THE MECHANISM OF ADENOSINERGIC REGULATION OF T-CELL MEDIATED ACUTE HEPATITIS

THESIS PRESENTED

BY

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Endogenous immune regulatory mechanisms exist to protect healthy tissue from collateral damage by overactive immune cells during inflammation. It has been found that extracellular adenosine can suppress pro-inflammatory activities through A2A adenosine receptors (A2ARs) on immune cells. This function of endogenous adenosine is non-redundant and has been regarded recently as a critical immune-regulatory negative feedback mechanism. Inflammatory responses in the liver have been shown to be under the control of A2AR-mediated anti-inflammatory pathway. The current study was designed to investigate the critical target of adenosine-mediated regulation of acute hepatitis. Experiments employing a murine model of acute hepatitis indicated exacerbated liver damage accompanied by exaggerated levels of cytokines TNF-α, IL-4 and IFN-γ in A2AR-deficient (A2ARKO) mice as compared to the wild type (WT) control animals. Since natural killer T cells (NKT cells) are responsible for the early production of these cytokines in this model of hepatitis, NKT cells were suspected to be under the physiological control of the ‘adenosine-A2AR anti-inflammatory pathway’. Indeed, activation of NKT cells was susceptible to A2AR stimulation, and more importantly, NKT cells from A2ARKO mice produced higher levels of cytokines in vivo and induced stronger liver damage as compared to WT NKT cells. Analysis of adenosine receptors in NKT cells suggested their expression of A2B adenosine receptors (A2BRs) along with A2AR. Studies using A2BRKO mice also showed exaggerated NKT cell responses in vivo as were observed in A2ARKO NKT cells. These results demonstrate the involvement of A2ARs and A2BRs in the physiological regulation of NKT cell activation. Levels of extracellular adenosine have been known to increase in response to hypoxia. Therefore, it was suspected that hypoxia could negatively regulate NKT cell activation through adenosine production. As expected, NKT cells were found to be susceptible to hypoxia both in
vivo and in vitro. The mechanism of hypoxic suppression of NKT cell activation was partially mediated by adenosine. Results obtained in the current study provide evidence that the immunoregulatory hypoxia-adenosine pathway negatively regulates NKT cell activation, at least in part via A2AR.
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**Abbreviations:**

A2AR (A2A adenosine receptor)

A2BR (A2B adenosine receptor)

A2ARKO mice (A2A adenosine receptor gene deficient mice)

A2BRKO mice (A2B adenosine receptor gene deficient mice)

ALT (alanine amino transferase)

CCPA (2-chloro-N⁶-cyclopentyladenosine)

CD40L (CD40 ligand)

CGS (CGS21680)

Cl-IB-MECA  1-[2-chloro-6-[(3-iodophenyl)methyl]amino]-9H-purin-9-yl]-1-deoxy-N-methyl-β-D-ribofuranuronamide

Con A (concanavalin A)

DCs (dendritic cells)

FasL (Fas ligand)

α-GalCer (α-galactosylceramide)

MRS (MRS1754)

NECA (5’-N-ethylcarboxamidoadenosine)

NKT cells (here, invariant natural killer T cells)

RAG1KO mice (recombinase activating gene 1 deficient mice)

WT mice (wild-type mice)

ZM-ZM241385
Statement of the problem

Improper or prolonged inflammation leads to tissue damage and is the leading cause of many diseases. Extracellular adenosine that accumulates in the inflamed tissue signals through A2ARs on immune cells and terminates inflammation (Sitkovsky and Ohta, 2005). ‘Adenosine-A2AR’ signaling was found to be critical for limiting immune responses in the liver and is hepatoprotective during acute hepatitis (Ohta and Sitkovsky, 2001). However, the critical cellular target of ‘adenosine-A2AR’ anti-inflammatory pathway in the liver was not known. Also, the involvement of other adenosine receptors in the regulation of hepatitis was not understood. The current study was designed to understand the mechanism of endogenous regulation of inflammation during acute hepatitis.
1. **SPECIFIC AIMS:**

**SPECIFIC AIM 1:** To investigate the critical target of the adenosine-A2AR mediated anti-inflammatory mechanism in acute hepatitis.

1. To pharmacologically evaluate A2ARs on NKT cells by combining receptor specific agonists and antagonists with in vitro activation of NKT cells.

2. To determine the physiological role of the ‘adenosine-A2AR’ pathway in activation of NKT cells using a NKT cell-specific ligand, α-galactosylceramide (α-GalCer).

3. To confirm the immunoregulatory role of A2AR on NKT cells in the induction of liver injury after intra-hepatic transfer of WT or A2ARKO NKT cells.

**SPECIFIC AIM 2:** To determine if the A2BR is involved in hepatic inflammation via the regulation of NKT cell activation.

1. To induce acute hepatitis in A2BR-deficient mice and examine the involvement of A2BR in the adenosine-mediated immunoregulatory mechanism.

2. To determine the expression of functional A2BRs and their role in NKT cell activation.

3. To confirm the role of A2BRKO NKT cells in acute hepatitis by intra-hepatic transfer of WT or A2BRKO NKT cells.

**SPECIFIC AIM 3:** To investigate hypoxia mediated regulation of NKT cell activation.

1. To determine if NKT cells are susceptible to hypoxia by culturing the cells under 1% oxygen.
2. To test NKT cell activation under hypoxia in vivo by whole body exposure of α-GalCer treated mice to 10% oxygen containing inspired gas mixture.

3. To test the involvement of A2AR in hypoxia mediated regulation of NKT cell function.

4. To test the involvement of A2BR in hypoxia mediated regulation of NKT cell function.
2. **BACKGROUND AND SIGNIFICANCE:**

The process of inflammation is a physiological response to an inciting stimulus such as infection or tissue injury. A microbial infection or chemical injury or surgical trauma to the tissue will activate the release of mediators of inflammation that provide chemical cues for leukocyte chemotaxis to the site of tissue injury (Serhan et al., 2007). Cytokines that are released at the site of infection or tissue injury cause the circulating immune cells to adhere to the vascular endothelium, roll over and extravasate into the tissue space (Kuby Immunology 6\textsuperscript{th} Edition). An ‘acute’ inflammation has rapid onset, lasts for a short duration followed by a resolution phase (Kuby Immunology 6\textsuperscript{th} Edition). The beginning of an acute inflammatory response is marked by neutrophilic infiltration into the tissue followed by monocytes, lymphocytes and other cells of the innate immune system. Immune cells secrete various pro-inflammatory cytokines and cytotoxic molecules in order to destroy pathogens or pathogen infected cells. This comprises the acute phase response during acute inflammation which is followed by a phase of resolution of inflammation and repair of the inflamed tissue so that it’s normal physiological function can be restored (Serhan et al., 2007). Certain conditions can result in ‘chronic’ inflammation such as persistence of pathogens due to incomplete clearance or presentation of an immunogenic self antigen leading to an auto-immune response (Kuby Immunology 6\textsuperscript{th} Edition). For instance, in humans the high frequency or persistence of hepatitis C virus (HCV) infection tends to cause chronic hepatitis (Nuti et al., 1998). Interestingly, the virus has been found to replicate in spite of the presence of cellular and humoral immune responses. Also, the HCV itself is not ‘cytopathic’ meaning the viral replication is found to occur in the hepatocytes without causing hepatocyte damage (Cerny and Chisari, 1999). However, an exaggerated necrotic inflammatory response develops during ‘chronic’ HCV resulting in damage to both virally infected and non-infected
liver cells which contributes largely to the liver damage associated with hepatitis C pathogenesis. (Nuti et al., 1998; Cerny and Chisari, 1999; Lucas et al., 2003).

The cellular organization of organs is not designed to sustain repeated inflammatory insults. Persistent activity of immune cells in the tissue stimulates fibroblasts to proliferate and produce collagen at the site of chronic inflammation. Fibrosis is an attempt to heal the inflamed tissue which results in loss of functional organ cells and compromised organ function in the long run: cirrhosis in the liver. Chronic inflammation underlies the pathophysiology of many disease states and is a vital factor predisposing to carcinogenesis. It is, hence, important to understand the physiological mechanisms that regulate inflammation.

**Hypoxia driven adenosine-mediated physiological regulation of inflammation**

The resolution of inflammation was thought to be a passive process that involves the normalization of chemokine gradients and decline of the survival signals from supporting stromal cells which causes leukocytes to retreat from the inflamed tissue (Serhan et al., 2007). However, resolution of inflammation is now thought to be a very tightly controlled and timed process (Sitkovsky and Ohta, 2005). The pharmacological evaluation of factors that could suppress activated immune cells led to the discovery that certain chemicals released as a response to neuroendocrine stress such as catecholamines, neuropeptides, prostaglandins of E and I series are immunosuppressive (Sitkovsky et al., 2004). Many of these molecules cause immunosuppression by activating adeynylyl cyclase and elevating cAMP in the immune cells. This led to further understanding that certain factors may be produced within the inflamed tissue microenvironment that can inhibit the activity of immune cells.
It has been found that the inflamed tissue microenvironment is often hypoxic due to the disruption of tissue microcirculation during inflammation (Linden 2001, Sitkovsky 2003). Several studies have demonstrated the anti-inflammatory effects of hypoxia (Eltzschig et al., 2005, Thiel et al., 2005, Rosenberger et al., 2009), where local hypoxia in the tissue was found to suppress inflammatory damage. For instance, mice exposed to 10% oxygen resisted lung damage by bacterial toxins, whereas exposure to 100% oxygen exacerbated the lung damage by abolishing the hypoxia driven tissue-protective mechanism (Thiel et al., 2005).

Deprivation of oxygen can cause dramatic changes in cellular metabolism. Hypoxia has been found to elevate the levels of extracellular adenosine in the inflamed tissue (Driver et al., 1993, Martin et al., 2000). Even brief duration of hypoxic exposure can lead to decreased production of ATP and a reciprocal accumulation of AMP which can be further metabolized to adenosine by cytosolic 5’-nucleotidase (Sitkovsky et al., 2004). Adenosine can shunt between intracellular and extracellular compartments by means of equilibrative nucleoside transporters (ENTs). Hypoxia has been shown to decrease adenosine uptake by the ENTs (Eltzschig et al., 2005). Also, ATP is thought to be released into the extra-cellular space during inflammatory tissue damage. In the extracellular space, ATP or ADP is metabolized to AMP by the activity of ectonucleoside triphosphate diphosphohydrolase (CD39) and AMP is further converted to adenosine in the extracellular space by ecto-5’-nucleotidase (CD73). The hypoxic microenvironment within the inflamed tissue is conducive to the activity of hypoxia inducible factor, HIF-1α, that upregulates the surface expression of CD73 (Synnestvedt et al., 2002). Hypoxia also induces CD39, and therefore promotes accumulation of extracellular adenosine. In addition, hypoxia inhibits adenosine kinase and prevents the reutilization of adenosine to produce AMP (Decking et al.
All these events lead to the enhanced production and accumulation of extracellular adenosine under hypoxia.

Extra-cellular adenosine is the endogenous ligand to purinergic G protein coupled receptors. Upon binding to the receptor, adenosine signals through heterotrimeric G proteins that can either
stimulate (Gs) or inhibit (Gi) the enzyme, adenylyl cyclase that catalyzes the formation of cAMP (Sitkovsky et al., 2004). Four adenosine receptors have been characterized: A1, A2A, A2B and A3. Depending upon the agonist potencies at these receptors with regard to intracellular production of cAMP, the receptors have been classified as high affinity A2AR and low affinity A2BR, that couple with Gs protein to induce cAMP formation and high affinity A1R and low affinity A3R, that couple with Gi to inhibit adenylyl cyclase activity (Sitkovsky et al., 2004). Of these, A2ARs are most abundantly expressed on T lymphocytes.

Adenosine can suppress a wide variety of immune responses such as the oxidative burst in neutrophils (Cronstein et al., 1990), activation of monocytes (Link et al., 2000). Also, adenosine induces immunosuppressive IL-10 from macrophages (Okusa et al., 1999). A2AR activation and subsequent elevation of intracellular cAMP leads to activation of protein kinase A (PKA). In T lymphocytes, PKA can modulate T cell receptor (TCR) signaling at multiple levels, most importantly, by the phosphorylation of carboxy terminal Src Kinase (CsK) which inhibits proximal TCR signaling by inactivating Lck and Fyn that play pivotal role in the initial events of T cell signaling. PKA also phosphorylates CREB (cAMP response element binding protein) that upon binding to CRE (cAMP response element) in the DNA prevents the transcription of NFkB and NFAT, nuclear factors needed to initiate T cell proliferation and cytokines production upon TCR stimulation (Tasken and Ruppelt, 2006). Combining A2AR stimulation with T cell activation can severely inhibit T cell effector functions namely, cytotoxicity and cytokines production and to a lesser extent, T cell proliferation (Ohta et al., 2009).

However, for immune cells to be regulated by the ‘adenosine–A2AR’ pathway, the level of extracellular adenosine in the inflamed tissue must be sufficiently high, which may be determined by the extent of tissue damage and resultant hypoxia. Also, this mechanism will be
affected by the number of A2ARs expressed on the immune cells. Thus, extra-cellular adenosine acts as a ‘reporter’ of the extent of tissue injury while A2ARs on immune cells act as ‘sensors’ for this anti-inflammatory signal in order to terminate the immune response only upon considerable inflammatory tissue damage, which perhaps allows time for immune cell mediated pathogen destruction (Sitkovsky et al., 2004).

2.2 Regulation of hepatitis by endogenous ‘adenosine-A2AR’ mediated anti-inflammatory signaling

![Diagram](image)

Fig. 2. A2ARs act as ‘sensors’ of the critical ‘tissue protective negative feedback signal’ in acute hepatitis: During liver inflammation, endogenous adenosine acts as a ‘reporter’ of the extent of tissue damage and A2ARs on immune cells can sense this signal to stop further inflammation (Ohta and Sitkovsky, 2001). (Left) Extracellular adenosine binds to high affinity Gs coupled A2ARs on immune cells and causes an elevation of intracellular cAMP. This leads to
inhibition of T cell effector functions. (Right) Immune cells at the inflamed tissue can be relieved of adenosine mediated inhibition by interfering with ‘adenosine-A2AR signaling’ by genetic deletion of A2ARs on immune cells or by pharmacological antagonism by receptor specific antagonist.

The above schematic representation (Fig. 2) summarizes the work done by Ohta and Sitkovsky in 2001. Using a model of acute hepatitis, the authors showed that sub-optimal doses of an inflammatory stimulus that caused only minimal tissue damage in the wild-type mice were sufficient to cause extensive liver damage as evidenced by exaggerated serum alanine amino transferase (ALT) levels and prolonged, elevated serum levels of pro-inflammatory cytokines: TNF-α, IFN-γ and IL-4 in the A2ARKO littermates (Ohta and Sitkovsky, 2001). Also, pharmacological antagonism of A2AR using ZM 241385 caused exaggerated liver damage in WT mice (Ohta and Sitkovsky, 2001). Tumor microenvironment also contains higher levels of extracellular adenosine than the surrounding normal tissue (Ohta et al., 2006). Improvement of tumor rejection in A2ARKO mice or by A2AR antagonist indicated a vital role of the ‘adenosine-A2AR’ pathway as an immunosuppressive mechanism in tumor microenvironment preventing successful tumor immunotherapy (Ohta et al., 2006). These experiments demonstrate that the lack of the adenosine-A2AR pathway results in much exaggerated inflammation because no other endogenous anti-inflammatory mechanism could fully compensate for the deficiency of A2AR.

Inflammatory responses in the liver are under the control of adenosine. Divergent roles of extracellular adenosine have been observed in the course of hepatitis. Anti-inflammatory nature of adenosine, mostly through A2AR, has been demonstrated by the suppression of acute hepatitis induced by Con A (Ohta and Sitkovsky, 2001), D-galactosamine+LPS (Odahima et al., 2005) or
ischemia-reperfusion (Harada et al., 2000). Formation of extracellular adenosine is indispensable as a physiological stop signal to prevent excess inflammation as evidenced by much exaggerated hepatitis in A2AR-deficient mice (Ohta and Sitkovsky, 2001). T cells, which play a major role in the pathogenesis of viral and autoimmune hepatitis, express A2ARs and T cell functions are susceptible to adenosine-A2AR inhibitory signal (Ohta et al., 2009). These studies can explain an anti-inflammatory mechanism of adenosine in T cell-dependent induction of acute hepatitis. In the resolution phase of hepatitis, however, the inactivation of adenosine-A2AR signaling prevented the induction of cirrhosis (Chan et al., 2006, Feoktistov et al., 2009). Indeed, adenosine was found to facilitate fibrosis through the induction of collagen (Chan et al., 2006). Thus, adenosine involves in hepatitis from the inhibition of immune responses in the priming phase to tissue remodeling in the resolution phase of inflammation.

A2BR is another Gs protein-coupled adenosine receptor, which is capable of increasing cAMP levels. Recently, adenosine signaling through A2BR has been reported to modulate inflammatory responses. Studies using A2BR-selective agents and A2BRKO mice, however, have been showing controversial roles of A2BR in inflammation. On one hand, A2BR stimulation can enhance IL-6 production (Ryzhov et al., 2008) and is reported to augment pulmonary inflammation and colitis (Sun et al., 2006, Mustafa et al., 2007, Kolachala et al., 2008). But, on the other hand, A2BR is demonstrated to attenuate vascular, pulmonary, and gastrointestinal inflammation (Yang et al., 2006, Eckle et al., 2008, Frick et al., 2009; Zhou et al., 2009). The reason underlying these controversial pro-/anti-inflammatory actions of A2BR is not clear.
2.3. **Natural Killer T cells:**

Natural Killer T (NKT) cells are a group of lymphocytes that express both functional T cell receptor and NK receptors (Kronenberg and Gapin, 2002, Swain, 2008). Most NKT cells bear invariant TCRα chain (Vα14Jα18 in mice and Vα24Jα18 in humans) and limited variations of TCRβ chain. These are called as invariant NKT cells (iNKT cells). In the current study, by mentioning NKT cells, I refer to iNKT cell population. They can co-ordinate between the adaptive and innate immunity (Swain, 2008). NKT cells rapidly activate upon recognition of self and foreign glycolipid antigens presented on MHC class I-like molecule, CD1d, and produce large amounts of cytokines including IL-4 and IFNγ. During a screen for molecules that could prevent metastases to the liver, α-galactosyl ceramide (α-GalCer) was first isolated from a sea sponge named *Agelas mauritianus* (Morita et al., 1995). The bacteria, *Sphingomonas* breeding in the sponge is thought to produce α-GalCer. α-GalCer is a glycolipid that binds to CD1d molecule and can selectively activate mouse and human NKT cells (Linsen et al., 2005). It is thought that α-GalCer mimics self glycolipid antigen recognized by NKT cells since α-glycosphingolipids cannot be synthesized in mammals (Gapin, 2010).

Though also present in other lymphoid organs with T cells, NKT cells are enriched in the liver. In both mice and humans, the liver has the highest NKT cell to conventional T cell ratio (Swain, 2008). α-GalCer loaded, soluble, CD-1d tetramers are used to identify the invariant NKT cells (Benlagha et al., 2000). Using such staining it has been reported that most of the NKT cells (approximately 50% of intrahepatic lymphocytes) bear the invariant TCR in the mouse. However, invariant NKT cells are rare in the human liver. 0.03%-0.34% invariant NKT cells are present in the human liver (Exley et al., 2002).
Extensive lipid metabolism occurs in the liver. Also, CD1d is found to be expressed on hepatocytes, Kupffer cells, hepatic dendritic cells and the endothelial cells lining the sinusoids (Exley et al., 2002). As NKT cells recognize and activate in response to endogenous or exogenous lipid antigens expressed on CD1d molecules, they are thought to be best suited immune cells for surveillance in the liver (Swain, 2010). Lysosomal glycosphingolipid, isoglobotrihexosylceramide (iGb3) has been found to activate mouse and human NKT cells (Gapin, 2010). However, endogenous glycolipid ligands for NKT cells have not yet been identified.

Leukocyte trafficking is controlled by small chemotactic proteins called as ‘chemokines’. NKT cells express chemokine receptors CXCR3 and CXCR6 which are important for the enrichment and perhaps, the retention of NKT cells in the liver (Swain, 2010; Geissmann et al., 2005). NKT cells perform immune surveillance while residing within the vasculature itself. In other lymphoid organs like the spleen and lymph nodes, the lymphocytes interact closely with antigen presenting cells in compartments that are protected from the flow rate of the blood. In the liver, however, most cells are in close contact with the blood. NKT cells do not extravasate into the liver tissue like other immune effector cells but patrol while residing within the vascular space. The NKT cells stop moving once their TCR is activated by a ligand (Geissmann et al., 2005).

NKT cells can initiate immune response to bacterial infections (Linsen et al., 2005). Gram negative, lipopolysaccharide (LPS) containing bacteria like Salmonella typhimurium indirectly activate NKT cells (Brigl et al., 2003). The LPS in the bacteria activates Toll like receptors on dendritic cells which then produce IL-12. This along with presentation of the endogenous lysosomal glycosphingolipid, iGb3, by LPS-activated dendritic cells causes CD1d dependent activation of NKT cells (Brigl et al., 2003, Mattner et al, 2005). Another mechanism of NKT
activation is observed in LPS negative bacterial infections. Glycosylceramides from the cell wall of gram negative, LPS negative bacteria belonging to the class of alpha-Proteobacteria like *Ehrlichia muris* and *Sphingomonas capsulata* cause direct activation of the T cell receptor of NKT cells (Mattner et al, 2005). Thus NKT cells extend the repertoire of lymphocytes that provide anti-microbial defense. NKT cells also involve in tumor immunesurveillance. IFNγ from activated NKT cells and secondary transactivation of anti-tumor effector cells such as NK cells and CD8+ T cells mainly contribute to NKT cell mediated anti-tumor immunity (Linsen et al., 2005).

2.4. **Role of NKT cells in regulating hepatic immune responses**


**Fig. 3 Effector functions of hepatic NKT cells:** NKT cells activate upon recognition of glycolipid antigen presented by CD1d on dendritic cells. Upon TCR activation, NKT cells
respond by rapid and explosive production of Th1 and Th2 cytokines (Wingender et al., 2011). Activation induces upregulation of CD40L on NKT cells that interacts with CD40 on dendritic cells (Kitamura et al., 1999). This interaction is important for dendritic cell maturation and IL-12 production (Swain, 2008). IL-12 produced from dendritic cells further activates NKT cells. IL-4 produced by NKT cells acts in an autocrine manner to upregulate FasL on NKT cells. FasL interacts with Fas expressed on hepatocytes leading to hepatocyte apoptosis (Kaneko et al., 2000). Also, NKT cells can cause cytotoxicity by release of preformed perforin and granzyme (Swain, 2010).

Cytokines produced from activated NKT cells can regulate subsequent immune responses by direct cellular effects or in an indirect manner by inducing production of chemokines that recruit inflammatory effector cells or regulatory cells into the liver (Swain, 2010). The scheme above (Fig. 3) explains the effector functions of hepatic NKT cells. Dendritic cells can process and present endogenous or exogenous glycolipid antigens on CD1d. The T cell receptor of NKT cells can recognize and activate to glycolipids presented in this way. Upon TCR activation, NKT cells can secrete copious amounts of various cytokines of Th1 (IFNγ and TNFα), Th2 (IL-4, IL-5, IL-10, IL-13) and Th17 (IL-17) lineages (Swain, 2010). IL-4 secreted from the activated NKT cells acts on the NKT cells itself causing an upregulation of Fas ligand (FasL). The FasL on NKT cells interacts with Fas expressed on hepatocytes and contributes largely to hepatocyte apoptosis in the induction of liver damage (Kaneko et al., 2000). IL-4 from activated NKT cells also augmented the expression of granzyme B in an autocrine manner which was important for NKT cell-mediated cytotoxicity (Kaneko et al., 2000). Also, IFNγ and IL-4 secreted by activated NKT cells stimulated IFNγ release from NK cells. IFNγ was found to be important in NKT cell mediated clearance of hepatitis C virus and also for anti-tumor immune responses in the liver.
Upon activation, NKT cells upregulate CD40 ligand (CD40L) which interacts with CD40 molecule expressed on dendritic cell surface. CD40-CD40L interaction is important for the maturation of dendritic cells which then secrete IL-12 (Kitamura et al., 1999). IL-12 secreted from dendritic cells in turn further activates NKT cells that upregulate IL-12 receptor on their surface (Kitamura et al., 1999).

NKT cells also have hepatoprotective functions. NKT cells activated by in vivo administration of α-GalCer produce IL-17, during the early phase of the immune response which regulates the recruitment of monocytes and neutrophils into the liver (Wondimu et al., 2010). Neutralization of IL-17 after α-GalCer administration causes extensive monocyte and neutrophil infiltration leading to extensive liver damage (Wondimu et al., 2010). Also, rapid production of IFNγ from NKT cells after α-GalCer stimulation induces chemokine CXCL10 that recruits regulatory T cells into the liver (Santodomingo-Garzon et al., 2009). Thus, depending on the stimulus for NKT cells and the immune context into which these cytokines are released, NKT cells can play both pro-inflammatory and anti-inflammatory roles and potently regulate hepatic immune response (Swain, 2010).

**Evidences for the involvement of hepatic NKT cells in various forms of hepatitis**

Direct activation of NKT cells by α-GalCer injection induces acute liver injury suggesting NKT cell-initiated pathogenesis of acute hepatitis (Osman et al., 2000). α-GalCer activated NKT cells produce IFNγ and transactivate NK cells that produce IFNα/β. These cytokines suppress replication of hepatitis B virus (HBV) within 24 hrs of α-GalCer injection to HBV transgenic mice (Kakimi et al., 2000). The study suggested the potential of NKT cells to inhibit HBV replication in the liver during a natural infection. One of the striking features of chronic HCV
infection is the presence of liver-infiltrating lymphocytes (Cerny and Chisari, 1999). The intrahepatic lymphocytes (IHLs) in the HCV infected patient livers largely contained Th1 polarized NKT cells (Nuti et al., 1998, Exley et al., 2002). Higher frequency of NKT cells have been observed in auto-immune liver diseases especially near the area of inflammation suggesting their role in auto-immune hepatitis. Higher frequency of NKT cells were found in the livers of patients with primary biliary cirrhosis as compared to the livers of healthy individuals (Kita et al., 2002). Wilson disease is an autosomal recessive disorder resulting in abnormal copper transport causing toxic accumulation of copper in the liver and brain. The absolute number and frequency of NKT cells was found to be increased in the patients with fulminant Wilsonian hepatitis (Kinebuchi et al., 2005). Chronic consumption of alcohol leads to an increase in hepatic NKT cells and also sensitizes hepatocytes to NKT cell mediated cytotoxicity (Minagawa et al., 2004). On the other hand hepatic NKT cells are found to be protective in high fat diet induced non-alcoholic steatohepatitis (Li et al., 2005). Thus hepatic NKT cells involve in most forms of hepatic disorders and this motivated the need to understand the mechanism of physiological regulation of NKT cells during acute hepatitis in the current study.
2.5. The T cell mediated model of acute hepatitis:

![Diagram of the T cell mediated model of acute hepatitis](image)

**Fig. 4. Schematic representation of the proposed mechanism of Con A induced hepatitis:**

Intravenous injection of concanavalin A (Con A) leads to activation of NKT cells and T cells in the liver. NKT cells participate in the early stages of inflammation producing large amounts of cytokines, IFN-γ and IL-4. The cytokines produced from NKT cells recruit other immune cells, including Kupffer cells which are resident macrophages in the liver to orchestrate an immune response leading to liver damage. Activated NKT cells also express FasL and cause Fas mediated apoptosis of hepatocytes. NKT cells are major producers of IL-4 and are indispensable for the induction of acute hepatitis by Con A.

Concanavalin A is a plant protein belonging to the class of ‘lectins’. The lectin specifically binds to certain alpha-mannosyl residues of oligosaccharides on the surface of T cells and is a T lymphocyte mitogen. Intravenous injection of Con A in mice causes acute hepatitis that resembles viral and autoimmune hepatitis, involving T cells, NKT cells and Kupffer cells (Tiegs...
et al., 1992, Toyabe et al., 1997, Kaneko et al., 2000, Herkel et al., 2005) (Figure 3).

Experiments using NKT cell-depleted/deficient mice have demonstrated that the lack of NKT cells completely abolished Con A-induced liver damage. In these experiments, IL-4 production from NKT cells was crucial in the induction of hepatitis (Toyabe et al., 1997, Kaneko et al., 2000).

In 2000, Harada et al. reported the reduction of ischemia/reperfusion injury of rat liver by A2AR agonist mediated inhibition of leukocyte activation (Harada et al., 2000). Subsequently, NKT cells were found to be essential to the induction of hepatic ischemia/reperfusion injury (Lappas et al., 2006). In the same study, A2AR specific agonist, ALT146e, could suppress ischemia/reperfusion injury in RAG-1KO mice that received adoptive transfer of NKT cells, thus confirming A2AR expression on NKT cells and its negative role in NKT cell activation (Lappas et al., 2006).

The current study focuses on investigating the mechanism of adenosine-mediated regulation of hepatic inflammation. Much exaggerated liver inflammation in A2ARKO has demonstrated the hepatoprotective role of endogenously produced adenosine; however, cellular target of A2AR-mediated anti-inflammatory effects is not clear. NKT cells could be the target because these cells play a critical role in the induction of acute hepatitis. NKT cells are reported to express A2AR; however, it is not known whether endogenously produced adenosine controls intensity of NKT cell activation. To address this question, the intensity of NKT activation and subsequent liver damage were studied in A2ARKO mice (Specific Aim #1). As it was observed in A2AR, similar immunoregulatory roles could be expected for A2BR. However, previous studies have provided controversial results and the role of A2BR in inflammation is not clear. To establish the role of A2BR in hepatic inflammation, extent of liver damage was compared between WT and
A2BRKO mice together with the A2BR-mediated regulation of NKT cell activation. (Specific Aim #2). The results from Specific Aim #1 and 2 showed exaggeration of NKT cell responses in the absence of A2AR or A2BR indicating NKT cell regulation by endogenous adenosine.

2.6. **Hypoxia mediated regulation of NKT cell activation**

Since hypoxia is conducive to the accumulation of extra-cellular adenosine, it was hypothesized that tissue hypoxia also regulates NKT cell activation. NKT cell activation was examined whether it is under control of the hypoxia-adenosine axis of immune regulation (Specific Aim #3).
3. MATERIALS AND METHODS:

3.1. Mice

C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, MA). C57BL/6-background A2ARKO mice were backcrossed 11 times to C57BL/6 mice (Chen J 1999). C57BL/6-background A2BRKO mice were developed by Ozgene Pty. Ltd. (Bentley, Australia) using C57BL/6 embryonic stem (ES) cells.

The mice were housed in the animal facility of Northeastern University and were used at 8-10 weeks of age in accordance with institutional animal care guidelines.

3.2. Concanavalin A (Con A)-induced hepatitis

Con A (Sigma, St. Louis, MO) was injected intravenously to induce acute liver injury (Tiegs et al., 1992). The blood samples were collected at 8 or 24 h after Con A injection and liver damage was evaluated by serum ALT levels. ALT activity was measured by using a kit from Teco Diagnostics (Anaheim, CA). Serum levels of TNF-α and IL-4 after 1.5 h and serum IFN-γ levels after 8 h were determined using ELISA kits obtained from R&D Systems (Minneapolis, MN). The liver tissue was fixed in 10% formalin-PBS 24 h after Con A injection, and the paraffin-embedded tissue slice was stained with hematoxyline-eosin (Mass Histology Service, Worcester, MA). Some wild-type mice received intraperitoneal injection of A2AR specific agonist CGS 21680 (2mg/kg) or A2BR antagonist MRS1754 (2 mg/kg) 10 min before Con A.
3.3. Flowcytometric analysis to evaluate NKT cell-surface expression of FasL, CD40L and IL-4 production from NKT cells.

Liver mononuclear cells 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 x 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 (Invitrogen), and washed using RPMI1640 medium containing 10 % FCS.

Activation-induced surface expression of CD40L and FasL on NKT cells were evaluated by staining with fluorochrome-conjugated antibodies for NK1.1, CD3, CD40L and FasL and analyzing by FACSCalibur (BD Biosciences).

IL-4 production from NKT cells was analyzed 2 h after Con A injection. Surface staining of the cells with FITC-conjugated anti-NK1.1 and allophycocyanin-conjugated anti-TCRβ mAbs was followed by fixation and permeabilization (Ohta et al., 2009). 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 IL-4 was stained with PE-conjugated anti-IL-4 mAb, and the IL-4 expression in NK1.1⁺ TCRβ⁺ cells was analyzed by FACSCalibur (BD Biosciences). All antibodies were obtained from BD Biosciences.

3.4. 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. After 2 days, splenocytes 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<sup>+</sup> cells using AutoMACS separator (Miltenyi Biotec). Purity of NK1.1<sup>+</sup> CD3<sup>+</sup> cells was higher than 90%.

3.5. Real-time PCR

RNA was extracted from unseparated lymph node cells, purified T cells, NK cells, NKT cells and dendritic cells using RNA STAT-60 (Tel-Test, Friendswood, TX). CD4<sup>+</sup> T cells were purified by a combination of FITC-conjugated anti-CD4 mAb and anti-FITC magnetic beads (purity: >98%). NK cells were also extracted from the spleen of RAG2<sup>−/−</sup> mice by positive selection of NK1.1<sup>+</sup> cells (purity: >97%). Dendritic cells were prepared from the bone marrow as described below. cDNA was synthesized with random hexaprimers using Superscript first-strand synthesis kit (Invitrogen, Carlsbad, CA). Real-time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) on the Applied Biosystems 7300 Real-Time PCR System (Lukashev et al., 2003). Levels of A2BR mRNA were normalized to the amount of L32 mRNA. Primers are as follows: A2BR, ACGTGGCCGTGGACTC and GCAGAAGCCCAAGCTGATG; L32, AGCAACAAGAAAAACCAAGCACAT and TTGACATTGTGGACCAGGAAC.

3.6. cAMP assay

Induction of cAMP in response to adenosine receptor agonists was measured as described previously (Ohta et al., 2009). Purified NKT cells (1 x 10<sup>5</sup>) 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).

3.7. Bone-marrow dendritic cells

The bone marrow cells were prepared from the femur bone of C57BL/6 mice by flushing out the bone marrow into a sterile petri dish using a syringe filled with PBS. The cells were collected in a tube and spun down. The pellet was re-suspended in 1ml of ACK buffer and incubated for approximately 3 min. After centrifugation, the bone marrow cells so obtained 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, the adherent cells were collected and washed at least twice with the culture media to remove any IL-4 or GM-CSF and used to stimulate NKT cells isolated from WT, A2ARKO, A2BRKO mice.

3.8. In vitro stimulation of NKT cells

Purified NKT cells (1.5 x 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 (3 x 10^5 cells). Following adenosine receptor agonists 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). Cells were briefly pretreated with 100nM concentration of antagonists, ZM241385 (ZM, A2AR/A2BR-selective antagonist) and MRS1754 (MRS, A2BR-selective antagonist)
before treatment with A2AR and A2BR agonists. CCPA, CGS, Cl-IB-MECA and ZM were obtained from Tocris (Ellisville, MO). NECA and MRS are from Sigma (St. Louis, MO). The cells were cultured for 24 h, and cytokine levels in the supernatant were determined by ELISA.

The affinity of the various adenosine receptor agonists and antagonists (Jacobson and Guo, 2006) used in the current study, at the four adenosine receptor subtypes is given in the table below:

<table>
<thead>
<tr>
<th>Receptor subtype</th>
<th>compound</th>
<th>Ki value for adenosine receptor (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Agonists</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A1</td>
<td>CCPA</td>
<td>0.83</td>
</tr>
<tr>
<td>A2A</td>
<td>CGS21680</td>
<td>27</td>
</tr>
<tr>
<td>A3</td>
<td>Cl-IB-MECA</td>
<td>1.4</td>
</tr>
<tr>
<td><strong>Antagonists</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A2A, A2B</td>
<td>ZM241385</td>
<td>1.6(A2AR) 75(A2BR)</td>
</tr>
<tr>
<td>A2B</td>
<td>MRS1754</td>
<td>2</td>
</tr>
</tbody>
</table>

3.9. α-galactosylceramide-induced hepatitis

α-GalCer (2 µg/mouse) was injected intravenously and serum IL-4 and IFN-γ levels were determined after 2 and 6 h, respectively (Kitamura H 1999). Liver damage was assessed 24 h after the injection by serum ALT levels and histochemistry as described for Con A-induced hepatitis.
3.10. NKT cell transfer into RAG-KO mice

WT, A2ARKO and A2BRKO mice were treated with intra-peritoneal injection of anti-asialo GM1 Ab (50 µg/mouse; Wako Chemicals, Richmond, VA) to deplete NK cells. On day 2 of anti-asialo GM1 Ab treatment, NK1.1+ cells were isolated from the splenocytes of WT and A2ARKO mice by magnetic cell sorting. Percentage purity of NK1.1+ cells was approximately 30-35 % each time. The cell suspension was completely depleted of T cells and NK cells. WT or A2ARKO NKT cells, thus isolated, were transferred intra-hepatically, as focal injection, into the livers of recipient RAG1-KO or RAG2-KO mice. Each RAG-KO recipient received 2.5*10^6 (for experiment with A2ARKO) or 3.5*10^6 (for experiment with A2BRKO) cells. The RAG-KO recipients were then challenged with Con A (20 mg/kg). The serum ALT levels were measured at 8 hrs and 24 hrs post Con A treatment.

3.11. Exposure to Hypoxic Atmosphere

WT mice were allowed to inspire 21% O₂ or 10% O₂ (hypoxic atmosphere) for 1 hr prior to α-GalCer injection (i.v.) and continued hypoxia treatment after α-GalCer treatment for the entire duration till the time point of NKT cell ex-vivo analysis. For exposure to hypoxic atmosphere, the mice were placed in air-tight modular incubation chambers (Billups-Rothenberg, San Diego, CA, USA). Ex-vivo analysis of splenic NKT cells for surface activation markers and intracellular cytokines production was conducted at 1 hr or 2 hrs post α-GalCer (2 µg) treatment. Serum IL-4 levels were measured at 2 hrs after α-GalCer treatment. For in vitro experiments, whole splenocytes from C57Bl/6 mice were stimulated with immobilized CD1d/Fc (2 µg/ml) + α-GalCer (2 µg/ml) and cultured at either 21% oxygen or 1% oxygen. Supernatant from the cultures were analyzed for IFN-γ after 48 hrs.
3.12. 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.
4. RESULTS:

4.1 NKT cell-dependent exacerbation of liver inflammation in A2A adenosine receptor-deficient mice

4.1.1 A2AR stimulation inhibits Con A-induced liver injury in wild type mice

With its T cell-dependent pathogenesis, Con A-induced hepatitis has been used as a model of acute viral hepatitis (Tiegs et al., 1992, Herkel et al., 2005). The concurrent administration of A2AR specific agonist, CGS21680 (2mg/kg) to the Con A treated WT mice significantly suppressed serum ALT levels (Fig. 5). After injection of Con A, serum levels of IL-4 and TNF-α peaked early preceding a rise of transaminase levels. CGS also significantly suppressed the early production (1.5hrs after Con A injection) of IL-4 and TNF-α (Fig. 5). Pharmacological stimulation of A2ARs can protect wild type mice from Con A induced liver damage by suppression of proinflammatory responses.

![Figure 5](image_url)

**Fig 5. A2AR stimulation inhibits induction of hepatitis.** Serum ALT levels were determined at 8 h. CGS treatment blocked early (1.5 h) upregulation of IL-4 and TNF-α, which are necessary to optimal induction of liver damage. **P < 0.001 vs Con A alone. Data represent average ± SD (n = 5).**
4.1.2 NKT cells are sensitive to adenosine-A2AR inhibitory signal

Since A2AR stimulation could suppress the early production of IL-4 and TNF-α, which are produced by NKT cells after Con A treatment (Tiegs et al., 1992, Ajuebor et al., 2003, Herkel et al., 2005), NKT cells were examined for the susceptibility to A2AR stimulation. Activation of NKT cells was analyzed by flowcytometry to evaluate the expression of CD40L and FasL. CGS co-treatment was found to decrease the expression of CD40L and FasL on NKT cells, thus causing the early inhibition of NKT cell activation (Fig. 6). The result suggests that NKT cells are sensitive to adenosine-A2AR signal and the early inhibition of NKT cell activation may be important to the observed suppression of Con A-induced liver injury by CGS.

![Fig 6. Suppression of NKT cell activation by the injection of CGS.](image)

**Fig 6. Suppression of NKT cell activation by the injection of CGS.** Panels represent CD40 ligand and Fas ligand expression on splenic NKT cells examined 1.5 h after Con A injection. The data shown here is gated for NK1.1⁺ CD3⁺ cells.

4.1.3 Pharmacological stimulation of A2AR suppresses NKT cell activation in vitro

To determine the adenosine receptor subtypes that are responsible for the regulation of NKT cell activation, NK1.1⁺ cells were isolated from the spleen of WT C57BL/6 mice. These cells were
then specifically stimulated with a NKT cell ligand, α-GalCer in the presence of A1R specific agonist (CCPA), A3R specific agonist (Cl-IB-MECA), A2AR specific agonist (CGS) and non-specific adenosine receptor agonist (NECA).

A2AR specific agonist, CGS, could suppress IFN-γ production by NKT cells and this could be almost completely reversed by specific antagonist, ZM241385 (Fig. 7). The non-specific agonist, NECA could also suppress cytokine production that was reversible by ZM (Fig. 7). This suggested that A2AR stimulation is inhibitory to NKT cell activation. However, A1 specific agonist, CCPA did not suppress cytokine production. A3 specific agonist, IB-MECA could suppress cytokine production to some extent but was not significant (Fig. 7). Forskolin that directly stimulates adenylate cyclase and increases intracellular cAMP could also significantly suppress IFN-γ production by NKT cells, indicating cAMP mediated suppression of NKT activation (Fig. 7). The data from this experiment indicates that NKT cells may bear functional A2ARs and that pharmacological stimulation of A2ARs on NKT cells can inhibit NKT cell activation.

<table>
<thead>
<tr>
<th>Adenosine receptors</th>
<th>IFN-γ (pg/ml)</th>
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<tbody>
<tr>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>CGS</td>
<td></td>
</tr>
<tr>
<td>CGS+ZM</td>
<td></td>
</tr>
<tr>
<td>NECA</td>
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</tr>
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<td></td>
</tr>
<tr>
<td>CCPA</td>
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<tr>
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Fig 7. Adenosine-A2AR signaling suppresses NKT cell activation. WT splenic NK1.1+ cells (1.5 x 10^5 cells) were culture-activated by α-GalCer (100 ng/ml) in the presence of bone marrow-derived dendritic cells (2 x 10^5 cells). Effects of adenosine receptor stimulation on NKT activation were tested using agonist of adenosine receptors (100 nM) and antagonists (100nM).

* p < 0.01, ** p < 0.001 vs Con A alone. Data represent average ± SD (n = 3-5).

4.1.4. Exacerbated liver injury accompanied by exaggerated cytokines induction in Con A-treated A2ARKO mice

To determine if endogenous adenosine regulates NKT cell activation through A2AR, A2ARKO mice were employed. Administration of Con A to A2ARKO mice produced serum ALT levels, approximately 5 times greater than that observed in WT mice (Fig. 8). Exacerbation of Con A-induced liver injury in A2ARKO mice implies that endogenous adenosine produced during hepatic inflammation can downregulate inflammation through A2AR signaling in the wild type mice. Correspondingly, early production of cytokines, IL-4 and TNF-α was also enhanced in A2ARKO mice (Fig. 8).
Fig 8. Pathophysiological regulation of inflammation by endogenously produced adenosine via A2AR. Serum ALT levels at 8 h are shown. IL-4 and TNF-α levels after Con A injection (1.5 h) were greater in A2ARKO mice. * P < 0.01, ** P < 0.001 vs Con A alone. Data represent average ± SD (n = 5).

4.1.5 NKT cell population in the liver

Since NKT cells plays a major role in Con A-induced liver injury, the exaggerated liver damage in A2ARKO mice might be due to a qualitative or quantitative difference in the NKT cells. Flowcytometric analysis of NK1.1+ CD3+ cell population in liver mononuclear cell preparation revealed that the proportion of NKT cell population was almost identical in WT and A2ARKO mice (Fig. 9). In addition, total cell numbers were comparable, ruling out the possibility of a quantitative difference being responsible for the observed exaggerated liver damage in the A2ARKO animals. This led us to suspect a qualitative difference in the NKT cells.
Fig 9. Flowcytometric analysis of NKT cells in the liver mononuclear cell preparation. The numbers represent percentages of NK1.1⁺ CD3⁺ cells.

4.1.6 Physiological adenosine controls NKT cell activation through A2AR signaling

To test if physiological levels of adenosine can regulate the activation of NKT cells through A2AR, NKT cells in WT and A2ARKO mice were directly activated in-vivo by intravenous injection of α-GalCer. α-GalCer injection produced higher serum levels of IL-4 and IFN-γ and exaggerated serum ALT levels in A2ARKO mice as compared to the WT controls (Fig. 10). The exaggerated NKT cell activation in vivo in the absence of A2AR signaling suggested the pathophysiological regulation of NKT cell activation by endogenous adenosine via A2AR.

Fig 10. Exaggerated activation of NKT cells in A2AR-deficient mice. WT and A2ARKO mice received intravenous injection of α-GalCer (2 µg/mouse). (A, B) Early production of IL-4 and IFN-γ from NKT cells. (C) Liver damage as indicated by serum ALT levels 24 hrs after α-GalCer injection. * P < 0.05, ** P < 0.01, *** P < 0.001 vs WT mice. Data represent average ± SD (n = 4-5).
4.1.7. *Role of A2AR deficient NKT cells in exaggerated Con-A induced liver injury*

In order to confirm the role of A2ARKO NKT cells in causing exacerbated Con A-induced hepatitis in A2ARKO mice, it was necessary to exclude the possibility of the involvement of other immune cells in this reaction. For this purpose, WT or A2ARKO NK1.1+ cells were transferred into the livers of recipient RAG1-KO mice, which lack T cells, B cells and NKT cells. The RAG1-KO recipients were then challenged with Con A (20 mg/kg). The RAG1KO mice that received the A2AR-deficient NKT cells had higher serum ALT levels (Fig. 11). The result confirms the crucial role of A2AR-deficient NKT cells in causing exacerbated liver damage in A2AR-deficient mice.

![Figure 11](image.png)

**Fig. 11** A2ARKO NKT cells caused exaggerated hepatitis in RAG1-KO recipient mice. WT or A2ARKO NKT cells were transferred intra-hepatically into RAG1-KO mice (2.5*10^6 cells/RAG1-KO mouse). The RAG1-KO recipients were then challenged with Con A (20 mg/kg). The serum ALT levels were measured at 8 hrs post Con A treatment. Data represent average ± SD of 3 mice and is representative of 2 independent experiments. *, p < 0.05
Based upon the results obtained in chapter 4.1, the following can be inferred:

1. NKT cells have functional expression of A2A adenosine receptor and the stimulation of A2AR results in down-regulation of NKT cell activation.

2. A2AR deficiency results in exaggerated activation of NKT cells during hepatitis.

3. Endogenous adenosine can regulate activation of NKT cells through A2A receptor signaling.

4.2 Immunoregulation by physiological levels of adenosine via A2B adenosine receptor

4.2.1 In vitro evidence of A2BR-mediated inhibition of NKT cell activation

To analyze the functional expression of A2BR on NKT cells, NKT cells were directly stimulated in vitro with α-GalCer together with various adenosine receptor agonists. In the WT NKT cells, a non-specific adenosine receptor agonist NECA strongly inhibited NKT cell activation as indicated by the suppression of IFN-γ production (Fig. 12A). Corresponding to A2AR expression in NKT cells as shown previously, A2AR selective agonist CGS blocked IFN-γ production from WT and A2BKO NKT cells, but not A2ARKO cells (Fig. 12B,C,D). In addition, A2AR/A2BR-selective antagonist ZM241385, but not A2BR-selective antagonist MRS1754, completely abolished the suppressive effect of NECA in wild-type NKT cells (Fig. 12B). These results again confirmed immunosuppressive role of A2AR on NKT cells.

To specifically determine A2BR-mediated response, NKT cells were examined to see if NECA was inhibitory even in the absence of immunosuppressive effect of A2AR. Interestingly, A2ARKO NKT cells were still vulnerable to NECA as shown by a 50 % reduction in IFN-γ production (Fig 12C). Since A1 and A3 adenosine receptors are not involved in this inhibition
(Fig12A), A2BR was suspected to be suppressive to NKT cell activation. (Fig 12B,C,D) Importantly, MRS1754 (A2BR-selective antagonist) blocked NECA-mediated inhibition of A2AR KO NKT cells supporting immunosuppressive role of A2BR in NKT cells.

**Fig 12.** A2BR stimulation is suppressive to the activation of NKT cells. (A) Purified WT NKT cells were stimulated by α-GalCer in the presence of DCs cells. IFN-γ levels in the culture supernatant were determined after 24 hrs. (B,C,D) NKT cells were purified from WT, A2AR KO, and A2BR KO mice. Data represent average ± SD of triplicate samples and are representative of 3 independent experiments. *, p < 0.05; **, p < 0.01 vs controls.

### 4.2.2 Expression of A2BR mRNA in NKT cells

Expression of A2BR mRNA was also tested in NKT cells. For verification of real-time PCR assay, dendritic cells which are known to express A2BR (Novitsky et al., 2008; Haskó et al.,
were employed as a positive control. Real-time PCR confirmed A2BR mRNA at high levels in dendritic cells, whereas the A2BR mRNA content was much lower in unseparated lymph node cells (Fig. 13). A2BR mRNA levels were further analyzed in purified lymphocyte subsets. Comparing to low levels of expression in conventional T cells and NK cells, A2BR mRNA levels were notably higher in NKT cells (Fig. 13) suggesting relatively abundant expression of A2BR in NKT cells.

**Fig 13. A2BR mRNA levels were quantified by real-time PCR.** Samples are unseparated lymph node cells (LN), purified CD4$^+$ T cells (Th), purified NK cells (NK), purified NKT cells (NKT) and dendritic cells (DC). A2BR mRNA levels were standardized on the basis of L32 mRNA levels.

### 4.2.3 Functional expression of A2BR on NKT cells

To test if NKT cells express functional A2BRs, cAMP responses after A2BR stimulation were tested. WT NKT cells increased cAMP in response to non-selective adenosine receptor agonist,
NECA (Fig. 14). A2AR/A2BR-selective antagonist ZM241385 blocked this increase. A2AR-selective stimulation by CGS21680 induced cAMP in WT NKT cells, but not in A2ARKO cells, confirming A2AR expression on NKT cells. A2ARKO NKT cells were still able to increase cAMP levels in response to NECA (Fig. 14) suggesting cAMP induction via A2BR. Since ZM241385 blocked this increase, it suggests that A2BRs expressed on NKT cells are functionally active and can signal by the upregulation of intracellular cAMP.

**Fig 14.** Functional expression of A2BR on NKT cells. Purified NKT cells were incubated with CGS21680 (A2AR-selective agonist) or NECA (non-specific adenosine receptor agonist), and cAMP levels were determined by ELISA. ZM241385 (A2AR/A2BR antagonist) blocked cAMP induction by NECA. Data represent average ± SD of triplicate samples and are representative of 2 independent experiments. *, p < 0.05 vs untreated NKT cells. †, p < 0.05 vs NECA-treated NKT cells.
4.2.4 *Exacerbation of inflammatory liver damage in the absence of A2BR*

NKT cells were found to express A2AR and A2BR and stimulation of both receptors are inhibitory to NKT cell activation. As it was observed with A2AR, similar immunoregulatory role could be expected for A2BR on NKT cells. Indeed, intravenous injection of Con A (13 mg/kg) induced quite stronger liver damage in A2BRKO mice as compared to intermediate liver damage in WT mice (Fig. 15).

The enhanced liver damage was reproducible in WT mice co-treated with A2BR-selective antagonist, MRS 1754. Intravenous injection of Con A into MRS 1754-treated C57BL/6 mice resulted in stronger liver damage than control mice (Fig. 15). These results suggested that Con A induced hepatitis is under the physiological regulation of adenosine-A2BR signaling and the interruption of this pathway by genetic deletion or pharmacological antagonism of the receptor resulted in exacerbation of liver damage.
Fig 15. Exacerbation of inflammatory liver damage in the absence of A2BR. (A) Exaggerated Con A-induced acute hepatitis in A2BRKO mice. A2BRKO and WT mice (n = 5) received intravenous injection of Con A (13 mg/kg). Serum ALT levels were determined after 8 and 24 h. (B) A2BR antagonist MRS1754 (2 mg/kg, i.p.) enhanced Con A-induced liver injury in WT mice. Serum ALT levels were determined 8 h after Con A injection. Data represent average ± SD of 5 mice and are representative of 4 independent experiments. The statistical significance was calculated by Student’s t-test: *, p < 0.05; **, p < 0.01.

4.2.5 Exaggerated cytokines induction in Con A-treated A2BR-KO mice

In Con A-injected A2BRKO mice, cytokines production was also found to increase (Fig 16), suggesting that exaggerated inflammatory responses in A2BRKO mice resulted in the exacerbation of liver damage. Robust increase of Con A-induced cytokines production including IL-4 in A2BRKO mice (Fig. 16) suggested the enhancement of NKT cell activation in A2BRKO mice as it was the case in A2ARKO mice (Fig 8).

Fig 16. Exaggerated inflammatory response in A2BRKO mice. Serum cytokine levels in Con A-injected mice were determined after 1.5 h (IL-4 and TNF-α) and 8 h (IFN-γ). Black bars, WT C57BL/6 mice; gray bars, A2BRKO mice. Data represent average ± SD of 5 mice and are
representative of 4 independent experiments. The statistical significance was calculated by
Student’s t-test: *, p < 0.05; **, p < 0.01.

4.2.6 Identical percentage of NKT cells in WT and A2BRKO mice

Flowcytometric analysis showed that WT and A2BRKO mice contain equivalent proportion of
NKT cells in the liver (Fig. 17). Since the total cell numbers were comparable, it was confirmed
that there was no numerical difference in NKT cells of these mice.

![Flowcytometric analysis of NKT cells from the liver](image)

**Fig 17. Flowcytometric analysis of NKT cells from the liver.** NKT cells were observed at
similar frequency in WT and A2BRKO mice. The numbers represent percentages of NK1.1⁺
CD3⁺ cells.

4.2.7 Enhanced IL-4 production from NKT cells in A2BRKO mice

Although no quantitative difference was observed, there was a qualitative difference in NKT cell
response. Two hours after the injection of Con A, cellular IL-4 production was immediately
analyzed by intracellular staining. Increases in IL-4 production were detectable in NKT cells
from both WT and A2BRKO mice, but Con A injection induced higher levels of IL-4 from
A2BRKO NKT cells (Fig. 18). The result suggested that early activation and resultant cytokine production from NKT cells is under the regulation of adenosine-A2BR signal.

![Graph showing enhanced production of IL-4 by A2BRKO NKT cells](image)

**Fig 18. Enhanced production of IL-4 by A2BRKO NKT cells.** Two hours after Con A injection, IL-4 was detected by intracellular staining ex vivo. The data shown here is gated for NK1.1\(^+\) TCR\(\beta^+\) cells. Control cells are from untreated WT mouse.

### 4.2.8 Physiological adenosine controls NKT cell activation through A2BR

To test whether physiological levels of adenosine can regulate NKT cell activation via A2BR stimulation, WT and A2BRKO mice received intravenous injection of \(\alpha\)-GalCer (2\(\mu\)g/mouse). It was found that direct stimulation of A2BRKO NKT cells in vivo induced stronger proinflammatory cytokines resulting in more extensive hepatic damage (Fig. 19). This implies the pathophysiological regulation of NKT cell activation by endogenous adenosine via A2BR on NKT cells.
Fig. 19 *Endogenous adenosine regulates NKT cells via A2BRs.* (Left and center panels) α-GalCer (2 µg/mouse) was injected intravenously and the increase of serum IL-4 and IFN-γ levels was monitored after 2hrs and 6 hrs, respectively. (Right panel) α-GalCer-induced hepatic damage was evaluated after 24 hrs by serum ALT levels. Black bars, WT C57BL/6 mice; gray bars, A2BRKO mice. Data represent average ± SD of 5 mice and are representative of 4 independent experiments. *, p < 0.05; **, p < 0.01.

4.2.9. *Role of A2BR deficient NKT cells in exaggerated Con A induced liver injury*

In order to confirm the role of A2BRKO NKT cells in causing exaggerated Con A-induced hepatitis in A2BRKO mice, WT or A2BRKO NK1.1+ cells were transferred into the livers of recipient RAG2-KO mice. After the injection of Con A, the RAG2KO mice that received the A2BR-deficient NKT cells had higher serum ALT levels (Fig. 20). The result confirms the crucial role of A2BR-deficient NKT cells in the exacerbated liver damage in A2BR-deficient mice.
Fig. 20. A2BRKO NKT cells caused exaggerated hepatitis in RAG2-KO recipient mice. WT or A2BRKO NKT cells were transferred intra-hepatically into RAG2-KO mice (3.5*10^6 cells/RAG2-KO mouse). The RAG2-KO recipients were then challenged with Con A (16 mg/kg). The serum ALT levels were measured at 8 hrs post Con A treatment. Data represent average ± SD of 3 mice and is representative of 2 independent experiments. *, p < 0.05.

Based upon the results for chapters 4.1 and 4.2, the following can be inferred:

1. NKT cells express functional A2A and A2B adenosine receptors.

2. Stimulation of A2AR and A2BR on NKT cells suppresses NKT cell activation.

3. Adenosine-A2AR/A2BR signaling pathway is involved in the physiological regulation of NKT cell activation during acute hepatitis.
4.3 Hypoxia mediated regulation of NKT cell activation.

4.3.1 In-vitro evidence for hypoxia mediated suppression of NKT cell activation.

The results from chapters 4.1 and 4.2 provide evidence for adenosine mediated regulation of NKT cell activation. Since hypoxia regulates extracellular adenosine levels, we investigated the effect of hypoxia on NKT cell activation. To determine the effect of hypoxia on NKT cell activation in vitro, whole splenocytes from C57Bl/6 mice were stimulated by immobilized CD1d/Fc + α Gal Cer in either 21% O₂ or 1% O₂. The NKT cell culture activated under hypoxia produced lower levels of IFN-γ (Fig. 21). Hypoxia was found to be suppressive to NKT cell activation in vitro.

Fig. 21. Hypoxia is suppressive to NKT cell activation. Whole splenocytes from C57Bl/6 mice were culture activated by immobilized CD1d/Fc + α Gal Cer (4:1). Cells were cultured in either 21% O₂ or 1% O₂. IFN-γ levels in the 48hr culture supernatant were measured by ELISA. Data represent average ± SD of triplicate samples and are representative of 2 independent experiments. * p < 0.05
4.3.2 Hypoxia is suppressive to the early activation of NKT cells *in vivo* after α-GalCer treatment.

To determine the effect of hypoxia on NKT cell activation in vivo, C57BL/6 mice were allowed to inspire 10% O₂ and received α-GalCer (2µg) injection. Control C57BL/6 mice were allowed to inspire 21% O₂. Activation markers CD69 and CD40L were analyzed on splenic NKT cells. Untreated C57BL/6 splenocytes were analyzed to estimate the basal levels of CD69 and CD40L expression on NKT cells. Approximately 35% of splenic NKT cells from untreated mice were found to constitutively express CD69 and 1-2% splenic NKT cells were found to express CD40L (Fig. 22). Surprisingly, it was found that hypoxia treatment alone upregulated CD69 expression on NKT cells from mice that were not treated with α-GalCer. CD40L expression was not affected by hypoxia alone (Fig. 23).

**Fig. 22 Basal level of CD69 and CD40L expression on splenic NKT cells.** Panel on the left represents basal level of CD69 expression on splenic NKT cells. Panel on right represents basal level of CD40L expression on splenic NKT cells. The numbers represent the percentage of NKT cells in each quadrant.
**Fig. 23 Hypoxia alone upregulates CD69 expression on NKT cells.** WT mice were exposed to 10% oxygen for 1 hr. Panel on the left represents CD69 expression on splenic NKT cells. Panel on right represents CD40L expression on splenic NKT cells. The numbers represent the percentage of NKT cells in each quadrant. Panels representative of 3 mice.

CD69 was upregulated within 1 hr of α-GalCer treatment and continues to increase at 2 hrs (Fig. 24). NKT cells in the hypoxia treated mice also activated in response to α-GalCer. However, hypoxic treatment suppressed the activation of NKT cells as seen by lower percentage of CD69+ NKT cells after hypoxic exposure (Fig. 24). It should be noted that hypoxia treatment alone could increase CD69 expression (Fig. 23). After subtraction of hypoxia-induced CD69 as background levels, normoxia versus hypoxia difference in CD69+ cells after α-GalCer might be more distinct.
Fig. 24. Hypoxia mediated regulation of NKT cell activation in vivo. C57Bl/6 mice were allowed to inspire 21% O₂ or 10% O₂ and were challenged with α-GalCer (2µg). CD69 upregulation on splenic NKT cells was analyzed at 1 hr and 2 hrs after α-GalCer injection. The numbers represent the percentage of NKT cells in each quadrant. The panel is representative of 3 mice. The percentage of CD69+ cells was calculated within NK1.1+ CD3+ cells. The statistical significance was calculated by Student’s t-test: * P< 0.05 vs 21% O₂.

α-GalCer treatment increased CD40L+ NKT cells after 1 hr and the levels remained almost constant when measured at 2 hrs (Fig. 25). However, hypoxic treatment significantly delayed the upregulation of CD40L on NKT cells after α-GalCer treatment (Fig. 25).
Fig. 25. Hypoxia mediated regulation of NKT cell activation in vivo. C57Bl/6 mice were allowed to inspire 21% O$_2$ or 10% O$_2$ and were challenged with α-GalCer (2µg). CD40L upregulation on splenic NKT cells was analyzed at 1 hr and 2 hrs after α-GalCer injection. The numbers represent the percentage of NKT cells in each quadrant. The panel is representative of 7 mice. The percentage of CD40L+ cells was calculated within NK1.1+ CD3+ cells. The statistical significance was calculated by Student’s t-test: * p< 0.05 vs 21% O$_2$.

4.3.3 Hypoxia is suppressive to cytokines production from NKT cells.

To determine the effect of hypoxia on NKT cell function in vivo, analysis for intracellular IL-4 and IFN-γ production from splenic NKT cells was done post α-GalCer treatment. IL-4 producing
NKT cells were detectable as early as 1 hr after α-GalCer treatment. The percentage of IL-4 producing NKT cells increased even more at 2 hrs after α-GalCer treatment (Fig. 26). However, hypoxic exposure suppressed IL-4 production from NKT cells. Correspondingly, hypoxia treated mice had lower serum IL-4 levels when measured at 2 hrs after α-GalCer treatment (Fig. 27).

**Fig. 26. Hypoxia mediated suppression of NKT cell function in vivo.** C57Bl/6 mice were allowed to inspire 21% O₂ or 10% O₂ and were challenged with α-GalCer (2µg). Intracellular IL-4 production from splenic NKT cells was analyzed at 1 hr and 2 hrs after α-GalCer injection. The numbers represent the percentage of NKT cells in each quadrant. The panel is representative of 3 mice. The percentage of IL-4+ cells was calculated within NK1.1+ CD3+ cells. The statistical significance was calculated by Student’s t-test: * p< 0.05 vs 21% O₂.
C57Bl/6 mice were allowed to inspire 21% O₂ or 10% O₂ and were challenged with α-GalCer (2µg). Serum IL-4 levels were measured at 2 hrs after α-GalCer injection by ELISA. Data represent average ± SD of 3 mice. The statistical significance was calculated by Student’s t-test: * p< 0.05 vs 21% O₂.

Intracellular IFN-γ production from NKT cells could be detected as early as 2 hrs post α-GalCer treatment. Hypoxia severely suppressed IFN-γ production from NKT cells in response to α-GalCer induced activation (Fig. 28).
Fig. 28. Hypoxia mediated suppression of NKT cell function in vivo. C57Bl/6 mice were allowed to inspire 21% O$_2$ or 10% O$_2$ and were challenged with α-GalCer (2µg). Intracellular IFN-γ production from splenic NKT cells was analyzed at 2 hrs after α-GalCer injection. The numbers represent the percentage of NKT cells in each quadrant. The panel is representative of 3 mice. The percentage of IFN-γ+ cells was calculated within NK1.1+ CD3+ cells. The statistical significance was calculated by Student’s t-test: * p< 0.05 vs 21% O$_2$.

The results obtained in the current study show hypoxia mediated suppression of NKT cell activation in vitro and in vivo in response to α-GalCer. The whole body exposure of C57Bl/6 mice to hypoxia inhibited α-GalCer induced early upregulation of activation markers and cytokines production.

4.3.4 Partial involvement of A2AR in the inhibition of NKT cells by hypoxia.

Since in the current study, NKT cells were found to be under the physiological regulation of adenosine-A2AR/A2BR pathway (chapters 4.1 and 4.2) and since whole body exposure to hypoxic air has been found to elevate blood adenosine levels (Chouker et al., 2008), it was reasonable to speculate that adenosine-A2AR/A2BR signaling could be one possible mechanism
by which hypoxia could exert suppressive effect on NKT cell activation. To test this hypothesis, WT and A2ARKO mice were allowed to inspire hypoxic atmosphere and received α-GalCer (2µg) injection. Activation marker CD40L was analyzed on splenic NKT cells 1 hr after α-GalCer treatment. As observed previously (Fig. 25), hypoxia was suppressive to the upregulation of CD40L on WT NKT cells as compared to 21% oxygen (Fig. 29). Interestingly, hypoxia also suppressed CD40L upregulation in A2ARKO NKT cells as compared to 21% oxygen (Fig. 29). The result implied that hypoxia is suppressive to NKT cell activation irrespective of A2AR expression.

![Fig. 29. Hypoxia regulates WT and A2ARKO NKT cell activation in vivo.](image)

WT and A2ARKO mice were allowed to inspire 21% O$_2$ or 8% O$_2$ and were challenged with α-GalCer (2µg). CD40L upregulation on splenic NKT cells was analyzed at 1 hr after α-GalCer injection. The numbers represent the percentage of NKT cells in each quadrant. The panel is representative
of 4 mice. The percentage of CD40L+ cells was calculated within NK1.1+ CD3+ cells. The statistical significance was calculated by Student’s t-test: * p< 0.05 vs 21% O₂.

The effect of hypoxia on cytokines production from WT and A2ARKO NKT cells was tested. In 21% oxygen group, A2ARKO NKT cells produced more IFNγ than WT NKT cells (Fig. 30). Correspondingly, serum IFNγ levels in A2ARKO mice were higher than WT mice (Fig. 31) which is consistent with the previous finding that A2AR deficiency causes exaggerated NKT cells activation (Fig. 10). In these experiments, exposure to hypoxic air very strongly suppressed IFNγ production from WT NKT cells (Fig. 30), however, there were still massive IFNγ-producing NKT cells in hypoxia-treated A2ARKO mice (Fig. 30). Consistent with this result, A2ARKO mice breathing hypoxic air showed significantly higher serum IFNγ levels as compared to the WT mice (Fig. 31). The fact that hypoxia could not reduce NKT cell function of A2ARKO mice to the levels of WT mice strongly suggests the involvement of A2AR-mediated mechanism downstream hypoxia. However, participation of A2AR-mediated mechanism in hypoxia-triggered NKT suppression was partial because hypoxia could largely suppress NKT cell activation even in the absence of A2AR.
Fig. 30. A2AR involves partly in the mechanism of hypoxia mediated suppression of NKT cell function in vivo. WT and A2ARKO mice were allowed to inspire 21% O₂ or 8% O₂ and were challenged with α-GalCer (2µg). Intracellular IFNγ production from splenic NKT cells was analyzed at 2 hrs after α-GalCer injection. The numbers represent the percentage of NKT cells in each quadrant. The panel is representative of 4 mice. The percentage of IFN-γ+ cells was calculated within NK1.1+ CD3+ cells. The statistical significance was calculated by Student’s t-test: * p<0.05 vs 21% O₂.
Fig. 31. A2AR involves partly in the mechanism of hypoxia mediated suppression of NKT cell function in vivo. WT and A2ARKO mice were allowed to inspire 21% O₂ or 8% O₂ and were challenged with α-GalCer (2µg). Serum IFNγ levels were measured at 2 hrs after α-GalCer injection by ELISA. Data represent average ± SD of 4 mice. The statistical significance was calculated by Student’s t-test: * p< 0.05 vs 21% O₂.
**A2BR does not involve in the regulation of NKT cells by hypoxia**

Another possible cause of hypoxia-mediated regulation of NKT cells activation was A2BR-mediated inhibition. To test this hypothesis, WT and A2BRKO mice were allowed to inspire hypoxic atmosphere and received α-GalCer (2µg) injection. As observed previously (Fig. 25), hypoxia was suppressive to the upregulation of CD40L on WT NKT cells as compared to 21% oxygen (Fig. 32). Hypoxia also suppressed CD40L upregulation in A2BRKO NKT cells as compared to 21% oxygen (Fig. 32). The result implied that hypoxia is suppressive to NKT cell activation irrespective of A2BR expression.
Fig. 32. Hypoxia regulates WT and A2BRKO NKT cell activation in vivo. WT and A2BRKO mice were allowed to inspire 21% O\(_2\) or 8% O\(_2\) and were challenged with α-GalCer (2µg). CD40L upregulation on splenic NKT cells was analyzed at 1 hr after α-GalCer injection. The numbers represent the percentage of NKT cells in each quadrant. The panel is representative of 4 mice. The percentage of CD40L+ cells was calculated within NK1.1+ CD3+ cells. The statistical significance was calculated by Student’s t-test: * p< 0.05 vs 21% O\(_2\).

The effect of hypoxia on cytokines production from WT and A2BRKO NKT cells was also tested. Exposure to hypoxic air strongly suppressed IFN\(\gamma\) production from both WT and A2BRKO NKT cells (Fig. 33.) The fact that hypoxia could suppress NKT cell function in A2BRKO mice to similar extent as in WT mice implies that A2BR does not involve in the hypoxia triggered downregulation of NKT cells.
**Fig. 33.** A2BR does not involve in the mechanism of hypoxia mediated suppression of NKT cell function in vivo. WT and A2BRKO mice were allowed to inspire 21% O\(_2\) or 8% O\(_2\) and were challenged with α-GalCer (2µg). Intracellular IFN\(\gamma\) production from splenic NKT cells was analyzed at 2 hrs after α-GalCer injection. The numbers represent the percentage of NKT cells in each quadrant. The panel is representative of 4 mice. The percentage of IFN-\(\gamma\)+ cells was calculated within NK1.1+ CD3+ cells. The statistical significance was calculated by Student’s t-test: * p< 0.05 vs 21% O\(_2\).
Discussion

For the induction of liver diseases, immunoregulatory roles for adenosine have been described in various experimental models. Treatment of animals with A2AR agonists blocked induction of hepatitis and upregulation of proinflammatory cytokines (Harada et al., 2000; Ohta and Sitkovsky, 2001; Odashima et al., 2005). Previous studies have demonstrated the exaggeration of hepatitis by the lack of adenosine-A2AR signaling by using pharmacological antagonism of A2AR as well as genetic deletion of A2AR, suggesting critical contribution of the adenosine-mediated physiological immunoregulatory mechanism in the liver (Ohta and Sitkovsky, 2001; Ohta et al., 2007). However, the critical cellular target of ‘adenosine-A2AR’ anti-inflammatory pathway in acute hepatitis is not known. The current study was conducted to understand the mechanism of adenosine mediated hepatoprotection during acute hepatitis.

Since NKT cells play a central role in the pathogenesis of acute hepatitis, it was hypothesized in the current study that NKT cells may be under the control of adenosine mediated anti-inflammatory signaling during hepatitis. The present findings provide evidence that endogenous adenosine signaling through A2AR is suppressive to the activation of NKT cells. Lappas et al. (2006) showed the suppression of NKT cell-dependent induction of hepatic ischemia-reperfusion injury by A2AR agonist. In an agreement with this report, A2AR agonists induced cAMP in NKT cells (Fig. 14) and inhibited NKT cell activation (Fig. 12A, 12B). The inhibition of cytokines production and NKT cell activation as shown by the decrease of CD40L and FasL expression showed that NKT cells are indeed sensitive to adenosine-A2AR inhibitory signal (Fig. 6).
However, it was not known whether A2AR plays role in pathophysiological regulation of NKT cells. Exaggerated pro-inflammatory cytokines production along with exaggerated liver damage in α-GalCer treated A2AKO mice demonstrated physiological control of NKT cell activation through A2AR (Fig. 10). The current study confirmed the important role of A2AR deficient NKT cells in the exacerbation of Con A induced liver injury (Fig. 11).

Although adenosine has been known to modulate liver diseases via A2AR, the involvement of A2BR was not known. The current study provides evidence that endogenous adenosine signaling through A2BR, in addition to A2AR, is suppressive to the induction of acute hepatitis. A series of in vitro studies strongly suggested the inhibitory role of A2BR on NKT cells. Being coupled to Gs-protein like A2AR, A2BR could inhibit NKT cells possibly through the upregulation of immunosuppressive cAMP. Indeed, cAMP induction by an adenylate cyclase activator forskolin strongly inhibited NKT cell activation (Fig. 12A), and A2BR stimulation increased cAMP levels in NKT cells (Fig. 14).

Exacerbation of Con A-induced liver injury by blocking adenosine-A2BR signalling demonstrated that A2BR is also involved in the physiological regulation of hepatitis (Fig. 15). Robust increase of Con A-induced cytokines production including IL-4 in A2BRKO mice (Fig. 16) suggested the enhancement of NKT cell activation in A2BRKO mice as it was the case in A2ARKO mice (Fig 8). This result was supported by the enhanced production of IL-4 from Con A stimulated A2BRKO NKT cells (Fig. 18). Exaggerated liver damage accompanied by enhanced production of cytokines upon direct activation of NKT cells in vivo by α-GalCer in A2BRKO mice demonstrated that physiological levels of adenosine controlled NKT cell activation through A2BR in the WT mice (Fig. 19). Importantly, the extent of NKT cell activation and subsequent liver damage in A2BRKO mice were augmented even though the mice
still express A2AR. It was further confirmed that A2BR-deficiency on NKT cells could, at least in part, account for the exacerbation of Con A-induced liver injury (Fig. 20). The results strongly suggest non-redundancy of A2BR in the negative regulation of NKT cells and hepatic inflammation.

For the confirmation of the role of A2AR-deficient and A2BR-deficient NKT cells, it was necessary to exclude immune cells that could involve in the reaction. For this purpose, WT or A2ARKO or A2BRKO NKT cells were isolated and transferred intra-hepatically into RAG1KO mice. The transferred cell suspension was completely devoid of NK cells and T cells and was enriched in NKT cells. However, the transferred cell suspension still contained about 65% non-NKT cells. Although these contaminated cells are not likely to contribute to the induction of Con A-hepatitis (Kaneko et al., 2000), purer NKT cell suspension should be used in order to completely rule out the possibility of participation of other cells.

Recent studies have revealed significant involvement of A2BR in vascular, pulmonary and intestinal inflammation (Sun et al., 2006; Yang et al., 2006; Mustafa et al., 2007; Eckle et al., 2008; Kolachala et al., 2008; Frick et al., 2009; Zhou et al., 2009); however, papers reported controversial roles for A2BR, i.e. pro- and anti-inflammatory. The reason for this discrepancy is not known, but it may be due to involvement of various cell types that respond to A2BR stimulation in different ways. A variety of cell types are expressing functional A2BR, e.g. endothelial cells, epithelial cells, fibroblasts, macrophages and mast cells (Haskó et al., 2009).

A2BR affects cellular functions in various ways dependent on cell types. Differences in the involvement of these cells in each disease model may affect overall pro- or anti-inflammatory roles of A2BR. For example, A2BR may play a proinflammatory role in mast cells because
A2BR stimulation may contribute to allergic asthma through the induction of IL-13 and vascular endothelial growth factor (VEGF) in mast cells (Ryzhov et al., 2008), although mast cells from A2BRKO mice are also reported to increase sensitivity to IgE-mediated anaphylaxis (Hua et al., 2007). In macrophages and dendritic cells, A2BR stimulation enhances IL-6 production in response to inflammatory stimuli (Ryzhov et al., 2008). However, it is important to note that the same treatment of dendritic cells also induces various immunoregulatory molecules including IL-10, TGF-β, cyclooxygenase-2 and indoleamine-2,3-dioxygenase (Novitskiy et al., 2008). Correspondent to the induction of anti-inflammatory molecules, A2BR stimulation reduced the capacity of antigen-presenting cells to activate T cells (Novitskiy et al., 2008). A2BR also positively regulate IL-10 production from intestinal epithelium (Frick et al., 2009).

The direct effect of A2BR on lymphocytes is not known. The current study demonstrates the expression of functional A2BR on NKT cells and inactivation of NKT cells by direct stimulation of A2BR. The elevation of intracellular cAMP by activation of Gs-coupled A2BR certainly seems to be one mechanism of negative regulation of NKT cell activation. Another mechanism could be A2BR mediated induction of IL-6 production. Pre-exposure to IL-6 is known to inactivate NKT cells and to suppress subsequent Con A-induced liver injury (Nishikage et al., 1999; Sun et al., 2004). Interestingly, A2BR stimulation can enhance IL-6 production (Ryzhov et al., 2008). Therefore, it is also possible that A2BR indirectly suppresses NKT cell activation by increasing IL-6 levels.

Taken together, NKT cells express functional A2AR and A2BR and endogenously produced adenosine signals through these receptors to negatively regulate NKT cell activation. Interruption of such adenosine signaling results in much exaggerated activation of NKT cells during hepatitis. Thus, adenosine can regulate activation of NKT cells in vivo.
The current study showed that both A2AR and A2BR are non-redundantly involved in the endogenous regulation of NKT during hepatitis. However, it is unclear why both A2AR and A2BR would be needed for regulating NKT cell activity in vivo. There is a question if A2AR and A2BR mediated suppression of NKT cell activation are two independent mechanisms or if the receptors can compensate for one another or if they can cooperate to cause an additive or synergistic suppression of NKT cell activation. It may be possible to speculate that since A2AR is a high affinity adenosine receptor, it may be recruited earlier during inflammation and as adenosine levels increase during the course of inflammation, low affinity-A2BR may also be recruited in the regulation of NKT cells. Together A2AR and A2BR may increase the threshold for NKT cell activation in the presence of endogenous adenosine which may critically suppress NKT cell-mediated hepatic inflammation. Another possibility is that A2AR and A2BR may form homo or heterodimers. The dimers may have different signalling pathway downstream as compared to the individual receptors. It is interesting to investigate if adenosine receptors dimerize and if receptor dimers can regulate NKT cells activation.

Hypoxic environment has been known to be suppressive to certain immune functions. The extent of activation of immune cells is thought to change depending on the oxygen tension surrounding the cells (Ohta et al, 2011). T cell proliferation was significantly decreased when cultured at physiologically relevant levels of oxygen (1-5% oxygen) when compared to proliferation at 21% oxygen (Loeffler et al., 1992; Naldini et al., 1997; Atkuri et al., 2005, 2007; Larbi et al., 2010). Low oxygen tension during culture suppressed IL-2 and IFNγ production from activated T cells (Zuckerberg et al, 1994; Caldwell et al., 2001; Kim et al., 2008; Roman et al., 2010). Also, the development of cytotoxic T cells in the mixed lymphocyte culture was suppressed by less oxygenated culture condition (Yang et al., 2009; Wang et al., 2010). A recent study
demonstrated that T cell activation in vivo correlated positively with oxygen tension. In the study, whole body exposure of mice to hypoxic air (8% oxygen) suppressed but hyperoxic air (100% oxygen) enhanced T cell activation as compared to 21% oxygen (Ohta et al., 2011).

In those animals exposed to hypoxic atmosphere, there was an increase in blood adenosine levels and strongly suppressed the induction of Con A-induced liver injury in an A2AR-dependent manner (Chouker et al., 2008). The current findings demonstrate that hypoxia can strongly suppress activation of NKT cells both in vivo and in vitro. Since NKT cell activation is crucial to the induction of Con A hepatitis, hypoxic immunosuppression targeting NKT cells is likely to play a role in the inhibition of hepatitis induction.

Hypoxia triggers the accumulation of extracellular adenosine by accelerating the breakdown of ATP (Sitkovsky et al., 2004). In the current study, NKT cells were found to be under the control of adenosine-A2AR/A2BR signaling. Therefore, the ‘adenosine-A2AR/A2BR’ pathway was suspected to contribute to the observed suppression of NKT cells activation by hypoxic exposure. A2AR was involved in the mechanism of hypoxic NKT cell suppression, but its contribution was limited. Indeed, hypoxia could suppress NKT cells activation irrespective of the presence of A2AR to some extent. This result suggested that hypoxia renders strong inhibitory effects on NKT cell activation partly through A2AR-dependent mechanism. For the ‘A2AR-independent’ portion of hypoxia-triggered NKT cell suppression, it was examined whether the ‘adenosine-A2BR’ pathway contributed or not. Previous studies have demonstrated hypoxia-mediated induction of A2BR on endothelial cells (Kong et al., 2006) and involvement of A2BR in the hypoxia-mediated inhibition of neutrophil transepithelial migration (Rosenberger et. al. 2008). Together with these previous studies, the current study suggested possible involvement of
A2BR downstream hypoxia; however, A2BR was not found to involve in hypoxia-mediated suppression of NKT cell activation.

The ‘A2AR-independent’ pathway of hypoxia-mediated NKT cell regulation may involve several other possibilities that need to be explored. One possibility is the involvement of the transcription factor, hypoxia inducible factor-1α, (HIF-1α). Under hypoxic conditions, HIF-1α is stabilized and it helps cells to adapt to hypoxia by switching the energy metabolism from oxidative phosphorylation to anaerobic metabolism (Majmundar et al., 2010). The reduced supply of ATP from the anaerobic pathway might be one reason why activation of lymphocytes is compromised under hypoxic condition. HIF-1α has been shown to suppress T cell signaling after TCR crosslinking (Neumann et al., 2005). Also, deletion of HIF-1α from T cells resulted in increased IFNγ production from these cells (Lukashev et al., 2006; Guo et al., 2009).

In conclusion, the current study demonstrates that NKT cells are under the control of endogenous adenosine during hepatitis. Endogenous adenosine signals through A2AR and A2BR to down regulate NKT cell activation during hepatitis. In addition, the current study also demonstrates that hypoxia is suppressive to NKT cell activation and function. Taken together, ‘hypoxia-adenosine anti-inflammatory pathway’ may play a crucial role in the pathogenesis of acute hepatitis.
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