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# Adenosine modulates LPS-induced cytokine production in porcine monocytes

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ABSTRACT

Adenosine plays an important role during inflammation, particularly through modulation of monocyte function. The objective of the present study was to evaluate the effect of synthetic adenosine analogs on cytokine production by porcine monocytes. The LPS-stimulated cytokine production was measured by flow cytometry and quantitative real-time PCR. Adenosine receptor expression was measured by quantitative real-time PCR. The present study demonstrates that adenosine analog N-ethylcarboxyamidoadenosine (NECA) down-regulates TNF-α production and up-regulates IL-8 production by LPS-stimulated porcine monocytes. The effect was more pronounced in $CD163^-$ subset of monocytes compared to the $CD163^+$ subset. Although both monocyte subsets express mRNA for A1, A2A, A2B and A3 adenosine receptors, the treatment of monocytes with various adenosine receptor agonists and antagonists proved that the effect of adenosine is mediated preferentially via A2A adenosine receptor. Moreover, the study suggests that the effect of NECA on porcine monocytes alters the levels of the cytokines which could play a role in the differentiation of naive T cells into Th17 cells. The results suggest that adenosine plays an important role in modulation of cytokine production by porcine monocytes.



## 1. Introduction

Adenosine is a ubiquitous purine nucleoside released rapidly from metabolically active cells and generated by degradation of adenosine triphosphate. Adenosine is present in virtually all cells, and it modulates numerous cell functions. Extracellular adenosine is released following cellular challenge or tissue injury and plays an important role during inflammation, ischemia or carcinogenesis [1,2].

Considering the important role of adenosine during inflammation, the modulatory effect of adenosine on various monocyte/macrophage functions has been thoroughly described in humans [2,3]. On the other hand, although the pig serves as an important animal model for understanding human innate immunity [4], the knowledge of the effect of adenosine on porcine innate immune cells is limited to NK cells [5]. As it was shown previously, monocytes play a crucial role during the inflammatory response in pigs [6]. Based on expression of CD163 molecule on their surface, porcine monocytes can be divided into two main subsets: the $CD163^-$ and $CD163^+$ [6]. From the functional point of view the $CD163^+$ subset is the one which migrates to the site of inflammation [6] where it becomes an important source of pro-inflammatory cytokines [unpublished data]. Therefore, from this perspective, it is important to explore and complete what is known about the effect of adenosine on cytokine production by porcine monocytes and their subsets. As our previous study showed that adenosine could modulate cytokine production by porcine lymphocytes [7] it could be assumed that adenosine might also modulate cytokine production by porcine monocytes.

The anti-inflammatory effect of adenosine is mediated via adenosine receptors (ARs) and subsequent intracellular pathways [8]. Four distinct AR subtypes (A1, A2A, A2B and A3) have been cloned and characterized [9]. The role of particular AR subsets in modulation of cytokine production by monocytes was evaluated many times and reviewed elsewhere [2,3]. From all the AR subsets, the A2A receptor is most commonly indicated as the one through which adenosine modulates the cytokine production by monocytes [10,11]. On the other hand, other AR could also be at least partially involved in such a modulatory effect [12–14]. In the pig, the role of AR in modulation of cytokine production by monocytes is completely unknown. However, with regard to the only available study which was performed on porcine NK cells [5] it could be presupposed that the signalling in pigs will also be mediated preferentially by A2A AR.

*Abbreviations:* 2-CADO, 2-chloroadenosine; ARs, adenosine receptors; CPT, cyclopenthyltheophylline; CWS, cell washing solution; MFI, median of fluorescence intensity; N6-CPA, N6-cyclopenthyladenosine; NECA, N-ethylcarboxyamidoadenosine.

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The present study demonstrates that adenosine influences TNF-α production by LPS-stimulated porcine monocytes via A2A AR. Moreover, the study suggests that the effect of NECA on porcine monocytes alters the levels of the cytokines which could play a role in the differentiation of naive T cells into Th17 cells.

## 2. Material and methods

### 2.1. Blood samples

Peripheral blood was collected from 12 healthy adult pigs by jugular vein puncture into heparin collection tubes.

### 2.2. Cell culture for intracellular cytokine detection by flow cytometry

Peripheral blood (50 μl) was cultivated in a total volume of 100 μl in complete RPMI (RPMI 1640 medium, Sigma–Aldrich, USA, supplemented with 100,000 i.u./l penicillin, 100 mg/l streptomycin and 4 mg/l gentamicin with 10% fetal calf serum from Invitrogen, Carlsbad, USA). First, the adenosine receptor antagonists (Table 1) at 10 μM concentration or the solvent (DMSO, Sigma, St. Louis, USA) alone were added for 15 min. Then, adenosine receptor agonists (Table 1) or the solvent (DMSO) alone were added for next 15 min. Finally, the cells were stimulated with 1 μg/ml LPS isolated from *Actinobacillus pleuropneumoniae* for 2 h. Moreover, 10 μg/ml of brefeldin A (Sigma–Aldrich, St. Louis, USA) was added together with the stimulators.

The cultivation was performed in 96-well plates at 37 °C in 5% $CO_2$ in duplicates. Staining of intracellular cytokines was performed immediately after finishing the cultivation.

### 2.3. Detection of TNF-α and IL-8 by flow cytometry

The cultivated cells were used for intracellular TNF-α and IL-8 detection. TNF-α and IL-8 were stained as described previously [15]. Briefly, red blood cells in the samples were lysed with ammonium chloride solution (154.4 mM $NH_4Cl$, 10 mM $KHCO_3$, 0.1 mM EDTA, all from Sigma–Aldrich, St. Louis, USA), leukocyte suspension was then washed with cell washing solution (CWS, phosphate buffered saline containing 1.84 g/L EDTA, 1 g/L sodium azide and 4 mL/L gelatin, all from Sigma–Aldrich, St. Louis, USA) and the final peripheral blood leukocyte count was ascertained using an auto hematology analyzer (BC-2800Vet, Shenzhen Mindray Bio-Medical Electronics, Shenzhen, People's Republic of China).

The intracellular cytokines produced by monocyte subpopulations were stained as follows: cells ($1 \times 10^6$) were stained with 10 μL of antibody cocktail containing anti-CD14 (clone MIL-2, IgG2a subclass, Serotec, Oxford, UK), anti-SWC8 (clone MIL-3, IgM class, a generous gift from Dr. J.K. Lunney, Animal Parasitology Institute, Beltsville, USA) and anti-CD163 (clone 2A10/11, IgG1 subclass, Serotec, Oxford, UK) antibodies and 10 μL of heat-inactivated goat serum. The cells were incubated for 20 min at 4 °C. Then, cells were rinsed, 25 μL of secondary antibody cocktail containing goat anti-mouse IgG2b: SPRD (Southern Biotech, Birmingham, USA), IgG1: AlexaFluor488 (Invitrogen, Carlsbad, USA) and IgM: AlexaFluor647 (Invitrogen, Carlsbad, USA) was added and cells were incubated for another 20 min at 4 °C. Then, cells were rinsed and 100 μl of mouse serum diluted 1:10 with CWS was added for 20 min. Then, cells were rinsed and 30 μl of solution A of IntraStain kit (DAKO, Glostrup, Denmark) was added for 15 min. The cells were rinsed and 20 μl of solution B of IntraStain kit containing anti-TNF-α: R-PE (clone MAb11, IgG1 subclass, eBioscience, San Diego, USA) or anti-IL-8 (clone 8M6, IgG1 subclass, Serotec, Oxford, UK) antibody was added. Before use the anti-IL-8 antibody was pre-labelled with mouse Zenon IgG1: R-PE kit (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. Finally, the cells were rinsed and immediately measured by BD FACSCalibur or BD LSRFortessa flow cytometer (Becton Dickinson, San Jose, USA). At least 200,000 events were acquired. The post-acquisition analysis was performed using Summit Software (DAKO, Glostrup, Denmark) or BD FACSDiva Software (Becton Dickinson, San Jose, USA).

The monocytes were identified according to their light scatter properties and cell surface molecules expression as large mononuclear $SWC8^-$ $CD14^+$ cells. Then TNF-α and IL-8 production by all monocytes or by $CD163^-$ and $CD163^+$ monocyte subpopulations was evaluated as % of TNF-α-producing cells and as median of fluorescence intensity (MFI) of TNF-α-producing cells.

### 2.4. Cell sorting

Peripheral blood monocytes and major monocyte subpopulations ($CD163^-$ and $CD163^+$ monocytes) were isolated by magnetic sorting. First, mononuclear leukocytes were isolated by gradient centrifugation on Histopaque density medium (Sigma–Aldrich, St. Louis, USA). Then, monocytes which, contrary to other mononuclear leukocytes, express CD14 molecule were sorted as follows. Freshly isolated mononuclear leukocytes ($1 \times 10^8$/100 μL DPBS) were stained with anti-CD14 antibody for 15 min at 4 °C. Then, they were thoroughly rinsed and stained with Anti-mouse IgG Micro Beads (Miltenyi Biotec, Bergisch Gladbach, Germany) for 20 min at 4 °C. The stained cells were passed through MACS LS Separation Columns using Quadro Macs Separator (Miltenyi Biotec, Bergisch Gladbach, Germany) and $CD14^+$ monocytes were collected.

**Table 1**
Adenosine receptor agonists and antagonists.

| | Abbreviation | Designation | Act via AR |
|---|---|---|---|
| Agonists | NECA | N-ethylcarboxyamidoadenosine | Non-selective |
| | 2-CADO | 2-Chloroadenosine | A1/A2 |
| | N6-CPA | N6-cyclopenthyladenosine | A1 |
| | CGS21680 | 2-p-(2-Carboxyethyl)phenethylamino-5′-N-ethylcarboxamidoadenosine | A2A |
| | IB-MECA | 1-Deoxy-1-[6-[((3-iodophenyl)methyl)amino]-9H-purin-9-yl]-N-methyl-β-D-ribofuranuronamide, N6-(3-iodobenzyl)adenosine-5′-N-methyluronamide | A3 |
| | AB-MECA | N6-(4-aminobenzyl)-9-[5-(methylcarbonyl)-β-D-ribofuranosyl]adenine, N6-(4-aminobenzyl)-N-methylcarboxamidoadenosine | A3 |
| | HEMADO | 2-(1-Hexynyl)-N-methyladenosine | A3 |
| Antagonists | CPT | Cyclopenthyltheophylline | A1 |
| | SCH58261 | 7-(2-Phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine | A2A |
| | MRS1754 | [N-(4-cyanophenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl)-phenoxy]acetamide] | A2B |
| | MRS1191 | 3-Ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate | A3 |