THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)

ROLE OF A_{2A} ADENOSINE RECEPTORS IN REGULATING SEPSIS

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1. INTRODUCTION

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. 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. 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. 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. However, 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. It appears that the excessive inflammatory response is only the cause of MOF and death in a small number of patients. On the other hand, 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. Furthermore, it has recently become evident that one of the most important factors contributing to the immune paralysis seen during MOF is macrophage dysfunction. Studies have indicated that while early on after the onset of sepsis there is initially an activation of macrophage pro-inflammatory cytokine release, over time this is followed by a prolonged and profound state of immunosuppression, which is characterized by substantially diminished macrophage effector functions.

Regulation and source of IL-10 production in response to PAMPs

Specific macrophage defects in septic patients include a substantial increase in the production of the crucial anti-inflammatory cytokine IL-10. 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. Much less is known about the regulation of production of IL-10. The induction of IL-10 production can be achieved by a broad spectrum of stimuli through multiple Pathogen Recognition Receptors (PRRs). TLR2 agonists such as lipopetides are potent inducers of IL-10, and prolonged ERK activation upon TLR2 stimulation seems to play a significant role in the production of a high level of IL-10. TLR6 has also a prominent role in the production of this anti-inflammatory cytokine in response to fungal infections. 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. Between the TLR-associated adapter molecules and the intracellular kinases, the TRAF3 seems a major regulator of the IL-10 expression, because 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. IL-10 is an inducible gene and several transcriptional factors have been implicated in its regulation, which include Sp1, Sp3, C/EBPδ and C/EBPβ, STAT3, and c-Maf. IL-10 gene expression also requires transient remodeling of the IL-10 promoter that occurs as a result of MAPK activation. In addition, it has shown that IL-10 production is also regulated at various posttranscriptional levels, including alterations in mRNA stability and translation efficacy.

Physiologic and pharmacologic roles of adenosine

Adenosine is produced in response to cellular stress and damage and elevations in extracellular adenosine are found in conditions of ischemia, hypoxia, inflammation, and trauma. 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 and CD73. 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, whereas plasma adenosine concentrations in healthy individuals are <1 μM.

Adenosine receptors

Adenosine mediates its effects via engaging A_1, A_{2A}, A_{2B}, and A_3 adenosine receptors. 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. All four adenosine receptors have been detected on both monocytes and macrophages. Whereas the expression of adenosine receptors is low on quiescent monocytes, their number increases during differentiation into macrophages. 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. 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.

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 increase or decrease cAMP accumulation, adenosine receptors were initially classified as A_1 and A_2, receptors. 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. Additionally, A_1 receptor activation can results in K^+ channel opening and Ca^+ channel inhibition. A_2 receptors have been divided into two groups: high-affinity A_{2A} receptors and low-affinity A_{2B} receptors. A_{2A} receptors, signal largely by the adenylate cyclase–cAMP–PKA pathway. A_{2B} receptor stimulation can elicit adenylyl cyclase activation via Gs and phospholipase C (PLC) activation via the Gq subunit. The signaling pathways associated with A_3 receptor activation comprise Gi-mediated inhibition of adenylyl cyclase and Gq-mediated stimulation of PLC. In addition, A_3 receptors can utilize the PLD, RhoA, WNT, MAP kinase and PI3 kinase pathways.

Adenosine receptors affect homeostasis through interfering with immune system function

Although the immune response to acute tissue injury has an essential role

in preventing 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. In certain scenarios and cell types, however, adenosine receptors are also able to provoke pro-inflammatory effects.

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, suggesting that A_1 receptor activation has a beneficial effect on the outcome of intra-abdominal sepsis.

The most potent anti-inflammatory and immunosuppressive effects of adenosine are generally attributed to occupancy of A_{2A} receptors expressed on neutrophils, monocytes/macrophages, and lymphocytes. 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. Multiple lines of evidence indicate that A_{2A} receptors are crucial for adenosine-mediated protection against pathophysiological conditions, such as ischemia-reperfusion injury and infectious diseases. 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. Furthermore, this protective effect disappeared in A_{2A} KO animals in this hyperacute model of sepsis.

A_{2B} adenosine receptors are increasingly recognized as important orchestrators of inflammation via modulating endothelial cell and dendritic cell function. *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. Additionally, 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.

The A_3 receptor subtype has a complex role in regulating inflammation, as both proinflammatory and anti-inflammatory effects have been demonstrated. Treatment of $ADA^{-/-}$ (adenosine deaminase deficient) 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. 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. 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.

2. AIMS

Based on the observations by our lab as well as others that A_{2A} receptors are immunosuppressive, 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 transcriptional regulation of IL-10 mRNA in macrophages challenged with *E. coli*

3. METHODS

Cecal ligation and puncture

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.

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 at 37 °C. After 24 h, the number of bacterial colonies was counted.

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 and according to the manufacturer's instructions.

Western blot analysis for markers of apoptosis

Samples of spleen and thymus were homogenized in a Dounce homogenizer in modified radio-immunoprecipitation (RIPA) buffer. A total of 40 µg of sample was separated on a 4-12 % Tris-glycine gel and transferred to nitrocellulose membrane. The membranes were probed with polyclonal rabbit anti-cleaved caspase-3, polyclonal rabbit anti-cleaved poly (ADP-ribose) polymerase (PARP), or polyclonal goat anti-β-actin antibody, and subsequently incubated with a secondary horseradish peroxidase-conjugated anti-rabbit or anti-goat antibody. Bands were detected using the ECL Western Blotting Detection Reagent.

Apoptosis detection by TUNEL staining

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.

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 glass ground to dissociate cells. The degree of apoptotic cell death was quantified using fluorescent-labeled Annexin V containing kit. MHC II expression was determined using anti-mouse APC-labeled MHC II antibody. Cell suspensions from peritoneal lavage and spleen were added to tubes pre-loaded with the corresponding fluorescent-labeled antibodies. Analyses were performed using a FACScan flow cytometer and CellQuest software.

Affymetrix genechip analysis of spleen samples and RT-PCR

Total RNA was prepared from spleen samples using TRIZOL and the samples were further purified using RNeasy mini kit. cRNA transcription, and cRNA hybridization to Affymetrix murine microarrays, which contain 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. 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. The reaction mix was incubated at 42 °C for 15 min for reverse transcription. Reverse transcriptase-generated DNA was amplified using Expand high fidelity PCR system.

Cell cultures and 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. TG-elicited mouse peritoneal macrophages, C/EBPβ-deficient and control macrophages, and RAW 264.7 macrophages 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.

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. Real-time PCR using SYBR Green I reaction was performed according to a protocol recommended by the manufacturer, and reactions were incubated in a Roche Lightcycler.

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

The mutated IL-10 promoter was prepared by gene synthesis by GenScript Co. The mutated DNA fragment was inserted into pGL2-Basic vector in HindIII-XhoI sites. The mutation sites were confirmed by DNA sequencing.

Transient transfection of RAW 264.7 cells

RAW 264.7 cells were transiently transfected using FUGENE 6.0 transfection reagent. 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, or C/EBP consensus mutant IL-10 promoter luciferase construct, or an A_{2A} adenosine receptor over-expressing pA_{2A}-CMV construct, or C/EBP reporter plasmid in 200 μl of medium per well. All transfections were performed at 37 °C overnight. For reporter assays, whole cell extracts were prepared using 80 μl of 1x passive lysis buffer. Luciferase activity was determined from 20 μl of cell extract.

Preparation of pSilencer plasmids with siRNA constructs and RAW 264.7 Cell Stable Transfectants

Specific hairpin siRNA oligonucleotide constructs for murine TRAF6 silencing were designed using the Ambion siRNA design algorithm, and cloned into the pSilencer3.1-H1neo expression vector. RAW 264.7 cells were transfected with siRNA-containing plasmids using Superfect® transfection reagent for 3 h. The medium was then removed and replaced with fresh medium supplemented with 0.8 % (w/v) G418. G418-resistant colonies were selected after about 2 weeks of growth. The expression level of TRAF6 was confirmed by real-time RT-PCR.

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

Cells were 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 and incubated for 15 min on ice with occasional vortexing. After centrifugation, supernatants (cytosolic extracts) were saved for further studies. Two pellet volume of nuclear extraction buffer was added to the nuclear pellet and incubated on ice for 30 minutes with occasional wortexing. Nuclear proteins were isolated by centrifugation. The C/EBP consensus oligonucleotide probe was labeled with [{γ}-^{32}P]ATP using T4 polynucleotide kinase and purified in MicroSpin G-50 columns. For the EMSA analysis, 10 µg of nuclear proteins was preincubated with C/EBP EMSA binding buffer 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 at room temperature for 45 minutes. Protein-nucleic acid complexes were resolved using a non-denaturing polyacrylamide gel consisting 4 % acrylamide and run in 0.5x Tris borate-EDTA buffer at constant current (35 mA). Gels were transferred to 3M paper, dried under vacuum at 80 °C for 40 min, and exposed to photographic film at –80 °C with an intensifying screen.

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. For phospho-p38 and phospho-p42/44 Western blotting peritoneal macrophages were homogenized in modified RIPA buffer. The lysates were centrifuged, and the supernatant was recovered. A total of 30 µg of protein samples were separated on 4-12 % Tris-Glycine gel and transferred to nitrocellulose membrane. The membranes were probed with polyclonal rabbit anti-phospho-p42/44, monoclonal mouse anti-phospho-p38, or polyclonal goat anti-β-actin antibody, and subsequently incubated with a secondary HRP-conjugated anti-rabbit IgG antibody. Bands were detected using ECL Western Blotting Reagent.

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.

4. RESULTS

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 ~70% when recorded on day 5 after the CLP procedure. The mortality rate of A_{2A} KO mice was significantly lower on each day with a ~35% mortality rate on day 5 after CLP. 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.

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

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. 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. Additionally, CLP-induced concentrations of MIP-2 were diminished in A_{2A} KO mice as compared with their WT counterparts when measured at 16 h. Cytokine concentrations subsided to comparable levels in septic A_{2A} KO and WT mice by 48 h.

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. Additionally, white blood cell counts, lymphocyte numbers, and platelet counts dropped significantly in CLP-subjected mice when compared with shams. However, there were no differences in the levels of these markers or

hematological parameters between the WT and KO groups either at 16 or 48 h after the CLP procedure.

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

Widespread lymphocyte depletion induced by apoptosis may contribute to the immunosuppression that occurs in sepsis. PARP is a major downstream target of activated caspase-3 and is cleaved by this enzyme during apoptosis. Therefore, we tested assessed 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. In contrast, the cleavage of both caspase-3 and PARP was markedly suppressed in A_{2A}R KO mice. Caspase-3 activation leads to the appearance of late apoptotic signs, such as phosphatidylserine exposure on the outer cell membrane. 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. We found that CLP significantly up-regulated phosphatidylserine exposure on thymocytes from both A_{2A}R KO and WT animals. Although, thymocytes from KO animals exhibited 34% lower phosphatidylserine exposure than those from WT animals, this difference did not reach statistical significance. Because phosphatidylserine exposure is only marginally detectable in the spleen of mice that have undergone CLP, we used TUNEL immunohistochemistry to quantify late apoptotic events in septic A_{2A}R KO and WT animals. CLP significantly increased the fraction of TUNEL-positive cells in WT mice and the percentage of TUNEL-positive cells in spleens of KO mice exposed to CLP was significantly lower.

Splenic gene expression profile in septic A_{2A}R 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. 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. Importantly, IL-10, IL-6, and MIP-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. 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 splenic and peritoneal macrophages from septic KO animals displayed markedly elevated MHC II expression levels as compared with cells from WT mice.

Pharmacological inactivation of A_{2A}R 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 starting at the time of resuscitation exhibited significantly improved survival compared with vehicle-treated mice. 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. 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. Finally, we observed that ZM241385 administration starting 2 h after resuscitation was still protective, indicating a potential clinical use of A_{2A}R blockade in acutely developing septic conditions.

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

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 begin to examine 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. Challenging *E. coli*–treated WT cells with adenosine dramatically increased IL-10 levels Moreover, the combination of adenosine and

E. coli was also ineffective in triggering IL-10 release by A_{2A} KO macrophages. We next treated macrophages with *E. coli* in the presence or absence of various adenosine receptor agonists. 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. 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. *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.

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

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. Exogenous adenosine synergistically upregulated *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 in TLR4 WT and TLR4 KO cells. 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. We observed that *S. aureus* increased IL-10 levels, which were further enhanced by adenosine. 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.

Because of the central role of MyD88 in many bacteria-induced macrophage responses, 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.

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. 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. In agreement with previous data, proinflammatory IL-6 release by *E. coli*–induced macrophages was decreased in TRAF6 shRNA–expressing cells compared with controls, 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, 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, indicating that TRAF6 is not required for the effect of *E. coli* and adenosine in inducing IL-10, but TRAF6 negatively modulates this synergistic interaction.

Both TLR ligands and adenosine have been reported to be able to activate both p38 and p42/44 in macrophages. Moreover, both p38 and p42/44 have been implicated in the regulation of IL-10 production in response to various TLR ligands. 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 found that *E. coli* increased p42/44 MAPK activation but not that of p38. When macrophages were treated with *E. coli* and adenosine together, p38 activation was increased, but the activation of p42/44 was not changed, compared with *E. coli* treatment alone. Additionally, 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.

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

First, we determined IL-10 mRNA levels from 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. In addition, 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.

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. 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*. Because RAW 264.7 macrophages express low levels of the A_{2A} receptor endogenously, we transfected these cells with an A_{2A} receptor-expressing construct. 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.

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. 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 the effect of adenosine was completely abolished by deletion of sequences between –438 and –376. 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.

Because the IL-10 promoter sequence between –438 and –376 contains binding sites for C/EBP transcription factors, we 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. 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. 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. The C/EBP family contains several members, and within this family C/EBPβ and C/EBPδ are both expressed in macrophages. 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, and we found that only the C/EBPβ antibody shifted the DNA–protein complex. 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. However, three C/EBPβ isoforms corresponding to the previously described 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. Furthermore, adenosine enhanced the nuclear accumulation of all three isoforms in the presence but not absence of *E. coli*. To provide further insight into the role of C/EBPβ in regulating IL-10 production, C/EBPβ WT and KO immortalized macrophage cell lines were stimulated with *E. coli* and adenosine, and IL-10 release was measured. 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.

5. 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.

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.

6. 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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Himer, L., **Csóka B.**, Selmeczy, Z., Koscso, B., Pócza, T., Pacher, P., Németh, Z.H., Deitch, E.A., Vizi, E.S., Cronstein, B.N., Haskó, G. (2010) Adenosine A2A receptor activation protects CD4+ T lymphocytes against activation-induced cell death. *FASEB J*. 24(8):2631-40. **IF: 6.791**

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Csóka, B. and Haskó, G. (2011) Adenosine, inflammation pathways and therapeutic challenges. *Joint Bone Spine* 78(1):4-6. **IF:2.250**

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