are unresponsive to repeat exposure to the initiating signals we reasoned that further signals are qualitatively different, with signals from tissue injury being attractive candidates. Adenosine is well known to regulate tissue responses to stress and injury via activation of four widely distributed receptors[9,11]. Adenosine levels rapidly increase in the extracellular environment in response to cell stress and death by release from cytosolic stores and sequential dephosporylation from ATP, and are rapidly reduced by uptake and metabolism[11,23,24]. Manipulations which increase adenosine signaling result in increased release of IL-1β from conventionally activated PEC, and this is not due to enhancement of an IL-1 mediated positive feedback loop (Fig. 1a–e). Adenosine has a role concurrent with, or after conventional inflammasome activation. The *in vivo* implications are that during and after inflammasome activation the ambient adenosine concentration regulates the duration of inflammasome activation, and can restimulate it. Our data are consistent with the original *in vivo* data from A_{2A} receptor deficient mice showing a lack of rise in serum IL-1β in response to LPS[10].

The contribution of A_{2A} receptor activation is to up-regulate the transcript levels of Pro-IL-1β, NLRP3 and others. This means that an adenosine stimulus can up-regulate both arms of signal 1 and 2 pathways, as demonstrated by greater levels of pro-IL-1β protein and active caspase-1 (Fig. 3e, i). Furthermore this is a broad response with adenosine increasing inflammasome activation in response to a range of TLR stimuli for signal 1, as well as ATP, monosodium urate and synthetic bead induced activation of signal 2 (Supplementary Fig. S2).

Initial activation of PEC by LPS results in high levels of Pro-IL1β, and subsequent exposure to LPS cannot stimulate a repeated elevation, which is the well characterized phenomenon of LPS tolerance[5,6]. We now show that after initial activation PEC will up-regulate Pro-IL-1β and produce IL-1β in response to adenosine signals without further exposure to conventional signals 1 and 2. These PEC are clearly not un-responsive in a global way, rather after initial activation by LPS and ATP they have switched their phenotype into a state in which Pro-IL1β production is regulated by adenosine signals (Fig. 3a). This is better characterized as a post-activation, rather than an un-responsive state. The effect of adenosine is however not uniform for all cytokines. In particular, adenosine results in downregulation of TNF-α production, and does not alter the genes for most anti-microbial proteins (Supplementary Fig. S5).

The initial steps downstream of the A_{2A} receptor which are required for the increased IL-1β production are the signaling molecules cAMP, and PKA (Fig. 3b–f)[28]. Detailed investigation using HIF-1α inhibitors, reporter constructs and macrophage specific knockouts demonstrates that the ability of adenosine signals to up-regulate pro-IL-1β is dependent on HIF-1α and CREB, with CREB being proximal to HIF-1α (Fig. 4b, c, d). This is distinct from the NF-kB pathway utilized for pro-cytokine up-regulation after initial TLR activation which has previously been shown not to be reduced by adenosine signaling[21,22,30].

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The prediction from these *in vitro* data is that in the absence of A_{2A} receptor signaling on macrophages there will be reduced inflammasome activation, with less tissue injury and fibrosis. This was the case in an acute and sustained model of LPS and TAA induced liver injury (Fig. 5). This provides valuable confirmation of the scale of the adenosine signal *in vivo*. In a number of experimental models, total A_{2A} receptor deficient animals have greater organ injury[31,32]. This apparent contradiction can be resolved if adenosine is simultaneously functioning to enhance macrophage based inflammatory responses, and providing parenchymal cell protection - with both being part of an integrated response to tissue injury by pathogens and sterile insults.

In summary we demonstrate that macrophages after receiving conventional signals 1 and 2, are dependent on adenosine via the A_{2A} receptor for initial and sustained inflammasome activity and IL-1β production. Conceptually this is significant as it changes our interpretation of a lack of cytokine response by macrophages after LPS exposure from the cell being in an unresponsive state, to it being in a post-activation state, which is regulated by a different set of signals. Mechanistically and therapeutically this is significant as it requires a clearer understanding if tissue injury is due to the initial inflammasome activation, or is due to maintenance of the activation state. Different antagonist and inhibitors will be effective to limit each of these.

METHODS

Animals and macrophages

C57BL/6 mice were purchased from the National Cancer Institute. *ASC*$^{-/-}$, *Caspase-1*$^{-/-}$, *Nlrp3*$^{-/-}$ and *P2xr7*$^{-/-}$ mice have been described previously (1). *A2ora2a*$^{flox/flox}$ mice and LysM^{cre} mice were purchased from Jackson Laboratories. *HIF-1a*$^{flox/flox}$ mice were kindly provided by Dr. Ruslan M Medzhitov (Yale University). *Adora2a*$^{-/-}$ mice were maintained at the animal facility of New York School of medicine according the regulations of Animal Care and Use Committee of the New York University. All procedures were performed in accordance with the regulations adopted by the National Institutes of Health and approved by the Animal Care and Use Committee of the Yale University. 7- to 12-week-old males with a variety of genetic manipulations were used in most experiments. The number of mice in each experimental group was chosen based on previous experience with these experimental models. Mouse peritoneal macrophages (PECs) were isolated by peritoneal lavage 3 days after intraperitoneal injection of 4% thioglycollate solution (B2551, Fluka,). Cells were plated at the density of 3×10^6 cells in 12-well dishes and non-adherent cells were removed after 3 h. Cells primed overnight with 100 ng/ml LPS or 10 ng/ml Pam3CSK4 were treated with various chemicals and followed stimulation. Cells were cultured in DMEM medium complemented with 10% FBS, penicillin/streptomycin and L-glutamine. Mouse bone marrow derived macrophages were isolated from bone marrow cells, and were differentiated for 7 days in complete RPMI-1640 medium supplemented with 2 mM L-glutamine, 100U/ml penicillin, 100 μg/ ml streptomycin, 50 μM 2-mercaptoethanol (all from Invitrogen), 10% heat-inactivated FBS and 20 ng/ml M-CSF (PeproTech). Kupffer cells (KC) were isolated by the density gradient separation of Optiprep (Sigma), and then

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plates were gently washed and media was replenished after seeding cells for 2 hrs to raise KC purity.

Reagents

ATP, LPS from *Salmonella minnesota* Re-595, Forskolin (cAMP analogue), SQ22536 (AC inhibitor), H-89 (PKA inhibitor), MRS1523 (A_3 antagonist), adenosine, EHNA (adenosine deaminase inhibitor), 5′-(N-ethylcarboxamido) adenosine (NECA) (nonselective adenosine receptor agonist), N^6,2′-O-Dibutyryladenosine 3′,5′-cyclic monophosphate sodium salt (db-cAMP) and Apyrase were obtained from Sigma (St. Louis, MO). Adenosine deaminase was obtained from Worthington Biochemical corporation (Lakewood, NJ). CGS21680 (A_{2A} agonist), DPCPX (A_1 antagonist), ZM241385 (A_{2A} antagonist), and MRS 1706 (A_{2B} antagonist) were obtained from TOCRIS (Ellisville, MI). Nigericin was purchased from Calbiochem, Pam3CSK4 and type B CpG oligonucleotide (ODN 1668, CpG-B) were purchased from Invivogen (San Diego, CA). CAY10585 (HIF-1α inhibitor) was purchased from Cayman Chemical (Ann Arbor, MI). TRIZOL and Dulbecco's modified Eagle's medium (DMEM) were purchased from GIBCO/Invitrogen (Carlsbad, CA). All reagents were of the highest quality grade commercially available. MSU crystals were produced as previously described (2). Briefly, 4 mg/ml uric acid (Sigma-Aldrich, St. Louis, MO) was dissolved in 0.1 M borate buffer by continuously adjusting the pH to 8.0. The solution was filtered, and the crystals precipitated after 7 days were washed twice with absolute alcohol and once with acetone and air dried in a tissue culture hood before use.

Quantitative real-time RT–PCR

Total RNA was extracted using TRIZOL reagent (Invitrogen), and cDNA was generated with an oligo (dT) primer and the Superscript II system (Invitrogen, USA) followed by analysis using LightCycler 480 system (Roche). q-PCR was performed for *Il1b* using commercial primer-probe sets (Applied Biosystems Inc.) with DNA master Mix (Roche). Expression of GAPDH was used to standardize the samples, and the results were expressed as a ratio relative to control; q-PCR was performed for *tnfa*, *Il6, Txnip, Nlrp3, Hif1a, Timp1*, using LightCycler 480 SYBR Green I master mix (Roche). Results were normalized based on the expression of β-actin. Primer sequences are listed in Supplementary Table S1.

Transfection and luciferase reporter assay

THP-1 cells (Sigma-Aldrich) were transiently transfected with human IL-1β promoter (−1 to −4000) luciferase or HRE-luciferase construct in the presence of CREB plasmid or empty vector. For each transfection, the total of 2.0 μg of plasmid was mixed with 200 μl of Opti-MEM® I medium (without serum and antibiotics) and 8.0 μl of X-tremeGENE HP DNA Transfection Reagent (Roche, Indianapolis, IN) according to the manufacturer's instructions. The mixture was incubated at room temperature for 20 minutes and added to 6-well plates containing cells and complete medium in a dropwise manner. The cells were incubated for 48 hrs and harvested using reporter lysis buffer (Promega) for determination of luciferase activity. Cells were co-transfected with β-galactosidase reporter plasmid to normalize experiments for transfection efficiency. 293T cells (InvivoGen) were transiently transfected with NFkB promoter luciferase reporter construct together with Renilla uciferase

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(Rluc) control reporter vector by Lipofectamine™ 2000 reagent. All the luciferase activities were measured and normalized to Rluc or β-galactosidase activity and the normalized value with the percentage of control group was indicated.

Flow cytometry

Liver non-parenchymal cells were isolated and antibodies were conjugated to FITC, PE, allophycocyanin (APC) specific for CD11b (1:200, M1/70), GR-1 (1:200, RB6-8C5) (BD Biosciences — Pharmingen), Ly6-C (1:200, HK1.4) and F4/80 (1:200, BM8, eBioscience), were used. Stained cells were analyzed using FACScalibur (BD Biosciences).

Cytokine ELISA measurements

Primed PECs were both pulsed for 20 min with 5 mM ATP or 10 μM nigericin, and left untreated until culture supernatants were collected. Secretion of IL-1β was determined by enzyme-linked immunoabsorbent assay (ELISA; R&D Systems).

Western blot analysis

Liver tissue lysates or PECs were lysed in RIPA buffer (10mM phosphate buffer pH 7.4, 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with complete protease inhibitor cocktail (Roche) and 2mM dithiothreitol. Lysates were resolved in 4–12% Tris-glycine gradient gels (Invitrogen) and transferred to nitrocellulose (Invitrogen) by electro-blotting. The following antibodies were used: rabbit anti-caspase-1 p10 (SC-514, Santa Cruz), goat anti-mouse IL-1β (BAF401, R&D Systems), Rabbit anti-mouse HIF-1α (NB100-449, Novus Biologicals, Littleton, CO).

Statistical analysis

All data were expressed as mean ±SD. Student's t test was used for statistical evaluation of the results. Significance was set at $p < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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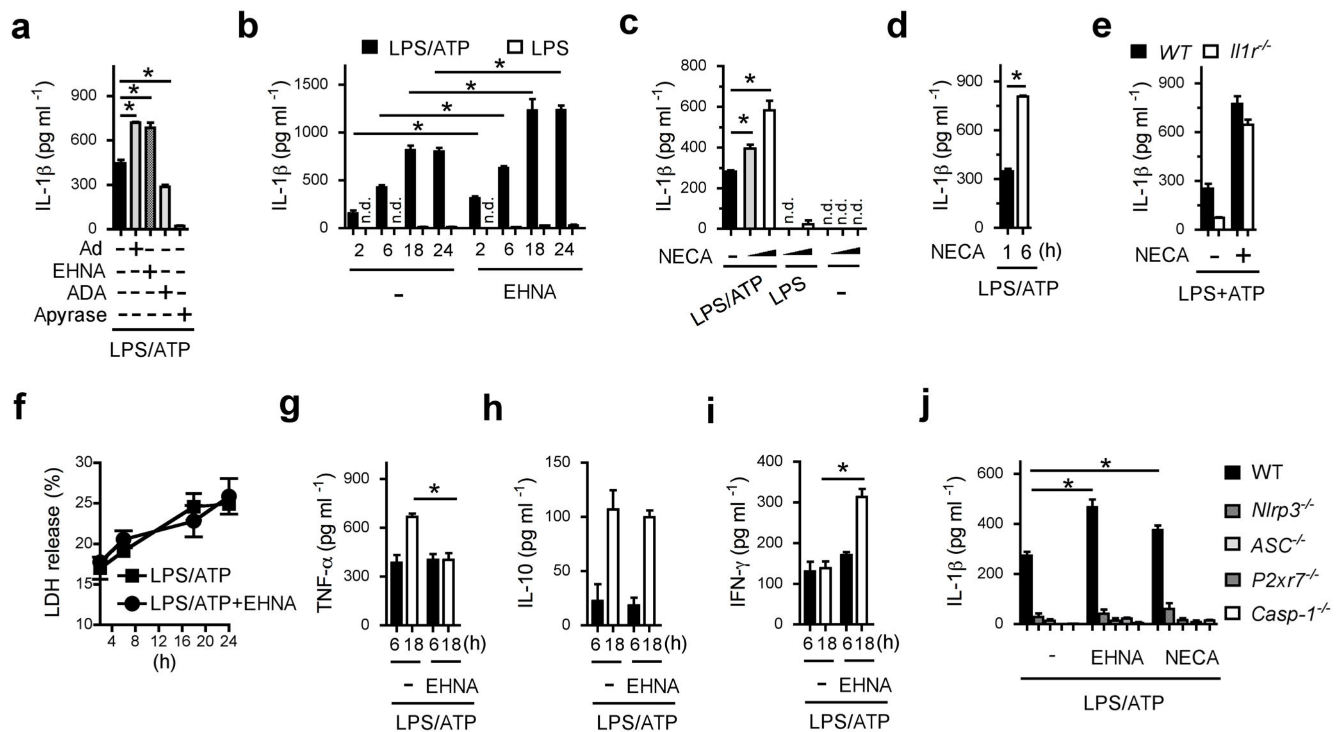

Figure 1.

Adenosine stimulates IL-1β production in NLRP3 inflammasome-dependent manner. Murine peritoneal macrophages (PECs) were obtained from wild type (WT) mice (**a, b, c, d, f, g, h, i**), *Il1r*$^{-/-}$ (**e**) or *Nlrp3*$^{-/-}$, *ASC*$^{-/-}$, *P2xr7*$^{-/-}$, or *Caspase-1*$^{-/-}$ mice (**j**) and were primed with LPS for 16 hrs. (**a**) This was followed by treatment for 1 hr with adenosine (Ad, 100 μM), adenosine deaminase inhibitor (EHNA, 10 μM), adenosine deaminase (ADA, 10 U ml^{-1}), or ATP diphosphohydrolase (Apyrase, 10 U ml^{-1}), (**b, f, g, h, i**). Pan adenosine agonist (NECA, 1 μM, 10 μM) (**c, e**), or (**d**) by NECA for 1 and 6 hrs, and were then pulsed with ATP for 20 min. Enzyme-linked immunosobent assay (ELISA) of IL-1β, TNF-α, IL-10 and IFN-γ secretion were measured in cell supernatants after pulsing with ATP for 5 hrs (**a**, **c**, **d**, **e, j**) or indicated time-course. (**b**, **g**, **h**, **i**). LDH assay was performed after pulsing with ATP as indicated time-course (**f**). Data are expressed as the mean ± SD from at least three independent experiments. * $p < 0.05$ determined by Student's *t*-test.

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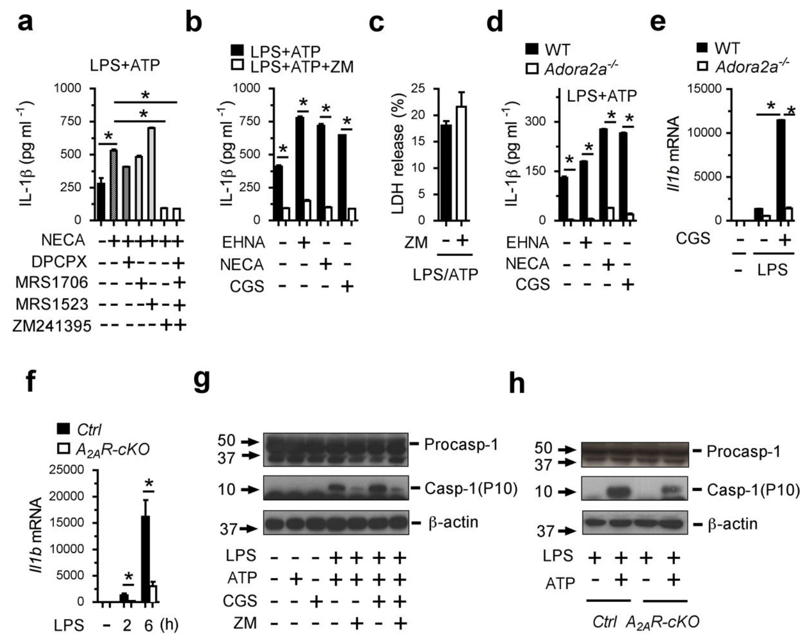

Figure 2.

Adenosine mediates increase in IL-1β via the A_{2A} receptor and amplifies signal 1 and signal 2 pathways. (**a**) LPS primed PECs were treated with NECA (10 μM) in the presence or absence of three different adenosine receptor antagonists for A_1 (DPCPX, 10 nM), A_{2A} (ZM 241385, 10 μM), A_{2B} (MRS1706, 10 nM), A_3 (MRS1523, 5 μM), or their combinations for 1hr, and pulsed with ATP for 20 min. **(b)** A_{2A} receptor specific agonist (CGS21680) and antagonist ZM 241385 increase and block IL-1β production respectively. (**c**) LDH release in PECs showed do difference in the presence or absence of ZM 241385 as sampled from **b.** **(d)** PEC's from A_{2A} receptor deficient cells have low IL-1β production, which is not increased by NECA and CGS 21680. **(e)** CGS21680 increases induction of *Il1b* mRNA expression, and this was decreased in A_{2A} receptor deficient cells. **(f)** Loss of the A_{2A} receptor in macrophages results in much lower levels of *Il1b* mRNA expression in response to LPS. **(g)** CGS21680 and ZM241395 increase and decreases production of cleaved caspase-1. **(h)** The production of cleaved caspase-1 is decreased in A_{2A} receptor deficient macrophages. Data are expressed as the mean ± SD from at least three independent experiments. Immunoblots shown are representative results from at least three independent experiments. * $p < 0.05$ determined by Student's *t*-test.

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Figure 3.

Adenosine supersedes LPS induced tolerance and increases IL-1β production by up-regulating transcription of pro-IL-1β via CAMP-PKA pathway. PECs were used in all of the following experiments. (**a**) Primed (pri) with LPS (100 ng/ml) for 16 hrs followed by stimulation (stim) with LPS (100 ng/ml), or NECA (10 μM) or CGS21680 (10 μM) in the presence or absence of LPS (100 ng/ml) for 6 hrs. (**b**) Treated with forskolin for 6 hrs as indicated doses with and without LPS priming. (**c**) Treated with or without NECA (10 μM), CGS21680 (10 μM) or db-cAMP (200 μM) for 6 hrs. (**d**) LPS primed and treated with NECA or CGS21680 in the presence and absence of SQ22536, H89 and PKi. (**e**) Primed with LPS for 16 hrs and treated with ATP for 20 min, followed by CGS 21680 in the presence or absence of the adenylyl cyclase and PKA inhibitors SQ22536 and H-89. (**f**) Primed with LPS and treated with or without NECA (10 μM) or db-cAMP (200 μM) for 6 hrs. (**g**) Treated with or without CGS21680 (10 μM) for 3 hrs, and transcription was stopped by adding 5 μg/ml actinomycin D, and RNA samples collected at indicated times-points. (**h**) LPS primed PECs obtained from wild type or $A_{2A}R$-*cKO* mice were stimulated with NECA (10 μM) for 6 hrs. The cells were harvested and RNA isolated after each treatment and the gene expression of *Il1b* and other genes as indicated quantified by real-time PCR using specific primers. (**i**) Primed with LPS for 16 hrs and treated with ATP for 20 min, followed

by CGS 21680 in the presence or absence of SQ22536 or H-89. Immunoblot analysis of the proIL-1β caspase-1 in cell lysate was performed by specific anti-IL-1β and anti caspase-1 p10 antibodies (**c**, e, **f, i**). Data are expressed as the mean ± SD from three independent experiments. Immunoblots shown are representative results from at least three independent experiments. * $p < 0.05$ determined by Student's *t*-test.

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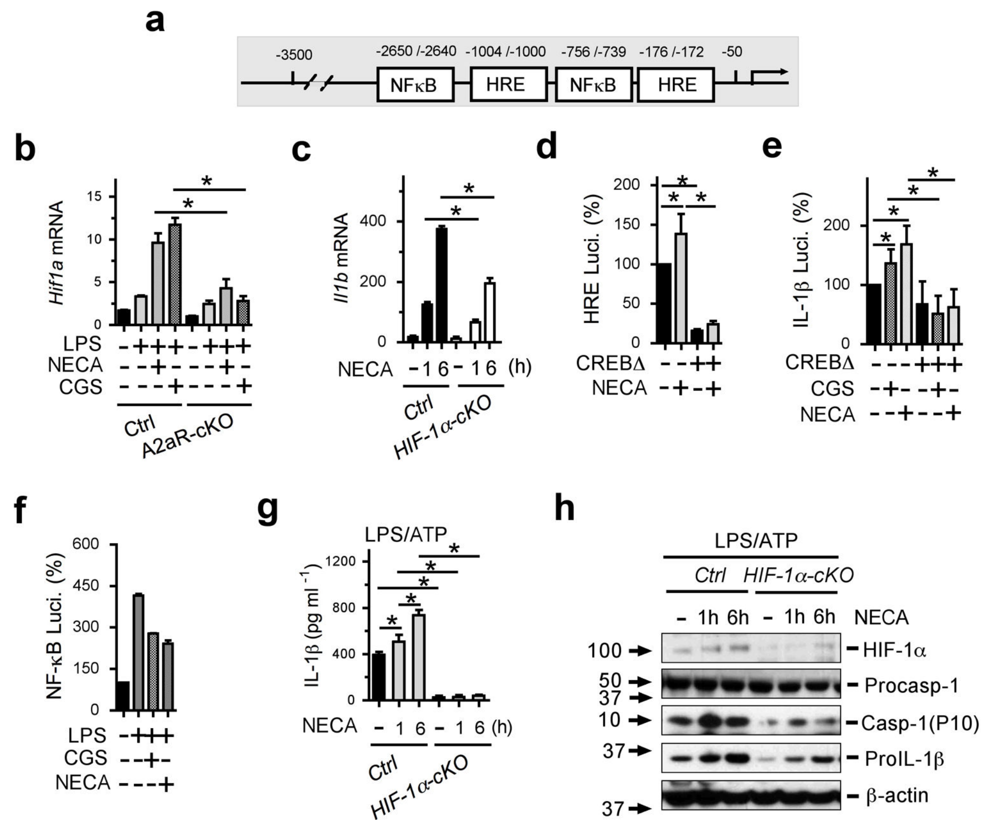

Figure 4.

Adenosine mediates increase in pro-IL-1β via a HIF-1α-dependent pathway. (**a**) Consensus NFκB and HRE binding sites in the IL-1β promoter. (**b**) LPS primed PECs obtained from *$A_{2A}R$-cKO* and controls were stimulated with or without NECA or CGS21680. (**c**) LPS primed PECs obtained from *HIF-1α-cKO* and controls were stimulated with or without NECA at time points as indicated. Cells were harvested and RNA isolated after each treatment and gene expression of *Il1b* and *Hif1α* quantified by real-time PCR. (**d**) THP-1 cells were transfected with HRE- promoter luciferase construct and β-galactosidase plasmid in the presence or absence of CREB dominant negative plasmid (CREB), and then primed with LPS/PMA followed by NECA. (**e**) THP-1 cells were transfected with human IL-1β promoter luciferase construct and β-galactosidase plasmid in the presence or absence of CREB , and then primed with LPS/PMA followed by CGS21680 or NECA. Luciferase activities were measured and normalized to β-galactosidase activity and normalized with controls. Data are mean± SD of triplicate cultures and are representative of three independent experiments. (**f**) CD14-MD2-TLR4- HEK 293 cells were transfected with NFκB promoter luciferase construct and Renilla luciferase (Rluc) control reporter vector, and then treated with CGS21680, NECA or ZM241395 in the presence of LPS, Luciferase activities were measured and normalized to Rluc activity and the normalized value with controls as indicated. Data are mean± SD of triplicate cultures and are representative of three independent experiments for **b-f. (g)** LPS primed PECs obtained from *HIF-1α-cKO* and controls were treated with or without NECA at different time points as indicated followed pulsing with ATP. Cell supernatants were collected in 5 hrs after ATP pulsing.

IL-1β was measured in cell supernatant by ELISA. Data are expressed as the mean ± SD from three independent experiments. **(h)** LPS primed PECs obtained from *HIF-1α-cKO* and control mice were treated with or without NECA at different time points as indicated followed by pulsing with ATP. Cell lysates were collected after ATP pulsing. HIF-1α, pro-caspase-1, Caspaspe-1 and pro-IL-1β was measured in cell supernatant by western blot. Immunoblots shown are representative results from at least three independent experiments.

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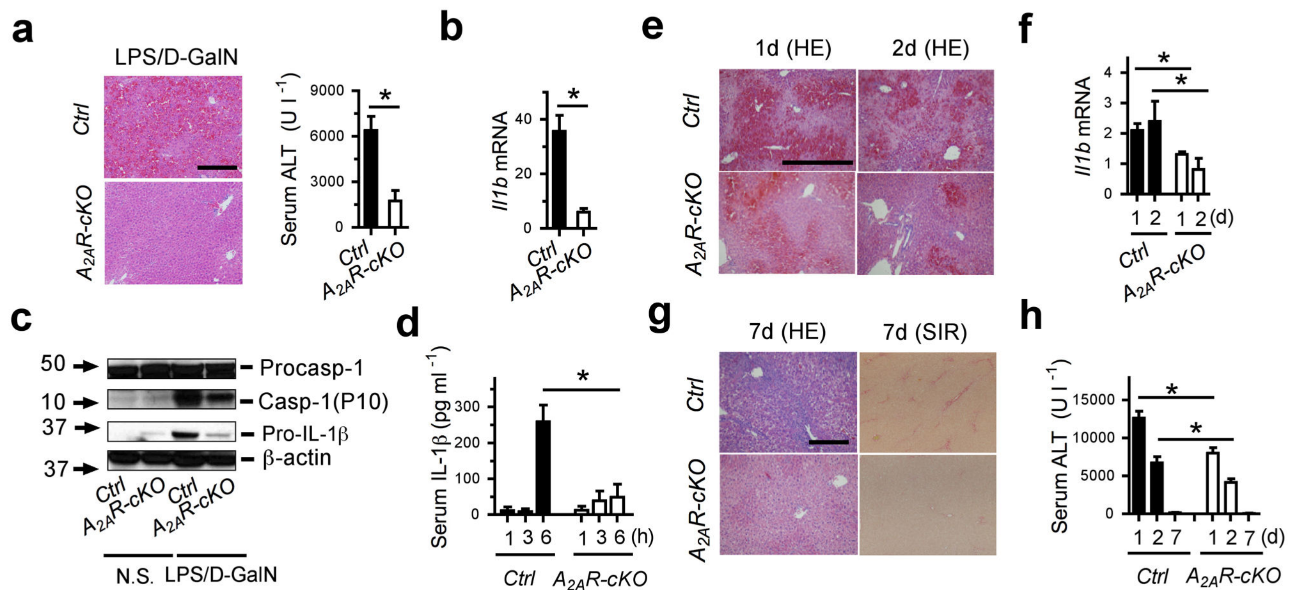

Figure 5. Liver injury and fibrosis is dependent on A_{2A} receptor signaling in macrophages

(a) *$A_{2A}R$-cKO* and control mice were injected intraperitoneally with LPS (1 mg/kg) and D-galactosamine (500 mg kg^{-1}) for 6 hrs followed by liver tissue and serum collection for H&E staining and ALT assay. **(b)** Liver RNA samples were collected and *Il1b* gene assayed by real-time PCR using specific primers. **(c)** Liver tissue lysates were assayed for pro-caspase-1, cleaved caspase-1 (p10), and β-actin protein level by immunoblot analysis using specific antibodies. Data are expressed as the mean ± SD from 10–11 mice from each group for **a-d. (d)** Serum was collected for measurement of IL-1β. **(e)** *$A_{2A}R$-cKO* and control mice were injected intraperitoneally with single dose of TAA followed by liver tissue collection as indicated for H&E staining. **(f)** Liver RNA samples were collected and *Il1b* gene was assayed by real-time PCR. **(g)** Liver tissue was also obtained at day 7 after TAA injection and stained for H&E and Sirius red for fibrosis. **(h)** Sera were collected and the serum ALT assay was performed (Data are expressed as the mean ± SD from 5 mice in each group). * $p < 0.05$ determined by Student's *t*-test. Scale bars correspond to 500μm.