Physiological control of NKT cell-dependent hepatitis induction by extracellular adenosine

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Summary

Extracellular adenosine regulates inflammatory responses via A2A adenosine receptor (A2AR). A2AR-deficiency results in much exaggerated acute hepatitis, indicating non-redundancy of adenosine-A2AR pathway in inhibitory mechanisms of immune activation. To identify a critical target of immunoregulatory effect of extracellular adenosine, we focused on NKT cells, which play an indispensable role in hepatitis. A2AR agonist abolished NKT cell-dependent induction of acute hepatitis by Con A or α-galactosylceramide (α-GalCer), corresponding to down-regulation of activation markers and cytokines in NKT cells and of NK cell co-activation. These results show that A2AR signaling can down-regulate NKT cell activation and suppress NKT cell-triggered inflammatory responses. Next, we hypothesized that NKT cells might be under physiological control of the adenosine-A2AR pathway. Indeed, both Con A and α-GalCer induced more severe hepatitis in A2AR$^{-/-}$ mice than in wild-type controls. Transfer of A2AR$^{-/-}$ NKT cells into A2AR-expressing recipients resulted in exaggeration of Con A-induced liver damage, suggesting that NKT cell activation is controlled by endogenous adenosine via A2AR, and this physiological regulatory mechanism of NKT cells is critical in the control of tissue-damaging inflammation. The current study suggests the possibility to manipulate NKT cell activity in inflammatory disorders through intervention to the adenosine-A2AR pathway.

Keywords

NKT cell; adenosine; A2A adenosine receptor; hepatitis; immunoregulation

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Conflict of interest

The authors declare no financial and commercial conflict of interest.

Introduction

Adenosine in the intracellular pool is at the center of energy and nucleic acid metabolism. However, extracellular adenosine plays a distinctive role in intercellular signaling through cell surface receptors. Four known adenosine receptors, A1, A2A, A2B and A3 receptors, are expressed in many cell types at various levels and regulate physiological functions in cardiovascular, respiratory and central nervous systems [1, 2]. Most immune cells express A2AR at high levels, and adenosine-A2AR interaction suppresses immune functions of granulocytes, macrophages, dendritic cells, NK cells and T cells [2, 3]. Therefore, effect of extracellular adenosine is generally anti-inflammatory and tissue-protective as it prevents inflammatory tissue injury in various experimental models.

Increase of extracellular adenosine levels has been observed in asthma, endotoxic/septic shock, and pulmonary and hepatic injury [4–6]. The increase of adenosine is presumably subsequent to inflammatory tissue damage. Damaged cells may release their intracellular adenosine and adenosine phosphates to extracellular space [7, 8]. Extracellular adenosine phosphates are catabolized to adenosine by ecto-nucleotidases, CD39 metabolizing ATP to AMP and CD73 further metabolizing AMP to adenosine [2, 3]. Extracellular production of adenosine by these cell surface enzymes is responsible for the elevation of adenosine concentration [9, 10]. Upregulation of this anti-inflammatory factor during inflammation implied that adenosine might be produced in inflamed tissue to stop exaggeration of inflammatory activities and prevent excessive damage to vital tissue. Indeed, adenosine's involvement in this negative feedback mechanism was demonstrated by much exaggerated inflammation in mice lacking A2AR [11–13]. Thus, the danger signal representing potential excessive tissue damage picked up by A2AR downregulates proinflamamtory activities and accelerates resolution of inflammation.

Inflammatory response in the liver is also under control of adenosine. Strong anti-inflammatory effect of A2AR agonists has been shown in acute hepatitis induction by Con A [11], D-galactosamine plus LPS [14] and ischemia-reperfusion [15, 16]. Conversely, antagonists of A2AR exacerbated acute hepatitis [11, 17]. This effect is consistent with exaggerated hepatitis in A2AR-deficient mice, demonstrating that endogenously formed adenosine via A2AR counteracts proinflammatory activities and decreases intensity of hepatic inflammation [11]. Therefore, spontaneous adenosine production plays a crucial role in pathophysiology of hepatitis; however, specific target of adenosine-mediated down-regulation of hepatitis is not clear.

NKT cells are one of the early responders to inflammatory stimuli. NKT cell activation involving robust cytokine production within hours after stimulation influences type and intensity of overall immune response [18–20]. NKT cells, a relatively more frequent population in the liver than in other tissues, play a pivotal role in the induction of hepatitis. To study pathogenesis of hepatitis, Con A-induced liver injury has been widely used for its resemblance to viral and autoimmune hepatitis. NKT cells play a key role in this hepatitis model by producing cytokines such as IL-4, IFN-γ and TNF-α that mediate the liver injury [21–24]. The lack of NKT cells completely abolishes Con A-induced liver injury [23, 24]. More recently, NKT cells were also shown to play an important role in the induction of

hepatic ischemia-reperfusion injury [16, 25, 26]. Hepatitis induction by the injection of α-GalCer, an antigenic ligand of NKT cells, indicated that NKT cell activation could be sufficient to trigger inflammatory response resulting in acute hepatitis [27, 28].

Importance of NKT cells in the pathogenesis of hepatitis suggests a possibility that NKT cells represent a critical target of adenosine-dependent regulation of hepatic inflammation. Indeed, A2AR agonist was shown to inhibit activation of NKT cells [16, 29], and suppresses NKT cell-dependent induction of ischemia-reperfusion injury [16, 25, 26]. In this study, we questioned whether NKT cell activation is under control of endogenous adenosine. To test the involvement of A2AR-mediated NKT cell inhibition in adenosinergic regulation of inflammation, we utilized NKT cell-dependent acute hepatitis models: Con A-induced and α-GalCer-induced liver injury. Results of the current study suggest that endogenous adenosine regulates NKT cell activation and thereby suppresses subsequent induction of hepatitis.

Results

A2AR-mediated inhibition of NKT cell activation

As previously reported in a few different models of acute hepatitis [11, 14–16], administration of A2AR agonist, CGS21680 (CGS), abolished Con A-induced liver injury (Fig. 1A). Suppression of liver injury accompanied remarkable decrease of early production of IL-4 and TNF-α (Fig. 1B, C), which are essential to the induction of hepatitis after Con A injection [21, 23, 24]. NKT cells play an indispensable role in Con A-induced liver injury through early IL-4 production [23, 24]. Extent of NKT cell activation was evaluated by flow cytometry. Consistent with previous reports [30], Con A treatment reduced the proportion of NKT cells to approximately 75 % of control level after 1.5 h. Co-injection of CGS down-regulated Con A-induced CD69 and CD40 ligand (CD40L) on NKT cells (Fig. 1D–F), suggesting that A2AR stimulation suppressed NKT cell activation after Con A injection.

Expression of functional A2AR on NKT cells was confirmed by cAMP induction in response to adenosine receptor agonists. Nonspecific adenosine receptor agonist NECA and A2AR-selective agonist CGS induced cAMP, and A2AR antagonist ZM241385 (ZM) blocked the increase (Fig. 2A). The addition of α-GalCer to cell culture stimulated NKT cells to produce IFN-γ, but NECA and CGS strongly suppressed the IFN-γ production (Fig. 2B). Agonists of other adenosine receptors, CCPA (A1 receptor agonist) and Cl-IB-MECA (A3 receptor agonist), did not significantly reduce IFN-γ levels. A2AR antagonist ZM reversed the inhibitory effect of CGS and NECA. Along with this result, CGS and NECA failed to inhibit IFN-γ production from NKT cells in A2AR-deficient (A2AR$^{-/-}$) mouse (Fig. 2C), confirming that stimulation of A2AR is strongly immunosuppressive to NKT cells.

Susceptibility of NKT cells to A2AR agonist was examined in vivo by direct stimulation of NKT cells with α-GalCer. Again, α-GalCer treatment downregulated NKT cell proportion as reported previously [31]. Number of NKT cells were reduced to approximately 75 % of control level after 2 h. α-GalCer induced activation of NKT cells within 2 h as evidenced by upregulation of activation markers, CD69 and CD40L (Fig. 3A, B). At the same time, these

NKT cells were producing IFN-γ and IL-4 (Fig. 3C, D). Co-treatment with CGS significantly inhibited upregulation of activation markers and cytokines production (Fig. 3A–D). Early activation of NKT cells is known to induce extensive co-activation of other immune cells [20, 32, 33]. In the current experiment, α-GalCer injection resulted in increases of CD69 and IFN-γ in NK cells; however, CGS also down-regulated such activation of NK cells (Fig. 3E, F). In A2AR$^{-/-}$ mice, CGS did not affect any of activation markers and cytokines in NKT and NK cells, confirming A2AR-dependence of the suppressive effect (Fig. 3A–F). In vivo activation of NKT cells by α-GalCer injection eventually results in hepatic injury. CGS blocked the induction of liver damage along with the increase of serum IL-4, IFN-γ and TNF-α levels after α-GalCer injection (Fig. 3G–J). In addition to the decrease of proinflammatory cytokines, CGS rather induced IL-10, an anti-inflammatory cytokine (Fig. 3K). Thus, A2AR stimulation suppressed NKT cell activation in vivo and consequently blocked propagation of inflammatory response that could cause acute hepatitis.

Regulation of NKT cell activation by endogenous adenosine

Experiments above showed that the adenosine-A2AR signaling pathway could block hepatitis induction by suppressing NKT cell activation. Next, we questioned whether endogenously produced adenosine is physiologically important in controlling NKT cell activation. To test this issue, we compared outcome of NKT cell activation in A2AR$^{-/-}$ mice with that of wild-type mice. As we previously reported [11], A2AR$^{-/-}$ mice were very susceptible to Con A-induced liver injury along with pronounced early increase of TNF-α and IFN-γ, but not IL-4 (Fig. 4). Since pathogenesis of Con A-induced hepatitis is NKT cell-dependent, we speculated a possible involvement of A2AR in the regulation of NKT cell activation.

If endogenous adenosine is vitally regulating NKT cells, stronger immune response will be expected in α-GalCer-injected A2AR$^{-/-}$ mice. Two hours after α-GalCer injection, upregulation of CD69 and CD40L in A2AR$^{-/-}$ NKT cells tended to be slightly higher than wild-type NKT cells, though the differences were not statistically significant (Fig. 3A, B). Such trend was the same for cytokine upregulation in NKT cells (Fig. 3C, D). Although exaggerated activation of A2AR$^{-/-}$ NKT cells was not evident from analyses after 2 h, significantly higher levels of IFN-γ production, but not IL-4, were observed in A2AR$^{-/-}$ mice thereafter (Fig. 5A, B). More than 2-fold increase of α-GalCer-induced serum IFN-γ levels after 4 and 6 hours suggests stronger NKT cell-mediated response in A2AR$^{-/-}$ mice. Although TNF-α levels were unchanged (Fig. 5C), serum IL-10 levels were largely decreased in A2AR$^{-/-}$ mice (Fig. 5D), suggesting attenuation of anti-inflammatory response in the absence of A2AR. Consistent with the changes in serum cytokine levels, NKT cell stimulation in A2AR$^{-/-}$ mice enhanced subsequent immune responses. Although extent of NK cell activation was at similar levels after 2 h (Fig. 3E, F), IFN-γ from NK cells became significantly enhanced in A2AR$^{-/-}$ mice after 4 h (Fig. 5E). NKT cell activation in A2AR$^{-/-}$ mice led to exacerbation of α-GalCer-induced acute hepatitis. More extensive hepatocellular damage was reflected in an increase of serum ALT levels (Fig. 5F) and tissue histology (Fig. 5G).

The different outcomes of direct NKT cell stimulation suggested an augmented NKT cell activity in A2AR$^{-/-}$ mice. Since NKT cells were proportional in wild-type and A2AR$^{-/-}$ mice (Fig. 6A), the difference was likely to be qualitative, not quantitative. To examine a difference in NKT cell activity, we injected wild-type or A2AR$^{-/-}$ NKT cells into RAG-1$^{-/-}$ mice and induced Con A hepatitis. Without NKT cell transfer, Con A induced no notable liver damage in RAG-1$^{-/-}$ mice, which lack T, B and NKT cells. Transfer of NKT cells made RAG-1$^{-/-}$ mice vulnerable to Con A-induced liver injury. However, transfer of A2AR$^{-/-}$ NKT cells induced much stronger liver damage (Fig. 6B, C). The lack of A2AR only in NKT cells sufficiently exaggerated acute hepatitis, indicating enhanced inflammatory activity of A2AR$^{-/-}$ NKT cells. This result suggests that activity of NKT cells is under regulation by endogenous adenosine through A2AR, and disruption of this physiological negative regulation can cause massive inflammatory hepatic damage.

Discussion

Divergent roles of extracellular adenosine have been observed in inflammation-related diseases in the liver. As evidenced by much exacerbated acute hepatitis in A2AR$^{-/-}$ mice, formation of extracellular adenosine is an indispensable physiological stop signal to prevent excess inflammation [11–13]. In the resolution of hepatitis, however, adenosine-A2AR interaction may promote induction of cirrhosis. Adenosine was found to facilitate collagen synthesis, and blockade of A2AR signaling prevented hepatic fibrosis [34, 35]. Furthermore, adenosine's involvement in steatosis was also suggested [36]. Thus, adenosine is a notable regulator of hepatic proinflammatory responses, remodeling of damaged tissue and energy metabolism in the liver, the imbalance of which will lead to disease state.

A2AR stimulation and subsequent cAMP induction strongly impair immune cell activation. Effector function of T cells, especially IFN-γ-producing activity, is very susceptible to A2AR agonist [37, 38]. NKT cells, as well as conventional T cells, were sensitive to A2AR stimulation. In vivo administration of A2AR agonist blocked CD69 and CD40L upregulation and cytokine production in NKT cells (Fig. 3), showing A2AR-mediated inhibitory pathway of NKT cell activation and their functions. Our experiment confirmed that NKT cells express functional A2AR and produce cAMP in response to agonists (Fig. 2) [16, 29]. NKT cells are susceptible to cAMP induction as shown by the impairment of NKT cell activation with cAMP inducers such as forskolin, phosphodiesterase inhibitor and dibutyryl cAMP [39].

NKT cells have been shown to play a major role in liver diseases. Induction of hepatic tissue damage after direct activation of NKT cells indicated their pathogenic role in hepatitis [27, 28]. NKT cells are essential to the induction of Con A hepatitis [23, 24] and ischemia-reperfusion injury [16]. To attenuate ischemia-reperfusion damage in the liver and in sickle cell disease, NKT cells were targeted to block their activity [16, 25, 26]. Indeed, to suppress NKT cell activity, these ischemia-reperfusion studies employed pharmacological stimulation of A2AR.

In the current study, A2AR-mediated suppression of NKT cell activation in vivo corresponded well with the inhibition of acute hepatitis by CGS (Fig. 1, 3). NKT cell

activation in a very early phase of immune response further activates diverse types of immune cells, orchestrating later expansion of inflammation [20, 32, 33]. A2AR agonist attenuated NKT cell activation as well as co-activation of NK cells (Fig. 3). These results show that the cascade of inflammatory responses triggered by NKT cells can be inhibited when exogenous A2AR agonist is introduced.

Spontaneous formation of extracellular adenosine in vivo has been shown to down-regulate inflammation and consequently protect tissues from excessive inflammatory damage [11]. This A2AR-mediated function of endogenous adenosine is non-redundant and may represent one of the most important immune-regulatory negative feedback mechanisms [2, 3, 12]. Then, we hypothesized that endogenous adenosine might be a physiological modulator of NKT cell activation. In vitro experiments using adenosine may underestimate adenosine concentration needed for NKT cell inhibition because adenosine deaminase activity present in cell culture media causes rapid degradation of adenosine [40]. Therefore, evaluation of pathophysiological significance of A2AR-mediated NKT cell regulation relies on in vivo assay, which reflects possible effects of local concentration of adenosine. Failure in A2AR$^{-/-}$ mice to receive immunoregulatory signal of endogenous adenosine exaggerated NKT cell-dependent induction of hepatitis. The results from A2AR$^{-/-}$ mice suggested A2AR-dependent negative regulation of NKT cells by physiological adenosine.

De-inhibition of NKT cells from A2AR-mediated suppression might be responsible for the exaggeration of acute hepatitis in A2AR$^{-/-}$ mice, though other immune effector cells could be also de-inhibited at the same time. To examine if the lack of A2AR only in NKT cells causes exacerbation of hepatitis, we injected purified NKT cells to A2AR-expressing recipients. Significant augmentation of hepatitis in mice received A2AR$^{-/-}$ NKT cells indicates that de-inhibition of NKT cells from A2AR-dependent regulation can sufficiently enhance acute hepatitis (Fig. 6). For endogenous immuno-regulators, molecules such as LECT2, CCR5 and BTLA are known to be capable of regulating NKT cells [41–43]. The current study suggests the pathophysiological significance of extracellular adenosine-dependent NKT cell regulation in liver inflammation.

Direct NKT cell activation by α-GalCer injection induced significantly higher levels of IFN-γ, but not IL-4, in A2AR$^{-/-}$ mice (Fig. 5). IL-10 levels were reduced in α-GalCer-injected A2AR$^{-/-}$ mice (Fig. 5), while CGS induced IL-10 in wild-type mice (Fig. 3). These changes correspond to a shift to Th2-type cytokine pattern in NKT cells exposed to adenosine [29]. Early and robust cytokine production from NKT cells is believed to change the course of inflammation. IFN-γ production from NKT cells elicits Th1-type immune response in the activation of anti-tumor immunity [44] and in the prevention of allergic disorders [45]. Enhanced IFN-γ production and NK cell activation after NKT cell stimulation in A2AR$^{-/-}$ mice (Fig. 5) implies promotion of cellular immune response, which may explain the exaggeration of inflammatory liver damage in these mice.

In the exacerbation of Con A-induced liver injury in A2AR$^{-/-}$ mice, levels of IFN-γ and TNF-α, proinflammatory cytokines essential to optimal liver damage [21, 22], were found to increase in the absence of A2AR (Fig. 4) [11]. TNF-α is also essential to hepatitis induction by α-GalCer [28], but TNF-α levels in A2AR$^{-/-}$ mice were comparable to wild-type mice

(Fig. 5). Although IFN-γ is not necessary to the induction of liver damage after α-GalCer injection, this cytokine is a strong inducer of IRF-1, which is indispensable to α-GalCer hepatitis [46]. Since IRF-1 increases dependent on IFN-γ concentration, augmented IFN-γ production in A2AR$^{-/-}$ mice may have contributed to the enhancement of liver damage.

In conclusion, we provide evidence for not only A2AR-mediated suppression of NKT cell activation but also augmented NKT cell activity in A2AR-deficient mice. These results strongly suggest physiological immunoregulatory mechanism controlling activation of NKT cells via adenosine-A2AR signaling pathway. Extracellular adenosine is known to spontaneously increase in inflamed tissues [4–6] and tumors [47, 48]. Tissue hypoxia in these environments is conductive to the increase of extracellular adenosine levels. Hypoxia-triggered induction of 5'-nucleotidases, CD39 and CD73, results in promotion of extracellular adenosine formation by increasing degradation of ATP [49, 50]. Hypoxia also suppresses adenosine kinase-dependent conversion of adenosine to AMP [51–53]. Since an increase of extracellular adenosine can directly affect NKT cell activities, attenuation of NKT cell activities are likely in tumors and tissues under inflammation. NKT cells play an important role in inflammatory disorders, infectious diseases, tumor immunity and vaccination [18–20, 54–56]. It may be possible to manipulate NKT cell activity in these immune responses through intervention to the adenosine-A2AR pathway by means of A2AR agonist/antagonist [11, 14–17], CD73 inhibitor [57–59] or atmosphere containing altered levels of oxygen [60, 61].

Materials and methods

Mice

C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, MA). A2AR$^{-/-}$ mice were backcrossed for 12 times to C57BL/6 mice [17]. C57BL/6-background RAG1$^{-/-}$ mice were obtained from the Jackson Laboratory (Bar Harbor, ME). The mice were housed in the animal facility of Northeastern University and were used at 8–12 weeks of age in accordance with the institutional animal care guidelines.

Concanavalin A (Con A)-induced hepatitis

Con A (Sigma, St. Louis, MO; 12 mg/kg) was injected intravenously to induce acute liver injury. Some mice received intraperitoneal injection of A2AR agonist CGS21680 (0.5 mg/kg) 10 min before Con A. Blood samples were collected at 8 h after Con A injection and liver damage was evaluated by serum ALT levels. ALT levels were determined by a kit from Teco Diagnostics (Anaheim, CA). Serum levels of TNF-α and IL-4 after 1.5 h were determined using ELISA kits from R&D Systems (Minneapolis, MN). CD69 and CD40L upregulation in NKT cells was analyzed 1.5 h after Con A injection by flow cytometry.

Preparation of liver mononuclear cells

LMNC were prepared by density centrifugation using Percoll (GE Healthcare, Upsalla, Sweden). The liver was pressed through stainless steel mesh (#200) and washed by centrifugation. The pellet was resuspended in 40 % Percoll and centrifuged at $500 \times g$ for 15 min at room temperature to isolate liver mononuclear cells from parenchymal hepatocytes

and cell debris. The pellet was collected, treated with ACK lysing buffer (Life Technologies, Grand Island, NY), and washed using RPMI1640 medium containing 10 % FCS.

Purification of NKT cells

Mice were pretreated by intraperitoneal injection of anti-asialo GM1 Ab (50 μg/mouse; Wako Chemicals, Richmond, VA) to deplete NK cells. Splenocytes isolated 2 days after the injection of anti-asialo GM1 Ab were labeled with FITC-conjugated anti-NK1.1 mAb (BD Biosciences, San Jose, CA) and with anti-FITC microbeads (Miltenyi Biotec, Auburn, CA). NKT cells were purified by positive selection of NK1.1$^+$ cells using AutoMACS separator (Miltenyi Biotec). Purity of NK1.1$^+$ CD3$^+$ cells was higher than 90 %.

cAMP assay

Induction of cAMP in response to adenosine receptor agonists was measured as described previously [62]. Purified NKT cells (1×10^5) were incubated with NECA or CGS21680 (10 μM) for 15 min at 37 °C in a total volume of 0.2 ml. Adenosine receptor antagonist, ZM241385 was used at 1 μM. After the incubation, 25 μl of 1N hydrochloric acid was added and the samples were stored at –80 °C. cAMP levels were determined by ELISA (GE Healthcare, Buckinghamshire, UK).

Bone marrow-derived dendritic cells

Dendritic cells were induced from bone marrow cells as described [63]. Bone marrow cells from C57BL/6 mouse femora were suspended in RPMI1640 medium containing 10 % FCS and cultured with GM-CSF (10 ng/ml) and IL-4 (10 ng/ml) in a plastic cell culture plate. Non-adherent cells were removed on the next day. After 5–7 days, adherent cells were used to stimulate NKT cells.

In vitro stimulation of NKT cells

Purified NKT cells (1×10^5 cells) were stimulated by α-GalCer (100 ng/ml; Biomol International, Plymouth Meeting, PA) in the presence of bone marrow-derived dendritic cells (1×10^5 cells). Following adenosine receptor agonists and antagonists were added in the culture at 100 nM: 2-chloro-N^6-cyclopentyladenosine (CCPA, A1 adenosine receptor-selective agonist), CGS21680 (CGS, A2AR-selective agonist), 5'-N-ethylcarboxamidoadenosine (NECA, non-specific adenosine receptor agonist), 1-[2-chloro-6-[[(3-iodophenyl)methyl]amino]-9H-purin-9-yl]-1-deoxy-N-methyl-β-D-ribofuranuronamide (Cl-IB-MECA, A3 adenosine receptor-selective agonist) and ZM241385 (ZM, A2AR antagonist). Forskolin was also used to induce receptor-independent cAMP increase. CCPA, CGS, Cl-IB-MECA and ZM were obtained from Tocris (Ellisville, MO). NECA and forskolin are from Sigma (St. Louis, MO). The cells were cultured for 24 h, and cytokine levels in the supernatant were determined by ELISA.

In vivo NKT cell activation by α-GalCer

α-GalCer (1 μg/mouse) was injected intravenously and serum IL-4, IFN-γ, TNF-α and IL-10 levels were determined after 2–6 h. Spleen cells were analyzed for the expression of CD69, CD40L, IFN-γ and IL-4. Liver damage was assessed 18–24 h after the injection by serum

ALT levels and histochemistry. For histochemistry, liver tissue was fixed in 10% formalin-PBS, and the paraffin-embedded tissue slice was stained with hematoxyline-eosin (Mass Histology Service, Worcester, MA).

Flowcytometric analysis of cytokine production from NKT cells

IFN-γ and IL-4 production from NKT cells were analyzed 2 h after Con A injection by intracellular staining [62]. After surface staining, cells were fixed with 4 % paraformaldehyde-PBS for 15 min. After washing with PBS, cells were treated with permeabilizing buffer (50 mM sodium chloride, 5 mM EDTA, 0.02 % sodium azide, 0.5 % Triton X-100, 10 mM Tris-HCl, pH 7.5) for 15 min. Intracellular cytokines were stained with anti-IFN-γ and anti-IL-4 mAbs, and cytokine expression in NK1.1^{+} CD3^{+} cells was analyzed by FACSCalibur (BD Biosciences). All antibodies were obtained from BD Biosciences.

Adoptive transfer of NKT cells

Immediately after purification, NKT cells (1×10^6 cells) were suspended in 50 μl saline and injected into RAG1^{−/−} mice at the lateral left lobe of liver [24]. One hour after the cell transfer, the recipient mice received intravenous injection of Con A (20 mg/kg). The liver damage was evaluated after 8 and 24 h.

Statistics

Data represent mean ± SD. Statistical calculations were performed using Student's t-test. Statistical significance was accepted for p values less than 0.05.

Acknowledgements

Abbreviation

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A2AR	A2A adenosine receptor
α-GalCer	α-galactosylceramide
CD40L	CD40 ligand
CGS	CSG21680
FasL	Fas ligand
ZM	ZM241385

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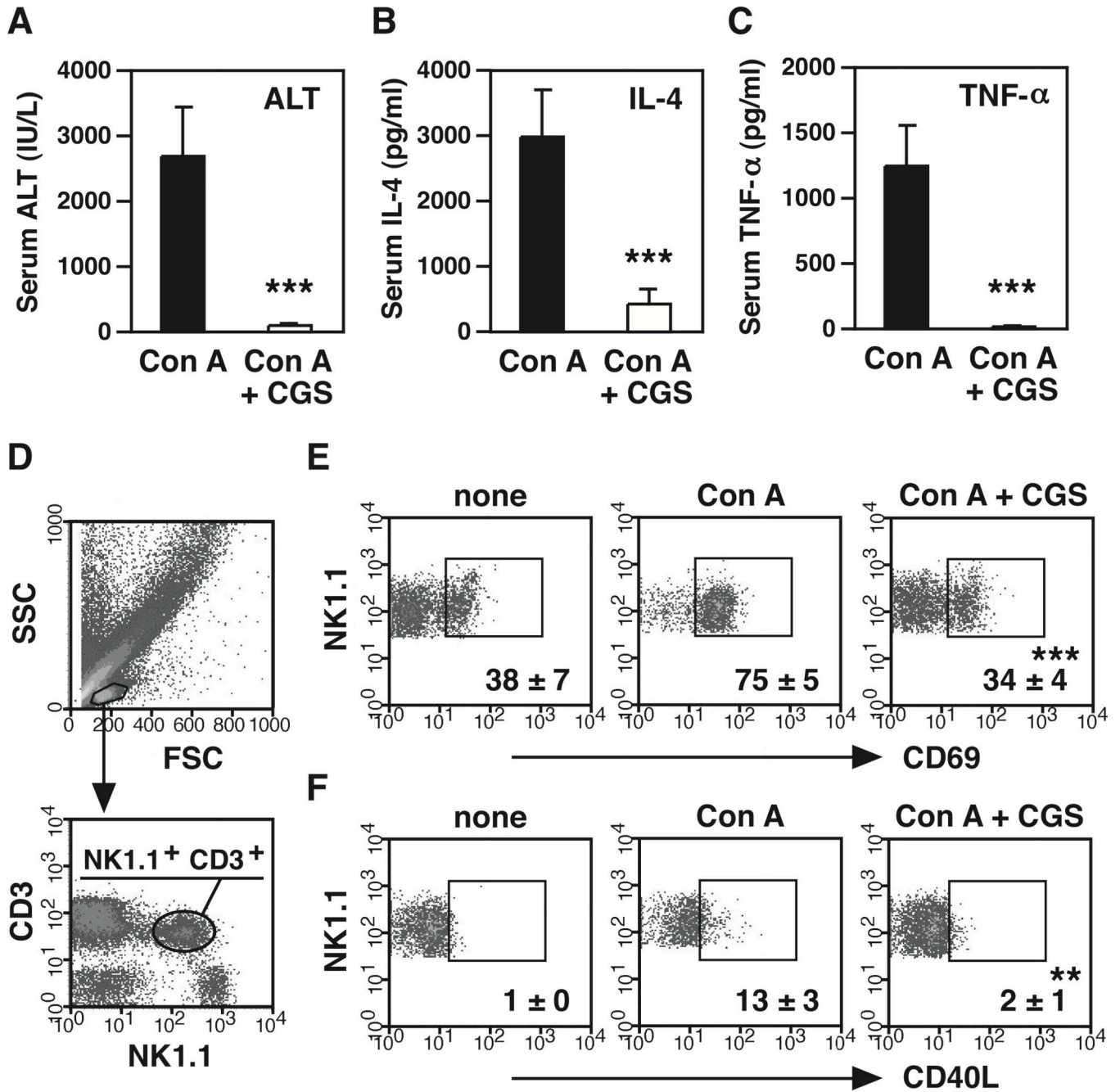
Figure 1.
A2AR stimulation inhibits induction of acute hepatitis and activation of NKT cells. (A–C) Effect of A2AR agonist CGS21680 (CGS) on Con A-induced liver injury. C57BL/6 mice received i.p. injection of CGS (0.5 mg/kg) 10 min prior to Con A administration (12 mg/kg i.v.). Serum ALT levels (A) were determined after 8 h. Serum IL-4 (B) and TNF-α (C) levels were determined after 1.5 h. (D) Gating strategy for flow cytometric analysis of NKT cells in liver mononuclear cells. (E, F) Representative density plots of CD69 (E) and CD40L (F) expression on NK1.1^{+} CD3^{+} cells in mice liver 1.5 h after Con A injection. "None" means untreated control. Numbers represent percentages of gated cells. ** P < 0.01, *** P <

0.001 vs Con A alone; Student's t-test. Data represent average ± SD of 5 mice (A–C) or 3 mice (E, F) and are representative of 3 (A–C) and 2 (E, F) independent experiments.
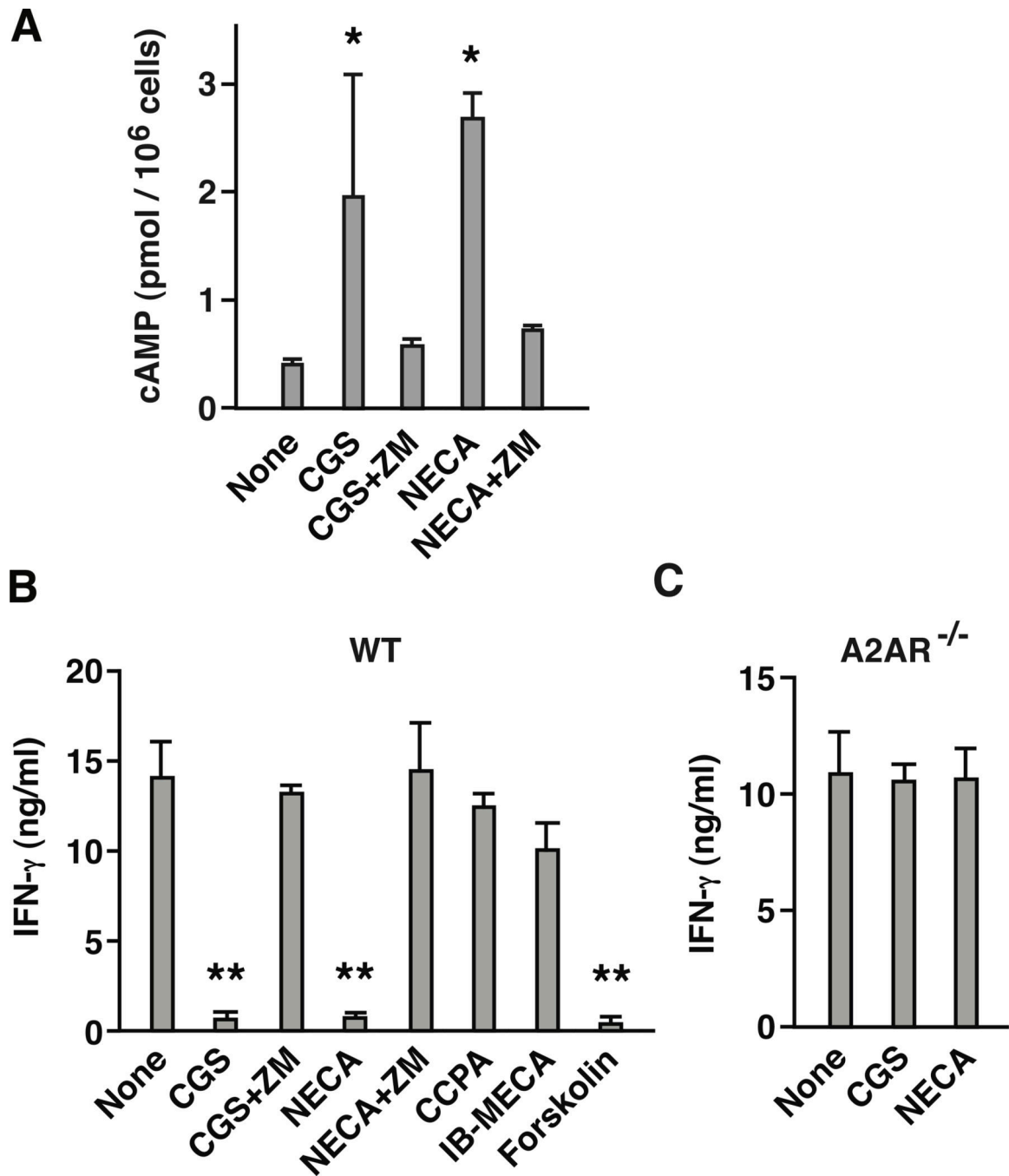
Figure 2.
NKT cells express functional A2AR, and stimulation of A2AR suppresses NKT cell activation. (A) cAMP upregulation in NKT cells in response to A2AR stimulation. Purified NKT cells (1×10^5 cells) were incubated with 10 μM CGS (A2AR agonist) and 10 μM NECA (non-specific adenosine receptor agonist) in the presence or absence of A2AR antagonist (ZM; 1 μM). After 15 min of incubation at 37 °C, cAMP levels were determined by ELISA. (B, C) IFN-γ production from wild-type (B) and A2AR$^{-/-}$ (C) NKT cells. Purified NKT cells (5×10^4 cells) were stimulated with α-GalCer (100 ng/ml) in the

presence of syngenic dendritic cells (2×10^5 cells). Effects of adenosine receptor stimulation were examined in the presence of various agonists and antagonist (100 nM): CGS (A2AR agonist), NECA (non-specific adenosine agonist), CCPA (A1 receptor agonist), IB-MECA (A3 receptor agonist), and ZM (A2AR antagonist). Forskolin (10 μM) was used to induce adenosine receptor-independent cAMP upregulation. IFN-γ levels in the culture supernatant were determined after 24 h by ELISA. * $P < 0.05$, ** $P < 0.01$ vs control; Student's t-test. Data represent average ± SD of triplicate samples and are representative of 2 (A) and 3 (B, C) independent experiments.
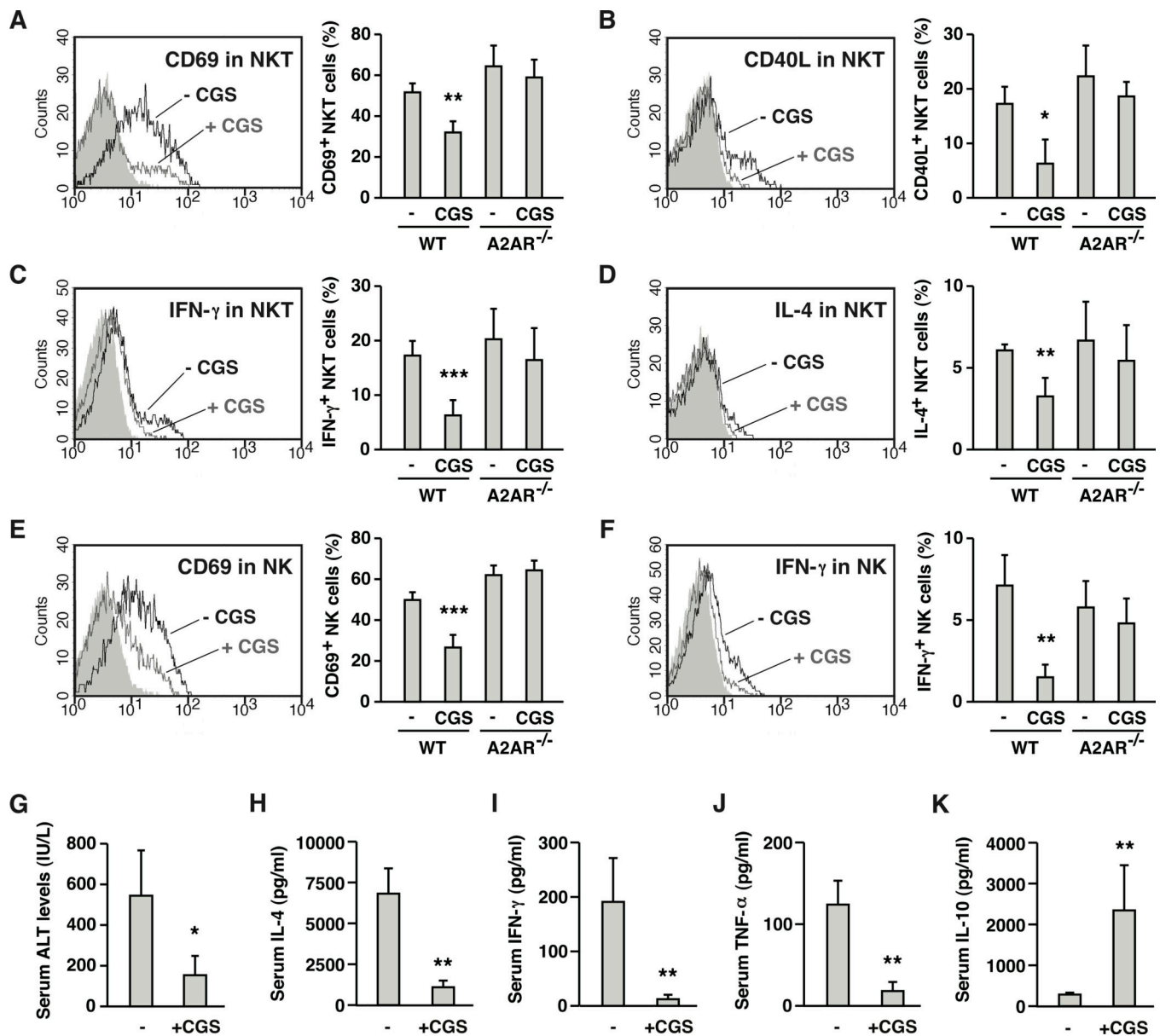
Figure 3.

A2AR agonist suppresses NKT cell-dependent immune responses in vivo and blocks inflammatory damage in the liver. Wild-type (WT) and A2AR^{−/−} mice received intravenous injection of α-galactosylceramide (α-GalCer; 1 μg/mouse) with or without CGS (0.5 mg/kg i.p.). After 2 h, NKT (NK1.1^{+} CD3^{+}) cells were analyzed for the expression of CD69 (A), CD40L (B), IFN-γ (C) and IL-4 (D) by flow cytometry. Black and grey lines represent α-GalCer alone and α-GalCer + CGS, respectively. Shaded peaks are background levels. Positive/negative signal threshold is the fluorescence intensity where >98% of background events are included. Fluorescence exceeding this level was defined as positive. To monitor co-activation of NK cells, CD69 (E) and IFN-γ (F) levels in NK1.1^{+} CD3^{−} cells were also analyzed after 2 h. The intensity of α-GalCer-induced liver damage was determined by serum ALT levels 18 h after α-GalCer injection (G). Serum levels of IFN-γ (H), IL-4 (I),
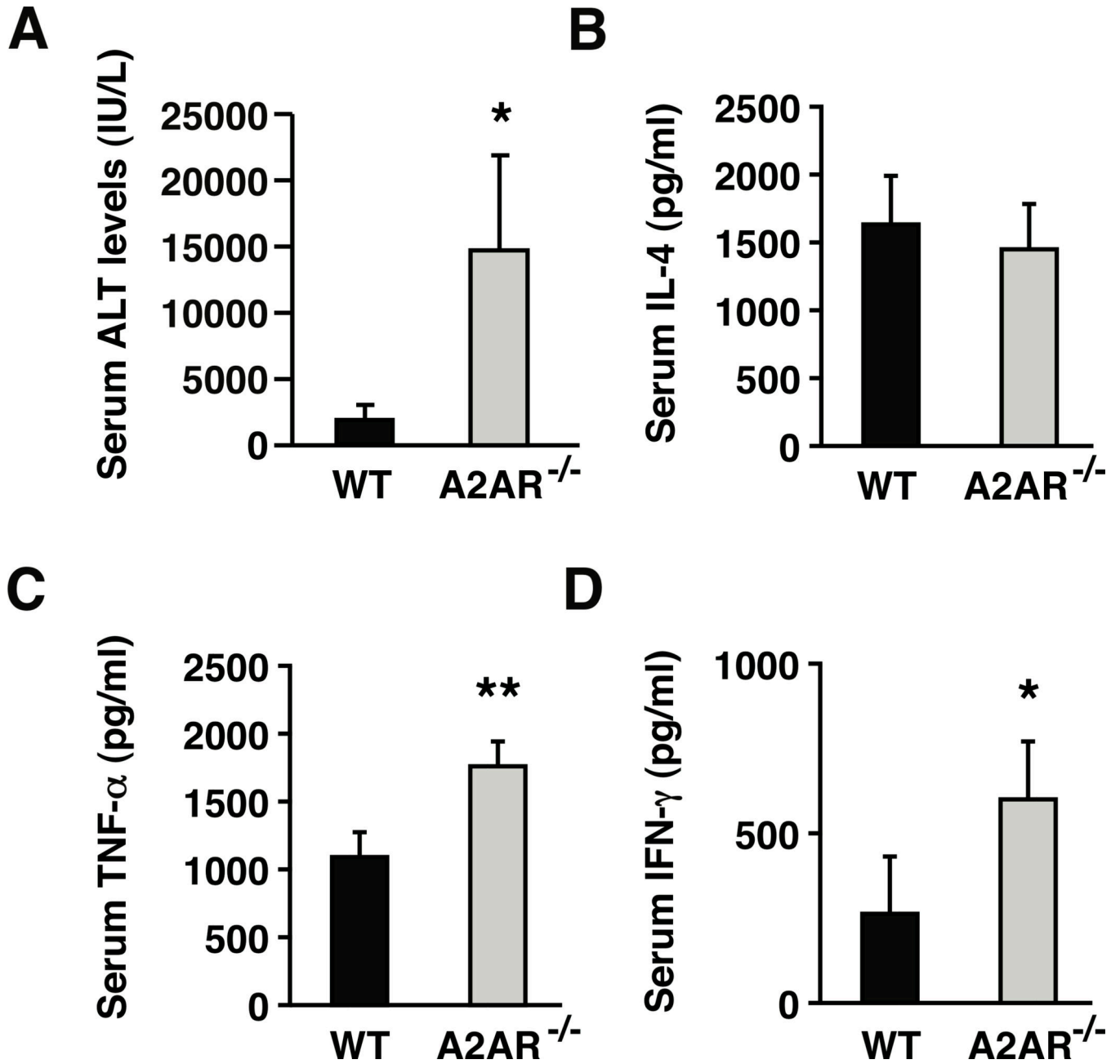
TNF-α (J) and IL-10 (K) were determined 2 h after α-GalCer injection. * P < 0.05, ** P < 0.01, *** P < 0.001 vs control; Student's t-test. Data represent average ± SD of 4 mice (A-F, H-K) or 3 mice (G) and are representative of 3 independent experiments.

Figure 4.
Exacerbation of Con A-induced liver injury in A2AR$^{-/-}$ mice. WT and A2AR$^{-/-}$ mice received intravenous injection of Con A (10 mg/kg). (A) Serum ALT levels 8 h after Con A injection. (B–D) Serum cytokine levels after Con A injection were determined by ELISA. Data shown here are IL-4 (B) and TNF-α (C) levels after 1.5 h and IFN-γ (D) levels 8 h after Con A injection. * P < 0.05, ** P < 0.01 vs WT mice; Student's t-test. Data represent average ± SD of 5 mice and are representative of 3 independent experiments.
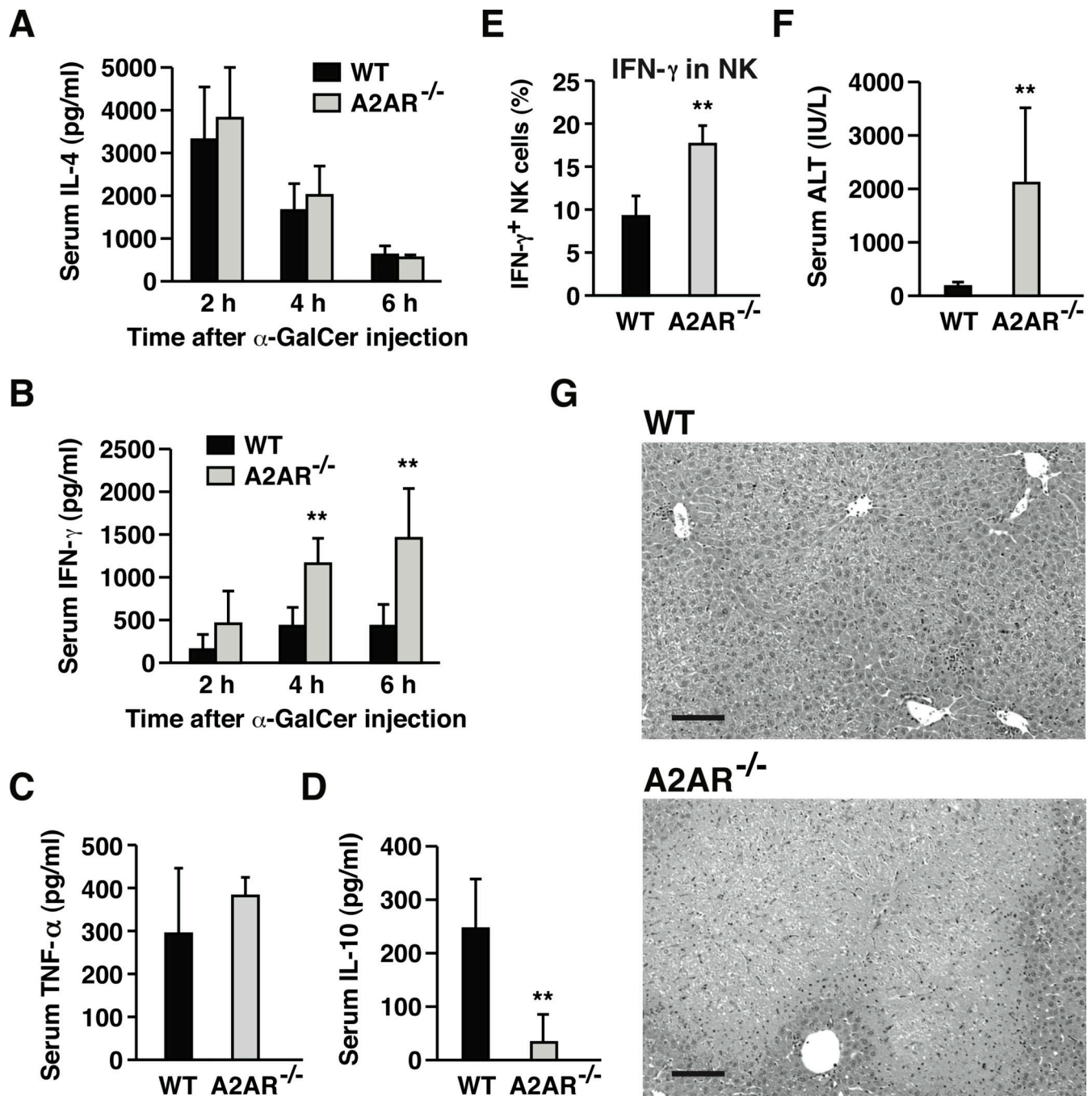
Figure 5.
Exaggerated inflammation and liver damage in A2AR$^{-/-}$ mice after direct activation of NKT cells. WT and A2AR$^{-/-}$ mice received intravenous injection of α-GalCer (1 μg/mouse), and serum IL-4 (A) and IFN-γ (B) levels were monitored between 2–6 h by ELISA. Serum TNF-α (C) and IL-10 levels (D) were also determined after 2 h by ELISA. (E) IFN-γ production from NK (NK1.1^{+} CD3^{-}) cells was determined 4 h after α-GalCer injection by flow cytometry. (F, G) Intensities of α-GalCer-induced liver damage were evaluated by serum ALT levels (F) and H-E staining of liver tissue (G) after 24 h. Bars represent 100 μm. ** P <
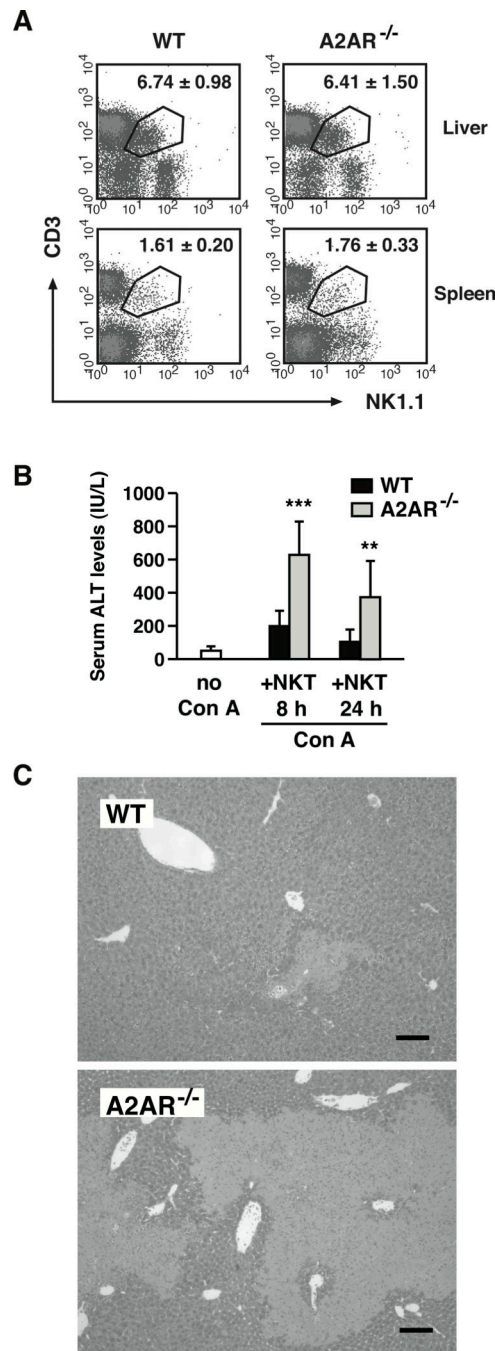
0.01 vs wild-type mice; Student's t-test. Data represent average ± SD of 4 (A–E) or 5 (F) mice and are representative of 3 independent experiments.

Figure 6.
Endogenous adenosine targets A2AR on NKT cells to down-regulate overall inflammatory tissue injury in the liver. (A) Representative density plots of NK1.1$^+$ CD3$^+$ cells in untreated WT and A2AR$^{-/-}$ mice. Numbers represent percentages of NK1.1$^+$ CD3$^+$ cells in the liver mononuclear cells and spleen cells. Data shown are average ± SD of 7 mice. (B, C) Induction of Con A hepatitis after the transfer of NKT cells. RAG-1$^{-/-}$ mice received intrahepatic transfer of NKT cells (1 × 10^6 cells) purified from WT or A2AR$^{-/-}$ mice. One hour after the cell transfer, Con A (20 mg/kg i.v.) was injected to induce hepatitis, and

serum ALT levels (B) were evaluated after 8 and 24 h. ALT levels in RAG-1$^{-/-}$ mice without NKT cell transfer did not increase by Con A injection (ALT 56 ± 4 IU/L after 8 h). Extent of tissue damage was also evaluated in H-E stained liver tissue 24 h after the injection of Con A (C). The images are representative of the liver in Con A-injected RAG-1$^{-/-}$ mice that received transfer of NKT cells from WT or A2AR$^{-/-}$ mice. Bars represent 100 μm. ** $P < 0.01$, *** $P < 0.001$ vs control; Student's t-test. Data represent average $\pm$ SD of 7 mice and are pooled from 3 experiments.