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

# Role of adenosine receptors in regulating classical and alternative activation of microglia and macrophages

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# 1. Introduction

## Microglia: mononuclear phagocytes of the brain

Mononuclear phagocytes in both the central nervous system and periphery are central to orchestrating innate immune responses to pathogens. Microglia, the main mononuclear phagocyte population in the central nervous system can be found in three morphological states, which are ramified, activated and amoeboid. Microglia, similar to other mononuclear phagocytes, become activated by recognizing molecules that are associated with invading pathogens or endogenous danger signals through pathogen recognition receptors. One of the most important families of pathogen recognition receptors is the Toll-like receptor (TLR) family, the members of which are expressed by microglia. Stimulation of microglial TLRs with bacterial cell wall products peptidoglycan (PGN) or lipopolysaccharide (LPS) results in secretion of proinflammatory cytokines such as interleukin (IL)-6 and tumor necrosis factor (TNF)-α, both of which are major players in neuroinflammation in addition to their homeostatic physiological roles.

Since prolonged inflammatory responses in the brain contribute to the pathology of a variety of brain disorders, and the regenerative ability of neural tissue is limited, the tight regulation of inflammation in the CNS is crucial. The anti-inflammatory cytokine IL-10 is produced by microglia, astrocytes and infiltrating T cells in the brain and it plays neuroprotective roles in animal models of numerous CNS disorders, including multiple sclerosis, traumatic brain injury and Parkinson's disease.

## Alternative activation of macrophages

Macrophages can be activated not only by pathogens but also by different cytokines. The T helper 1 ($T_H1$) cytokine interferon (IFN)-γ induces a proinflammatory phenotype in macrophages. This population is called classically activated or M1 macrophages. Classically activated macrophages produce reactive nitrogen and oxygen species (nitric oxide, peroxynitrite, hydrogen peroxide, superoxide) and proinflammatory cytokines, such as TNF-α, IL-6, and IL-12, and they participate in the defense against microbial infections. In contrast, alternative macrophage activation occurs in a $T_H2$ cytokine environment, arising for example during parasitic disease or wound healing, which imparts immunomodulatory and anti-inflammatory

rather than proinflammatory properties on macrophages. The most important $T_H2$ cytokines that induce the alternatively activated phenotype of macrophages are IL-4 and IL-13. IL-4 and IL-13 induce a unique, partly overlapping gene expression signature in macrophages through intracellular signaling pathways involving transcription factors such as signal transducer and activator of transcription (STAT)-6 and CCAAT enhancer binding protein β (C/EBPβ). One of the hallmark genes induced by IL-4 or IL-13 in macrophages is *arginase-1*. Arginase-1 in alternatively activated macrophages contributes to wound healing by facilitating the deposition of extracellular matrix and also regulates IFN-γ-induced NO secretion. Additional proteins upregulated by IL-4 or IL-13 that are appreciated as markers of alternative activation are tissue inhibitor of metalloproteinase (TIMP)-1, which regulates extracellular matrix turnover and cell growth, and macrophage galactose-type C-type lectin (mgl)-1, which binds glycoproteins expressed by helminthes and tumor cells.

**Adenosine is an extracellular signaling molecule**

Adenosine is a purine nucleoside, which is an essential component of intracellular metabolic processes and is a precursor of both nucleic acid and adenosine 5'-triphosphate. Adenosine can be released into the extracellular space in response to both metabolic disturbances and other types of insults, which include inflammation, physical damage, and apoptosis. It has become clear in the last few decades that extracellular adenosine has wide-ranging regulatory effects in all organs and tissues. The cellular effects of adenosine are mediated by ARs, which are members of the G protein-coupled family of receptors. The four subtypes of ARs are the $A_1$, $A_{2A}$, $A_{2B}$ and $A_3$AR. The $A_1$AR and $A_3$AR are coupled to $G_i$ proteins, which inhibit the enzyme adenylyl cyclase thereby decreasing intracellular cyclic AMP (cAMP) concentrations, while the $A_{2A}$AR and $A_{2B}$AR couple to $G_s$ proteins which activate adenylyl cyclase followed by the increase of intracellular cAMP concentration.

Adenosine influences a wide range of immune functions of a variety of cell types, including mononuclear phagocytes, lymphocytes, natural killer cells, and mast cells. Microglial functions regulated by ARs include proliferation, migration and secretion of proinflammatory mediators and neurotrophic factors. Adenosine also decreases the expression of proinflammatory cytokines, such TNF-α and IL-12 by classically activated peripheral monocytes and

macrophages. In addition to the inhibition of proinflammatory functions of macrophages, adenosine augments the production of IL-10 through $A_{2A}$ARs and $A_{2B}$ARs.

## 2. Aims of the study

The last 2 to 3 decades have seen a dramatic increase in our understanding of the role of ARs in regulating the function of mononuclear phagocytes. However, several gaps remained. For example, while the role of ARs in regulating proinflammatory cytokine production by microglia had been addressed prior to our studies, the effect of adenosine on IL-10 production by microglia had not been studied. In addition, prior to our studies, the effect of adenosine had only been studied in conjunction with classically activated macrophages and the role of adenosine in regulating alternative macrophage activation had not been explored. To begin to fill these gaps, we sought to address the following aims:

1. Determine the effect of adenosine on IL-10 production by microglia activated with TLR ligands. Compare the effect of adenosine on IL-10 production with its effect on IL-6, IL-12 and TNF-α production.
2. Identify the AR subtype mediating the effect of adenosine on IL-10 production by microglia
3. Examine the intracellular signaling pathways that mediate the effect of adenosine on IL-10 production by microglia
4. Delineate the effect of adenosine on IL-4 or IL-13-induced alternative macrophage activation
5. Elucidate the AR subtypes responsible for the effect of adenosine on alternative macrophage activation
6. Determine the intracellular signaling pathways that mediate the effect of adenosine on alternative macrophage activation

## 3. Materials and Methods

### Experimental animals and cell cultures

Primary microglia were obtained from cerebral cortices of 1- to 3-day-old C57BL/6J mice. Peritoneal macrophages were obtained from mice injected intraperitoneally with 3 ml of sterile thioglycollate broth. In addition, BV-2 murine microglial cell line, RAW 264.7 macrophages and C/EBPβ KO or WT immortalized macrophages were used for the experiments.

### Enzyme-linked immunosorbent assay (ELISA) for determining cytokine production

IL-10, TNF-α, IL-6, IL-12 and TIMP-1 levels were determined from supernatants of BV-2 cells, primary microglial cells, RAW 264.7 macrophages or peritoneal macrophages after different treatments using ELISA Duoset kits.

### RNA extraction, cDNA synthesis, and real-time polymerase chain reaction (PCR)

Total RNA was prepared from peritoneal macrophages, BV-2 or RAW 264.7 cells using Trizol reagent according or from primary microglia using RNeasy Mini Kit according the manufacturer's protocol and reverse-transcribed using High Capacity cDNA Reverse Transcription kit. For detection of IL-10, ARs, arginase-1, TIMP-1, and mgl-1 mRNA, a real-time PCR commercial kit was used, and all data were normalized to constitutive rRNA values (18S).

### Transient transfection of cells and luciferase assay

BV-2 or RAW 264.7 cells were transiently transfected using Polyfect or FUGENE 6.0 transfection reagents respectively. BV-2 cells were transfected with IL-10 or CREB reporter plasmids and RAW 264.7 cells were transfected with arginase-1 or C/EBP reporter plasmid. After different treatment cell extracts were prepared from transfected cells, and Luciferase activity was determined using 20 μl of cell extract.

### Whole cell protein isolation and Western blotting

For Western blotting peritoneal macrophages, BV-2 cells or RAW 264.7 cells were homogenized in modified RIPA buffer followed by the separation of samples on 10 % Tris-

Glycine gel and the transfer to nitrocellulose membranes. The membranes were probed with different antibodies, and subsequently incubated with a secondary HRP-conjugated anti-rabbit IgG antibody. Bands were detected using ECL Western Blotting Reagent.

**Silencing CREB using lentivirally-delivered shRNA**

BV-2 or RAW 264.7 cells were transduced with Mission Lentiviral particles containing CREB-specific shRNA or non-targeting shRNA. Stable shRNA expressing cell lines were selected by culturing in the presence of the antibiotic puromycin. The efficiency of silencing was tested by Western blotting using CREB specific monoclonal antibodies.

**Chromatin immune precipitation (ChIP)**

ChIP was performed using a ChIP assay kit from Millipore according to the manufacturer's protocol using phospho-CREB-specific monoclonal antibodies. PCR reaction was performed using DNA purified from ChIP samples using primers specific for the IL-10 promoter region between -376 and -158 bp relative to the transcription start site.

**Determination of arginase activity from macrophage cell extracts**

Cell extracts were prepared from RAW 264.7, peritoneal macrophages or C/EBPβ KO or WT cells in 10 mM Tris-HCl (pH 7.4) containing 0.4% Triton X-100. Arginase activity in the cell extracts was determined using a commercially available arginase assay kit.

## 4. Results

**Adenosine augments IL-10 and inhibits IL-6, TNF-α and IL-12 production by activated microglia**

Our results show that PGN enhanced dramatically IL-10, IL-6 and TNF-α production by primary microglia. Adenosine treatment augmented the PGN-induced secretion of IL-10 and inhibited the PGN-induced secretion of IL-6 and TNF-α. Next, we investigated the effect of adenosine on cytokine production by BV-2 cells stimulated with PGN or LPS. The combination of adenosine and PGN or LPS synergistically induced IL-10 production by BV-2 cells. PGN or

LPS induced IL-6, TNF-α, and IL-12 production by BV-2 cells, all of which was inhibited by adenosine.

**The effect of adenosine on IL-10 production by BV-2 cells is $A_{2B}$AR-dependent**

To determine which AR is responsible for the IL-10-increasing effect of adenosine, we first treated BV-2 cells with increasing concentration of various AR agonists immediately followed by treatment with PGN or LPS for 24 hours. Our results showed that the non-selective AR agonist NECA was the most potent IL-10 enhancer in BV-2 cells. In the next step, we tested which antagonist could reverse the increasing effect of NECA. We found that only the specific $A_{2B}$AR antagonist MRS1754 inhibited the stimulatory effect of NECA on IL-10 production by PGN- or LPS-treated cells. We conclude that the stimulatory effect of adenosine on IL-10 production by BV-2 cells is $A_{2B}$AR-dependent. We next studied the level of expression of AR mRNA in BV-2 cells and primary microglia using real-time PCR We found that the levels of $A_{2B}$AR and $A_3$AR were highest in BV-2 cells, and in primary microglia, the $A_{2B}$AR was the dominantly expressed receptor. PGN and LPS treatment increased the level of $A_{2B}$AR mRNA in both cell types.

**AR activation augments IL-10 mRNA accumulation in a CREB-dependent manner**

Our results showed that NECA augmented IL-10 mRNA levels in BV-2 cells exposed to PGN, which effect was completely abolished in cells pre-treated with the transcription inhibitor actinomycin D. Using mutant IL-10 promoter constructs we showed that the region between -376 and -158 bp relative to transcription start site was necessary for the augmenting effect of NECA on IL-10 promoter activity. Since there is a cAMP responsive element binding protein (CREB)-binding site in this region, we next tested the effects of NECA in cells transfected with CREB-reporter construct in which luciferase activity was driven by tandem CREB-binding sequences (pCRE). NECA augmented luciferase activity in cells transfected with pCRE but failed to do so in cells transfected with a control plasmid indicating that NECA can upregulate CREB-dependent transcription. The role of CREB was confirmed by chromatin immunoprecipitation analysis and RNA interference. In addition, we showed that activation of p38 mitogen-activated

protein kinase and phosphatidylinositol 3-kinase by adenosine was necessary for the stimulatory effect of adenosine on IL-10 production.

**Adenosine augments IL-4- and IL-13-induced alternative macrophage activation by a TLR4-independent mechanism**

To study the effects of AR stimulation on alternative activation of macrophages we treated RAW 264.7 and peritoneal macrophages with adenosine or NECA before challenging the cells with IL-4 or IL-13. Our results showed that adenosine or NECA augmented arginase activity as well as mRNA and protein levels of arginase-1, and TIMP-1 in IL-4- or IL-13-treated macrophages, but not in control cells. Adenosine enhanced IL-4-induced TIMP-1 release by both wild-type (WT) and TLR4 knockout (KO) macrophages showing that the effect is TLR4-independent. Additionally, adenosine or NECA augmented mgl-1 mRNA accumulation in IL-4-challenged macrophages.

**Role of $A_{2A}$ and $A_{2B}$ARs in mediating the stimulatory effect of adenosine on alternative macrophage activation**

We found that NECA was the most efficacious AR agonist in augmenting IL-4-induced arginase-1 and TIMP-1 expression, indicating the role of $A_{2B}$ARs. Both genetic blockade and pharmacological antagonism of $A_{2B}$ARs inhibited the NECA enhancement of both arginase activity and TIMP-1 production. Our results indicated that the $A_{2A}$AR also contributed, although to a lesser degree, to the upregulation of TIMP-1 production, as both adenosine and NECA were less efficacious in upregulating TIMP-1 production by macrophages obtained from $A_{2A}$AR KO mice than from their WT littermates.

**C/EBPβ is required but CREB and STAT-6 are dispensable for the stimulatory effect of adenosine on arginase-1 expression in IL-4-stimulated macrophages**

Using a previously described arginase-1 promoter luciferase construct we showed that either adenosine or NECA enhanced IL-4-induced arginase-1 promoter activity. We then focused our effort on 3 transcription factors that have been implicated in regulating alternative macrophage activation, C/EBPβ, CREB and STAT-6. We found that either adenosine or IL-4

alone was unable to increase C/EBP transcriptional activity; however, adenosine and IL-4 together synergistically induced C/EBP transcriptional activity. To provide further insight into the role of C/EBPβ in regulating arginase-1 expression, C/EBPβ WT and KO immortalized macrophage cell lines were stimulated with adenosine and IL-4, and then arginase activity was measured. IL-4 increased arginase activity in both WT and KO macrophages, and adenosine upregulated this activity by approximately 2-fold in WT but not KO macrophages. In contrast, adenosine augmented IL-4-induced arginase-1 activity in RAW 264.7 macrophages after downregulation of CREB using lentivirally-delivered shRNA, indicating that CREB is not necessary for the stimulatory effect of adenosine on arginase expression. Additionally, we excluded a role for STAT-6 in mediating the augmenting effect of adenosine on arginase activity, as we found that IL-4-induced STAT-6 activation was not increased but actually decreased by adenosine.

We next sought to dissect the role of intracellular kinases in mediating the stimulatory effect of adenosine on alternative macrophages activation. We found using Western blot analysis that adenosine alone and in combination with IL-4, but not IL-4 alone, upregulated p38 activation. Pharmacological inhibition of p38 pathway prevented the increasing effect of adenosine on both arginase activity and TIMP-1 release, confirming that p38 mediates the stimulatory effect of adenosine on alternative macrophage activation.

## 5. Summary

In conclusion, we have shown that the $A_{2B}$AR regulates the function of both classically activated microglia and alternatively activated macrophages. $A_{2B}$AR stimulation upregulates IL-10 production by microglia activated with TLR ligands. $A_{2B}$ARs augment arginase-1 expression and activity in alternatively activated macrophages. $A_{2B}$ARs, and to a lesser extent $A_{2A}$ARs, also increase IL-4-induced TIMP-1 production. As the $A_{2B}$AR exerts a regulatory role in wide range of macrophage populations with different activation profiles, targeting this receptor has a widespread therapeutic potential in neuroinflammatory and neurodegenerative diseases and conditions involving inappropriate $T_H2$ responses and dysfunctional alternatively activated macrophages.

## 6. List of publications

This dissertation is based on the following publications:

**Koscsó B**, Csóka B, Selmeczy Z, Himer L, Pacher P, Virág L and Haskó G: Adenosine augments IL-10 production by microglial cells through an A2B adenosine receptor-mediated process. *Journal of Immunol*. 2012 Jan 1;188(1):445-53. **IF: 5.745**

Csóka B, Selmeczy Z, **Koscsó B**, Németh ZH, Pacher P, Murray PJ, Kepka-Lenhart D, Morris SM Jr, Gause WC, Leibovich SJ and Haskó G: Adenosine promotes alternative macrophage activation via A2A and A2B adenosine receptors. *FASEB Journal*. 2012 Jan; 26(1):376-86. **IF: 6.515**

Other publications:

Csóka B, Németh ZH, Selmeczy Zs, **Koscsó B**, Pacher P, Vizi ES, Deitch EA and Haskó G: Role of $A_{2A}$ adenosine receptors in regulation of opsonized *E. coli* induced macrophage function. *Purinergic Signalling*. 2007. 3(4):447-452.

Himer L, Csóka B, Selmeczy Z, **Koscsó B**, Pócza T, Pacher P, Németh ZH, Deitch EA, Vizi ES, Cronstein BN, Haskó G: Adenosine A2A receptor activation protects CD4+ T lymphocytes against activation-induced cell death. *FASEB Journal.* 2010 Aug;24(8):2631-40. **IF: 6.515**

Csóka B, Németh ZH, Rosenberger P, Eltzschig HK, Spolarics Z, Pacher P, Selmeczy Z, **Koscsó B**, Himer L, Vizi ES, Blackburn MR, Deitch EA, Haskó G: A2B adenosine receptors protect against sepsis-induced mortality by dampening excessive inflammation. *Journal of Immunology*. 2010 Jul 1;185(1):542-50. **IF: 5.745**

**Koscsó B**, Csóka B, Pacher P and Haskó G: Investigational $A_3$ adenosine receptor targeting agents. *Expert Opinion on Investigational Drugs.* 2011 Jun; 20(6):757-68. **IF: 4.337**

Haskó G, Csóka B, **Koscsó B**, Chandra R, Pacher P, Thompson LF, Deitch EA, Spolarics Z, Virág L, Gergely P, Rolandelli RH and Németh ZH: Ecto-5'-nucleotidase (CD73) decreases mortality and organ injury in sepsis. *Journal of Immunology*. 2011 Oct 15;187(8):4256-67. **IF: 5.745**