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Role of A_{2A} Adenosine receptors in regulating sepsis

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1. ÖSSZEFOGLALÁS

A szepszis során megfigyelhető csökkent immunfunkció és bakteriális elimináció hátterében meghúzódó mechanizmusok nem teljes mértékben ismertek. Az adenozin szintje jelentősen megemelkedik a szepszishez kapcsolódó szöveti sérülések hatására. Emellett az adenozinnak erős immunszupreszív hatásai vannak, amelyek jelentős része az immunsejteken kifejeződő A_{2A} receptorok aktivációjához kötődik. Ezért kíváncsiak voltunk, hogy vajon az A_{2A} receptor aktivációja hozzájárul-e a szepszisben tapasztalható immunfunkciók regulációjának megváltozásához. Azt tapasztaltuk, hogy az A_{2A} KO (knock-out) egerek halálozási aránya lecsökkent a vad-típusú egerekéhez képest a CLP (cecal ligation and puncture) által előidézett szepszisben. Megvizsgálva a jellemző apoptotikus markereket azt találtuk, hogy az A_{2A} KO állatok lépében az apoptozis mértéke jelentősen lecsökkent. A génchip és a flow cytometriás vizsgálatok megemelkedett MHC II génexpressziót mutattak ki a makrofágokon. Továbbá azt találtuk, hogy a CLP-t követően az immunszupreszív citokin, interleukin (IL)-10 szintje jelentősen alacsonyabb volt az A_{2A} KO állatok, valamint az A_{2A} receptor antagonistával kezelt állatok vérében és hasüregében, ami arra utalt, hogy a szepszis során felszabaduló endogén adenozin az A_{2A} receptoron keresztül képes megnövelni az IL-10 szintjét. Ezenfelül azt tapasztaltuk, hogy az A_{2A} receptor antagonistával történő gátlása szintén javította az egerek túlélését, még abban az esetben is, ha a sebészeti beavatkozást követően 2 óra múlva adtuk be az egereknek. Mivel eredményeink arra engedtek következtetni, hogy az endogén módon felszabaduló adenozin az A_{2A} receptor aktiválásán keresztül képes megnövelni az IL-10 termelést, ezért figyelmünket a továbbiakban a folyamat mögött meghúzódó lehetséges molekuláris mechanizmusok feltérképezése fele fordítottuk. Megerősítettük, hogy az A_{2A} receptornak alapvető szerepe van az adenozin stimulációs hatásának közvetítésében a makrofágok *Escherichia coli* által kiváltott IL-10 termelésben. Kimutattuk, hogy az adenozin stimulációs hatása független a toll-like receptor (TLR)4-től és a myeloid differentiation factor (MyD)88-tól, azonban negatív módon szabályozódik a TRAF6 fehérje által, és gátolható volt a p38 kináz inhibíciójával. Bebizonyítottuk, hogy az adenozin és *E. coli* szinergisztikusan aktiválja az IL-10 transzkripcióját. Az IL-10 gén promoterének deléciós és mutációs analízisével kimutattuk, hogy az adenozin a promoter CCAAT-enhancer binding protein (C/EBP) kötődésért felelős régióján keresztül fejti ki a hatását az *E. coli* által indukált IL-10 transzkripcióra. Továbbá az adenozin megnövelte mind a C/EBPβ sejtmagi felhalmozódását mind a DNS kötődését. Emellett a C/EBPβ deficiens makrofágok nem termeltek IL-10-et sem *E. coli*, sem *E. coli*/adenozin kombinált kezelést követően. Eredményeink azt sugallják, hogy az A_{2A} adenozin receptor- C/EBPβ tengely alapvető fontosságú a bakteriális fertőzést követő IL-10 termelésben. Megfigyeléseink tükrében, miszerint az A_{2A} adenozin receptor aktiváció az IL-10 termelés szabályozásán keresztül részt vesz a fertőzéshez kapcsolódó immunszupresszált állapot kialakulásában, úgy gondoljuk, hogy a receptor farmakológiai gátlása hasznos lehet a szepszis kezelésében.

2. ABSTRACT

The mechanisms governing the impairment of bacterial clearance and immune function in sepsis have not been elucidated. Adenosine levels are elevated during tissue hypoxia and damage associated with sepsis. Adenosine has strong immunosuppressive effects, many of which are mediated by A_{2A} receptors ($A_{2A}R$) expressed on immune cells. Therefore, we tested the hypothesis that activation of $A_{2A}R$ contributes to the dysregulation of immune function in sepsis. A_{2A} receptor knockout (KO) mice were protected from the lethal effect of cecal ligation and puncture (CLP)-induced polymicrobial sepsis and had improved bacterial clearance when compared to wild type (WT) mice. cDNA microarray analysis and flow cytometry revealed increased MHC II expression in $A_{2A}R$ KO mice. Apoptosis was attenuated in the spleen of A_{2A} KO mice indicating preserved lymphocyte function. Levels of the immunosuppressive cytokine interleukin (IL)-10 were markedly lower in A_{2A} KO vs. WT mice and in mice treated with an $A_{2A}R$ antagonist indicating that endogenous adenosine upregulates IL-10 production via $A_{2A}R$. Additionally, the $A_{2A}R$ antagonist increased survival even when administered in a delayed fashion. Since we found that $A_{2A}R$ activation by endogenous adenosine contributes to IL-10 production in polymicrobial sepsis, we next investigated the molecular mechanisms underpinning this interaction between adenosine receptor signaling and infection. We confirmed in vitro that A_{2A} receptor activation is critically required for the stimulatory effect of adenosine on IL-10 production by *Escherichia coli*–challenged macrophages. The stimulatory effect of adenosine on *E. coli*–induced IL-10 production did not require toll-like receptor (TLR)4 or myeloid differentiation factor (MyD)88, but was blocked by p38 inhibition. Additionally, *E. coli* and adenosine synergistically activate IL-10 transcription. Sequential deletion analysis and site-directed mutagenesis of the IL-10 promoter revealed that a region harboring CCAAT-enhancer-binding protein (C/EBP) binding elements was responsible for the stimulatory effect of adenosine on *E. coli*–induced IL-10 promoter activity. Moreover, adenosine augmented *E. coli*–induced nuclear accumulation and DNA binding of C/EBPβ and C/EBPβ-deficient macrophages failed to produce IL-10 in response to adenosine and *E. coli.* Therefore, these studies demonstrate that the A_{2A} receptor–C/EBPβ axis is critical for IL-10 production after bacterial infection. In summary, based on our observations that $A_{2A}R$ contributes to immunosuppression during infection we propose that the blockade of this receptor may be useful in the treatment of sepsis.

3. ABBREVIATIONS

$A_{2A}R$	adenosine A_{2A} receptor
Ag	antigen
CARS	compensatory anti-inflammatory response syndrome
CCPA	A_1 receptor agonist (2-chloro-N^6-cyclopentyladenosine)
C/EBP	CCAAT-enhancer-binding protein
CGS-21680	A_{2A} receptor agonist (2-*p*- (2-carboxyethyl)phenethyl-amino-5'-N-ethyl-carboxamidoadenosine)
CLP	cecal ligation and puncture
DMSO	dimethylsulphoxide
HRP	horseradish peroxidase
IB-MECA	A_3 receptor agonist (N^6-(3-iodobenzyl)adenosine-5'-N-methyluronamide)
IL-	interleukin-
KO	knockout
LPS	lipopolysaccharide
LTA-SA	*Staphylococcus aureus* Lipoteichonic acid
MAPK	mitogen-activated protein kinase
MOF	multiple organ failure
MyD88	myeloid differentiation factor 88
NECA	A_{2B} receptor agonist (5'-N-ethylcarboxamidoadenosine)
NF-κB	nuclear factor-κB
PD98059	p42/44 inhibitor (2′-Amino-3′-methoxyflavone)
RIPA buffer	radioimmunoprecipitation buffer
SB203580	p38 inhibitor (4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole)
shRNA	short hairpin RNA
SIRS	systemic inflammatory response syndrome
TLR	toll-like receptor
TNF	tumor necrosis factor

TRAF6	tumor necrosis factor receptor–associated factor 6
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling
ZM241385	A_{2A} receptor antagonis (4-(2- [7-amino-2-(2-furyl)[1.2.4]triazolo[2.3-a][1.3.5]triazin-5-ylamino] ethyl) phenol)

4. INTRODUCTION

4.1. Pathophysiology of sepsis

Sepsis is defined as systemic illness caused by microbial invasion of normally sterile parts of the body. Despite advances in antibiotic, hemodynamic, and ventilatory support, the incidence of sepsis and the numbers of sepsis-related deaths are increasing. In fact, an annualized increase of sepsis from 1979-2000 of 8.7 % resulting in three times as many deaths from sepsis in 2000 when compared to 1979 has been reported (Martin et al., 2000). Sepsis and the resultant multiple organ failure (MOF) are the cause of 50% to 80% of all deaths in intensive care units, which places sepsis as the tenth leading cause of death in the developed world (Hoyert et al., 2006). Although MOF was first described more than 20 years ago, the cause of the condition is still poorly understood, treatment is still largely supportive, and the mortality rate (30-70 % in most series) is little improved (Angus et al., 2001). Although initially proposed as a sign of occult or uncontrolled infection, MOF has now been documented to occur after a number of diverse clinical conditions, including mechanical and thermal trauma, pancreatitis and shock (Deitch, 1992). In 1992, the term systemic inflammatory response syndrome (SIRS) was introduced to describe the underlying massive inflammatory reaction of the body that contributes to the development of sepsis and MOF. Although, since then the term SIRS has been shown to lack specificity in predicting MOF, the underlying concept that uncontrolled inflammation predisposes to MOF remains valid. The SIRS, or hyper-inflammatory theory of sepsis and MOF states that in response to an inciting event, the body unleashes an inflammatory reaction consisting of an overproduction of a host of pro-inflammatory mediators. In the past few years, multiple clinical trials have been carried out aiming to counteract many of the SIRS-inducing pro-inflammatory mediators. Unfortunately, all of these trials failed to demonstrate any benefit from treatment (Opal and Cross, 1999). Based on the failure of these trials, it was suggested that the SIRS model of sepsis had a fundamental flaw, because it did not take into consideration the fact that although the clinical symptoms follow a relatively predictable consistent course, diverse pathophysiological processes underlie the manifestation of sepsis and MOF (Bone, 1996). Most importantly, it was overlooked that in response to the original insult, the body also mounts a compensatory anti-inflammatory response. It was postulated that it is the balance of pro and anti-

inflammatory events that is lost in MOF resulting in either a destructive systemic inflammatory state or a state of excessive immunosuppression. The excessive production of pro-inflammatory mediators leads to MOF via a variety of pro-inflammatory processes including widespread endothelial damage, edema resulting from vascular permeability, and impaired availability of oxygen. It appears however, that this excessive inflammatory response is only the cause of MOF and death in a small number of patients. On the other hand, the immune paralysis that develops as a result of an overwhelming anti-inflammatory response manifests itself as anergy and an increased susceptibility to infection. In fact, the subsequent development of infection, secondary to this immunosuppressed, compensatory anti-inflammatory response syndrome (CARS) state is a major cause of the development of MOF and sepsis in the majority of patients. There is even an oscillation between periods of severe inflammation and periods of immunosuppression in some patients.

While numerous studies have investigated the events of the pro-inflammatory component of MOF, the anti-inflammatory response of the host culminating in immune paralysis has been largely neglected. Nevertheless, it has recently become evident that one of the most important factors contributing to the immune paralysis seen during MOF is macrophage dysfunction (Ayala and Chaudry, 1996; Benjamim et al., 2004; Hotchkiss and Karl, 2003; Riedemann et al., 2003a). Studies have indicated that while early on after the onset of sepsis there is initially an activation of macrophage pro-inflammatory cytokine release, is followed by a prolonged and profound state of immunosuppression, which is characterized by substantially depressed macrophage effector functions. Specific macrophage defects in septic patients include a striking decrease in the ability to produce important pro-inflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-12, and IL-1β (Docke et al., 1997; Munoz et al., 1991; Reddy et al., 2001), and a substantial increase in the production of the crucial anti-inflammatory cytokine IL-10 (Ayala et al., 1994; Song et al., 1999). Other macrophage effector functions that are impaired in septic patients include histocompatibility protein expression, antigen-presenting capacity, phagocytosis, and bacterial clearance (Le Tulzo et al., 2004; Reddy et al., 2001; Tschaikowsky et al., 2002).

4.2 Macrophages are the major source of IL-10

IL-10 was initially described as a T helper 2 (Th2) product that reduced the production of cytokines by T helper 1 (Th1) T cell clones (Mosmann et al., 1990). Subsequently, it has become clear that monocytes/macrophages are the predominant cell types secreting IL-10 following LPS administration, *in vivo* (Barsig et al., 1995). Macrophages exposed to LPS secrete IL-10 with a later onset when compared to proinflammatory cytokines. This belated production of IL-10 represents an essential autoregulatory mechanism that limits excessive production of the proinflammatory cytokines TNFα IL-1, and IL-12 (Moore et al., 2001), thereby contributing to both the limitation and resolution of inflammation (Gilroy et al., 2004). A gradual long-lasting increase in IL-10 production, however is a pivotal factor contributing to the potentially lethal impairment in immune function and anti-bacterial defense observed in the CLP model. Using anti-IL-10 antibody administration, Steinhauser et al. (1999a) showed that endogenous IL-10 suppressed the immune response to *Pseudomonas pneumonia* after CLP in mice. In the presence of high levels of IL-10, the immune system cannot initiate an appropriate acquired immune response to the first septic insult and may be even more susceptible to additional nosocomial infections that contribute to an adverse outcome. In another study consisting only of the CLP insult, an anti-IL-10 antibody administered at 12 h following CLP protected animals against the lethal effect of sepsis (Song et al., 1999).

4.3. Role of pathogen recognition receptors in inducing inflammatory cytokine production by macrophages

Twenty years ago, Janeway proposed the hypothesis that the immune system detects pathogen infection using pattern recognition receptors (PRRs), which are able to recognize molecular signatures known as “pathogen-associated molecular patterns (PAMPs)” (Janeway, 1989). The first piece of evidence supporting this hypothesis was provided in 1996, when it was revealed that *Drosophila* carrying mutations in a receptor called “Toll” exhibit high susceptibility to fungi infection (Lemaitre et al., 1996). Subsequently, a human homologue of Toll was discovered and its ability to induce innate responses, such as the production of inflammatory cytokines was demonstrated (Medzhitov et al., 1997). This observation paved the

way for the discovery of a family of membrane-bound TLRs (TLR1-13), which serve as PRRs to recognize a wide range of PAMPs, which include lipids, lipoproteins, glycans, and nucleic acids and play a crucial role in initiating immune responses (Medzhitov et al., 1997; Rock et al., 1998; Brightbill and Modlin, 2000; Akira et al., 2006; West et al., 2006). The TLR family members can be divided into two groups. TLR1, TLR2, TLR4, TLR5, TLR6, and TLR11 are found on the cell surface and recognize microbial membrane/wall components. TLR3, TLR7, TLR8, and TLR9 are localized in intracellular vesicles and predominantly recognize nucleic acid components. TLR2 recognizes a wide range of PAMPs including triacyl lipopeptides from bacteria and mycobacteria, diacyl lipopeptides from mycoplasma, peptidoglycan (PGN) and lipoteichoic acid (LTA) from Gram-positive bacteria, porin from *Neisseria*, zymosan from fungi (Takeuchi et al., 1999). TLR2 generally forms heterodimer with TLR1, TLR6. TLR2-6 discerns the mycobacterial diacylated lipopeptide, LTA, and zymosan, whereas TLR2/1 recognizes triacylated lipopeptide. TLR4 is essential for responses to lipopolysaccharide (LPS), a major constituent of the outer membrane of Gram-negative bacteria (Poltorak et al., 1998). TLR5 senses flagellin, a protein component of bacterial flagella (Hayashi et al., 2001). Mouse TLR11 recognizes uropathogenic bacterial components and parasite products (Zhang et al., 2004). TLR3 recognizes genomic RNA from double-stranded (ds)RNA viruses and dsRNA produced during the course of replication of single-stranded (ss)RNA viruses (Alexopoulu et al., 2001; Wang et al., 2004). TLR7 was originally identified as a sensor of imidazoquinoline derivates, such as imiquimod and guanine analogues such as loxoribine (Hemmi et al., 2002), but subsequent studies have identified guanosine- and uridine-rich ssRNA derived from influenza virus, as well as silencing (si)RNAs as ligands for this receptor (Diebold et al., 2004; Heil et al., 2004). Human TLR8 recognizes bacterial RNA and ssRNA from influenza A virus; however, TLR8-/- mice respond normally to these microbial components, suggesting a species-specific function for these receptors (Gilliet et al., 2008). TLR9 senses unmethylated 2'-deoxyribo cytidine-phosphate-guanosine (CpG) DNA motifs that are frequently present in bacterial genomes (Hemmi et al., 2000).

The engagement of pattern recognition receptors by microbial components triggers the activation of signaling cascades leading to the induction of genes involved in antimicrobial host defense such as cytokines and chemokines (Brightbill et al., 1999; Takeuchi et al., 1999; Hemmi et al., 2000). TLR signaling is initiated by the ectodomain-mediated dimerization of TLRs, which

then facilitates the recruitment of TIR (Toll/IL-1 receptor) domain-containing cytosolic adapter molecules. These adapter molecules are the following: MyD88 or TIRAP (TIR domain containing adapter protein) - also known as MyD88 adapter like (MAL)-, TRIF (TIR-containing adapter inducing IFNβ) - also known as TIR domain-containing adapter molecule (TICAM) 1 - and TRAM (TRIF-related adapter molecule) - also known as TIR domain-containing adapter molecule (TICAM)-2 - to the receptor complex (Kawai and Akira, 2007). These adapters are selectively recruited to their respective TLRs, eliciting appropriate responses depending on the type of PAMP. Myeloid differentiation factor 88 (MyD88) has been shown to be a critical adaptor protein linking TLRs to several downstream intracellular pathways (Akira et al., 2006; West et al., 2006). Many of the activating signals originating from TLRs and MyD88 converge on tumor necrosis factor receptor–associated factor 6 (TRAF6), which transduces the signals toward the nuclear factor-κB (NF-κB) system and mitogen-activated protein kinases (MAPKs), resulting induction of genes involved in pro-inflammatory responses (Akira et al., 2006; West et al., 2006) (Figure 1). TIRAP is recruited to TLR2 or TLR4 and functions as a sorting adapter that recruits MyD88. TRIF, in contrast, is used by TLR3 and TLR4 and initiates an alternative pathway through TBK1 (TANK [TNF receptor-associated factor family member-associated nuclear factor κB activator] binding kinase 1) leading to IRF (IFN regulatory factor)3, NF-κB and MAPK to induce type I IFN and inflammatory cytokines (Akira et al., 2006; West et al., 2006) (Figure 1). TRAM selectively serves to link TRIF to TLR4, but not TLR3.

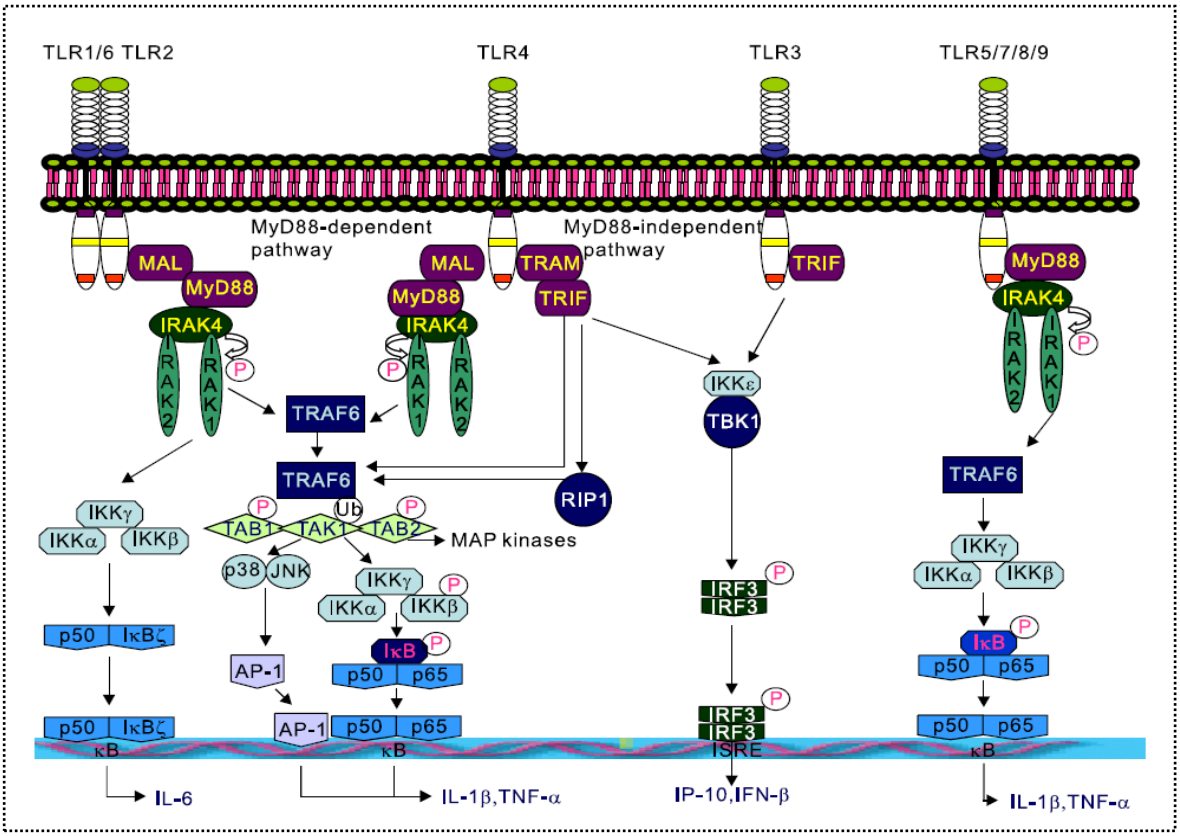

Figure 1.The signaling pathways of Toll-like receptors (TLRs) (West et al, 2006) Signaling mediated by TLR is broadly classified as MyD88-dependent and independent pathways. All TLRs utilize MyD88 with the exception of TLR 3. TLR 7, 8 and 9 pathways are predominant in pDCs. MyD88 binds with the TIR domain of the receptor and phosphorylates IRAK4 which in turn phosphorylates IRAK1. IRAK1 phosphorylates TRAF6 leading to the ubiquitination of TAK complex. Activation of IKK, JNK and p38 pathways leads inflammatory and antiviral responses. ISRE, interferon stimulatory response element; κB, κB site; p, phosphorylation; ub, ubiquitination.

4.4. Regulation of IL-10 production in response to PAMPs

Much less is known about the regulation of anti-inflammatory cytokines and their genes, the most prominent of which is IL-10 (Fiorentino et al., 1989; de Waal Malefyt et al., 1991). The induction of IL-10 production can be achieved by a broad spectrum of stimuli through multiple PRRs. TLR2 agonists such as lipopetides are potent inducers of IL-10 (Agarwal et al., 2003). In dendritic cells (DCs), prolonged ERK activation upon TLR2 stimulation seems to play a significant role in the production of a high level of IL-10 (Dillon et al., 2004). Furthermore, the

PI-3K (Phosphoinositide-3 Kinase)-Akt pathway may also contribute to IL-10 production in DCs. This PI-3K-Akt pathway-dependent IL-10 expression is mediated via suppressing GSK (Glycogen synthase kinase) 3 (Martin et al., 2005; Xu et al., 2006). TLR6 has also a prominent role in the production of this anti-inflammatory cytokine in response to fungal infections, since TLR6 KO mice display a defective IL-10 response to *Candida albicans* stimulation (Netea et al., 2008). Recently it was also found that MyD88-dependent TLRs, such as TLR4, 7, 9, as well as TLR3 can trigger a robust IL-10 response in murine macrophages and myeloid dendritic cells (Boonstra et al., 2006). Furthermore, NOD2 also can mediate Gram+ bacteria–induced IL-10 production by macrophages (Kapetanovic et al., 2007; Moreira et al., 2008). Additionally, lectins derived from pathogens stimulate IL-10 production by a TLR-independent signaling pathway (Geijtenbeek et al., 2003; Rogers et al., 2005). These C-type lectin receptors (CLRs) can be divided into two groups: group I CLRs belong to the mannose receptor family and group II CLRs belong to the asialoglycoprotein receptor family and include the DC-associated C-type lectin 1 (dectin-1) and DC immunoreceptor (DCIR) subfamilies (Geijtenbeek and Gringhuis, 2009). CLRs recognize most classes of human pathogens; mannose specificity allows the recognition of viruses, fungi and mycobacteria, fucose structures are more specifically expressed by certain bacteria and helminths and glucan structures are present on mycobacteria and fungi (van Kooyk and Rabinovich, 2008). CLR triggering by different pathogens can induce diverse immune responses. Dectin-1, DC-SIGN triggering activates the serine/threonine protein kinase RAF1, which induces the phosphorylation and subsequent acetylation of TLR-induced p65, which increases TLR-induced cytokine expression, including that of IL-10 (Gringhuis et al., 2007 and 2009; Rogers et al., 2005). Moreover, the SYK pathway modulates TLR signalling by inducing the NF-κB subunits Rel and RelB. Because RAF1 signalling counteracts RelB activation by sequestering RelB in inactive dimers, the crosstalk between the RAF1 and SYK pathways increases the production of TLR-induced IL-10 (Gringhuis et al., 2007). Additionally, *M. tuberculosis* and *M. leprae* induce the production of IL-10 because they trigger TLRs and DC-SIGN, which enhances TLR-induced NF-κB activity through the RAF1 signalling pathway as described above.

Between the TLR-associated adapter molecules and the intracellular kinases, the TRAF3 seems a major regulator of the IL-10 expression. Häcker et al. (Häcker et al., 2006) showed that TRAF3 has an important role in TRIF-dependent signaling to the IL-10 gene, probably by

marshalling TBK1 into the TIR signaling complex leading to activation of IL-10 expression. Moreover, TRAF3 interact with TRIF and IRAK1, components that define the MyD88-independent pathway via TLR signaling.

IL-10 is an inducible gene and several transcriptional factors have been implicated in its regulation, which include Sp1 (Brightbill et al., 2000; Tone et al., 2000; Ma et al., 2001), Sp3 (Tone et al., 2000), C/EBPδ and C/EBPβ (Liu et al., 2003), STAT3 (Benkhart et al., 2000), and c-Maf (Cao et al., 2005). IL-10 gene expression also requires transient remodeling of the IL-10 promoter that occurs as a result of MAPK activation (Zheng et al., 2006). In addition, it has shown that IL-10 production is also regulated at various posttranscriptional levels, including alterations in mRNA stability and translation efficacy (Powell et al., 2000; Németh et al., 2005).

4.5. Physiologic and pharmacologic roles of adenosine

Adenosine is produced in response to cellular stress and damage. Elevations in extracellular adenosine are found in conditions of ischemia, hypoxia, inflammation, and trauma (Haskó and Cronstein, 2004; Haskó et al., 2002; Haskó et al., 2004; Linden, 2001). Adenosine, was first recognized as a physiologic regulator of coronary vascular tone by Drury and Szent-Györgyi (1929), however it was not until 1970 that Sattin and Rall showed that adenosine regulates cell function via occupancy of specific receptors on the cell surface (Sattin and Rall, 1970). It is now clear that there are at least four different subtypes of adenosine receptor, A_1, A_{2A}, A_{2B}, and A_3 (Fredholm et al., 2001; Fredholm et al., 2000). These receptors have all been cloned and the deduced sequence reveals that all four are members of the large family of 7-transmembrane spanning, G protein-coupled receptors. Three of the adenosine receptor subtypes, A_1, A_{2A} and A_{2B} are highly conserved throughout evolution (80-95 % sequence homology) whereas the sequence of A_3 receptors varies considerably among species (Burnstock, 2006).

Adenosine receptor-mediated effects have been demonstrated in virtually every tissue or organ examined. Some of the more prominent physiologic or pharmacologic effects mediated by adenosine receptors include: neurotransmission (A_1 and A_{2A} receptors, the central nervous system effects of caffeine are result from adenosine receptor antagonism); modulation of cardiac pacemaker automaticy (A_1 receptors, intravenous preparations of adenosine are licensed for clinical use for the treatment of supraventricular tachycardia); coronary vasodilation (A_{2A}

receptors, infusions of adenosine are licensed for clinical use as a coronary vasodilator for pharmacologic stress testing); regulation, indirectly, of airway tone (A_{2B} receptors); inhibition of inflammation (mediates the anti-inflammatory effects of low-dose methotrexate, the most commonly used second line agent for the treatment of rheumatoid arthritis).

Adenosine receptors are expressed on a wide range of immune cell types. Neutrophils can express all adenosine receptor subtypes, and their expression pattern may be affected by the presence of an inflammatory environment, as evidenced by changes in neutrophil A_{2A}R expression in rheumatic disease (Martini et al., 1991). All four adenosine receptors have been detected on both monocytes (Merril et al., 1997; Broussas et al., 1999; Suzuki et al., 2000) and macrophages (Eppel et al., 1989; Murphree et al., 2005; Nemeth et al., 2005). Whereas the expression of adenosine receptors is low on quiescent monocytes (Sajjadi et al., 1996; Fossetta et al., 2003), their number increases during differentiation into macrophages (Eppel et al., 1989; Merril et al., 1997; Sajjadi et al., 1996). Adenosine receptor expression also changes upon inflammatory activation. A_{2A} receptor in human THP-1 monocytes was shown to be up-regulated by IL-1 and TNFα, but to be down-regulated by IFNγ (Khoa et al., 2001). In macrophages, LPS was shown to increase both A_{2A} and A_{2B} receptor expression, whereas IFNγ selectively up-regulated A_{2B} receptor expression in these cells (Németh et al., 2003a; Németh et al., 2005; Murphree et al., 2005). Adenosine receptor expression on DCs also appears to depend on their maturation state. Whereas immature human DCs have been shown to express functional A_1, A_{2B} and A_3 receptors, as well as low levels of the A_{2A} receptor, mature DCs predominantly express functional A_{2A} and A_{2B} receptors (Panther et al., 2001; Hofer et al., 2003; Schnurr et al., 2004). Both peripherial cytotoxic T cells (CTLs) and Th cells express A_{2A}, A_{2B} and A_3 receptors, but these cells are thought to express little or no A_1 receptors (Antonysamy et al., 1995; Huang et al., 1997; Varani et al., 1997; Koshiba et al., 1999; Mirabet et al., 1999; Gessi et al., 2001; Hoskin et al., 2002; Zhang et al., 2004; Lappas et al., 2005; Csoka et al., 2008). B lymphocytes express mainly A_{2A} receptors (Conigrave et al., 2001; Napieralski et al., 2003), and functional A_1, A_{2A} and A_3 receptors have been found on Natural Killer (NK) cells (Priebe et al., 1990; MacKenzie et al., 1994).

4.6. Adenosine metabolism

The dominant pathway leading to high extracellular adenosine levels during metabolic stress is release of precursor adenine nucleotides (mostly ATP) from the cell followed by extracellular catabolism to adenosine by a cascade of ectonucleotidases, including CD39 (nucleoside triphosphate diphosphohydrolase, NTPDase; (Figure 2) and CD73 (5'-ectonucleotidase, Ecto5'NTase; (Figure 2) (Eltzschig et al., 2004; Linden, 2001; Thompson et al., 2004; Zimmermann, 2000).

CD39 is a transmembrane lipoprotein that was originally characterized as an obscure activation marker, identified on B cells, subsets of activated natural killer cells, T lymphocytes, macrophages and endothelial cells. Nucleotides are present in extracellular fluids as a consequence of several mechanisms: cell lysis, selective permeabilization of the plasma membrane and exocytosis of secretory vesicles, such as from platelet dense bodies. CD39/ATPDase sequentially converts extracellular purine nucleotides in the extracellular space, such as ATP and ADP, to the monophosphate form (5'-AMP). CD73 is a 70-kDa glycosyl phosphatidylinositol-anchored cell surface protein with ecto-5´-nucleotidase enzyme activity that catalyzes the dephosphorylation of 5´-AMP to adenosine. Therefore, this molecule plays a key role in the generation of extracellular adenosine by catalyzing the last step in the cascade of ATP breakdown initiated by CD39. In fact, CD73 has been proposed to be the rate-limiting enzyme in the generation of adenosine during metabolic stress (Lennon et al., 1998; Volmer et al., 2006). CD73 is ubiquitously expressed in most tissues and its expression on B and T cells correlates with cell maturity (Thompson and Ruedi, 1988; Thompson and Ruedi, 1989). Adenosine bioavailability is limited by its catabolism to inosine by adenosine deaminase or its recycling into AMP by adenosine kinase (ADK).

Since most nucleosides and their analogues are relatively hydrophilic and thus cannot readily pass the plasma membrane, nucleoside transporters are essential in adjusting extra- and intracellular adenosine levels (Cass et al., 1999). Concentrative nucleoside transporters (CNT) take up nucleosides from the extracellular space in an energy-dependent manner (Gray et al., 2004; Smith et al., 2007) while equilibrative nucleoside transporters (ENT) passively equilibrate pools of extra- and intracellular adenosine (Baldwin et al., 2004). Under physiological conditions, ENTs appear to play a predominant role in adjusting extra- and intracellular pools of

adenosine, and the extracellular concentration of adenosine can be enhanced by inhibition of ENTs with dipyridamole, dilazep, nitrobenzylthioinosine, or cannabidiol (Kong et al., 2004).

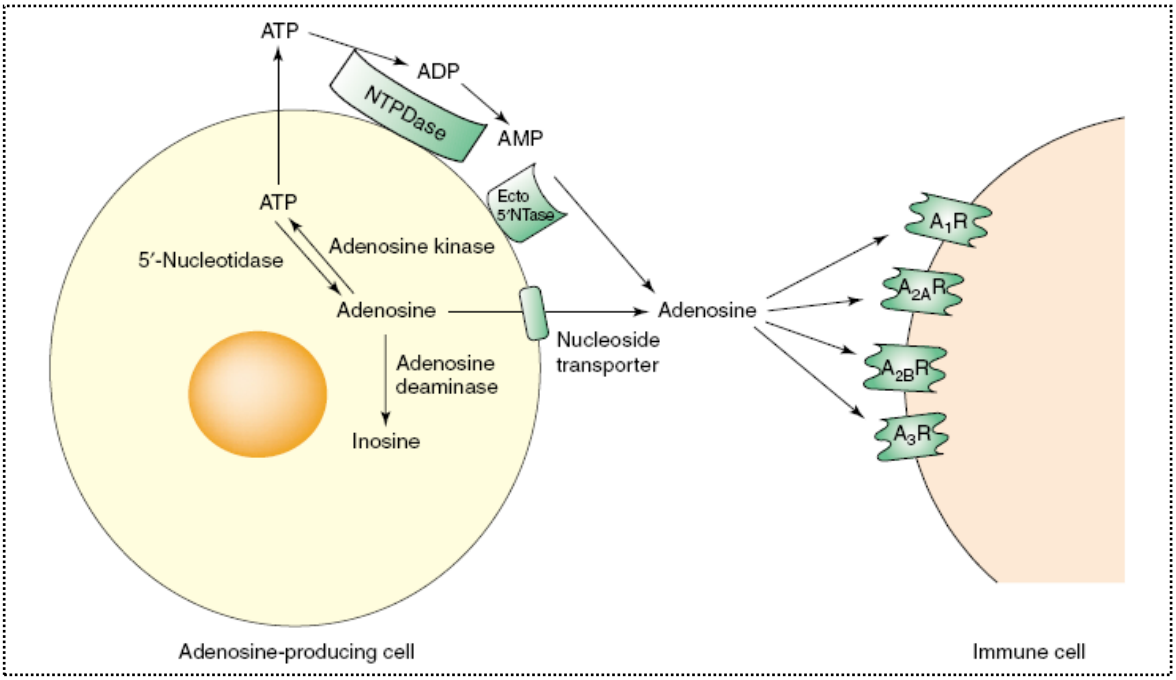

Figure 2. Major pathways involved in adenosine metabolism. (Haskó and Cronstein, 2004) Adenosine is formed from its precursor ATP in both the intracellular and extracellular spaces. Intracellular adenosine is shunted into the extracellular space through nucleoside transporters. The extracellular formation of adenosine is the result of an enzymatic cascade consisting of CD39 and CD73. Extracellular adenosine ligates adenosine receptors, all of which are expressed on the surface of immune cells.

It is generally thought that the concentrations of extracellular adenosine are below 0.1 μM in unstressed tissues, whereas adenosine levels in inflamed or ischemic tissues can be as high as 10 μM. For example, a recent study has documented that systemic (plasma) adenosine levels reach 10 μM in patients with sepsis, a condition associated with both ischemia and inflammation, whereas plasma adenosine concentrations in healthy individuals are <1 μM (Martin et al., 2000).

4.7 Adenosine receptor signaling pathways

Traditionally, adenosine receptor signaling is associated with the stimulation or inhibition of adenylyl cyclase with a concomitant increase or decrease in intracellular cAMP

concentrations. Based on their ability to decrease or increase cAMP accumulation, adenosine receptors were initially classified as A_1 and A_2, receptors (van Calker et al., 1979).

A_1 receptor activation was initially linked to Gi-mediated inhibition of adenylyl cyclase. However, it is now known to be also linked to various kinase pathways including protein kinase C (PKC), PI-3K and MAPKs (Jackobson and Gao, 2006). Additionally, A_1 receptor activation can result in K^+ channel opening and Ca^+ channel inhibition.

cAMP-increasing A_2 receptors have been divided into two groups: high-affinity A_{2A} receptors and low-affinity A_{2B} receptors (Bruns et al., 1986). A_{2A} receptors, dominantly activates the adenylate cyclase–cAMP–protein kinase A (PKA) pathway, but they can also signal through the activation of an exchange factor that is directly activated by cAMP (EPAC) (Fredholm et al., 2007). Signaling downstream from PKA proceeds through phosphorylation of the transcription factor CREB on Ser133, resulting in its activation (Németh et al., 2003b). Activated CREB can mediate gene expression directly by interacting with gene promoters or indirectly by competing with the major inflammatory transcription factor, NF-κB. A_{2B} receptor stimulation can elicit adenylyl cyclase activation via Gs and phospholipase C (PLC) activation via the Gq subunit (Feoktistov an Biaggioni, 1997). Cross-talk between these two pathways is essential for the upregulation of IL-4 production by human mast cells following A_{2B} receptor activation (Rhyzov et al., 2006).

The A_3 receptor is the only adenosine receptor subtype which was cloned before its pharmacological identification. It was originally isolated as an orphan receptor from rat testis, having 40% sequence homology with canine A_1 and A_{2A} subtypes (Meyerhof et al., 1991) and was found to be identical to the A_3 receptor later cloned from rat striatum (Zhou et al., 1992). Homologues of the rat A_3 receptor have since been cloned from sheep and human, revealing large interspecies differences in A_3 receptor structure. The signaling pathways associated with A_3 receptor activation comprise Gi-mediated inhibition of adenylyl cyclase and Gq-mediated stimulation of PLC (Gessi et al., 2008). In addition, A_3 receptors can utilize the PLD, RhoA, WNT, MAP kinase and PI3 kinase pathways.

4.8. Adenosine receptors affect homeostasis through interfering with immune system function

Although the immune response to acute tissue injury has an essential role in altering tissue homeostasis, uncontrolled inflammation or immune activation can inflict further damage on the affected tissue. It appears that the release of adenosine followed by its binding to adenosine receptors on immune cells represents a potent endogenous immunosuppressive pathway that regulates the exuberant immune response to harmful external insults (Haskó et al., 2008). In certain scenarios and cell types, however, adenosine receptors are also able to provoke pro-inflammatory effects. One of the most compelling of these pro-inflammatory effects is that A_{2B} receptor stimulation can enhance inflammatory mediator production by mast cells (Rhyzov et al., 2008). In addition, A_{2B} receptors stimulation enhanced pro-inflammatory effector production by human bronchial smooth muscle cells (Zhong et al., 2004), bronchial epithelial cells (Zhong et al., 2006) and lung fibroblasts (Zhong et al., 2005), which observations point to a role for A_{2B} receptors in the pathophysiology of asthma and chronic obstructive pulmonary disorder (COPD).

The recent availability of mouse models engineered to harbor a deletion of adenosine receptors has proven critical in elucidating the *in vivo* function of these receptors. On the basis of these genetic studies, it is becoming increasingly recognized that these receptors represent major immunoregulatory roles in the immune system. The next chapters will summarize our knowledge on how adenosine receptors influence the immune immune system gained through animal studies employing adenosine receptor KO mice.

4.9. Role of A_1 receptors in regulating immunity

Our knowledge of A_1 receptors in regulating immune responses is limited. A recent study employing A_1 receptor KO mice showed that inactivation of this receptor increased mortality in CLP-induced sepsis, an effect that was correlated with enhanced inflammation-induced hepatic and renal injury (Gallos et al, 2005). These results suggest that A_1 receptor activation has a beneficial effect on the outcome of intra-abdominal sepsis.

4.10. Adenosine is immunosuppressive through A_{2A} receptors

The most potent anti-inflammatory and immunosuppressive effects of adenosine are generally attributed to occupancy of A_{2A} receptors expressed on neutrophils (Cronstein et al., 1990), monocytes/macrophages (Pinhal-Enfield et al., 2003; Bshesh et al., 2002), and lymphocytes (Lappas et al., 2005; Zhang et al., 2004; Csóka et al., 2008; Fredholm et al., 1996). A_{2A} receptor activation inhibits neutrophil adhesion, oxygen radical production (Cronstein et al., 1985), degranulation (Richter, 1992), and TNFα production (Their and Chouker, 1995). In addition, A_{2A} receptor stimulation inhibits macrophage proinflammatory cytokine production (TNF-α, IL-6 and IL-12) and enhances release of the anti-inflammatory cytokine IL-10 (Khoa et al., 2001; Reinstein et al., 1994; Haskó et al., 1996; Sajjadi et al., 1996; Szabó et al., 1998; Haskó et al., 2000). Adenosine also inhibits lymphocyte proliferation, and induces lymphocyte suppressor function, anergy, and apoptosis (Lappas et al., 2005; Csóka et al., 2008; Huang et al., 1997; Apasov et al., 2001 Armstrong et al., 1997; Erdmann et al., 2005).

Multiple lines of evidence indicate that A_{2A} receptors are crucial for adenosine-mediated protection in pathophysiological conditions, such as ischemia-reperfusion injury and infectious diseases (Haskó and Pacher, 2008). Myocardial infarction results in high morbidity and mortality worldwide, and it is suggested that the inflammatory response during reperfusion is an important contributor of myocardial ischemia-reperfusion injury. The protective role of A_{2A} receptors during the reperfusion phase is well documented. For example, treatment with the selective A_{2A} receptor agonist ATL146e immediately after reperfusion of the coronary artery reduces infarct size in A_{2A} receptor wild-type (WT) but not knockout (KO) mice (Yang et al., 2005). This A_{2A} receptor-mediated protection is achieved by prevention of the proinflammatory/immune response, and $CD4^+$ T lymphocytes appear to be the major targets of this protective effect (Yang et al., 2005).

Ischemia-reperfusion injury of the liver is a clinically important manifestation of various surgical interventions, including liver transplantation, trauma repair, or partial hepatic resection. Inflammatory events that take place during hepatic reperfusion lead to disruption of the vascular endothelium, platelet aggregation, activation of immune cells, and cytokine and chemokine secretion. Pharmacological activation of A_{2A} receptors with ATL146e during this reperfusion phase strongly suppressed liver inflammation, as demonstrated by reduced neutrophil infiltration,

as well as decreased up-regulation of cytokine and chemokine gene expression (Day et al., 2004). Subsequent studies using a bone marrow chimeric approach revealed that protection could be attributable to A_{2A} receptors on bone marrow-derived cells (Day et al., 2005).

Ischemia reperfusion injury in kidney is a leading cause of acute kidney injury, and is associated with prolonged hospitalization, higher morbidity and mortality. A_{2A} receptor activation by exogenous agonists or endogenous adenosine produced a dramatic reduction in renal injury following ischemia-reperfusion in mice (Okusa et al., 2001; Day et al., 2003), and the protective effect of A_{2A} receptor stimulation was correlated with decreased endothelial cell adhesion molecule expression and neutrophil sequestration in the kidney parenchyma (Okusa et al., 2000).

A_{2A} receptors are expressed at high levels on cells of the central nervous system, such as inflammatory cells and glial cells. Nevertheless, an important contribution of A_{2A} receptors on bone marrow-derived cells were recently documented in ischemic brain injury. Selective inactivation of A_{2A} receptors on bone marrow cells in chimeric mice protected against ischemic brain injury following middle cerebral arterial occlusion (Yu et al., 2004). This detrimental effect of A_{2A} receptor activation on bone marrow cells in the ischemic brain is unique, as A_{2A} receptor activation on bone marrow-derived cells is protective in the spinal cord and other peripheral organs (Li et al., 2006).

Similar to the data with ischemia-reperfusion in peripheral organs, pharmacologic studies using exogenous A_{2A} receptor agonists in mice show that the activation of A_{2A} receptors decreases organ injury and mortality, which is secondary to the overwhelming inflammation triggered by endotoxin (Sullivan et al., 2004; Reutershan et al., 2007). Furthermore, in the same study by Sullivan et al. (Sullivan et al., 2004) has shown that A_{2A} receptor stimulation protects against endotoxin-induced mortality, and this protective effect disappeared in A_{2A} KO animals in this model of sepsis. The recent demonstration of the tissue protection by endogenous adenosine acting at A_{2A} receptors in this hyperinflammatory model of sepsis (Ohta and Sitkovsky, 2001) further suggests the relevance of A_{2A} receptors to protection from hyperacute, systemic inflammation.

4.11. A_{2B} receptors in regulation of immunity

A_{2B} adenosine receptors are increasingly recognized as important orchestrators of inflammation via modulating endothelial cell and dendritic cell function (Haskó et al., 2009). Both innate and adaptive immune responses are dependent on the migration of leukocytes across endothelial cells (Muller, 2003). *In vivo* studies utilizing A_{2B} KO mice have shown that A_{2B} receptors inhibit the expression of the adhesion molecules ICAM-1 and E-selectin, which results in decreased leukocyte rolling and adhesion (Yang et al., 2006). A_{2B} receptor KO mice exposed to hypoxia exhibit increased neutrophil infiltration into tissues revealing an inhibitory role for A_{2B} receptors in neutrophil transmigration *in vivo* (Eckle et al., 2008). In addition, Ravid and coworkers (Yang et al., 2006; Yang et al., 2008) showed that A_{2B} receptors are important in moderating vascular injury to guidewire-induced endothelial denudation of the femoral artery, a model of human restenosis after angioplasty. Thus, endothelial A_{2B} receptors prevent endothelial cell-mediated inflammatory events and increase endothelial barrier function at the early stages of tissue injury. Recent studies employing A_{2B} KO mice and selective A_{2B} receptor agonists and antagonists have defined a novel role for A_{2B} receptors in regulating dendritic cell function. Human monocytes differentiated towards dendritic cells using IL-4 and GM-CSF in the presence of adenosine develop into a cell type which resembles DC morphologically but is different phenotypically (Novitskiy et al., 2008). These ‘adenosine-differentiated’ cells represent an intermediate phase between monocytes and dendritic cells and are impaired in their ability to induce T-cell proliferation and IFN-γ production (Novitskiy et al., 2008). However, these intermediate cells are highly angiogenic and secrete VEGF and IL-8, and are also anti-inflammatory because of their robust production of IL-10 and TGF-β. Furthermore, A_{2B} KO mice demonstrated that adenosine triggering of this receptor inhibits IL-12 p70 production by TLR-activated bone-marrow-derived dendritic cells (Ben Addi et al., 2008). Taken together, A_{2B} receptor activation reshapes the function of antigen-presenting cells in a way that produces an anti-inflammatory and tolerance-inducing phenotype.

4.12. The enigmatic inflammatory role of the A_3 receptor

The A_3 receptor subtype has a complex role in regulating inflammation, as both proinflammatory and anti-inflammatory effects have been demonstrated. Genetic defects in the adenosine deaminase (ADA) gene are among the most common causes for severe combined immunodeficiency (SCID). Lack of ADA causes accumulation of adenosine in plasma and lymphoid tissues and this adenosine is thought to contribute to the immunodeficiency. ADA-SCID patients, and genetically ADA deficient (ADA-/-) mice suffer from lymphopenia, absent cellular and humoral immunity, and exhibit pulmonary inflammation, increased mucus production, alveolar air-space enlargement, and pulmonary fibrosis. Treatment of $ADA^{-/-}$ mice with MRS 1523, a selective A_3 antagonist, prevented airway eosinophilia and mucus production. Similar results were obtained in the lungs of ADA/A_3 double KO mice, suggesting that A_3 signaling plays an important role in regulating chronic lung disease (Young et al., 2004).

On the other hand, there is also evidence for the anti-inflammatory role of the A_3 receptors. It was recently demonstrated that A_3 receptor activation decreases mortality and renal and hepatic injury in murine septic peritonitis (Lee et al., 2006). Higher levels of endogenous TNF-α were observed in A_3 KO mice after sepsis induction, in comparison to wild-type animals and the A_3 receptor agonist IB-MECA significantly reduced mortality in mice lacking the A_1 or A_{2A} but not the A_3 receptor, confirming the specificity of this A_3 agonist in mediating protection against sepsis-induced mortality (Lee et al., 2006; Lee et al., 2003).

4.13. Aims of the study

Our laboratory had focused for a long time on investigating the role of A_{2A} adenosine receptors in regulating immune functions. We hypothesized that adenosine might contribute to the sepsis-induced onset of immune paralysis via occupancy of A_{2A} receptors. To address this hypothesis, we evaluated whether targeted genetic deletion or pharmacological inactivation of A_{2A} receptor would reverse the immune-compromised phenotype of septic mice using the clinically relevant cecal ligation and puncture (CLP) model of sepsis. Our scientific goals were the following:

1. Determine the effect of genetic A_{2A} receptor deficiency on sepsis-induced mortality

and bacterial clearance

2. Delineate the effect of genetic A_{2A} receptor inactivation on cytokine production in septic mice
3. Determine the effect of genetic A_{2A} receptor inactivation on apoptotic markers in lymphoid organs of mice undergoing sepsis
4. Examine the impact of genetic A_{2A} receptor inactivation on splenic gene expression profile in septic mice
5. Uncover the effect of pharmacological inactivation of A_{2A} receptors on the response of mice to sepsis
6. Examine the effect of A_{2A} adenosine receptor engagement on IL-10 production by *E. coli*-activated macrophages
7. Determine the role of TLR4, MyD88, TRAF6, and MAP kinases in regulating IL-10 production by macrophages exposed to adenosine and *E. coli*
8. Elucidate the effect of A_{2A} receptor activation on the transciptional regulation of IL-10 mRNA in macrophages challenged with *E. coli*

5. MATERIALS AND METHODS

5.1. Drugs and Reagents

Adenosine, adenosine 5'-monophosphate, adenosine 5'-triphosphate, the selective A_1 receptor agonist 2-chloro-N^6-cyclopentyladenosine (CCPA), A_{2A} receptor agonist 2-*p*- (2-carboxyethyl)phenethyl-amino-5'-N-ethyl-carboxamidoadenosine (CGS-21680), A_3 receptor agonist N^6-(3-iodobenzyl)adenosine-5'-N-methyluronamide (IB-MECA), and non-selective A_{2B} receptor agonist 5'-N-ethylcarboxamidoadenosine (NECA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The selective A_{2A} receptor antagonist 4-(2- [7-amino-2-(2-furyl)[1.2.4]triazolo[2.3-a][1.3.5]triazin-5-ylamino] ethyl) phenol (ZM241385) was purchased from Tocris Cookson (Ellisville, MO, USA). Actinomycin D was obtained from Sigma-Aldrich. The selective TLR2 agonist prepared from *Staphylococcus aureus* (LTA-SA) was from Invivogen (San Diego, CA, USA). Stock solutions of the various agonists and antagonists, were prepared using dimethylsulphoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA).

5.2. Experimental animals

A_{2A} receptor KO mice and their WT littermates, which were on the CD-1 background, were a kind gift from Catherine Ledent (Institut de Recherche Interdisciplinaire en Biologie Humaine et Nucléaire, Université Libre de Bruxelles, Brussels, Belgium). Male CD-1 mice were purchased from Charles River Laboratories (Wilmington, MA, USA). MyD88 KO and WT mice on the C57Bl/6J background, kindly provided by Dr. Shizuo Akira (Osaka University, Japan) were bred in a specific pathogen-free facility, using founder heterozygous male and female mice. Male TLR4 KO (C57BL/10ScNJ) and WT (C57BL/10ScSnJ) littermates mice were obtained from the Jackson Laboratories (Bar Habor, ME, USA). A_{2B} receptor KO mice were acquired from Deltagen Inc. (San Mateo, CA, USA). These mice were backcrossed 10 generations onto a C57BL/6J background. All mice were maintained in accordance with the recommendations of the "Guide for the Care and Use of Laboratory Animals", and the experiments were approved by the New Jersey Medical School Animal Care Committee.

5.3. Cecal ligation and puncture

Polymicrobial sepsis was induced by subjecting mice to CLP. Eight-to-twelve week-old male A_{2A} receptor KO or WT mice were anesthetized with Pentobarbital (50 mg/kg), given i.p. Under aseptic conditions, a 2-cm midline laparotomy was performed to allow exposure of the cecum with adjoining intestine. Approximately two-thirds of the cecum was tightly ligated with a 3.0 silk suture, and the ligated part of the cecum perforated twice (through and through) with a 20-gauge needle. The cecum was then gently squeezed to extrude a small amount of feces from the perforation sites. The cecum was then returned to the peritoneal cavity and the laparotomy closed in two layers with 4.0 silk sutures. Sham-operated animals underwent the same procedure without ligation or puncture of the cecum. The mice were resuscitated with 1 ml of physiological saline injected subcutaneously (s.c.), and returned to their cages with free access to food and water. One group of mice was monitored daily and survival recorded for 10 days. Another group of mice was reanesthetized with Pentobarbital (50 mg/kg; i.p.) 16 or 48 h after the operation, and blood, peritoneal lavage fluid, and various organs were harvested as described below.

The effect of pharmacological inactivation of A_{2A} receptors in mice subjected to CLP was evaluated using CD-1 mice in a similar fashion to that described for the A_{2A} receptor KO or WT mice. In this set of experiments, the mice were injected immediately before or 2 h after the CLP operation and every 12 h thereafter with ZM241385 (15 mg/kg, s.c.) or its vehicle (DMSO).

5.4. Collection of blood, peritoneal lavage fluid, and organs

Blood samples were obtained aseptically by cardiac puncture using heparinized syringes after opening the chest and placed on ice into heparinized Eppendorf tubes until further processing for hematological and bacteriological analysis. Aliquots of whole blood were analyzed for hematology by flow cytometry (CELL-DYN 3200 System, Abbott Laboratories, Abbot Park, IL, USA). Then the blood samples were centrifuged at 2,000 x *g* for ten minutes and the recovered plasma stored at –70 °C until further use. For peritoneal lavage, the abdominal skin was cleansed with 70 % ethanol and the abdominal wall exposed by opening the skin. Four milliliters of sterile physiological saline was then installed into the peritoneal cavity via an 18-gauge needle. The abdomen was massaged gently for 1 minute while keeping the tip of the

needle in the peritoneum, after which procedure peritoneal fluid was recovered through the needle. Recovered peritoneal lavage fluid was placed on ice until processed for bacteriological examination. After serially diluting the peritoneal lavage fluid to determine colony forming unit (CFU) numbers (see below), the peritoneal lavage fluid was centrifuged at 5,000 x *g* for ten minutes and the supernatant stored at –70 °C until further analysis. Samples from spleen and thymus were excised and either immediately frozen in liquid nitrogen or placed in 4 % paraformaldehyde for subsequent histological analysis. Snap-frozen tissue samples were transferred to a –70 °C refrigerator until analyzed for gene expression and apoptotic markers.

5.5. Quantification of bacterial CFUs from peritoneal lavage fluid and blood

100 µl of blood or peritoneal lavage fluid was diluted serially in sterile physiological saline. 50 µl of each dilution was aseptically plated and cultured on trypticase blood agar plates (Becton Dickinson) at 37 °C. After 24 h, the number of bacterial colonies was determined. Number of colonies are expressed as CFUs per milliliter of blood or peritoneal lavage fluid.

5.6. Determination of cytokine levels in plasma and peritoneal fluid

Concentrations of IL-10, IL-6, IL-12p70, TNFα and MIP-2, in plasma or peritoneal lavage fluid were determined using commercially available ELISA DuoSet kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

5.7. Western blot analysis for markers of apoptosis

Samples of spleen and thymus were homogenized in a Dounce homogenizer in modified radioimmunoprecipitation (RIPA) buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.25 % Na-deoxycholate, 1 % NP-40, 1 µg/ml pepstatin, 1 µg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM Na_3VO_4). A total of 40 µg of sample was separated on a 4-12 % Tris-glycine gel (Invitrogen, Carlsbad, CA, USA) and transferred to nitrocellulose membrane. The membranes were probed with polyclonal rabbit anti-cleaved caspase-3 (1:1,000) (Cell Signaling, Danvers, MA, USA), polyclonal rabbit anti-cleaved poly

(ADP-ribose) polymerase (PARP) (1:1,000) (Cell Signaling), or polyclonal goat anti-β-actin antibody (1:20,000) (Santa Cruz Biotechnologies, Santa Cruz, CA, USA), and subsequently incubated with a secondary horseradish peroxidase-conjugated anti-rabbit (1:5,000) or anti-goat antibody (1:5,000) (Santa Cruz). Bands were detected using the ECL Western Blotting Detection Reagent (GE Healthcare, Piscataway, NJ, USA).

5.8. Apoptosis detection by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL)

Paraffin blocks containing spleen tissue specimens were cut in 5-μm-thick sections and the sections processed and stained for the detection of apoptosis using the TACS^{TM} In Situ Apoptosis Detection Kit (TACS Klenow DAB) obtained from Trevigen Inc (Gaithersburg, MD, USA) according to the manufacturer's instructions. When viewed under a standard light microscope, apoptotic nuclei can be clearly distinguished by brown staining. Quantification of the number of apoptotic cells was performed using an Olympus IX71 microscope.

5.9. Flow cytometric detection of thymocyte apoptosis and MHC II expression on splenic and peritoneal macrophages

To quantitate thymocyte apoptosis, tissue sections from thymi were gently homogenized to dissociate cells. Tissue debris were then removed from cell suspensions using a 70-μm nylon cell strainer (BD Biosciences, San Jose, CA, USA) and the cells washed twice and then resuspended in ice cold PBS. The degree of apoptotic cell death was quantified using a commercially available, fluorescein labeled Annexin V containing kit (Annexin V-FITC Apoptosis detection Kit I, BD Biosciences Pharmingen). Thymocytes (3×10^5) were stained with fluorescein isothiocyanate (FITC)-labeled Annexin V and propidium iodide according to the manufacturer's instruction. Cells were analyzed using a FACScan Flow Cytometer equipped with a 488 nm laser, 530/30-nm and 585/42-nm band pass filters and a 650-nm long-pass filter (BD Biosciences). Instrument calibration was performed daily employing Calibrate Beads (BD Biosciences) and also by sphero beads (Spherotech Inc.Lake Forest, IL, USA). Data were analyzed using Cytomation Summit computer software. Electronic compensation of the

instrument was carried out to exclude overlapping of the two emission spectra. Cell counts in regions of doublets for annexin V positive only, propidium iodide positive only, double-positive, and double-negative were determined and compared.

MHC II expression on splenic and peritoneal macrophages was also determined using flow cytometry. Macrophages were identified using phycoerythrin (PE)-labeled anti-mouse F4/80 antibodies (eBiosciences, San Diego, CA, USA). MHC II expression was determined using anti-mouse APC-labeled MHC II antibody (eBiosciences, San Diego, CA, USA). Cell suspensions from peritoneal lavage and spleen were added to tubes pre-loaded with the corresponding fluorescent-labeled antibodies. After gentle mixing, the tubes were kept at room temperature in the dark for 15 min. Red blood cells were then lysed with 2.0 ml of BD FACS Lysing Solution (BD Biosciences). After two washes the cells were fixed in 0.3 ml of 3 % formaldehyde and kept at 4 °C in the dark until acquisition. Analyses were performed using a FACScan flow cytometer and CellQuest software (BD Biosciences).

5.10. Affymetrix genechip analysis of spleen samples and reverse transcriptase (RT)-PCR

Total RNA was prepared from spleen samples using TRIZOL (Invitrogen) and the samples were further purified using RNeasy mini kit (QIAGEN, Valencia, CA, USA). cRNA transcription, and cRNA hybridization to Affymetrix murine microarrays, which contained probe sets for the whole mouse genome, scanning, and data analysis were performed at the Affymetrix Gene Chip Core Facility in the W. M. Keck Foundation Biotechnology Resource Laboratory at Yale University, New Haven, CT, USA (technical details are available at http://info.med.yale.edu/wmkeck/affymetrix/). Differentially expressed genes were identified by the Biostatistics Resource Laboratory at the W. M. Keck Foundation by comparing data from spleens taken from CLP-induced A_{2A} WT and KO mice 16 h after the operation (n = 3 per group).

RT-PCR for IL-10, IL-6, MIP-2, and 18S was performed using RNA isolated for the microarray experiment. RNA (5 µg) was transcribed in a 20-µl reaction containing 10.7 µl of RNA (5 µg), 2 µl of 10x PCR buffer, 2 µl of 10 mM dNTP mix, 2 µl of 25 mM $MgCl_2$, 2 µl of 100 mM dithiothreitol, 0.5 µl of RNase inhibitor (20 U/µl; PerkinElmer Life Sciences, Boston, MA), 0.5 µl of 50 mM oligo d(T), and 0.3 µl of reverse transcriptase (Roche Diagnostics, Nutley,

NJ, USA). The reaction mix was incubated at 42 °C for 15 min for reverse transcription. Thereafter, the reverse transcriptase was inactivated at 99° C for 5 min. Reverse transcriptase-generated DNA was amplified using Expand high fidelity PCR system (Roche Diagnostics). The reaction buffer (25 µl) contained 2 µl of cDNA, water, 2.5 µl of PCR buffer, 1.5 µl of 25 mM $MgCl_2$, 1 µl of 10 mM dNTP mix, 0.5 µl of 10 µM oligonucleotide primer (each), and 0.2 µl of *Taq* polymerase. cDNA was amplified using the following primers and conditions: IL-10 — 5'-AAGGAGTTGTTTCCGTTA-3' (sense) and 5'-AAGGGTTACTTGGGTTGC-3' (antisense); IL-6 — 5'-GGTCCTTAGCCACTCCTTCTGTG-3'(sense) and 5'-GATGCTACCAAACTGGATATAATC-3'(antisense); MIP-2 — 5'-ATGGCCCCTCCCACCTGCCGGCTCC-3' (sense) and 5'-TCAGTTAGCCTTGCCTTTGTTCAGTATC-3' (anti-sense); and 18S — 5'-GTAACCCGTTGAACCCCATT-3' (sense) and 5'-CCATCCAATCGGTAGTAGCG-3' (anti-sense), an initial denaturation at 94 °C x 5 min, 30, 29, and 26 cycles of 94 °C x 30 s for IL-10, IL-6, and MIP-2, respectively, 59 °C x 45 s, 72 °C x 45 s; a final dwell at 72 °C x 7 min. PCR products were resolved on a 1.5 % agarose gel and stained with ethidium bromide.

5.11. Cell cultures

Thioglycolate-elicited mouse peritoneal macrophages (see below), C/EBPβ-deficient and control macrophages (kind gifts from Valeria Poli, Department of Genetics, Biology, and Biochemistry, University of Turin, Turin, Italy) (Gorgoni et al., 2002; Albina et al., 2005), and RAW 264.7 macrophages (ATCC, Manassas, VA, USA) were grown in Dulbeccos's modified Eagle's Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), 50 U/ml penicillin, 50 µg/ml streptomycin, and 1.5 mg/ml sodium bicarbonate in a humified atmosphere of 95 % air and 5 % CO_2.

5.12. Isolation of peritoneal macrophages

Mice were injected intraperitoneally with 3 ml of sterile Brewer's thioglycolate (TG) broth (2% w/v). Four days later, the mice were sacrificed and peritoneal exudate cells were harvested using 10 ml of DMEM. Cells were centrifuged at 300 x *g* for 10 minutes at 4 °C,

washed twice with DMEM, and re-suspended in DMEM containing 10 % FBS, 50 U/ml penicillin / 50 μg/ml streptomycin, and 1.5 mg/ml sodium bicarbonate. Cells were seeded into Falcon Multiwell tissue culture plates (BD Biosciences). Dishes were then incubated at 37° C in a humidified incubator for 5 h, to allow the cells to adhere. Non-adherent cells were removed by washing with serum-free DMEM, and the cells were re-fed with DMEM containing 10 % FBS. 18 h following initial plating, the various test compounds were added to the macrophages.

5.13. Preparation of heat-killed E. coli

E. coli (K-12 strain) were grown in LB medium (LB broth was obtained from Invitrogen) at 37 °C with shaking at 220 rpm overnight. Cells were centrifuged at 3,000 x *g* for 15 minutes at 4 °C, resuspended in PBS at a density of 10^8/ml and incubated at 65 °C for 90 minutes. After heat inactivation, the killed *E. coli* cells were collected by centrifugation at 3,000 x g for 15 minutes at 4 °C and resuspended in PBS at density of 6 x 10^9/ml for treatment of mouse macrophages.

5.14. RNA extraction, cDNA synthesis and real-time PCR

Total cellular RNA was extracted from peritoneal macrophages by using TRIzol reagent. The RT reaction was performed with 5 μg of purified total RNA by using MuLV RTase (GeneAmp RNA PCR Core Kit), (Applied Biosystems, Foster City, CA, USA) according the manufacturer's protocol. Real-time PCR using SYBR Green I reaction was performed according to a protocol recommended by the manufacturer. 2 μl of cDNA was added to 8 μl of reaction mixture including 3 mM $MgCl_2$, 20 pM of each primer and 2 μl of a Lightcycler-DNA Master SYBR Green I (Roche Diagnostic) and incubated in a Lightcycler™ under the following conditions: at 95 °C for 10 min, followed by 50 cycles of 94 °C for 10 s, 60 °C for 10 s, and 72 °C for 10 s. The following primers were used in the experiments: IL-10 — 5'-AAGGAGTTGTTTCCGTTA-3' (sense) and 5'-AAGGGTTACTTGGGTTGC-3' (antisense); 18S — 5'-GTAACCCGTTGAACCCCATT-3' (sense) and 5'-CCATCCAATCGGTAGTAGCG-3' (anti-sense).

5.15. Generation of C/EBP consensus mutant IL-10 promoter luciferase construct

The mutated IL-10 promoter was prepared by gene synthesis by GenScript Co (Piscataway, NJ, CA). The following nucleotides were changed in the C/EBP consensus sequences of the 5'-flanking region of the IL-10 gene between -410/-390 relative to the transcription start site: -410-TGGAGGAAACAATTATTTCTC-390, The mutated construct had the following sequence: -410 TGGActgtgCAATTAgtaCTC-390. The mutated DNA fragment was inserted into pGL2-Basic vector in HindIII-XhoI sites. The mutation sites were confirmed by DNA sequencing.

5.16. Transient transfection of RAW 264.7 cells with IL-10 promoter-luciferase constructs, C/EBP luciferase construct and A2A receptor over-expressing construct, and luciferase assay

RAW 264.7 cells were transiently transfected using FUGENE 6.0 transfection reagent (Roche). For transfection, 0.5 x 10^6/ml cells were plated in a 24-well plate. The following day, the cells were transfected with 0.4 µg of IL-10 reporter plasmids (kind gifts from Stephen T. Smale, University of California, Los Angeles, School of Medicine, Los Angeles), and C/EBP consensus mutant IL-10 promoter luciferase construct, and an A_{2A} adenosine receptor over-expressing pA_{2A}-CMV construct (Origene, Rockville, MD, USA) and C/EBP reporter plasmid (Stratagene, La Jolla, CA, USA) in 200 µl of medium per well. All transfections were performed at 37 °C overnight, after which procedure the cells were washed with DMEM and treated with heat-killed *E. coli* and/or adenosine for 8 h. For reporter assays, whole cell extracts were prepared using 80 µl of 1x passive lysis buffer (Promega, Madison, WI, USA). Luciferase activity was determined from 20 µl of cell extract.

5.17. Preparation of pSilencer plasmids with siRNA constructs

Specific hairpin siRNA (shRNA) oligonucleotide constructs for murine TRAF6 silencing were designed using the Ambion siRNA design algorithm, synthesized in the New Jersey Medical School Biotechnology Central facility, and cloned into the pSilencer3.1-H1neo

expression vector (Ambion Inc. Austin, TX, USA), following the manufacturer's protocol. siRNA constructs were designed targeting both the 5' end and the 3' of the gene. As controls, non-specific siRNA with the same nucleotide composition as the specific inserts, but lacking significant homology with any sequence in the murine genome database, were also prepared.

The specific complementary siRNA sequences for TRAF6 and the scrambled sequences were the following:

5' end (specific):

Upper: 5'gat ccg tac tga tgc ggg ggt gta ttc aag aga tac acc ccc gca tca gta ctt ttt tgg aaa 3'
Lower: 5'agc ttt tcc aaa aaa gta ctg atg cgg ggg tgt atc tct tga ata cac ccc cgc atc agt acg 3'

3'-end (specific):

Upper: 5'gat ccg tat gag ggc gac tgt tgg ttc aag aga cca aca gtc gcc ctc ata ctt ttt tgg aaa 3'
Lower: 5'agc ttt tcc aaa aaa gta tga ggg cga ctg ttg gtc tct tga acc aac agt cgc cct cat acg 3'

5' end (scrambled):

Upper: 5'gat ccg cag tct agc gta cat cat ttc aag aga atg atg tac gct aga ctg ctt ttt tgg aaa 3'
Lower: 5' agc ttt tcc aaa aaa gta tga ggg cga ctg ttg gtc tct tga acc aac agt cgc cct cat acg 3'

3' end (scrambled):

Upper: 5' gat ccg tat gag ggc gac tgt tgg ttc aag aga cca aca gtc gcc ctc ata ctt ttt tgg aaa 3'
Lower: 5' agc ttt tcc aaa aaa gta tga ggg cga ctg ttg gtc tct tga acc aac agt cgc cct cat acg 3'

The plasmids were transfected into XL10-Gold ultracompetent cells (Stratagene), and plasmid DNA was isolated using the MAXIPREP GFII Endo-Free kit (Sigma). Plasmids were sequenced to ensure the fidelity of the cloned inserts.

5.18. Preparation of RAW 264.7 Cell Stable Transfectants

RAW 264.7 cells were transfected with siRNA-containing plasmids using Superfect® transfection reagent (Qiagen Inc.), following the manufacturer's protocol. Briefly, cells were plated 1 day prior to transfection in 60-mm culture dishes. Cells were then incubated with Superfect transfection complex with siRNA plasmids in growth medium for 3 h. The medium was then removed and replaced with fresh medium supplemented with 0.8 % (w/v) G418. This selection medium was replaced every two days. G418-resistant colonies were selected after about 2 weeks of growth. Six single clones of each siRNA transfection were selected and grown up and G418 was maintained in the medium for the growth of the clones. The expression level of TRAF6 was confirmed by real-time RT-PCR.

5.19. Nuclear protein extraction and C/EBP Electromobility Shift Assay (EMSA) and supershift assay

RAW 264.7 cells were stimulated with adenosine and *E. coli* for varios periods and nuclear protein extracts were prepared as described previously (Németh et al., 2003a). All nuclear extraction procedures were performed on ice with ice-cold reagents. Cells were washed with PBS and harvested by scraping into 1.5 ml of PBS and pelleted at 300 x *g* for 10 minutes. The pellet was resuspended in 50 μl of cytosolic buffer [20% (v/v) glycerol, 10 mM HEPES pH 8.0, 10 mM KCl, 0.5 mN EDTA pH 8.0, 1.5 mM $MgCl_2$, 0.5% (v/v) Nonidet P-40, 0.5 mM dithiothreitol (DTT), 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A] and incubated for 15 min on ice with occasional vortexing. After centrifugation at 4,500 x *g* for 10 min, supernatants (cytosolic extracts) were saved for further studies. Two pellet volume of nuclear extraction buffer [20% (v/v) glycerol, 20 mM HEPES pH 8.0, 420 mM NaCl, 0.5 mM EDTA pH 8.0, 1.5 mM $MgCl_2$, 50 mM glycerol-phosphate, 0.5 mM dithiothreitol (DTT), 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A] was added to the nuclear pellet and incubated on ice for 30 minutes with occasional wortexing. Nuclear proteins were isolated by centrifugation at 14,000 x *g* for 15 min. The nuclear protein extracts were aliquoted and stored at –80 °C until used for EMSA. The C/EBP consensus oligonucleotide probe used for EMSA was

purchased from Santa Cruz Biotechnology. The oligonucleotide probe was labeled with [{γ}-^{32}P]ATP using T4 polynucleotide kinase (Invitrogen) and purified in MicroSpin G-50 columns (GE Healtcare). For the EMSA analysis, 10 μg of nuclear proteins was preincubated with C/EBP EMSA binding buffer [5% glycerol (v/v), 10 mM Tris-HCl pH 8.0, 0.5 mM DTT, and 15 ng/ml poly(dI)-poly(dC)] at room temperature for 10 min before addition of the radiolabeled oligonucleotide for an additional 30 min at 37 °C. For supershift studies, samples were preincubated with 3 μg of C/EBPβ or C/EBPδ antibody (Santa Cruz Biotechnology) at room temperature for 45 minutes. Protein-nucleic acid complexes were resolved using a non-denaturing polyacrylamide gel consisting 4 % acrylamide (29:1 ratio of acrylamide/bysacrylamide) and run in 0.5x Tris borate-EDTA buffer (44.5 mM Tris-base, 44.5 mM boric acid, and 1 mM EDTA) for approximately 2.5 h at constant current (35 mA). Gels were transferred to 3M paper (Whatman), dried under vacuum at 80 °C for 40 min, and exposed to photographic film at –80 °C with an intensifying screen.

5.20. Western blot analysis of C/EBPβ and C/EBPδ, phospho-p38 and phospho-p42/44 proteins

C/EBPβ and C/EBPδ protein levels were analyzed using 10 μg of nuclear extracts prepared as described for the EMSA protocol. C/EBPβ and C/EBPδ polyclonal antibodies were purchased from Cell Signaling Technology. For phospho-p38 and phospho-p42/44 Western blotting peritoneal macrophages were washed in PBS and then removed from culture dishes by scraping. Cells were then homogenized in modified RIPA buffer. The lysates were centrifuged at 14,000 x *g* for 15 min, and the supernatant was recovered. A total of 30 μg of protein samples were separated on 4-12 % Tris-Glycine gel (Invitrogen) and transferred to nitrocellulose membrane. The membranes were probed with polyclonal rabbit anti-phospho-p42/44 (Cell Signaling), monoclonal mouse anti-phospho-p38 (Cell Signaling), or polyclonal goat anti-β-actin antibody (Santa Cruz Biotechnology), and subsequently incubated with a secondary HRP-conjugated anti-rabbit IgG antibody (Santa Cruz Biotechnology). Bands were detected using ECL Western Blotting Reagent (GE Healthcare).

5.21. IL-10 and IL-6 ELISA

Peritoneal macrophages in 96-well plates (2 x 10^5/ml) were treated with adenosine or various adenosine receptor agonists followed by addition of *E. coli*. IL-10 and IL-6 levels in cell supernatants were determined by DuoSet ELISA (R&D Systems).

5.22. Statistical analysis

Survival curves were analyzed using the two-tailed Fisher's exact test. Two–tailed *t* testing was used to compare cytokine concentrations, CFUs, and other laboratory parameters. Statistical significance was assigned to *p* values less than 0.05. Values in the figures are expressed as mean ± SEM of *n* observations. Statistical analysis of the data was performed by Student *t* test or one-way analysis of variance followed by Dunnett's test, as appropriate.

6. RESULTS

6.1. Genetic $A_{2A}R$ deficiency protects against CLP-induced mortality and improves bacterial clearance

We initially examined the effect of A_{2A} adenosine receptor deficiency on mortality from CLP-induced septic peritonitis by comparing 5-day survival statistics for A_{2A} WT, and A_{2A} KO mice. Control (WT) mice had a mortality rate of 67% when recorded on day 5 after the CLP procedure (Figure 3). This mortality rate was the result of a gradual process, which was characterized by 10–20% of the mice dying every day. No changes in mortality were detected when the mice were followed for an additional 5 days (data not shown). The mortality rate of A_{2A} KO mice was significantly lower on each day with a ~35% mortality rate on day 5 after CLP (Figure 3). There were no additional deaths in this group until the termination of the experiment (10 days after the surgery, data not shown).

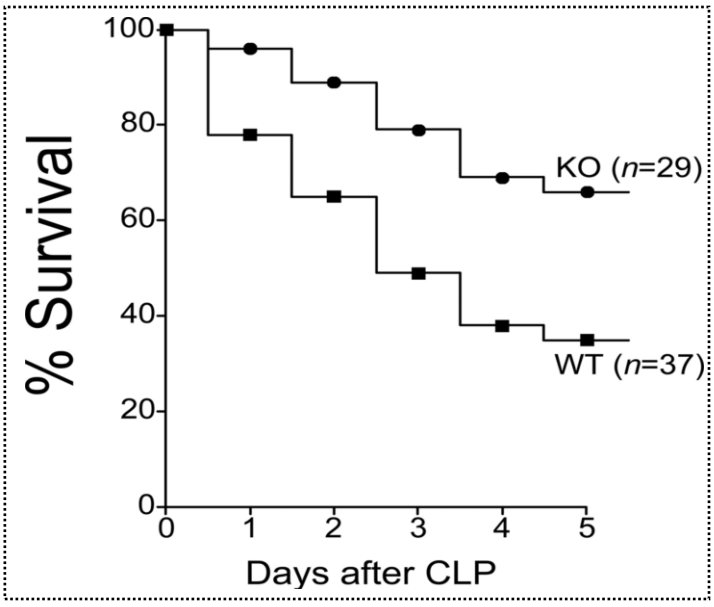

Figure 3. $A_{2A}R$ KO mice are protected from death in septic peritonitis. $A_{2A}R$ WT and KO mice were subjected to CLP (2/3 ligation and through and through puncture with a 20-gauge needle), and survival was monitored for 5 days ($p < 0.05$, two-tailed Fisher's exact test).

Because persistent local bacterial infection and bloodstream invasion play important roles in mortality in the CLP model, we next assessed the impact of $A_{2A}R$ inactivation on bacterial levels at the primary peritoneal site of infection and in the bloodstream. We found markedly decreased numbers of bacteria in both the blood and peritoneal lavage fluid of $A_{2A}R$ KO mice when compared with WT animals at 16 h (Figure 4, *A* and *B*). Bacterial numbers fell substantially by 48 h after surgery in both the blood and peritoneal lavage fluid and there were no differences in CFUs between A_{2A} KO and WT mice at this point (Figure 4, *C* and *D*). Blood and peritoneal lavage fluid remained sterile in sham-operated $A_{2A}R$ KO and WT mice (data not shown).

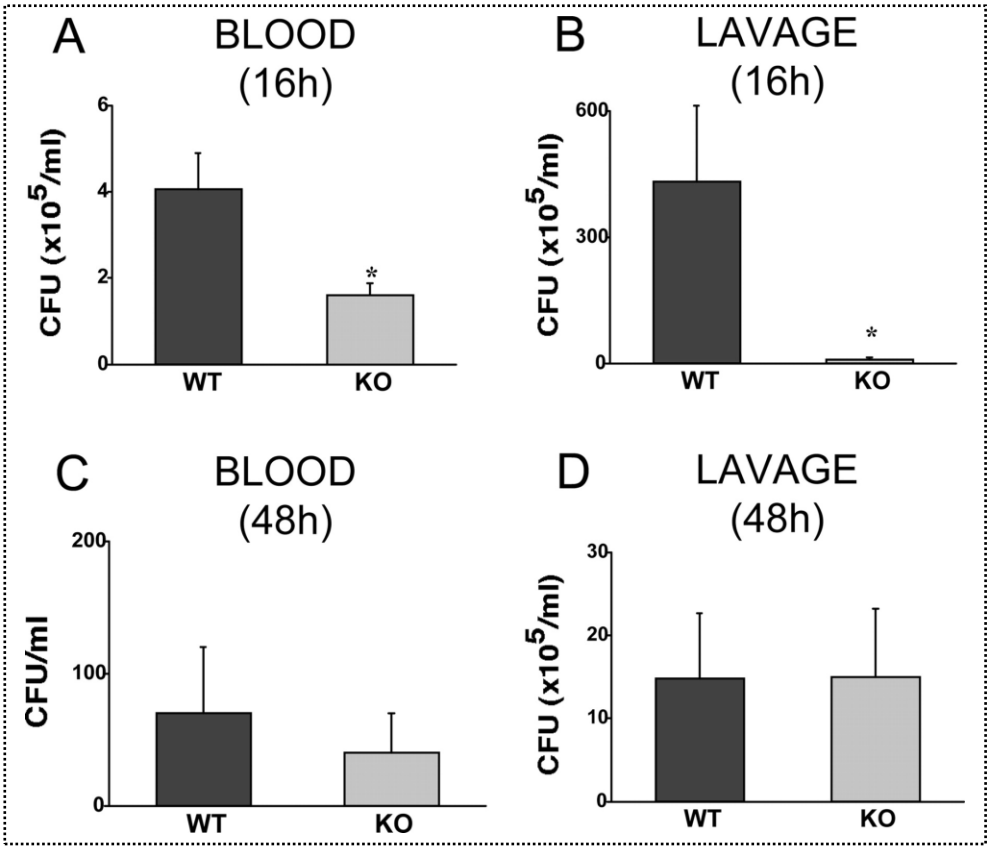

Figure 4. Effect of $A_{2A}R$ deficiency on bacterial load in mice subjected to CLP at 16 (*A* and *B*) or 48 (*C* and *D*) hours after surgery. Dilutions of blood (***A*** and ***C***) or peritoneal lavage fluid (***B*** and ***D***) were cultured on tryptose blood agar plates, and the number of bacterial colonies was counted. Data are the mean ± SEM of $n = 6–9$ mice/group. Results are representative of at least three separate experiments. $*$, $p < 0.05$.

6.2. Effect of genetic $A_{2A}R$ inactivation on cytokine production and markers of organ injury

Since IL-10 appears to be an essential mediator in sepsis-induced impairment in antibacterial host defense (Lyons et al., 1997; Song et al., 1997; Steinhauser et al., 1999a), we compared IL-10 concentrations in the plasma and peritoneal lavage fluid obtained from $A_{2A}R$ KO and WT mice subjected to CLP or sham operation. Sham-operated $A_{2A}R$ WT or KO mice had no detectable levels of IL-10 in their plasma or peritoneal lavage fluid (data not shown). While CLP elevated IL-10 concentrations in both the plasma and peritoneal lavage fluid in both $A_{2A}R$ KO and WT mice, A_{2A} KO mice exhibited markedly lower levels of IL-10 at 16 h after the CLP procedure (Figure 5A). IL-10 concentrations subsided to comparable levels in septic A_{2A} KO and WT mice by 48 h (Figure 5B).

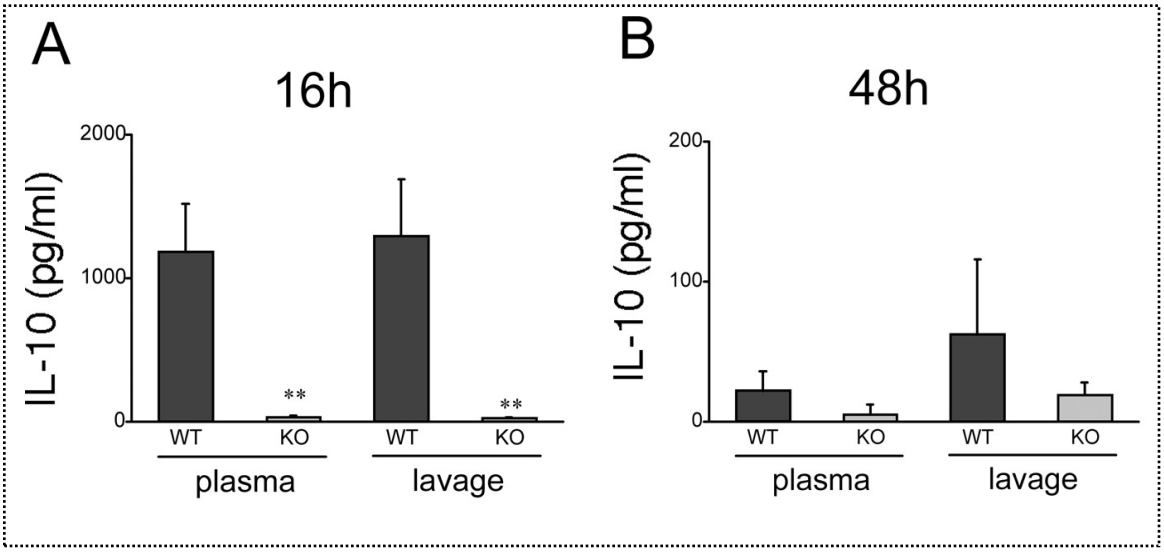

Figure 5. Effect of $A_{2A}R$ deficiency on IL-10 levels in the plasma or peritoneal lavage fluid of mice subjected to CLP. Concentrations of these cytokines were measures at 16 **(*A*)** or 48 **(*B*)** hours after surgery using ELISA. Data are the mean ± SEM of n = 6–9 mice/group. Results are representative of at least three separate experiments. **, $p < 0.01$.

Since IL-6 has been shown to be deleterious in CLP-induced sepsis (Riedemann et al., 2003b), we next explored the role of $A_{2A}R$ in regulating IL-6 production during sepsis. Although IL-6 levels in sham-operated $A_{2A}R$ WT and KO mice were low and comparable between the two

groups (38 ± 20 pg/ml in the WT vs 19 ± 0.6 pg/ml in the KOs for the peritoneal lavage fluid and 2.53 ± 0.01 ng/ml in the WT vs 2.53 ± 0.03 ng/ml in the KOs for the plasma), CLP-induced levels of IL-6 were significantly and markedly higher in the peritoneal lavage fluid but not plasma of A_{2A}R WT mice than in the A_{2A} KO animals (Figure 6A). IL-6 concentrations decreased by 48 h after the CLP procedure and no differences were seen in IL-6 concentrations between the A_{2A} KO and WT mice at this point (Figure 6B).

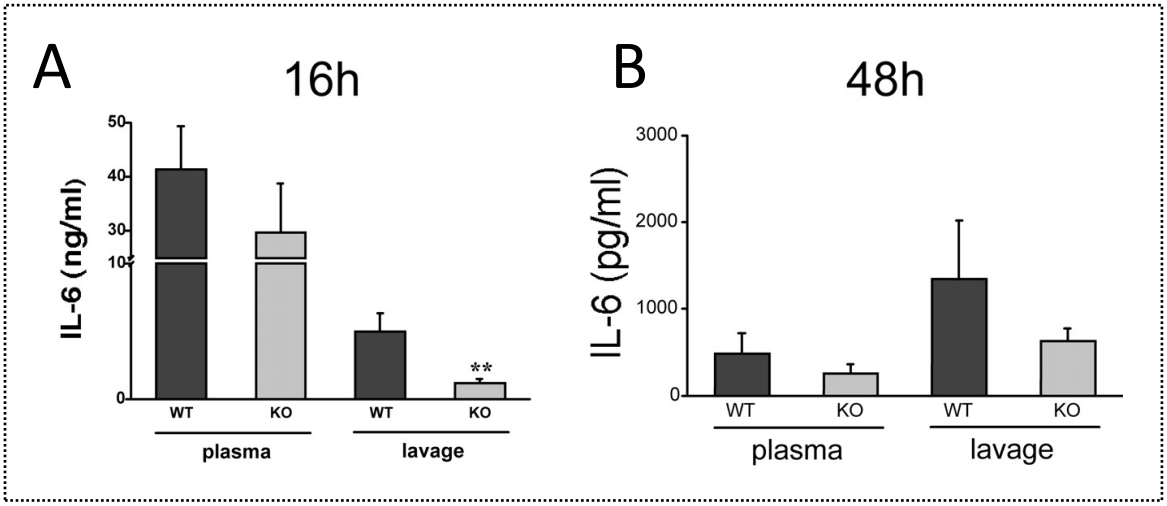

Figure 6. Effect of A_{2A}R deficiency on IL-6 levels in the plasma or peritoneal lavage fluid of mice subjected to CLP. Concentrations of these cytokines were measures at 16 ***(A)*** or 48 ***(B)*** hours after surgery using ELISA. Data are the mean ± SEM of n = 6–9 mice/group. Results are representative of at least three separate experiments. **, $p < 0.01$.

To investigate whether A_{2A}R deficiency altered the levels of classical proinflammatory cytokines, we next determined concentrations of TNF-α, IL-12 p70, and MIP-2 in plasma and peritoneal lavage fluid. We found that concentrations of IL-12 p70 and TNF-α were below the detection limit for our assays in all groups of mice, including sham- and CLP-operated A_{2A}R WT and KO mice (data not shown). Although the chemokine MIP-2 was not detectable in sham-operated WT or KO animals (data not shown), CLP-induced concentrations of MIP-2 were lower in A_{2A} KO mice as compared with their WT counterparts when measured at 16 h (Figure 7A) but not at 48 h (Figure 7B).

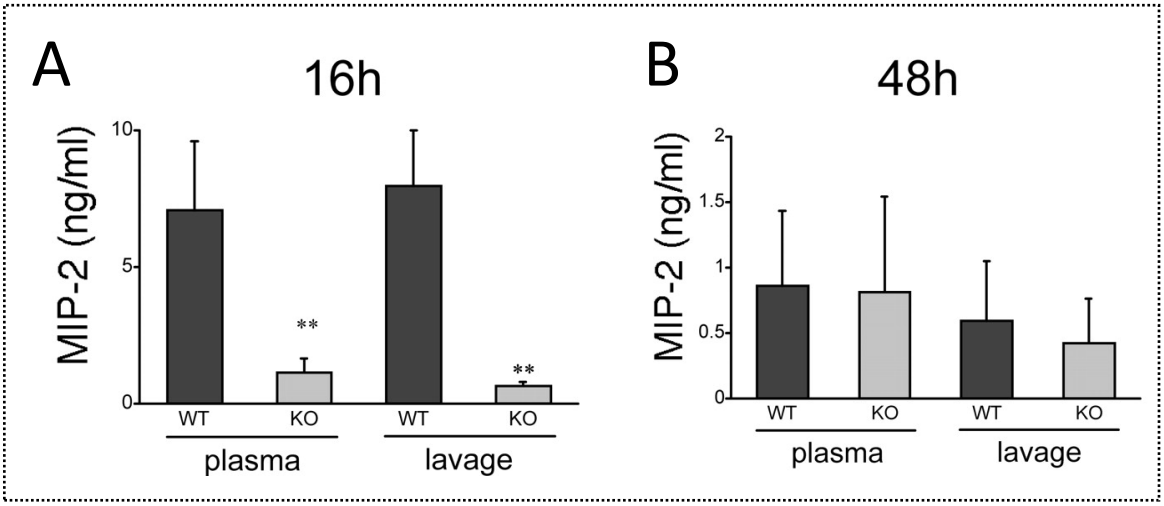

Figure 7. Effect of $A_{2A}R$ deficiency on MIP-2 levels in the plasma or peritoneal lavage fluid of mice subjected to cecal ligation and puncture. Concentrations of these cytokines were measures at 16 ***(A)*** or 48 ***(B)*** hours after surgery using ELISA. Data are the mean ± SEM of n = 6–9 mice/group. Results are representative of at least three separate experiments. **, $p < 0.01$.

We next assessed the markers of organ injury and hematological parameters. CLP induced an increase in markers of kidney (BUN) and liver (AST and ALT) injury, when compared with sham-operated animals (Table I). Additionally, white blood cell counts, lymphocyte numbers, and platelet counts dropped significantly in CLP-subjected mice when compared with sham operated (Table I). However, there were no differences in the levels of these markers or hematological parameters between the WT and KO groups either at 16 (Table I) or 48 h after the CLP procedure.

Table I. Laboratory markers in A_{2A} KO and WT mice 0, 16, and 48 h after cecal ligation puncture. Data are the mean ± SEM of n = 5–6 mice. The results are representative of three separate experiments. [b]$p < 0.05$ compared with respective (WT or KO) sham.

Laboratory Parameter	WT (Sham)	KO (Sham)	WT (16 h after CLP)	KO (16 h after CLP)	WT (48 h after CLP)	KO (48 h after CLP)
White blood cells/µl	3010 ± 105	3340 ± 675	1027 ± 269[b]	890 ± 138[b]	1223 ± 215[b]	1790 ± 310[b]
Lymphocytes/µl	930 ± 134	1610 ± 585	375 ± 52[b]	525 ± 77[b]	405 ± 60[b]	830 ± 298
RBC (million/µl)	8.5 ± 0.25	9 ± 0.25	9.70 ± 0.19	9.3 ± 0.2	9.12 ± 0.22	9.24 ± 0.19
Hemoglobin (g/dl)	12.65 ± 0.395	13.1 ± 0.39	14.85 ± 0.25	14.05 ± 0.31	13.59 ± 0.28	13.71 ± 0.39
Hematocrit (%)	40.5 ± 1.3	42.1 ± 1.15	47.25 ± 1	44.80 ± 0.9	43 ± 0.9	43.7 ± 1.11
Platelet (thousands/µl)	990 ± 19.45	995 ± 52.6	824.65 ± 85.4	915.85 ± 67.5	549.87 ± 42.76[b]	637.53 ± 54.53[b]
Blood urea nitrogen (mg/dl)	28.2 ± 3.81	24.2 ± 3.01	69.33 ± 14.15[b]	51.38 ± 6.92[b]	33.71 ± 12.15[b]	38.29 ± 10.2[b]
Aspartate aminotransferase (U/L)	428 ± 219.97	209.4 ± 26.07	939 ± 120.9[b]	726.83 ± 102.5[b]	424.14 ± 67.23	539 ± 160.08[b]
Alanine aminotransferase (U/L)	85.8 ± 27.68	42.6 ± 3.8	340 ± 36.62[b]	338.17 ± 52.12[b]	126.29 ± 17.78	179.29 ± 78.6[b]

6.3. A_{2A} receptor deletion attenuates CLP-induced apoptosis in lymphoid organs

Increasing evidence shows that widespread lymphocyte depletion induced by apoptosis may contribute to the immunosuppression that occurs in sepsis. In addition, A_{2A}R activation has been reported to induce lymphocyte apoptosis (Koshiba et al., 1999; Apasov et al., 2000; Armstrong et al., 2001). Previous studies have documented that the cleavage/activation of caspase-3 is an important early indicator of apoptosis in the spleen (Javadi et al., 2004) and thymus (Guo et al., 2000) of animals subjected to CLP-induced sepsis. PARP is a major downstream target of activated caspase-3 and is cleaved by this enzyme during apoptosis (Soldani and Scovassi, 2002). Therefore, we tested the hypothesis that A_{2A}R deficiency would prevent the cleavage of caspase-3 and PARP in the spleen and thymus of mice subjected to CLP. We found that 16 h after the onset of sepsis, WT mice exhibited substantial cleavage of caspase-3 and PARP (Figure 8). In contrast, the cleavage of both caspase-3 and PARP was markedly

suppressed in $A_{2A}R$ KO mice (Figure 8 *A–D*). These indicators of apoptosis were absent in both $A_{2A}R$ WT and KO mice at 48 h, as well as in sham-operated mice (data not shown).

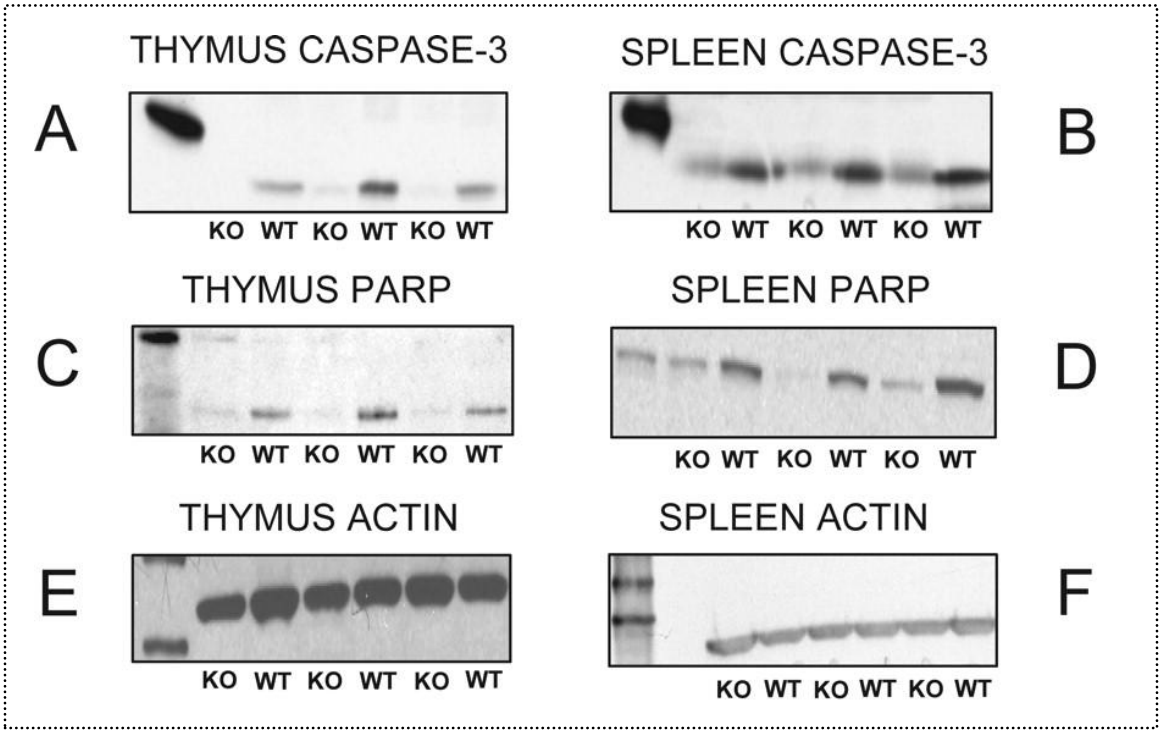

Figure 8. Lessened cleavage of caspase-3 and PARP in $A_{2A}R$ KO mice. Cleaved forms of caspase-3 (***A*** and ***B***) and PARP (***C*** and ***D***) were detected using antibodies raised against the cleaved forms of these enzymes by Western blotting of thymus (***A*** and ***C***) and spleen (***B*** and ***D***) samples taken from $A_{2A}R$ WT and KO mice 16 h after CLP. Approximately equal loading of proteins is demonstrated by β-actin Western blotting (***E*** and ***F***). Results are representative of three separate experiments for each group.

Caspase-3 activation leads to the appearance of late apoptotic signs, such as phosphatidylserine exposure on the outer cell membrane (Soldani and Scovassi, 2002). We therefore examined whether the decreased caspase-3 cleavage/activation in the thymi of A_{2A} KO mice translated into decreased phosphatidylserine exposure 16 h after the onset of sepsis. Using FITC-labeled Annexin V staining and flow cytometry of thymocytes, we found that CLP significantly up-regulated phosphatidylserine exposure on thymocytes from both $A_{2A}R$ KO and WT animals (Figure 9). Nevertheless no difference was found between the two groups.

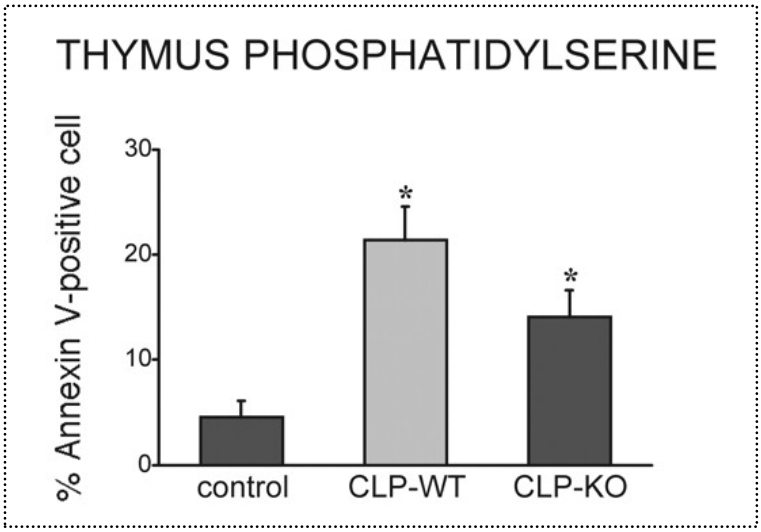

Figure 9. Average percentage of annexin V-positive thymocytes by flow cytometry. Thymocytes were isolated 16 h after the onset of CLP-induced sepsis. Data are the mean ± SEM *of* n = 3–5 mice/group. Results are representative of three separate experiments. *, $p < 0.05$.

Because phosphatidylserine exposure is only marginally detectable in the spleen of mice that have undergone CLP (Ayala et al., 1996), we used TUNEL immunohistochemistry to quantify late apoptotic events in septic $A_{2A}R$ KO and WT animals. The percentage of TUNEL-positive cells in spleens of nonseptic control mice was very low 0.13 ± 0.13 % (n = 3; mean ± SEM) (Figure 10). CLP significantly increased the fraction of TUNEL-positive cells in WT mice to 6.59 ± 1.32 % (n = 6, $p < 0.001$). The percentage of TUNEL-positive cells in spleens of KO mice exposed to CLP was significantly lower (4.08 ± 0.72 %; n = 6, p < 0.05) (Figure 10).

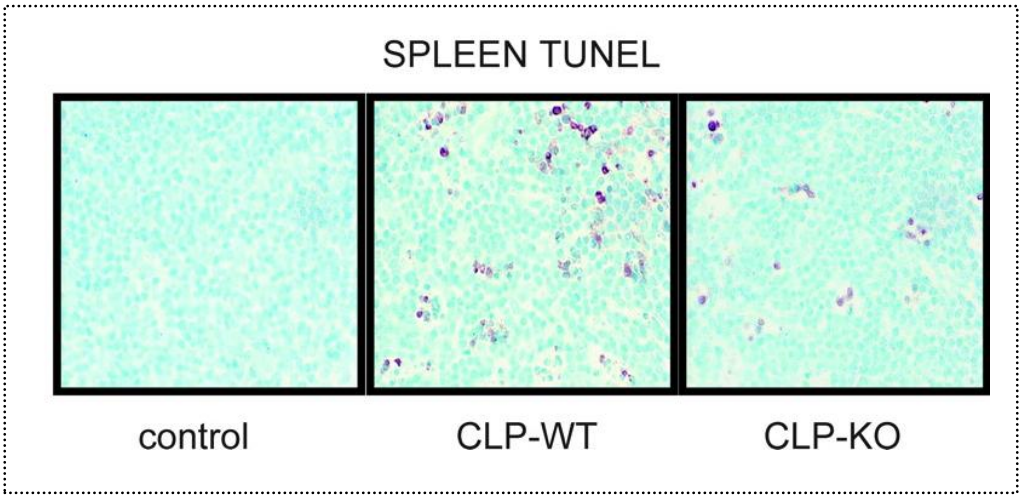

Figure 10. Decreased DNA fragmentation in $A_{2A}R$ KO mice. DNA fragmentation was quantitated using TUNEL immunohistochemistry (light microscopy, x600) of spleen samples obtained 16 h after the CLP procedure.

6.4. Splenic gene expression profile in septic A2AR KO vs WT mice

To further assess the potential cellular and molecular mechanisms that are associated with the decreased mortality of A_{2A} KO vs WT mice during sepsis, we compared splenic gene expression profiles of these animals. We used oligonucleotide microarray analysis using Affymetrix chips representing the entire mouse genome. There were ~330 genes that were significantly up-regulated and nearly 700 genes that were down-regulated in A_{2A} KO vs WT mice at least 2-fold (Figure 11*A)*. Importantly, IL-10, IL-6, and MIP-2 (chemokine (CXC motif) ligand 2) were among the down-regulated genes in A_{2A} KO vs WT mice. RT-PCR confirmed that mRNA levels of IL-10, IL-6, and MIP-2 were decreased in spleens of A_{2A} KO mice when compared with their WT controls (Figure 11*B*). Of the up-regulated genes in A_{2A} KO vs WT mice, the most notable differences were observed with members of the *MHC II* locus. To test whether these changes manifested at the cellular phenotypic level, we compared MHC II expression of septic KO and WT animals using flow cytometry. We found that F4/80$^+$ splenic (Figure 11*C*) and peritoneal (Figure 11*D*) macrophages from septic KO animals displayed markedly elevated MHC II expression levels as compared with cells from WT mice. These data indicate that there are concordant decreases in the protein and mRNA levels of IL-10, IL-6, and MIP-2, as well as a concordant increase in protein and mRNA of MHC II in septic A2A KO vs WT mice.

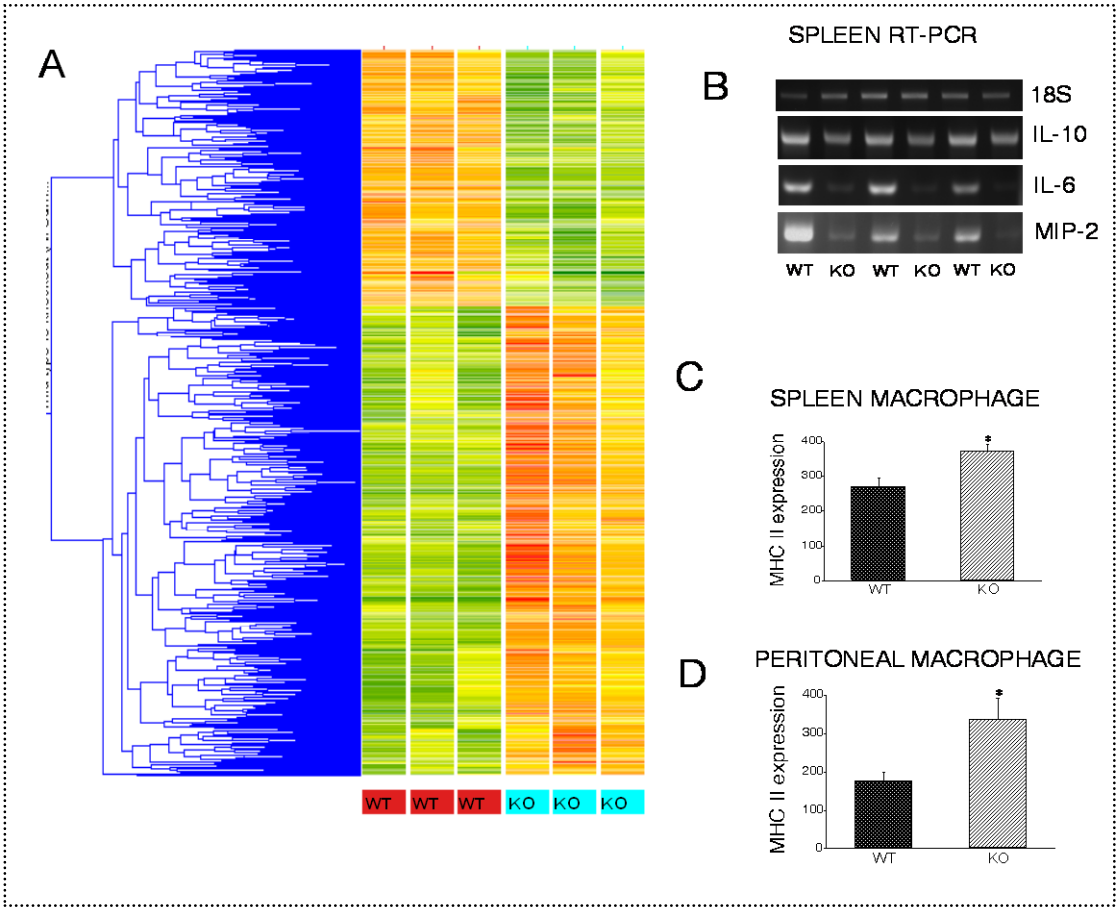

Figure 11. Different gene expression patterns in spleen of A_{2A} receptor WT versus KO mice subjected to cecal ligation and puncture. ***(A)*** Spleens were taken 16 hours after CLP. Hierarchical clustering of the data was based on genes that were differentially expressed by at least twofold with *p* values < 0.05 (*n* = 3 for both WT and KO spleens). Rows and columns represent genes and samples, respectively. Colors red, green, and yellow indicate that the expression level for a gene is above, below, and the same as the median measurement of the gene across all samples. Color saturation reflects the magnitude of the ratio relative to the median**.** ***(B)*** RT-PCR analysis demonstrates that levels of IL-10, IL-6, and MIP-2 mRNA are decreased in spleens of A_{2A} KO mice when compared to WT mice. $F4/80^{+}$ macrophages from spleens ***(C)*** or peritoneal cavity ***(D)*** of A_{2A} KO mice (*n*=5) exhibit increased MHC II expression (mean fluorescence intensity) when compared to WT (*n*=8) animals. Spleens or peritoneal cells were taken 16 hours after cecal ligation and puncture.

6.5. Pharmacological inactivation of A2AR decreases CLP-induced mortality

We further examined the role of $A_{2A}R$ in mediating CLP-induced mortality using a pharmacological approach. CD-1 mice treated with the selective $A_{2A}R$ antagonist ZM241385 (15 mg/kg, s.c., twice daily) starting at the time of resuscitation exhibited significantly improved survival compared with vehicle-treated mice (Figure 12).

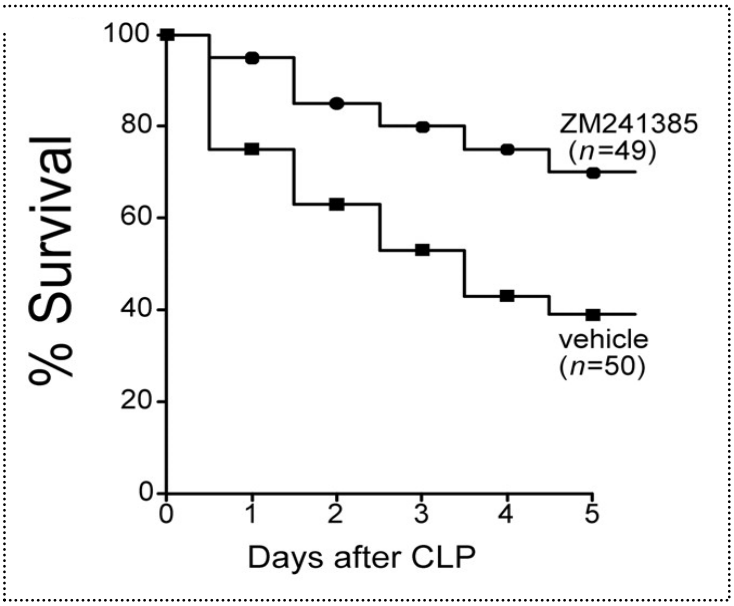

Figure 12. Treatment with the selective $A_{2A}R$ antagonist ZM241385 protects mice from mortality induced by CLP. ZM241385 (15 mg/kg, s.c, twice daily) - or vehicle-treated mice were subjected to CLP (2/3 ligation and through and through puncture with a 20-gauge needle), and survival was monitored for 5 days ($p < 0.05$, two-tailed Fisher's exact test).

To explore whether this improved survival of ZM241385-treated vs. vehicle-treated mice was associated with a similar cytokine pattern to that one observed in A_{2A} KO vs WT mice, we measured IL-10, IL-6, and MIP-2 concentrations in the plasma and peritoneal lavage fluid at 16 h. Levels of IL-10 and MIP-2 in both the plasma and peritoneal lavage fluid were decreased in ZM241385-treated mice as compared with vehicle-treated animals (Figure 13, *A* and *B*). Similar to genetic inactivation of $A_{2A}R$, levels of IL-6 were lower in the peritoneal fluid of ZM241385-treated mice than in the peritoneal fluid of vehicle-treated mice, however, IL-6 concentrations in the plasma were comparable between the two groups (Figure 13*C*).

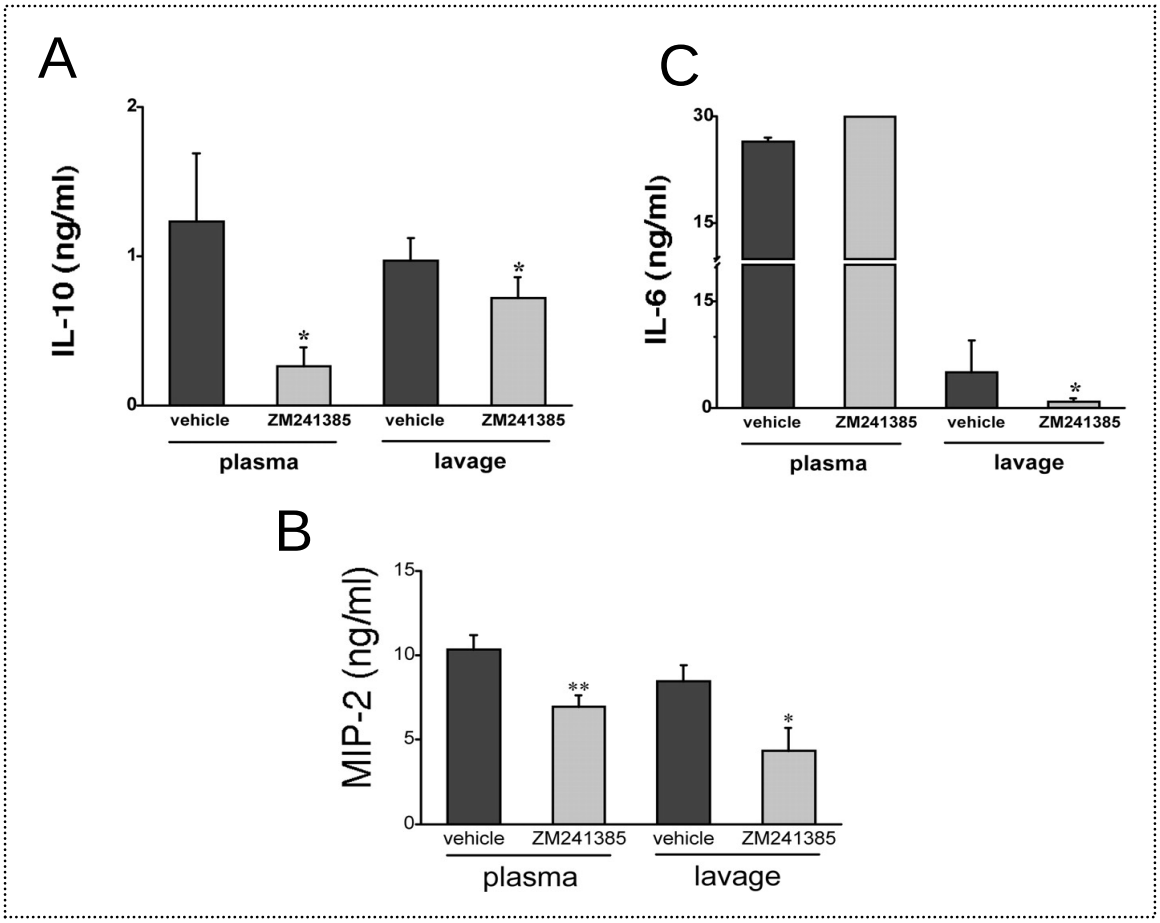

Figure 13. Cytokine levels in the plasma and peritoneum of mice treated with the selective A_{2A} receptor antagonist ZM241385. IL-10 ***(A)*** and MIP-2 **(*B*)** levels are decreased in the plasma and peritoneum of mice treated with the selective A_{2A}R antagonist ZM241385 (15 mg/kg, s.c., twice daily). IL-6 levels are attenuated in the peritoneum of ZM241385-treated mice as compared with vehicle-treated mice. ***(C)*** Concentrations of IL-10, MIP-2, and IL-6 were measured by ELISA in plasma and peritoneal lavage fluid that were obtained 16 h after cecal ligation and puncture. Data are the mean ± SEM of $n = 6–9$ mice per group. Results are representative of three separate experiments. $*$, $p < 0.05$; $**$, $p < 0.01$.

Finally, we explored the effect of the delayed administration of ZM241385 relative to resuscitation. We observed that ZM241385 administration starting 2 h after resuscitation (15 mg/kg, s.c., twice daily) was still protective (Figure 14), indicating a potential clinical use of A_{2A}R blockade in acutely developing septic conditions.

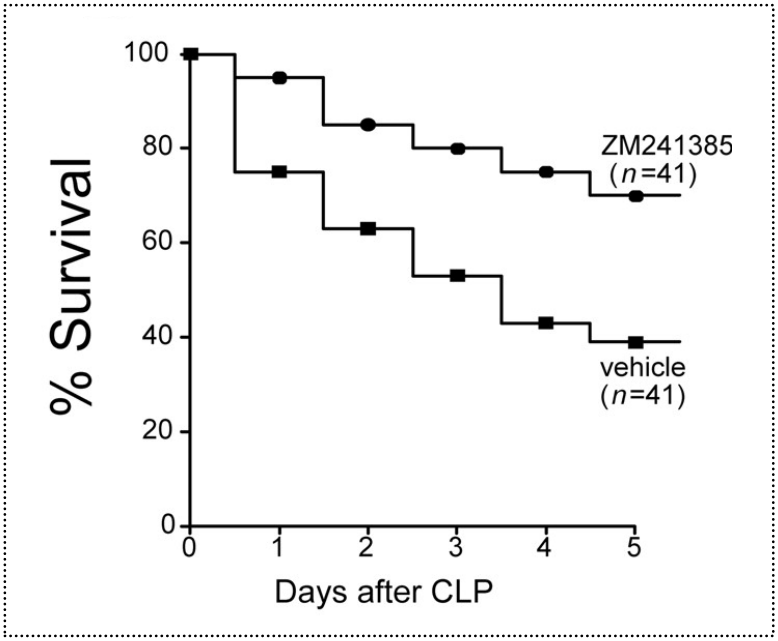

Figure 14. Treatment with the selective A_{2A}R antagonist ZM241385 at time 2 h after resuscitation protects mice from death induced by CLP. ZM241385 (15 mg/kg, s.c, twice daily)- or vehicle-treated mice were subjected to CLP (2/3 ligation and through and through puncture with a 20-gauge needle), and survival was monitored for 5 days ($p < 0.05$, two-tailed Fisher's exact test).

6.6. A_{2A} adenosine receptor activation and *E. coli* synergistically induce IL-10 release by macrophages

One of the most striking findings of our in vivo studies was that genetic inactivation of the A_{2A}R almost abolished IL-10 production in the CLP model of murine polymicrobial sepsis, which suggested that A_{2A} receptor activation is critically required for IL-10 production after bacterial stimuli. Because IL-10 appears to be pivotal in mediating immune dysregulation during sepsis, the molecular mechanisms by which this induction of IL-10 production occurs following A_{2A}R receptor activation in the cotext of bacterial sepsis were likely to be vitally important to our understanding of clinical sepsis. Thus, in the next series of experiments, we turned our attention toward the intracellular signaling mechanisms by which A_{2A} receptor activation augments IL-10 production by macrophages following exposure bacterial stimuli.

To reveal the nature of the interaction of bacterial stimuli and A_{2A} receptor signaling *in vitro*, peritoneal macrophages were obtained from A_{2A}R KO and WT mice and treated with *E.*

coli and adenosine. Macrophages from A_{2A}R receptor WT mice produced low levels of IL-10 after exposure to *E. coli* but not adenosine (Figure 15*A*). Challenging *E. coli*–treated WT cells with adenosine dramatically increased IL-10 levels (Fig. 15*A*). However, neither adenosine (Figure 15*A*) nor *E. coli* (Figure 15*A*) was capable of eliciting IL-10 release by A_{2A} KO macrophages. Moreover, the combination of adenosine and *E. coli* was also ineffective in triggering IL-10 release by A_{2A} KO macrophages (Figure 15*A*).

We next treated macrophages with *E. coli* in the presence or absence of various adenosine receptor agonists. Both the selective A_1 receptor agonist CCPA and A_3 receptor agonist IB-MECA failed to mimic the stimulatory effect of adenosine on IL-10 release. However, both the selective A_{2A} receptor agonist CGS21680 and nonselective agonist NECA increased IL-10 release by *E. coli*–challenged macrophages, with CGS21680 being the most potent (Fig. 15*B*).

We then investigated the role of A_{2B} receptors using A_{2B} receptor KO and WT mice, because previous studies have implicated A_{2B} receptors in regulating IL-10 release (Németh et al., 2005; Yang et al., 2006). *E. coli* induced the release of IL-10 by macrophages from both A_{2B} receptor WT and KO mice to the same extent. Adenosine enhanced substantially this *E. coli*–induced IL-10 release in A_{2B} WT mice, which was slightly (by approximately 10%) but consistently decreased in A_{2B} KO mice (Figure 15*C*).

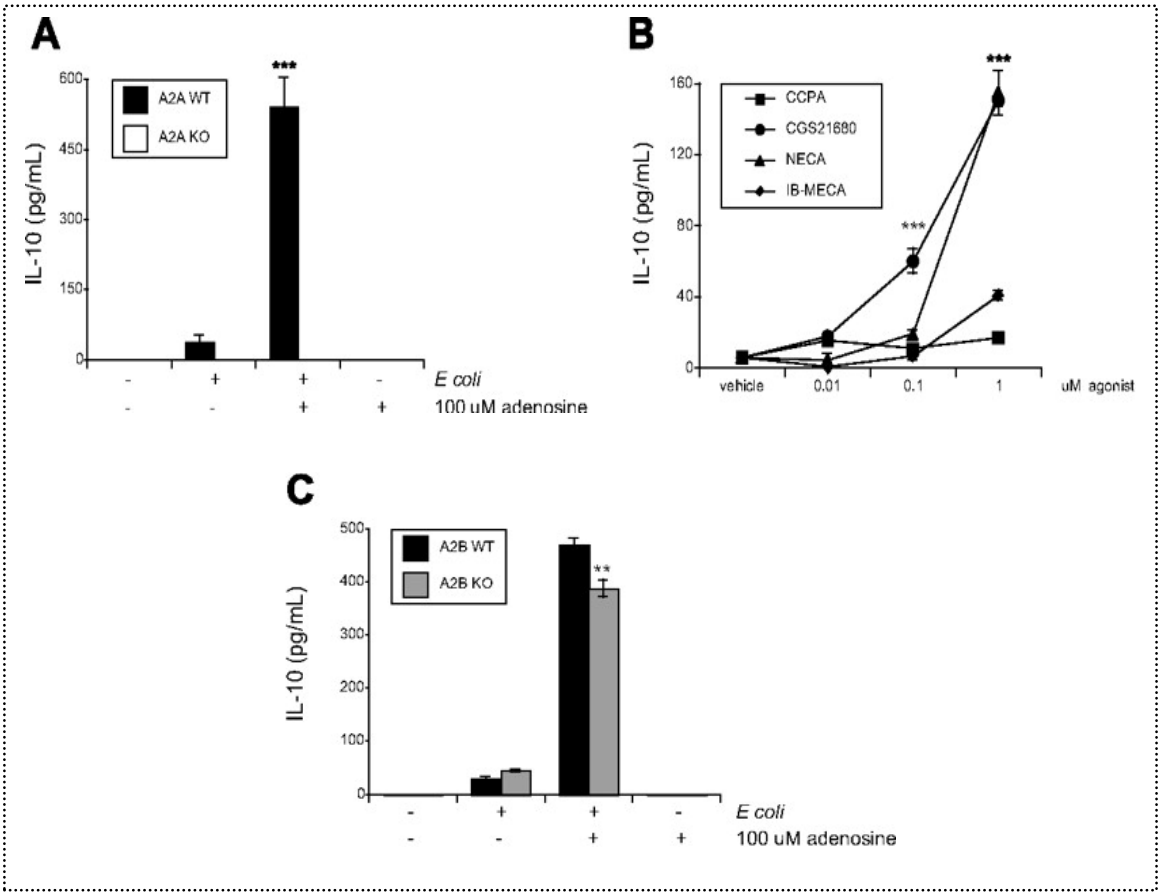

Figure 15. $A_{2A}R$ are critical for IL-10 production by *E. coli*-challenged macrophages. (*A*) $A_{2A}R$ KO and WT peritoneal macrophages were treated with heat-killed *E. coli* (at a macrophage:bacterium ratio of 1:15) or co-treated with 100 µM adenosine and heat-killed *E. coli* for 5 hours, and then IL-10 was measured from the supernatant using ELISA. ***indicates $p<0.001$ vs. *E. coli* A_{2A} WT group. **(*B*)** Adenosine agonists were added to macrophages before stimulating with *E. coli*. IL-10 concentrations were measured from the supernatants taken 5 hours after *E. coli* stimulation. ***indicates $p<0.001$ vs. vehicle. **(*C*)** A_{2B} KO and WT peritoneal macrophages were treated with heat-killed *E. coli* or co-treated with 100 µM adenosine and heat-killed *E. coli* for 5 hours, and then IL-10 was measured from the supernatant using ELISA. **indicates $p<0.01$ vs. *E. coli* A_{2B} WT group. Results (mean ± SEM) shown are representative of at least three experiments with $n = 6$ in each experiment.

6.7. The stimulatory effect of adenosine on *E coli*–induced IL-10 production did not require TLR4 or MyD88, but negatively regulated by TRAF6 and inhibition of p38.

Recent studies have illustrated that bacteria contain a number of components that are able to elicit IL-10 secretion independently of TLR4 (Ramakers et al., 2006). To determine whether bacterial components that are not TLR4 ligands could be involved in the synergistic upregulation

of IL-10 release after combined administration of *E. coli* and adenosine, we treated peritoneal macrophages obtained from TLR4 KO and WT mice with adenosine and heat-killed *E. coli*. We found that *E. coli* was capable of inducing low levels of IL-10 in TLR4 WT macrophages, which was decreased in TLR4 KO cells (Figure 16*A*). Exogenous adenosine synergistically upregulated (> 10-fold) *E. coli*–induced IL-10 release in TLR4 WT cells. Although the combined exposure of adenosine and *E. coli* induced lower levels of IL-10 in TLR4 KO macrophages than the same treatment in TLR4 WT cells, adenosine upregulated *E. coli* induced IL-10 release to the same degree (> 10-fold) in TLR4 WT and TLR4 KO cells (Figure 16*A*). We next examined whether adenosine affected the release of IL-6, a pro-inflammatory cytokine, independently of TLR4. *E. coli* triggered IL-6 release in TLR4 WT and to a lesser extent in TLR4 KO macrophages, and adenosine decreased this IL-6 release in both WT and KO cells approximately to the same degree (by approximately 30%; Figure 16*B*).

To confirm that adenosine can upregulate bacteria-induced IL-10 release independently of TLR4, we challenged peritoneal macrophages with heat-killed *S. aureus*, a Gram-positive bacterium, which, unlike Gram-negative *E. coli*, is thought to act in a TLR4-independent fashion to elicit macrophage activation. We observed that *S. aureus* increased IL-10 levels, which were further enhanced by adenosine (Figure 16*C*). We then investigated the effect of adenosine on IL-10 release that was induced by the specific TLR2 agonist lipoteichoic acid prepared from *S. aureus*. This TLR2 agonist elicited the release of IL-10 by macrophages, and adenosine boosted the TLR2 agonist-induced IL-10 level (Figure 16*D*). Collectively, our results demonstrate that *E. coli* increases IL-10 release in macrophages, and this effect is partially dependent on TLR4. Moreover, adenosine upregulates bacteria-induced IL-10 secretion and down-regulates IL-6 release by a mechanism that does not require TLR4.

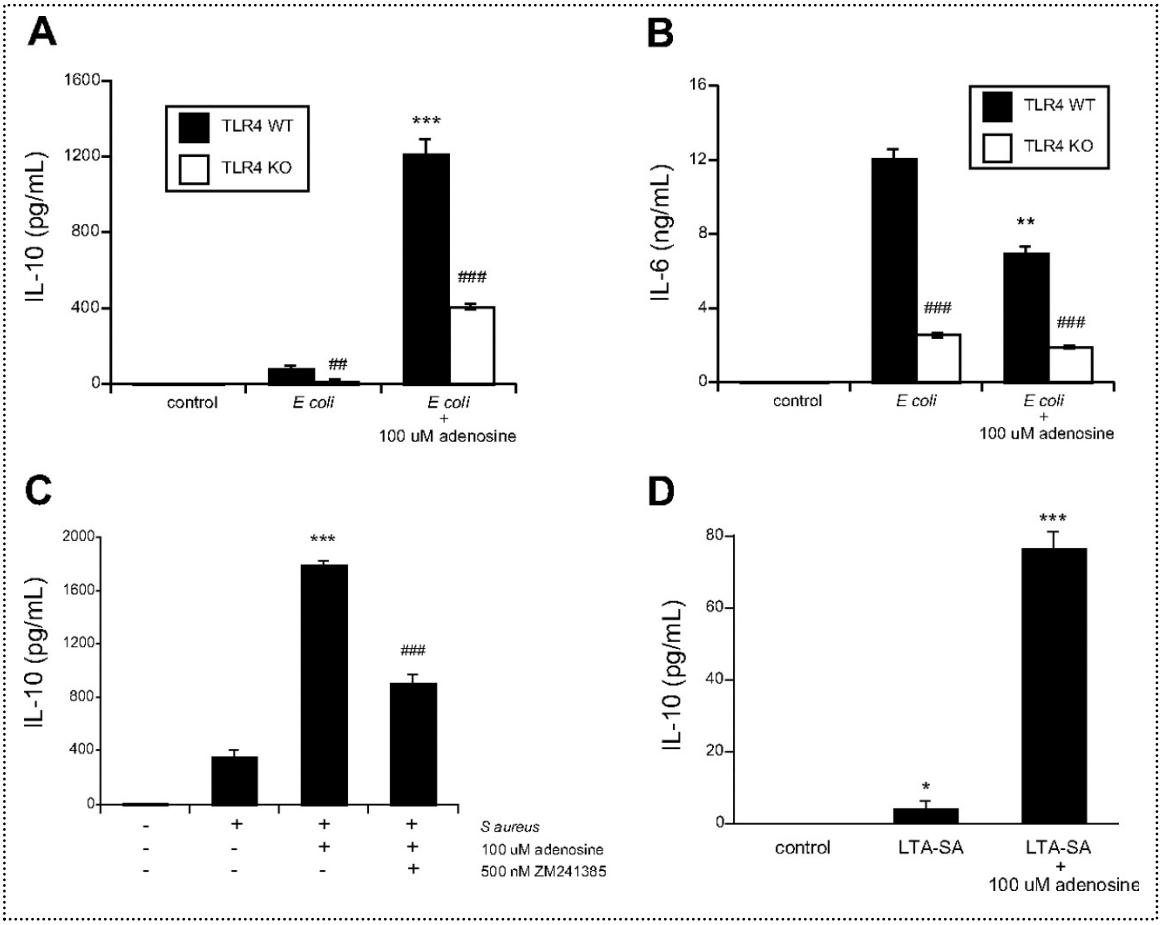

Figure 16. Effects of *E. coli* and adenosine on IL-10 and IL-6 production by TLR4 WT and KO macrophages. (*A*) Peritoneal macrophages were obtained from TLR4 KO and WT mouse and treated with heat-killed *E. coli* (at a macrophage:bacterium ratio of 1:15) or *E. coli* plus 100 μM adenosine for 5 hours, after which procedure IL-10 production was determined from the supernatant. *** and ### indicate p<0.001 vs. corresponding *E. coli*-treated groups, ##indicates p<0.01 vs. TLR4 WT group **(*B*)** IL-6 levels were determined from the same supernatant that was used for measuring IL-10 production. ###indicates p<0.001 vs. corresponding TLR4 WT groups, **indicates p<0.01 vs. *E. coli*-stimulated group. **(*C*)** Peritoneal macrophages were obtained from CD-1 mice. IL-10 production was measured after 5 hours of stimulation with *S. aureus* and/or 100 μM adenosine. ***indicates p<0.001 vs. *S. aureus* alone, ###indicates p<0.001 vs. 100 μM adenosine. **(*D*)** Peritoneal macrophages were obtained from CD-1 mice and treated with 1μg/ml LTA-SA or LTA-SA plus 100 μM adenosine for 5 hours, after which IL-10 release was determined from the supernatant. *indicates p<0.05 vs. vehicle and ***indicates p<0.001 vs. vehicle. Results (mean ± SEM) shown are representative of at least three experiments with $n = 6$ in each experiment.

MyD88 has been documented to contribute to intracellular signaling from all TLRs except TLR3. In addition, recent evidence indicates that TLR signaling consists of both MyD88-dependent and a MyD88-independent pathways (Akira et al., 2001). Because of the central role

of MyD88 in many bacteria-induced macrophage responses (Lembo et al., 2003; Takeuchi et al., 2000), we first studied whether MyD88 regulated IL-10 release in macrophages challenged with *E. coli*, adenosine, or the combination of *E. coli* and adenosine. There was no difference in IL-10 release by peritoneal macrophages obtained from MyD88 WT and KO mice after *E. coli* treatment and the IL-10 level was upregulated to the same degree in WT and KO macrophages after adenosine treatment (Figure 17*A*).

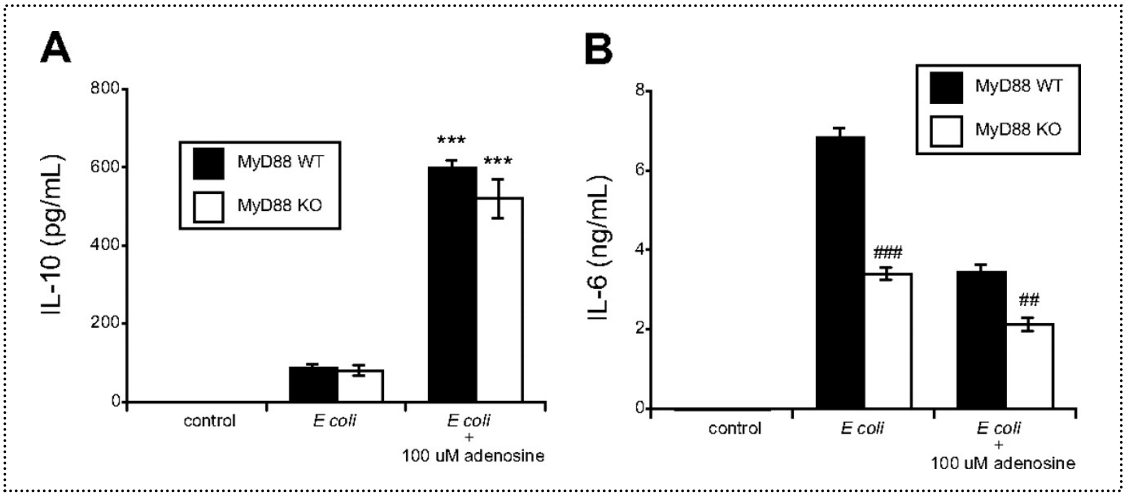

Figure 17. Role of MyD88 in regulating IL-10 and IL-6 production by *E. coli*–treated and adenosine-stimulated macrophages. ***(A)*** MyD88 is not necessary for the synergistic effect of adenosine and *E. coli* on IL-10 production. Peritoneal macrophages were taken from MyD88 KO and WT mice. After 5 hours of stimulation with heat-killed *E. coli* or *E. coli* plus adenosine IL-10 enzyme-linked immunosorbent assay was performed. ***$P < .001$ vs. corresponding adenosine-untreated, *E. coli*–stimulated groups. ***(B)*** MyD88 is partially required for *E. coli*–induced IL-6 production. IL-6 cytokine levels were determined from the same supernatants that were used for IL-10 detection. $^{###}P < .001$ vs. *E. coli*–treated MyD88 WT group, $^{##}p < .01$ vs. adenosine/*E. coli*–treated MyD88 WT group. Results (mean ± SEM) shown are representative of at least 3 experiments with n = 6 in each experiment.

Previous work has shown that in the absence of MyD88 the production of IL-6 is decreased or completely abolished depending on which TLR activates IL-6 gene expression (Kawai et al., 1999; Takeuchi et al., 2000). In addition, contrary to its stimulatory effect on IL-10 production, adenosine has been shown to suppress IL-6 production by TLR4-stimulated macrophages (Sajjadi et al., 1996). IL-6 release in *E. coli*–stimulated MyD88 KO macrophages was decreased compared with WT macrophages, and adenosine decreased the IL-6 level in both MyD88 KO and WT peritoneal macrophages (Figure 17*B*). These results indicate that the release

of the anti-inflammatory cytokine IL-10 and pro-inflammatory IL-6 were regulated differentially by adenosine, and that while IL-10 release is completely MyD88-independent, IL-6 release is partially MyD88-dependent.

TRAF6 has been shown to be a crucial intracellular protein for the induction of pro-inflammatory genes in macrophages in response to bacteria or TLR agonists (Schnare et al., 2000; Kobayashy et al., 2004; Gohda et al., 2004). Therefore, we assessed using an shRNA approach whether TRAF6 would be required for the effect of *E. coli* and/or adenosine also on IL-10 release. First, we confirmed that TRAF6 shRNA–expressing cells expressed 83% less TRAF6 mRNA than cells stably transfected with a control vector expressing scrambled TRAF6 sequences (Figure 18*A*). Then, we treated cells with heat-killed *E. coli* and/or adenosine and measured cytokine levels. In agreement with previous data (Schnare et al., 2000), IL-6 release by *E. coli*–induced macrophages was decreased in TRAF6 shRNA–expressing cells compared with controls (scrambled shRNA-expressing cells; Figure 18*B*), indicating that IL-6 release after inflammatory stimuli requires TRAF6. Moreover, adenosine decreased IL-6 release in control cells, but did not reduce it in TRAF6 shRNA–expressing cells (Figure 18*B*), indicating that the suppressive effect of adenosine was TRAF6-dependent. In contrast, IL-10 levels were markedly higher in TRAF6 shRNA–expressing cells than in control cells after both *E. coli* and *E. coli*/adenosine treatment (Figure 18*C*). The fact that adenosine was less efficacious in potentiating the stimulatory effect of *E. coli* on IL-10 release in RAW 264.7 cells (Figure 18) is probably a reflection of the low expression of A_{2A} receptors on RAW cells (Németh et al., 2003a). In summary, these findings indicate that TRAF6 is not required for the effect of *E. coli* and adenosine in inducing IL-10, but TRAF6 negatively modulates this synergistic interaction.

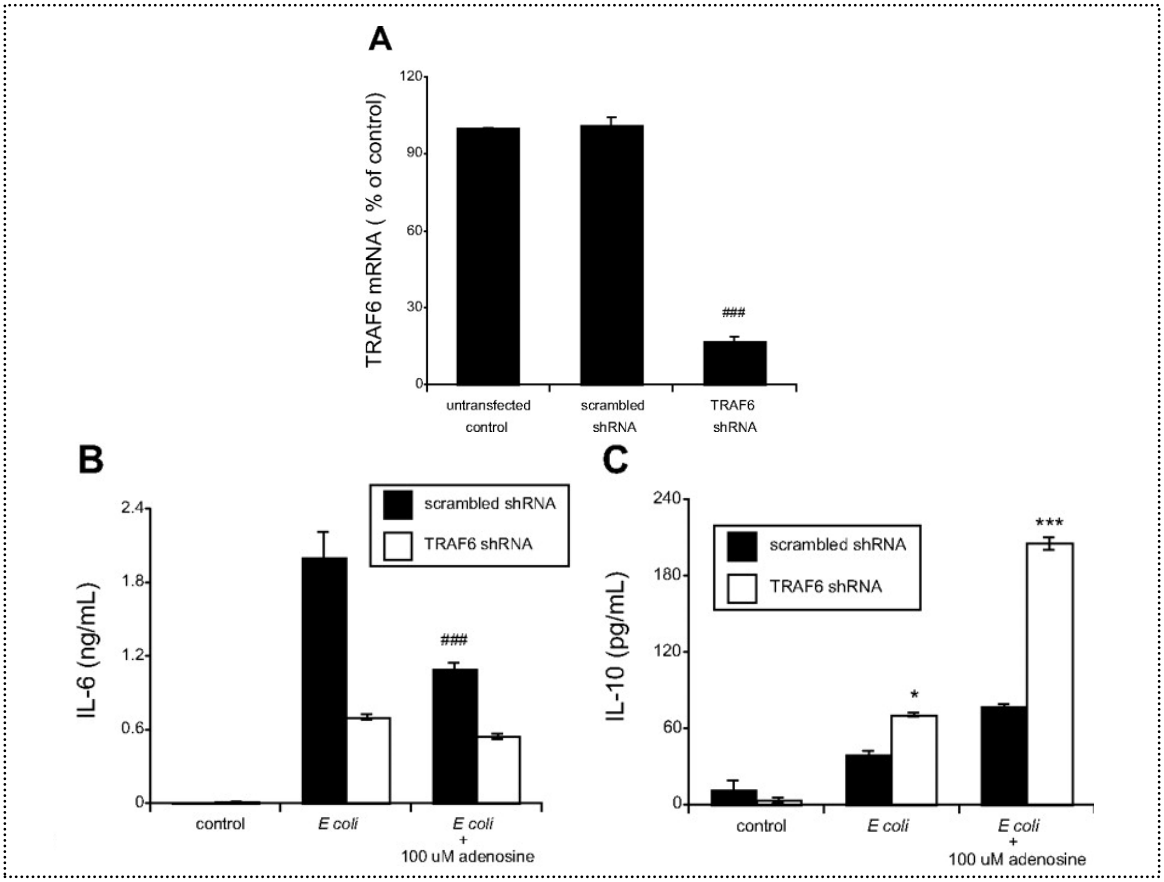

Figure 18. Effect of TRAF6 gene silencing on cytokine production by *E. coli*–treated and adenosine-stimulated macrophages. ***(A)*** TRAF6 mRNA level was decreased in RAW 264.7 macrophages stably transfected with TRAF6 shRNA. TRAF6 mRNA level was measured by real-time RT-PCR reaction using total RNA isolated from RAW 264.7 macrophages. Results (mean ± SEM) shown are representative of at least 3 experiments with n = 4 in each experiment. $^{###}P < .001$. ***(B)*** IL-6 production was decreased by TRAF6 gene silencing and the suppressive effect of adenosine is TRAF6-dependent in *E. coli*-stimulated macrophages. IL-6 production was determined from the supernatants that were obtained from stably transfected RAW 264.7 cells after 5 hours of exposure to *E. coli* or *E. coli* plus adenosine. d. $^{###}p < .001$ vs. scrambled. ***(C)*** Effect of TRAF6 silencing on IL-10 production by macrophages exposed to *E. coli* or *E. coli* plus adenosine. IL-10 production was determined from the supernatants that were obtained from stably transfected RAW 264.7 cells after 5 hours of exposure to *E. coli* or *E. coli* plus adenosine. $*p < .05$ vs. scrambled. $***P < .001$ vs. scrambled. Results (mean ± SEM) shown are representative of at least 3 experiments with n = 4 in each experiment.

Both TLR ligands and adenosine have been reported to be able to activate both p38 and p42/44 in macrophages (Németh et al., 2003b; Aderem and Ulevitch, 2000; Feoktistov et al., 1999). Moreover, both p38 and p42/44 have been implicated in the regulation of IL-10 production in response to various TLR ligands (Quian et al., 2006, Chi et al., 2006). Therefore, we tested the possibility that either p38 or p42/44 MAPK was involved in mediating the

stimulatory effect of *E. coli* or the combination of *E. coli* and adenosine on IL-10 release. We first examined whether *E. coli* or the combination of *E. coli* and adenosine triggered the activation of p38 and p42/44 MAPKs. The results of these experiments showed that *E. coli* increased p42/44 MAPK activation (Figure 19*A*) but not that of p38 (Figure 19*B*). When macrophages were treated with *E. coli* and adenosine together, p38 activation was increased (Figure 19*B*), but the activation of p42/44 was not changed (Figure 19*A*), compared with *E. coli* treatment alone.

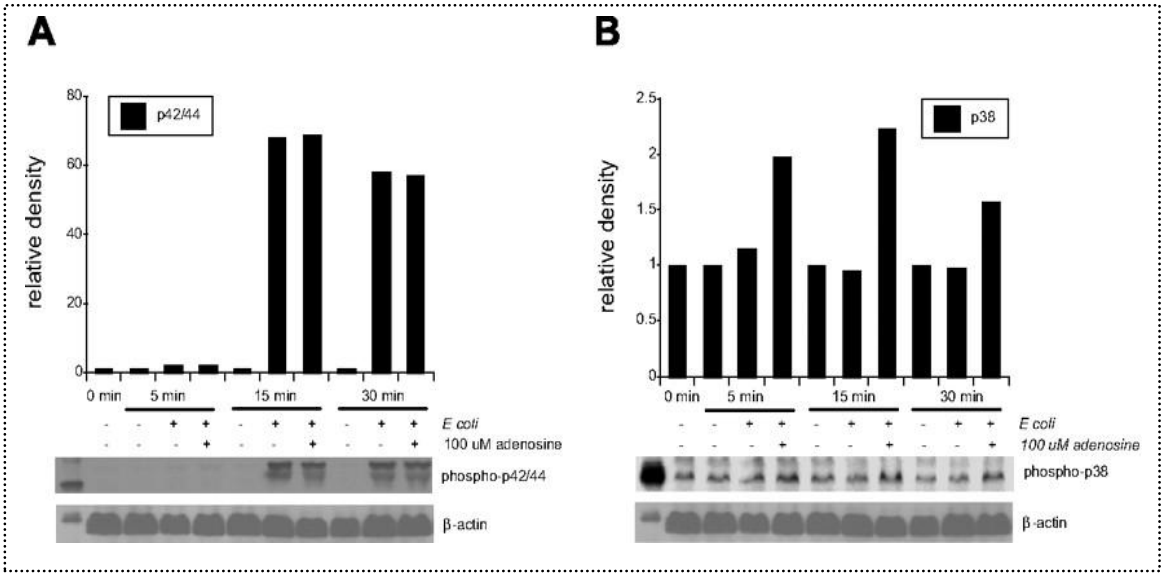

Figure 19. Effect of adenosine on MAPK activation in *E. coli*-stimulated macrophages. ***(A)*** *E. coli* but not adenosine increases p42/44 activation. Peritoneal macrophages were challenged with *E. coli* in the presence or absence of adenosine for 5, 15, and 30 minutes. p42/44 MAPK activation was determined from cytosolic extracts taken at the end of the 5-, 15-, and 30-minute incubation periods using Western blotting with antibodies raised against the active, doubly phosphorylated form of p42/44. Bands were detected using autoradiography. Relative densities are fold increase vs. control. This figure is representative of 3 separate experiments. ***(B)*** Adenosine but not *E. coli* increases p38 activation. Peritoneal macrophages were challenged with *E. coli* in the presence or absence of adenosine for 5, 15 and 30 minutes. p38 MAPK activation was determined from cytosolic extracts taken at the end of the 5-, 15-, and 30-minute incubation periods using Western blotting with antibodies raised against the active, doubly phosphorylated form of p38. Bands were detected using autoradiography. Relative densities are fold increase vs. control. This figure is representative of 3 separate experiments.

To examine whether this activation of p38 caused by adenosine contributed to the stimulatory effect of adenosine on *E. coli*–induced IL-10 release, we investigated whether MAPK inhibition decreased the adenosine-stimulated IL-10 cytokine level. Treatment of peritoneal macrophages with the selective p38 MAPK pathway inhibitor SB203580 but not p42/44 MAPK inhibitor PD98059 completely abolished the IL-10 response to adenosine (Figure 20).

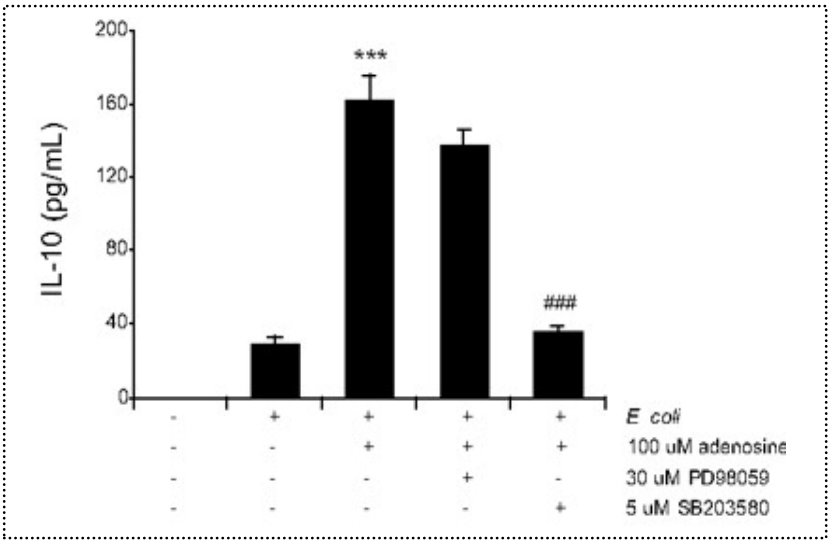

Figure 20. Inhibition p38 but not p42/44 MAPK prevents the stimulatory effect of adenosine on IL-10 production in *E. coli*–treated macrophages. Peritoneal macrophages were pretreated for 30 minutes with 5 μM SB203580 (p38 inhibitor) or 30 μM PD98059 (p42/44 inhibitor) before adding 100 μM adenosine and heat-killed *E. coli.* After 5 hours, supernatants were harvested and IL-10 levels measured using enzyme-linked immunosorbent assay. Results (mean ± SEM) shown are representative of at least 3 experiments with n = 6 in each experiment. ***$p < .001$ vs. *E. coli* alone. $^{###}P < .001$ vs. 100 μM adenosine plus *E. coli.*

6.8. The stimulatory effect of adenosine on *E. coli*–induced IL-10 release is associated with increased IL-10 mRNA accumulation and C/EBPβ transcription factor activation on the IL-10 promoter

To investigate the mechanisms that are responsible for the increased release of IL-10 by bacteria-activated macrophages both in the absence and presence of adenosine, we determined IL-10 mRNA levels from CD-1 peritoneal macrophages using real-time PCR. We found that *E. coli* increased IL-10 mRNA levels by approximately 4-fold, and adenosine further augmented by approximately 8-fold the *E. coli*–induced accumulation of IL-10 mRNA (Figure 21*A*). In

addition, adenosine had no effect on IL-10 mRNA levels in the absence of *E. coli* (Figure 21*A*). The transcriptional inhibitor actinomycin D prevented the synergistic effect of *E. coli* and adenosine in inducing IL-10 mRNA and protein accumulation arguing for the transcriptional nature of this synergism (Figure 21*A* and *B*).

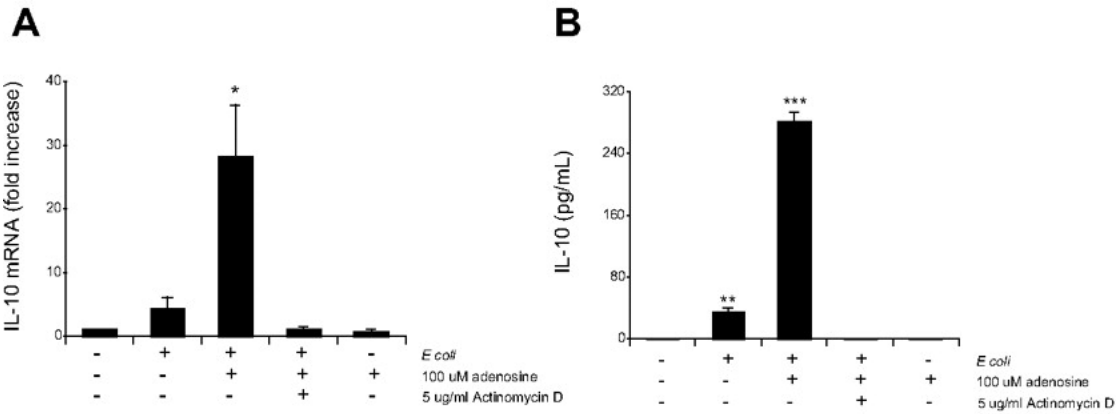

Figure 21. The stimulatory effect of adenosine on IL-10 production is transcriptional. ***(A)*** Adenosine enhances *E. coli*–induced IL-10 mRNA accumulation. Peritoneal macrophages were pretreated for 2 hours with 5 µg/mL Actinomycin D before adding 100 µM adenosine and heat-killed *E. coli.* IL-10 mRNA concentrations were measured by real-time polymerase chain reaction using RNA isolated 5 hours after stimulating with *E. coli* and adenosine. Results (mean ± SEM) shown are representative of at least 3 experiments with n = 4 in each experiment. **P* < .05 vs. *E. coli* alone. ***(B)*** Actinomycin D inhibits the stimulatory effect of *E. coli* and adenosine on IL-10 protein release. Supernatants were harvested from these treatments and IL-10 levels were measured using enzyme-linked immunosorbent assay. Results (mean ± SEM) shown are representative of at least 3 experiments with n = 4 in each experiment. **p< .005 vs. unstimulated control. ****P* < .001 vs. *E. coli* alone.

We also studied the effect of adenosine on *E. coli*–induced IL-10 promoter activity by transfecting RAW 264.7 cells with a construct in which luciferase expression was driven by the full-length IL-10 promoter (Brightbill et al., 2000). We found that *E. coli* increased IL-10 promoter activity, and adenosine enhanced IL-10 promoter activity approximately 2-fold in *E. coli*-induced but not control macrophages that were not exposed to *E. coli* (Figure 22). Because RAW 264.7 macrophages express low levels of the A_{2A} receptor endogenously (Németh et al., 2003a), we transfected these cells with an A_{2A} receptor-expressing construct (Figure 22). This enforced expression of the A_{2A} receptor resulted in a more pronounced increase in IL-10 promoter activity both after *E. coli* and after the combination of *E. coli* plus adenosine, underscoring the importance of A_{2A} receptors in augmenting IL-10 production.

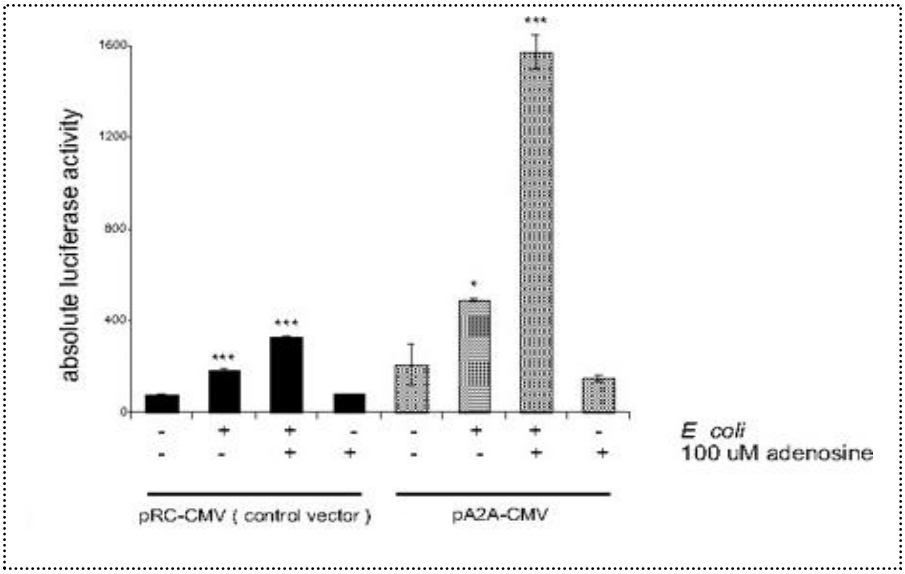

Figure 22. Adenosine up-regulates IL-10 promoter luciferase activity in RAW 264.7 macrophages exposed to *E. coli*. To measure IL-10 promoter activity, cells were transiently transfected with an IL-10 promoter-luciferase reporter construct and an A_{2A} receptor-expressing (pA2A-cytomegalovirus) or control (pRC-cytomegalovirus) plasmid. Cells were treated with *E. coli* in the presence or absence of adenosine for 8 hours. Results (mean ± SEM) shown are representative of at least 3 experiments with n = 3 in each experiment. *$p < .05$ and ***$p < .001$ vs. control.

To identify the DNA sequences that are necessary for adenosine to increase IL-10 promoter activity in *E. coli*–challenged cells, a series of promoter mutants that contain successive deletions from the 5' end were inserted upstream of the luciferase reporter gene (Brightbill et al., 2000) (Figure 23*A*). After transfection of RAW 264.7 cells with these constructs, luciferase activity was detected after adenosine/*E. coli* treatment. Analysis of luciferase activity from the 5' deletion mutants revealed that deletion of sequences between –1538 and –438 from the transcription start site did not affect the effect of adenosine in stimulating promoter activity. In contrast, the effect of adenosine was completely abolished by deletion of sequences between –438 and –376 (Figure 23*A*). These results show that DNA sequences in the IL-10 promoter between –438 and –376 are sufficient for the enhancing effect of adenosine on IL-10 promoter activity in *E. coli*–treated macrophages. Using Searching Transcription Factor Binding Sites program we found that there were two potential binding sites for C/EBP (–410/–399 and –398/–385) in this promoter region.

To confirm that these two potential C/EBP binding sites are necessary for the stimulatory effect of adenosine on IL-10 promoter activity, we mutated these sites, and those modified

sequences were inserted upstream of the luciferase reporter gene into pGL2. Analysis of luciferase activity from this construct revealed that mutating the C/EBP consensus sites prevented the stimulatory effect of adenosine on *E. coli*–induced promoter activity (Figure 23*B*).

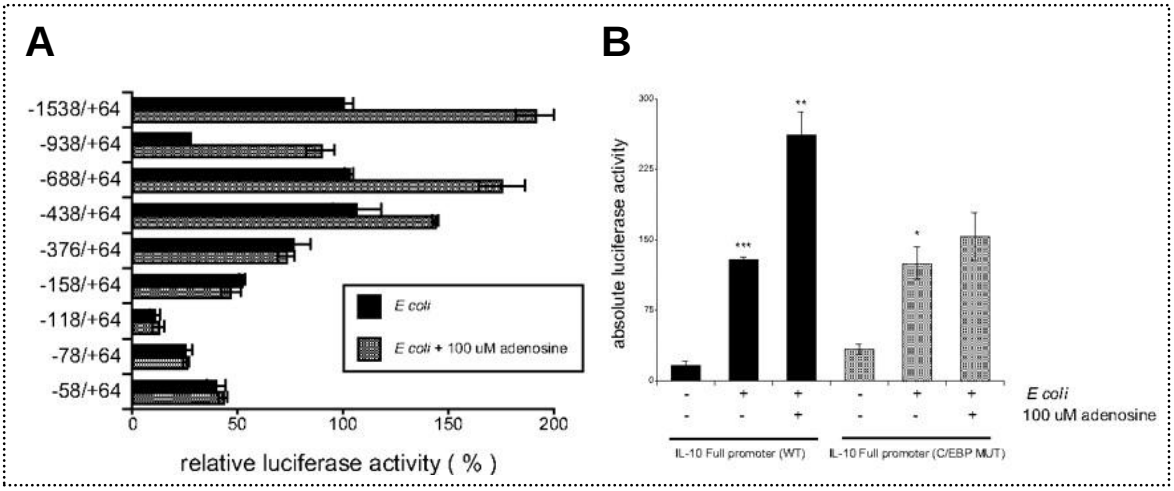

Figure 23. C/EBP consensus sequences in the 5' flanking region between –410/–385 of the IL-10 promoter are crucial for the stimulatory effect of adenosine. ***(A)*** Sequences between –438 and –376 from the transcription start site in the IL-10 promoter are necessary for the stimulatory effect of adenosine on IL-10 promoter activity. RAW 264.7 cells were transfected with a series of IL-10 promoter deletion mutants that were inserted in the pGL2B luciferase reporter vector. Transfected cells were stimulated with *E. coli* or *E. coli* plus adenosine for 8 hours. Luciferase activities are expressed as the mean activity and SEM relative to the activity of the full-length promoter (–1538/ + 64; 100%) after *E. coli* stimulation followed by normalization to protein concentration. Results (mean ± SEM) shown are representative of at least 3 experiments with n = 3 in each experiment. ***(B)*** C/EBP consensus sequences in the 5' flanking region between –410/–385 of the IL-10 promoter are crucial for the stimulatory effect of adenosine. RAW 264.7 cells were transfected with a C/EBP consensus mutant of the IL-10 promoter that was inserted in the pGL2B luciferase reporter vector. Transfected cells were treated with *E. coli* in the presence or absence of adenosine for 8 hours. Luciferase activities were normalized to protein concentration. C/EBP MUT: mutant. Results (mean ± SEM) shown are representative of at least 3 experiments with n = 3 in each experiment. **p* < .05, ***p* < .005 vs *E. coli* alone, and ****p* < .001 vs. control.

Because the IL-10 promoter sequence between –438 and –376 contains binding sites for C/EBP transcription factors, we first determined the effect of *E. coli* as well as *E. coli* plus adenosine on C/EBP transcriptional activity. To this end, RAW 264.7 macrophages were transfected with a construct in which luciferase expression is driven by C/EBP. The transfected cells were treated with adenosine and *E. coli*, and luciferase activity was measured after 8 h of treatment. We found that *E. coli* stimulated C/EBP luciferase activity by approximately 2-fold

and adenosine upregulated this activity by another approximately 2.5-fold (Figure 24). Adenosine alone had no effect on C/EBP luciferase activity (Figure 24).

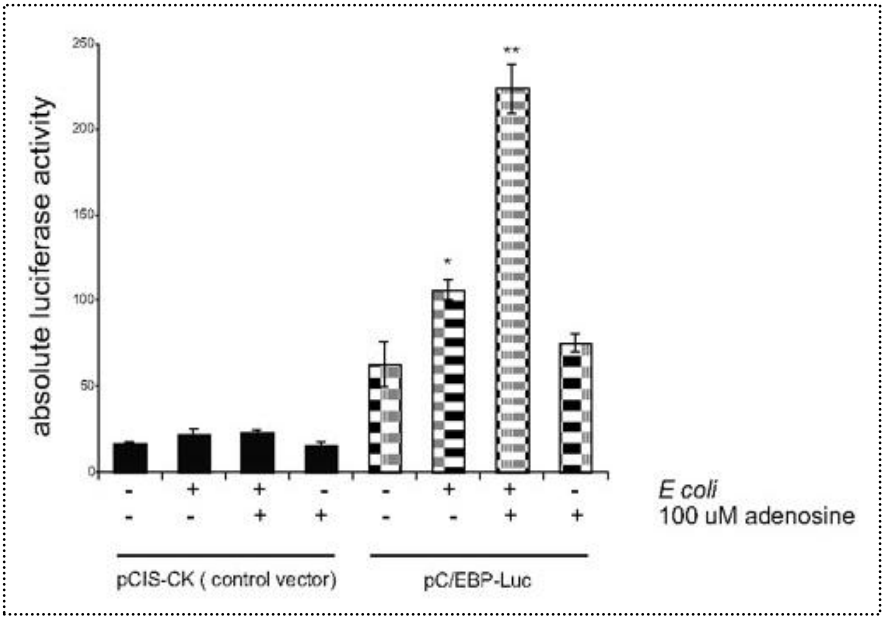

Figure 24. Adenosine up-regulates C/EBP–luciferase activity in macrophages exposed to *E. coli.* RAW 264.7 macrophages were transfected with a luciferase reporter vector driven by C/EBP (pC/EBP-luc) or a control vector (pCIS-CK). Cells were treated with *E. coli* and adenosine (100 μM) for 8 hours, after which period the cells were lysed and luciferase activity determined. Luciferase reporter activities were normalized to protein concentration. Results (mean ± SEM) shown are representative of at least 3 experiments with n = 3 in each experiment. **p* < .05 and ***P*p< .01 vs. control.

We then determined the effect of *E. coli* and adenosine on C/EBP DNA binding. Using nuclear extracts from *E. coli*–stimulated macrophages, we observed a significant increase in protein binding to a C/EBP consensus sequence at 30 and 60 minutes compared with untreated cells, and adenosine further enhanced this induction of C/EBP DNA binding (Figure 25). In competition binding assay, a 50-fold molar excess of cold C/EBP consensus probe completely abrogated the binding of the radiolabeled C/EBP probe (Figure 25).

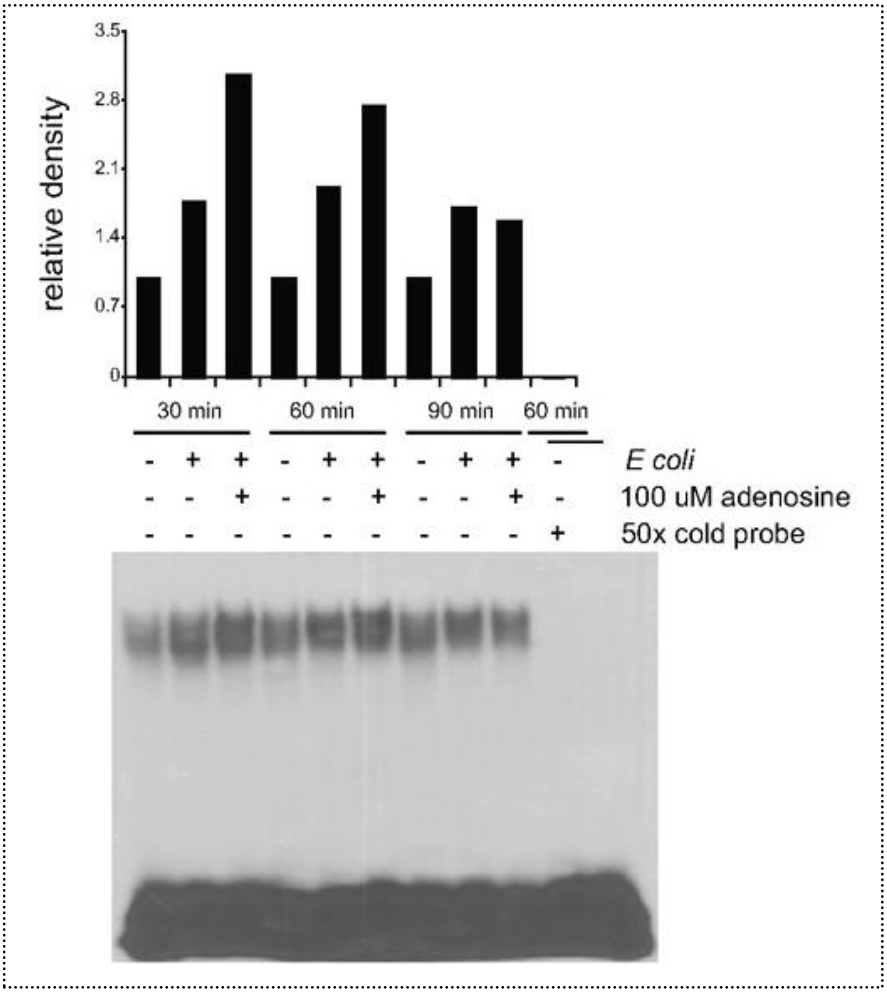

Figure 25. Adenosine enhances *E. coli*–induced C/EBPß DNA binding. RAW 264.7 cells were treated with heat-killed *E. coli* or simultaneously with 100 µM adenosine and heat-killed *E. coli*, and nuclear proteins were extracted at 30, 60, and 90 minutes thereafter. C/EBPß DNA binding of nuclear proteins was measured using electrophoretic mobility shift assay. The upper panel shows densitometric analysis of intensities of complexes (fold increase vs. control) observed on the gel (lower panel).

The C/EBP family contains several members, and within this family C/EBPβ and C/EBPδ are both expressed in macrophages (Lekstrom-Himes and Xanthopoulos, 1998). Therefore, we determined the nature of the different C/EBP isoforms binding to the C/EBP consensus oligo using antibodies specific for C/EBPβ or δ in supershift studies using nuclear extracts from *E. coli*/adenosine–treated RAW 264.7 macrophages. Figure 26 shows that only the C/EBPβ antibody shifted the DNA–protein complex.

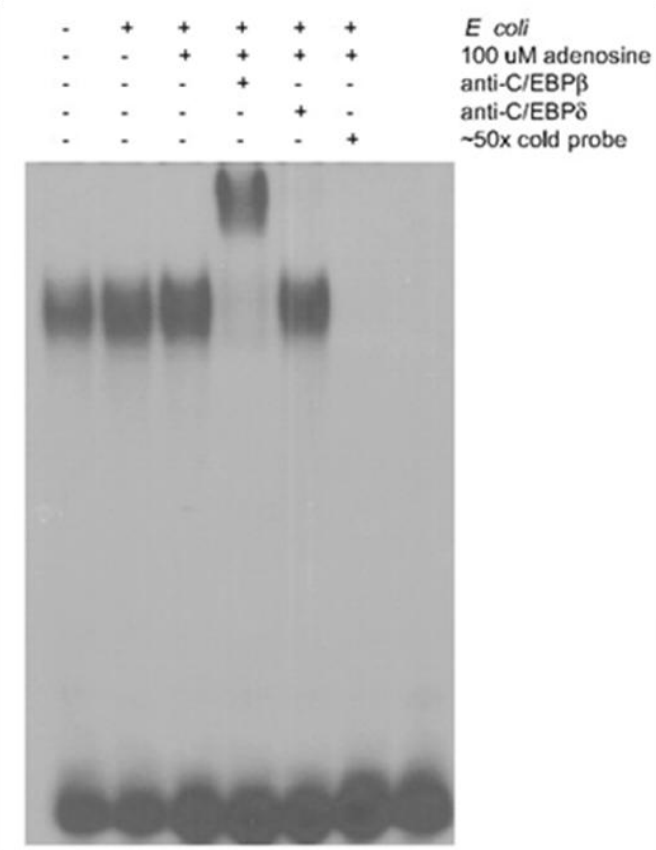

Figure 26. An antibody against C/EBPß shifts the adenosine/*E. coli*–induced (for 60 minutes) DNA–protein complex. For supershift studies, nuclear extracts were preincubated with C/EBPß and C/EBPδ antibodies before the binding reaction, and the complexes were separated by electrophoretic mobility shift assay and visualized using autoradiography. The figure shown is representative of 3 separate experiments.

To obtain additional evidence for the role of C/EBP, we performed immunodetection of C/EBPβ and C/EBPδ from nuclear and cytosolic fractions of macrophages after adenosine and/or *E. coli* treatment using antibodies raised against C/EBPβ and C/EBPδ. This analysis revealed that C/EBPδ did not accumulate in the nuclear fraction after either *E. coli* or adenosine treatment (data not shown). However, three C/EBPβ isoforms corresponding to the previously described (Descombes and Schibler, 1991; Joo et al., 2004) liver-enriched inhibitory protein, liver-enriched activating protein and full-length liver-enriched activating protein accumulated in nuclear fractions obtained from *E. coli*-stimulated macrophages (Figure 27*A* and *B*). Furthermore,

adenosine enhanced the nuclear accumulation of all three isoforms in the presence but not absence of *E. coli* (Figure 27*A* and *B*).

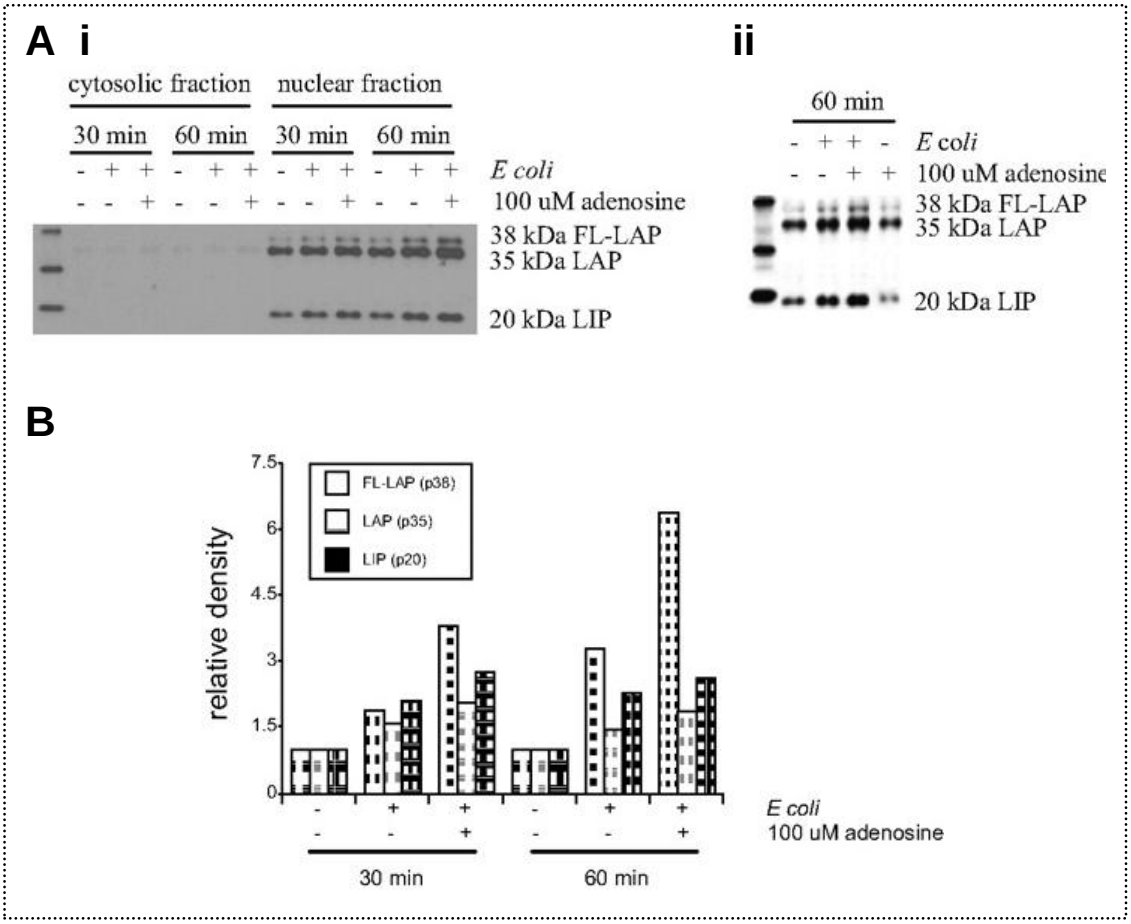

Figure 27. Adenosine increases *E. coli*–induced C/EBPß protein accumulation in the nuclear fraction of RAW 264.7 cells. ***(Ai)*** Cytosolic and nuclear protein extracts were taken at 30 and 60 minutes after exposure to *E. coli* or *E. coli* plus adenosine (100 µM) and C/EBPß expression was determined by Western blotting and autoradiography. ***(Aii)*** Adenosine alone fails to affect C/EBPß protein accumulation in the nuclear fraction of RAW 264.7 cells. The figures shown are representative of 3 separate experiments. ***(B)*** Densitometric analysis of the 3 C/EBPß isoforms detected by autoradiography on the blot shown in (***Ai***; fold increase vs. control). The figure is representative of 3 separate experiments.

To provide further insight into the role of C/EBPβ in regulating IL-10 production, C/EBPβ WT and KO immortalized macrophage cell lines (Gorgoni et al., 2002; Albina et al., 2005) were stimulated with heat-killed *E. coli* and adenosine for 5 h, and IL-10 release was measured by enzyme-linked immunosorbent assay. We found that *E. coli* or the combination of

E. coli and adenosine failed to induce IL-10 release by C/EBPβ KO cells, whereas these stimuli efficiently triggered IL-10 production by C/EBPβ WT macrophages (Figure 28). These data identify C/EBPβ as the major transcription factor mediating the stimulatory effect of adenosine on IL-10 release in *E. coli*–challenged cells.

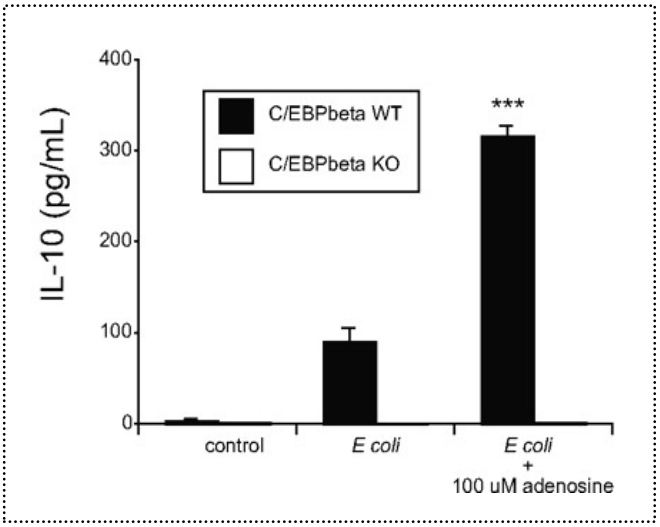

Figure 28. *E. coli* both in the absence and in presence of adenosine fails to increase IL-10 production by C/EBPß-deficient macrophages. C/EBPß WT and KO macrophages were challenged with *E. coli* or *E. coli* plus adenosine and IL-10 production was determined from the supernatants after 5-hour stimulation. Results (mean ± SEM) shown are representative of at least 3 experiments with n = 6 in each experiment. ***p< .001 vs. *E. coli.*

7. DISCUSSION

7.1. A_{2A} receptors contribute to immortality and immune dysfunction in sepsis

We have shown that A_{2A}R blockade either genetically or pharmacologically protects mice from the lethal effect of CLP-induced sepsis. This protection following A_{2A}R blockade is paralleled by a decrease in bacterial burden as well as an increase in macrophage MHC II expression. Concurrently, A_{2A}R blockade leads to decreased apoptosis in the spleen as well as decreased levels of the cytokines IL-10, IL-6 and MIP-2. Our results, therefore, are the first to demonstrate a harmful role of A_{2A}R stimulation in invasive bacterial infection. Several recent studies have addressed the role of A_{2A}R in regulating injury in animal models of systemic overwhelming inflammation. Pharmacological studies using exogenous A_{2A} receptor agonists show that the activation of A_{2A}R protects both organs and the organism from early overwhelming inflammation triggered by endotoxin (Sullivan et al., 1999; Sullivan et al., 2004). The recent demonstration of the tissue protection by endogenous adenosine acting at A_{2A}R in acute endotoxemia (Ohta and Sitkovsky, 2001; Lukashev et al., 2004) further suggests the relevance of A_{2A}R to protection from hyperacute systemic inflammation. The mechanistic link between A_{2A}R stimulation and protection from inflammatory organ injury is suggested by studies showing that A_{2A} receptor KO mice injected with endotoxin have increased activation of NF-κB and higher plasma levels of proinflammatory TNFα compared with similarly treated WT controls (Ohta and Sitkovsky, 2001; Lukashev et al., 2004).

Thus, at first glance, our observations that A_{2A}R activation is harmful during CLP-induced sepsis might seem contradictory to observations that A_{2A}R activation is beneficial in acute inflammation due to endotoxicosis. However, we believe the data seen as a whole suggests that differences in outcome in the two models are mainly due to immunosuppression being beneficial in acute endotoxemia but detrimental in more clinically relevant models of infection-induced sepsis where mortality depends more upon the loss of control of bacterial growth (Riedemann et al., 2003a; Deitch, 1998, Kelly et al., 1997). Consistent with the distinct pathophysiology of endotoxicosis models and the CLP model, LPS is not a major contributor to the mortality of mice subjected to CLP, because genetic ablation of TLR4, the most important LPS receptor, fails to influence the survival of mice undergoing CLP (Echtenacher et al., 2001). Circulating LPS levels in the CLP model were found to be very low, an observation that is also

consistent with minute amounts of LPS found in human sepsis (Benjamim et al., 2004). A gradual long-lasting increase in IL-10 production is a pivotal factor contributing to the potentially lethal impairment in immune function and antibacterial defense observed in the CLP model (Steinhauser et al., 1999a; Kalechman et al., 2002). One potential explanation for the decreased bacterial load in mice lacking $A_{2A}R$ is a decrease in the levels of the immunosuppressive IL-10 leading to a better-preserved phagocytic response (Steinhauser et al., 1999a; Kalechman et al., 2002). IL-6 levels are up-regulated in septic humans and are correlated with poor outcome (Calandra et al., 1991; Waage et al., 1989). Although controversial because IL-6 KO mice do not show improved survival in CLP-induced sepsis (Leon et al., 1998), high IL-6 levels may be disadvantageous in CLP-induced sepsis. Riedemann et al. (Riedemann et al., 2003b) demonstrated a dose-dependent beneficial effect of IL-6 blockade in this sepsis model, using anti-IL-6 in mice. These authors suggested that although IL-6 blockade is beneficial for the outcome in CLP-induced sepsis in mice, some low levels of IL-6 might be necessary for an appropriate acute-phase response. Because the levels of IL-6 were substantially, but not completely, inhibited in the A_{2A} KO mice at the 16-h time point, it can be proposed that $A_{2A}R$ inactivation improves survival, at least in part, by decreasing IL-6 production. Interestingly, IL-6 concentrations were decreased in the peritoneum, but not in the systemic circulation of A_{2A} KO mice. It is possible that the cellular sources of IL-6 are different in the peritoneal cavity and blood. We also found that levels of the CXC chemokine MIP-2 were highly elevated in $A_{2A}R$ WT but not KO mice at 16 h after CLP. Because the neutralization of MIP-2 was protective in a similar CLP model to ours (Walley et al., 1997), it is plausible that the salutary effect of $A_{2A}R$ deficiency on survival following CLP was partially due to the decreased MIP-2 levels.

CLP-induced sepsis triggers extensive apoptosis in the thymus and spleen (Javadi et al., 2004; Guo et al., 2000; Tinsley et al., 2000). Guo et al. (Guo et al., 2000) found that caspase-3 activity elevated to a greater extent than other caspases in the thymus and these authors suggested that caspase-3 plays an important role in sepsis-induced thymocyte apoptosis. Similarly, a high degree of caspase-3 activation occurs in CLP-induced sepsis in the spleen (Javadi et al., 2004). The mechanisms triggering the apoptotic machinery in lymphoid organs during sepsis are not well understood. Our data showing decreased levels of caspase-3 cleavage as well as PARP cleavage indicate that $A_{2A}R$ are essential contributors to early apoptotic processes in both the spleen and thymus. Interestingly, although late apoptotic events in the spleen were also

suppressed in A_{2A} KO mice, this was not as evident in the thymus. A similar divergence between early and late apoptotic events in the septic thymus has been previously reported in mice overexpressing Bcl-2 (Méthot et al., 2004). As both caspase-dependent and -independent pathways may be involved in thymocyte apoptosis during sepsis, our results suggest that caspase-independent pathways may dominate caspase-dependent pathways in the thymus following sepsis.

In a recent study, Jenner and Young (2005) have collated and compared published transcriptional-profiling data from 32 prior studies that involved 77 different host-pathogen interactions, and have defined a common host-transcriptional response. Using cluster analysis, a common host-transcriptional program was defined that is shared among different cell types in response to a range of pathogen species (Jenner and Young, 2005). In addition, several functional groups of gene products were identified. The group of genes that was most strongly and most consistently up-regulated across the various studies was termed the inflammatory/chemotactic cytokine cluster, which consisted of genes that encoded *TNF*, *IL-1*β, *IL-6*, *IL-8*, *CSF3*, *CCL3*, *CCL4*, *CXCL1*, *CXCL2*, *CXCL3*, and *PTGS2* (*COX2*). Our microarray study demonstrated that with the exception of TNF, CCL20, and CXCL3, expression of all members of this cluster of genes was down-regulated at least 2-fold in spleens of CLP-induced A_{2A} KO mice as compared with their WT counterparts. Another group of genes contained mostly IFN-inducible genes, including chemokines, four metallothionein (*M1T*) genes, as well as a number of other genes, which included members of the 2',5'-oligoadenylate cyclase group (Jenner and Young, 2005). A_{2A}R KO mice had decreased levels of expression of all of these IFN-inducible genes. Such concerted down-regulation of inflammatory and IFN-regulated genes suggests that A_{2A}R may modulate a pathway(s) that is intrinsic to the induction of inflammatory responses in bacterial sepsis.

It is less clear whether any gene expression patterns can be defined based on the up-regulated genes in A_{2A} KO mice. Our analysis identified two functional clusters using the gene ontology nomenclature, in which most genes were up-regulated: lysosomal and endosomal clusters. The most notable of these genes were at least four members of the *MHC II* locus. Importantly, expression of the CIITA, which is the master regulator of MHC class II genes (Reith and Mach, 2001), was also increased in A_{2A} KO mice. We confirmed increased MHC II expression on macrophages from A_{2A} KO animals using flow cytometry. Because MHC II

proteins expressed on cells of the innate immune system are major players in Ag presentation to $CD4^+$ lymphocytes, cells that are crucial for adaptive immune responses against bacteria, increased expression of MHC II molecules in A_{2A} KO mice might constitute an important mechanism resulting in an improved antibacterial defense in these mice. This idea is supported by the observation that patients suffering from bare lymphocyte syndrome, caused by genetic defects in the *MHC2TA* gene encoding CIITA, often die of severe infectious and septic complications (Reith and Mach, 2001).

Our findings that markers of organ damage are similar in $A_{2A}R$ KO and WT mice demonstrate that the survival advantage conferred by A_{2A} receptor deficiency does not result from protection of the liver, kidney, or lung. Two key determinants in sepsis are bacterial clearance and the inflammatory response to the infection. $A_{2A}R$ deficiency potentiated bacterial clearance and decreased the expression of IL-6, which is a major initiator of the acute phase response, suggesting that the mechanisms leading to improved survival in $A_{2A}R$ KO mice may be several-fold. These mechanisms may also include various effects on the cardiovascular system or the CNS, as the function of all of these organ systems can be influenced by the ubiquitously expressed $A_{2A}R$ (Fredholm et al., 2001; Ralevic and Burnstock, 1998).

In addition to our results showing that adenosine via $A_{2A}R$ regulates immunity and mortality in sepsis, it was recently reported that A_1 and A_3 receptors are also important in governing mortality in mice subjected to CLP (Gallos et al., 2005; Lee et al., 2006). These results confirm the hypothesis that endogenous adenosine is an important regulator of immune events in mice undergoing sepsis.

From a therapeutic standpoint, it is worth emphasizing that pharmacological blockade of $A_{2A}R$ was as protective as genetic deletion in preventing CLP-induced death. In addition, pharmacological blockade of $A_{2A}R$ using a selective A_{2A} antagonist produced a similar change in the cytokine profile of CLP-induced mice to that achieved by targeted deletion of the A_{2A} gene. Even more importantly, delayed administration of the A_{2A} antagonist was also protective. Recent studies have demonstrated that a variety of targets hold promise as possible therapies of sepsis. These include activated protein C (Bernard et al., 2001), high-mobility group box 1 protein (Wang et al., 1999), macrophage migration inhibitory factor (Calandra et al., 2000), C5a (Ward, 2004), and the cholinergic system (Wang et al., 2004). Our results reveal that blockade of $A_{2A}R$ may offer a new strategy for the management of patients with sepsis and septic shock.

7.2. A_{2A} receptor-C/EBPβ axis is critical for IL-10 production after bacterial infection

In the second series of our experiments we have documented, that endogenous adenosine acting at A_{2A} receptors represents a crucial second signal in inducing the production of IL-10. In addition, our results unequivocally demonstrate that C/EBPβ is a central intracellular mediator on which signals from both *E. coli* and adenosine receptors converge to induce IL-10 gene expression.

Prior to our studies, stimulation of adenosine receptors had been shown to convert the phenotype of TLR4 (LPS)-stimulated macrophages from a pro-inflammatory to an anti-inflammatory one (Haskó and Cronstein, 2004; Yang et al., 2006; Ohta and Sitkovsky, 2001; Huang et al., 1997; Lukashev et al, 2004). Most notably, adenosine receptor activation had been documented to up-regulate the TLR4-induced production of IL-10 (Haskó et al., 1996; Haskó et al., 2000a; Khoa et al., 2001). In addition to TLR4-stimulated macrophages, adenosine enhanced IL-10 production in TNF-α- or hydrogen peroxide-stimulated human monocytes (Le Moine et al., 1996). Since during infection the host is challenged with whole microorganisms that possess multiple components capable of triggering IL-10 production, prior to our studies the relevance of these findings with stimuli such as TLR4 ligands, TNF-α, or hydrogen peroxide to bacterial infections was not clear.

As we demonstrated, genetic inactivation of A_{2A}R almost abolished (by > 95%) CLP-induced IL-10 production, which suggested that A_{2A}R activation by endogenous adenosine is required for IL-10 production after bacterial stimuli in vivo. By showing that *E. coli* is unable to trigger IL-10 production by macrophages isolated from A_{2A} KO mice, we provide in vitro evidence for the first time that A_{2A}R have a crucial role in inducing IL-10 production in conjunction with a physiologic stimulus, such as *E. coli.* In addition, we provide indirect proof that macrophages can serve as a source of endogenous adenosine at concentrations that are sufficient to engage A_{2A} receptors. Although macrophages have been documented to release adenosine even under resting conditions as well as in response to inflammatory stimuli, such as LPS (Ferrari et al., 1997; Sperlágh et al., 1998) the current study establishes that this endogenously released adenosine has immunomodulatory effects in vitro. In addition, the current results demonstrate that although A_{2B} receptors have a minor regulatory role in mediating the

stimulatory effect of exogenous adenosine on IL-10 production, A_{2A} receptors are crucially required for the effect of both exogenous and endogenous adenosine.

Recent data have shown that IL-10 production by macrophages was induced via TLR-mediated MyD88-dependent or TRIF-dependent pathways, as well as via non-TLR signals (Boonstra et al., 2006). For example, LPS induction of IL-10 via TLR4 was dependent on MyD88 and TRIF. Triggering through TLR9 by CpG showed that the induction of high levels of IL-10 was completely MyD88-dependent in macrophages. Unlike with LPS and CpG, our results clearly indicate that IL-10 production in *E. coli*–stimulated or *E. coli*/adenosine stimulated macrophages is completely MyD88-independent. Because of the complexity of *E. coli*, it is unclear at this juncture what *E. coli* components trigger IL-10 production by macrophages. It is interesting to note that a NOD2 ligand had synergistic effect on the induction of IL-10 on co-stimulation with a TLR agonist (Netea et al., 2005), indicating that NOD2 may have also contributed to the effect of *E. coli.* It is also worth noting that because *S aureus* also elicited IL-10 secretion, ligands common to both Gram-negative and Gram-positive bacteria, such as peptidoglycan and muramyl dipeptide, may have had a role in triggering IL-10 production. However, because adenosine synergistically augmented IL-10 release induced by lipoteichoic acid prepared from *S aureus*, a true TLR2 agonist, it is also possible that the adenosine–*S aureus* interaction was mediated by the specific Gram-positive bacterial product lipoteichoic acid prepared from *S aureus*.

We have shown recently that adenosine through A_{2B} receptors enhanced LPS-induced IL-10 production, and this effect was posttranscriptional, because adenosine had no effect either on LPS-induced IL-10 mRNA accumulation or on IL-10 promoter activity (Németh et al., 2005). In contrast, our current results indicate that the mechanism of action of adenosine in upregulating IL-10 production by *E. coli*–treated macrophages is transcriptional. Specifically, adenosine increased *E. coli*–induced IL-10 mRNA accumulation and upregulated IL-10 promoter activity, and the effect of adenosine was blocked by actinomycin D. Our data have clearly demonstrated that C/EBPβ is critical for the stimulatory effect of adenosine on IL-10 production in *E. coli*–treated murine macrophages. First, sequential deletion analysis of the IL-10 promoter as well as site-directed mutagenesis showed that a region containing C/EBPβ binding elements was responsible for the potentiating effect of adenosine in stimulating IL-10 promoter activity. Second, adenosine stimulated *E. coli*–induced nuclear accumulation and DNA binding of

C/EBPβ. Third, C/EBPβ-deficient macrophages failed to produce IL-10 in response to adenosine and *E. coli.* Interestingly, C/EBPβ was also important for the stimulatory effect *E. coli* even in the absence of exogenous adenosine, because C/EBPβ-deficient macrophages failed to respond to *E. coli* alone, and *E. coli* alone induced increases in the nuclear levels and DNA binding of C/EBPβ.

From studies performed with different cell types a number of different transcriptional, translational and posttranslational mechanisms have been proposed to regulate C/EBPβ activity. The most frequently reported mechanism is the increased induction of C/EBPβ gene transcription (Alam et al., 1992; Cardinaux et al., 2000). Regulation by nuclear translocation (Yin et al., 1996) and alternative translation initiation (An et al., 1996), phosphorylation (Trautwein et al., 1994), and acetylation (Joo et al., 2004; Xu et al., 2003) have also been described. Our data suggest that C/EBPβ activity is not regulated by nuclear translocation, because C/EBPβ was undetectable in cytoplasmic fractions both before and after adenosine and/or *E. coli* treatment.

The fact that adenosine alone, unlike *E. coli*, was not able to induce IL-10 production, mRNA accumulation, and C/EBPβ activation suggests that adenosine and *E. coli* act by different mechanisms on the C/EBPβ transcription factor system. Previous studies demonstrated that C/EBPβ promoter activity is induced during monocyte activation, and binding of CREB to C/EBPβ promoter elements is critical for the activation of C/EBPβ transcription (Berrier et al., 1998). Moreover, we have recently found that adenosine enhances CREB transcriptional activity in macrophages (Németh et al., 2003b). Thus, it is plausible that adenosine up-regulates *E. coli*-induced C/EBPβ accumulation via CREB activation. This explanation is supported by our findings that adenosine stimulates both CREB activation (Németh et al., 2003b) and IL-10 production in a p38-dependent manner. The fact that in contrast to the slow (4 h) accumulation of C/EBPβ in response to LPS reported previously (Bradley et al., 2003), we observed a rapid (30 minutes) increase in C/EBPβ abundance after *E. coli* exposure of macrophages suggests that the mechanism by which *E. coli* activates C/EBPβ is fundamentally different from not only that of adenosine but also that of LPS. Because adenosine can stimulate the activation of CREB (Németh et al., 2003b) but not C/EBPβ in the absence *E. coli*, it is plausible that this CREB activation by exogenous or endogenous adenosine plays a permissive role in activating C/EBPβ in response to *E. coli*. Further studies are warranted to investigate the mechanisms by which *E. coli* and adenosine activate the C/EBPβ system.

8. SUMMARY

Preclinical studies using both KO and pharmacological approaches have provided insights into the role of the various adenosine receptors in regulating the physiological response of the organism to sepsis. A_{2A} receptor inactivation by either gene deletion or administration of ZM241385 prevented CLP-induced mortality by a mechanism that involved decreased bacterial dissemination that appeared to be secondary to sustained immune system function.

Although adenosine receptor agonists have powerful immunomodulatory actions, the wide tissue distribution of adenosine receptors may limit their usefulness in the treatment of inflammatory diseases. Adenosine receptor antagonists, however, represent an ideal target for the therapy of certain immune-related disorders because their action is selectively targeted to the site of injury, where endogenous adenosine is released. For example, when there is need to enhance immune/inflammatory responses to rid the body of infections, such as in the immune-suppressed phase of sepsis, A_{2A} antagonists might be useful in enhancing the immune system's ability to fight and defeat invading pathogens. Potential side-effects with the A_{2A} receptor antagonist might include increased blood pressure and inflammation; however, based on the results of recent trials with A_{2A} antagonists to treat patients with Parkinson's disease, A_{2A} antagonists seem to be well-tolerated and devoid of side-effects (Chase et al., 2003; Schwarzschild et al., 2006).

Taken together, endogenously released adenosine contributes to the immune paralysis via activation of C/EBPβ/IL-10 axis through A_{2A} receptors, an effect that prevents the host from an effective response to the infectious challenge represented by sepsis. A_{2A} receptor antagonists, thus, have a potential as therapeutic agents in the treatment of this devastating disease.

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10. **LIST OF PUBLICATIONS**

This thesis is based on the following publications:

Németh, Z.H., **Csóka, B.**, Wilmanski, J., Xu, D., Lu, Q., Ledent, C., Deitch, E.A., Pacher, P., Spolarics, Z., Haskó, G. (2006) Adenosine A2A receptor inactivation increases survival in polymicrobial sepsis. Journal of Immunology, **176**, 5616-5626**. IF: 6.293**

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Other publications:

Csóka, B., Zeke, T., Kókai, E., Doonan, J., Fox, H., Fehér, Z., Dombrádi, V. **(2002)** Expression of the bimG gene from Aspergillus nidulans in Neurospora crassa. Fungal Genetic Newsletters, **49**, 13-14.

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11. KEYWORDS

adenosine; adenosine receptors; inflammation; innate immunity; sepsis; inflammatory cytokines; interleukin-10; C/EBPβ

adenozin; adenozin receptorok; gyulladás; öröklött immunitás; szepszis; gyulladásos citokinek; interleukin-10, C/EBPβ

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