Reduced ligand affinity leads to an impaired function of the adenosine A2A receptor of human granulocytes in sepsis

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Abstract

The enhanced release of reactive oxygen species by excessively activated polymorphonuclear leucocytes (PMN) is a key step in the pathogenesis of sepsis. Potent action of adenosine in inhibiting cytotoxic PMN functions has been documented. Recent data, however, provide evidence that in sepsis a diminished capability of adenosine to inhibit the generation of oxygen radicals by PMN occurs. Here, we investigated the underlying mechanisms in an in vitro sepsis model and in PMN of sepsis patients. We report that lipopolysaccharide (LPS)-incubation of human PMN elicited the same increase in the half-maximal inhibitory concentration (IC50) of adenosine as observed in patients with septic shock. Coupling to adenylyl cyclase was impaired as well, as indicated by a decreased potency of adenosine to stimulate cyclic adenosine monophosphate (cAMP) accumulation. Ligand-binding studies conducted with native, LPS-stimulated PMN, and with PMN of sepsis patients revealed that, despite an increased adenosine A2A receptor (A2AR) expression, the receptor function declines due to a diminished ligand-binding affinity most likely caused by allosteric modulators within the inflammatory environment. A2AR function obviously is highly dependent upon the cellular environment and thus, further functional characterization of A2AR responses in sepsis may be a promising approach to develop new adenosine or A2AR agonists based therapeutic strategies.

Keywords: sepsis • human PMN • A2A receptor • ligand affinity

Introduction

Sepsis describes a complex clinical syndrome resulting from excessive and uncontrolled host responses to infection. Many of the components of the innate immune response that are initially recruited to fight infection can, under some circumstances, cause life-threatening cell and tissue damage leading to multiple organ failure, the clinical hallmark of sepsis [1, 2].

Polymorphonuclear leucocytes (PMN) or neutrophils serve as the ‘first line’ of innate defence against infection by eliminating pathogens. They contribute to the pathophysiology of sepsis as they are potent in their antimicrobial defences but non-selective in their targets and fulfil the task of eradicating an infection or responding to local tissue injury at the cost of significant collateral damage [3]. In sepsis, circulating PMN develop a dysfunction syndrome characterized by the impairment of potentially microbicidal functions, whereas potentially tissue toxic functions get enhanced:

Spontaneous respiratory burst activity is enhanced in resting PMN and even more after activation with soluble stimuli like fMLP (N-formyl-methionyl-leucyl-phenylalanine) and lipopolysaccharide (LPS), whereas distinct microbicidal functions of PMN like phagocytosis and the associated radical production elicited by unopsonized zymosan particles are suppressed [4, 5].

Adenosine is an endogenous purine nucleoside that is released from metabolically active cells and formed extracellularly by degradation of ATP [6]. Thus, extracellular adenosine accumulates in inflamed and hypoxic tissues [7], and elevated adenosine plasma levels have been detected in patients with sepsis and septic shock [8]. Adenosine is a potent mediator that modulates numerous cell functions and exerts its effects via binding to four subtypes of G-protein coupled adenosine receptors: A1, A2A, A2B and A3 [9]. Activation of high-affinity A1 and low-affinity A3 receptor inhibits adenylyl cyclase activity, whereas activation of high-affinity A2A and low-affinity A2B adenosine receptors causes accumulation of intracellular cAMP, which has strong immunosuppressive effects [10-12]. Tissue hypoxia-associated accumulation of extracellular adenosine and subsequent signalling through the A2A receptors (A2AR) are well known as important mechanisms inhibiting overactive immune cells, limiting inflammation and protecting normal tissues from excessive collateral damage [13].

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damage. Consequently, up-regulation of A2A receptors, as shown for PMN of sepsis patients, for human granulocytes in vitro stimulated with LPS or tumour necrosis factor (TNF)-α [13, 14], and for human peripheral blood cells in patients with chronic heart failure [15] might be a physiological mechanism to increase the anti-inflammatory effects of adenosine and to control inflammation. Furthermore, recent findings suggest that in activated human PMN, adenosine, acting via A2A receptors, selectively inhibits the unwanted release of tissue-toxic oxygen radicals without compromising phagocytosis or the phagocytosis-associated production of oxygen radicals. Thus, the adenosine-A2A-R-pathway is indispensable to hold the balance between the microbicidal response to infectious agents on the one hand, and the protection of own tissues from an overwhelming inflammatory reaction on the other hand. This has conclusively been shown in mouse models of inflammation: A2AR inactivation has been shown to be detrimental in states of acute hyperinflammation [16], whereas it revealed as beneficial in situations where the inflammatory process is appropriate in order to respond to microbial challenges [17].

In septic patients, the equilibrium between pro- and anti-inflammation obviously is disturbed: Despite an increased expression of A2A receptor mRNA, the favourable tissue protective effects of adenosine diminish, as the dose–response curves for the effect of adenosine shift towards increased IC50 values, which means higher plasma adenosine concentrations are necessary to achieve a comparable inhibition of the toxic H2O2 release. Furthermore, the maximum inhibition decreases [8]. These findings indicate a diminished anti-inflammatory potency of adenosine in sepsis that may contribute to the observed PMN dysfunction syndrome, thereby enhancing inflammatory reactions and facilitating further inflammatory tissue damage.

We hypothesized that an A2A receptor dysfunction might account for the decreased anti-inflammatory action of adenosine in septic patients. In this study we investigated these issues in an in vitro model comparing native to LPS-stimulated human granulocytes. Furthermore, we confirmed the clinical impact of our in vitro data by investigating the detected changes in A2AR parameters in PMN of septic patients compared to healthy volunteers. Our results indicate that in sepsis, the up-regulation of A2A receptors as a physiological mechanism to control inflammation is unaffected, whereas the A2A-receptor affinity is significantly decreased, most likely due to allosteric effects arising in the highly inflamed environment. This leads to the observed diminished anti-inflammatory potency of adenosine promoting the further exacerbation of septic processes.

Materials and methods

Patients

The study was approved by the University of Munich Institutional Ethics Committee and informed consent for blood withdrawal was obtained from healthy volunteers, from patients or their relatives, respectively. Patients were considered eligible for this study if they met the criteria for severe sepsis or septic shock as defined by the members of the American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference Committee [18]. Healthy volunteers of comparable age and sex served as controls.

Materials

Fluorescence-activated cell sorting (FACS) Lysing Solution was obtained from Becton Dickinson (Heidelberg, Germany). Hanks buffered salt solution (HBSS) was manufactured from soluble ingredients by the hospital’s own pharmacy. Dihydrorhodamine 123 (DHR 123) was obtained from Mobitec GmbH (Göttingen, Germany). Hydrochloric acid (HCL) was bought from Merck GmbH (Darmstadt, Germany). The following agents were purchased from Sigma-Aldrich GmbH (Taufkirchen, Germany); Ficoll-Histopaque, IMLP, LPS (E. coli, EOS585), and the specific A2AR-agonist 2-[p-(2-carboxyethyl)-phenethyl-amino]-5’-N-ethylcarboxamidoadenosine (GOS 21680). The radiolabelled A2A-R-antagonist 4-2-[7-amino-2-(2-furyl)-[1 2 4]-triazolo-[2 3-a]-[1 3 5]-triazin-5-ylamino]ethylphenol ([^3]H) ZM 241385, 27.4 Ci/mmol) was purchased from American Radiolabeled Chemicals, Inc. (Saint Louis, MO, USA). PCR primers were synthesized by MWG Biotech (Ebersberg, Germany).

Preparation of blood peripheral neutrophils

Granulocytes were separated from whole blood of sepsis patients and from healthy volunteers as previously described [19] using a continuous Percoll density gradient. PMN leucocytes were harvested and washed with Hanks’ buffered salt solution number of cells was counted by a Coulter Counter®T540 (Coulter Electronics, Hialeah, FL, USA).

Determination of adenosine concentrations

Concentrations of the purine nucleoside adenosine were analysed by dual-column switching high-affinity performance/reversed phase high-performance liquid chromatography technique using an internal surface nitrophenylboronic acid precolumn as previously described [8].

Cell stimulation

Cells were cultured in RPMI-1640 medium (Biofluids, Rockville, MD, USA) supplemented with 10% (v/v) foetal calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate and 1 mM Heps in 12-well plates at a density of 5 × 10⁶ cells/well. For stimulation, one of the following factors was added to each well: 10 μg/ml LPS, 100 pg/ml IL-1, 500 pg/ml TNF-α, 15 ng/mg IFN-γ and 5 ng/ml IL-8. After incubation for 6 hrs, cells were harvested by centrifugation and washed twice with HBSS.

Preparation of neutrophil membranes

Human neutrophils were suspended in 1.5-mI Tris buffer (50 mM Tris HCl pH 7.4 containing 10 mM MgCl₂), sonicated on ice for 2 × 10 sec. at
minimum power, and centrifuged at 800 × g for 5 min. at 4°C. The cell
pellet was resuspended in 1.5-ml Tris buffer, sonicated and centrifuged a
second time. Supernatants were collected and centrifuged at 20,000 × g
for 30 min. at 4°C. The resulting pellet was suspended again at a concentra-
tion of 150–180 μg protein/100 μl and this homogenate was used for
the binding assays. Protein concentrations were determined by Bradford
protein assay (Bio-Rad Laboratories, München, Germany) with bovine
albumin as reference standard.

RNA isolation and cDNA RT

Total cellular RNA was isolated from native and LPS-treated granulocytes
using the RNeaQuous Kit (Ambion, Austin, TX, USA) with subsequent
DNAse treatment (TURBO DNase, Ambion) following the manufacturer’s
protocol. RNA was quantified using a spectrophotometer, and 1000 ng
from each samples were transcribed into cDNA. The RT reaction was car-
ried out using mixed random and oligo-dT primers and Superscript III RT
(Invitrogen, Carlsbad, CA, USA), as per manufacturer’s instructions.

RT-PCR

RT-PCR was performed using the primer pairs listed in Table 1. The PCR
was performed under the following conditions: 94°C for 5 min. denaturing,
35 cycles of 94°C for 30 sec., 58°C for 30 sec., 72°C for 45 sec. and a final
extension at 72°C for 10 min. The PCR products were separated on 2%
agarose gels and stained with ethidium bromide.

Determination of fMLP-induced production of
hydrogen peroxide in human neutrophils

Isolated PMN (1 × 10^6) were incubated with DHR 123 (1 μM) in the pres-
ence or absence of different concentrations of adenosine (10^{-12} to 10^{-5} M)
in 1 ml of HBSS at 37°C for 5 min. Then PMN were stimulated with fMLP
(10^{-7} M). After 15 min. activation of cells was stopped by putting tubes on
dry ice. Production of H2O2 was determined by flow cytometry using a Becton
Dickinson FACScan (Becton Dickinson, San Jose, CA, USA) equipped with
an argon laser emitting light at 488 nm, as previously described [5]. Dead
cells were excluded from analyses after identification by propidium iodide
staining (3 × 10^{-5} M).

Measurement of cyclic AMP levels in human
neutrophils

Human neutrophils (1 × 10^6 cells) were suspended in 0.9 ml HBSS,
containing 0.5 mM Ro 20–1724 as phosphodiesterase inhibitor and, for
the CGS 21680 experiments, 1.0 IU adenosine deaminase/ml and were pre-
incubated for 5 min. in a shaking water bath at 37°C. Then eight different
concentrations of adenosine or CGS 21680 were added to the mixtures and
incubations continued for a further 5 min. Hereafter, fMLP in a concentra-
tion of 10^{-7} M was added and the incubations were continued for another
2 min. The reaction was stopped by the addition of 12.5 μl 30% HCl and
transferred to −70°C. After thawing and centrifugation at 10,000 g to
remove cellular debris, supernatants were assayed for cAMP using a cAMP
EIA kit from Biomol (Hamburg, Germany).

[3H]-ZM241385 binding assays

Saturation-binding experiments were performed by incubating PMN mem-
brane preparations with 12–14 different concentrations of [3H] ZM 241385
ranging from 0.125 to 12 nM. Non-specific binding was determined in the
presence of 1 μM of ZM 241385. After 60 min. incubation at 4°C samples were
filtered through Whatman GF/B glass fibre filters (Springfield Mill, UK) using a
Brandel cell harvester (Gaithersburg, MD, USA). The filter bound radioactivity
was counted on a scintillation counter 1219 LKB (efficiency 55%).

Statistical analysis

All data were analysed using SigmaPlot 9.0 and SigmaStat 3.5 software. If
not stated otherwise, values given represent means ± S.E.M. Intergroup
comparisons were performed by unpaired t-test or Mann-Whitney test, if
data were normally or not normally distributed, respectively. Two-tailed
levels of statistical significance are indicated by *: P < 0.05.

Results

Down-regulation of the adenosine-induced
inhibition of H2O2 production in ex vivo
stimulated neutrophils

A reduced anti-inflammatory potency of adenosine in PMN of
sepsis patients has been reported previously [5]. Intending to
establish an appropriate in vitro model to further investigate these
findings, we examined whether the changes in adenosine-
mediated effects also occur in neutrophils in vitro incubated with
LPS, TNF-α, IL-8, IFN-γ and IL-1. Stimulation times and mediator
concentrations were tested out previously (for reproducible maxi-
mum effects and high cell viability, data not shown). Treatment
with these mediators did neither affect the spontaneous nor the
fMLP-stimulated H2O2 production of PMN (Table 1).

We determined the effects of increasing adenosine concentra-
tions on fMLP-stimulated H2O2 production in native compared to
LPS- or cytokine-treated PMN. As shown in Fig. 1, adenosine
inhibited the generation of H2O2 in a dose-dependent manner,
reaching maximum inhibitory effects at a concentration of 10^{-5} M
in untreated neutrophils, whereas maximum inhibition in LPS-
incubated PMN was accomplished at 10^{-6} M. IC50 values changed
from 75 to 332 nM in LPS-treated PMN. These results completely
agree with the data described for PMN from patients with septic
shock. Incubation with TNF-α, IL-8, IFN-γ and IL-1 did not change
the effects of adenosine on the H2O2 production significantly
(Table 2). To exclude any artefacts provoked by unphysiological
adenosine levels potentially arising during cell stimulation, we

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determined the adenosine concentrations being present in the culture medium alone and in supernatants after incubating PMN with or without LPS. As shown in Fig. 2, cell culture medium itself contained 40-nM adenosine. After incubation for 6 hrs with and without LPS, adenosine concentrations slightly increased to 49 and 70 nM, respectively. The comparison of these adenosine levels with plasma adenosine concentrations found in healthy volunteers

Table 1 Influence of inflammatory mediators on vitality and on spontaneous and fMLP-stimulated H2O2 production of human PMN: spontaneous and fMLP-stimulated oxidative activity, dead cells after stimulation and number of independent experiments

|                | Max. inhibition (6 S.E.M.) [%] | IC50 [nM] | n  |
|----------------|--------------------------------|-----------|----|
| Control        | 78.8 (± 1.5)                   | 80 (65–97)| 31 |
| LPS            | 75.2 (± 2.5)                   | 332 (189–584)* * * | 14 |
| TNF-α          | 84.8 (± 3.6)                   | 106 (62–179)| 9  |
| IFN-γ          | 86.8 (± 4.3)                   | 123 (72–213)| 7  |
| IL-1           | 78.6 (± 2.5)                   | 40 (25–64) | 12 |
| IL-8           | 76.3 (± 5.8)                   | 43 (20–96) | 8  |

Human PMN were incubated in culture medium with different cytokines or without stimulation (control) for 6 hrs. Spontaneous and fMLP (10⁻⁷ mol/l)-stimulated H2O2 production was assessed by flow cytometric analysis. Data represent the mean (± S.E.M.) of n independent experiments (***P < 0.001, one-way ANOVA followed by Student’s t-test for comparison of cytokine-stimulated with control).
(42 nM) and septic patients (120 nM) (we already described in one of our previously published studies [8]) clearly showed that our in vitro model lies within physiologically relevant adenosine ranges.

**Down-regulation of the adenosine-induced stimulation of the A2A receptor transduction system in ex vivo stimulated neutrophils**

Based on our previously reported findings that A2A receptor mRNA expression increases upon LPS stimulation whereas the expression of the other adenosine receptors does not change significantly [13], we evaluated the stimulatory effect of adenosine and the selective A2AR agonist CGS 21680 on the adenylyl cyclase activity to test for changes in receptor function. In LPS-stimulated neutrophils, adenosine concentration-response curves were significantly shifted to the right with respect to native PMN (Fig. 3a). EC50 values changed from 165 nM in native to 801 nM in LPS-stimulated human granulocytes, indicating a decreased potency of adenosine to stimulate cAMP formation. Furthermore, in LPS-treated PMN the rise of cAMP levels only reached 90% of the maximum cAMP accumulation elicited in native PMN. The use of the selective A2AR agonist CGS 21680 exhibited identical results in stimulating adenylyl cyclase activity: dose–response curves shifted to the right and EC50 values increased from 64 nM to 893 nM after LPS treatment (Fig. 3b), thereby confirming that the differences in stimulatory effects were essentially A2A receptor mediated.

**In ex vivo stimulated neutrophils affinity of the A2A receptor is decreased**

These results compelled us to focus on further studies of A2A adenosine receptors. In order to determine the effect of LPS stimulation on their binding parameters, we measured affinity and density of A2A adenosine receptors on human neutrophil membranes using the specific A2AR antagonist [3H]ZM 241385. In native PMN, the affinity (Kd) was 1.02 ± 0.22 nM and the receptor density (Bmax) was 64.2 ± 17.5 fmol/mg protein (Fig. 4a, Table 3). After LPS stimulation, neutrophil membranes exhibited increased Kd (2.48 ± 0.27) and Bmax (87.6 ± 26.9) values, indicating an increase in receptor density but a decrease in receptor affinity (Fig. 4b, Table 3). To exclude effects of the pro-inflammatory A1 receptors, binding parameters of A1 receptors using [3H]DPCPX were determined exhibiting no differences between native and LPS-stimulated PMN (data not shown).
In neutrophils of sepsis patients affinity of the A2A receptor is decreased

In concordance with the in vitro data, sepsis patients exhibited an altered binding of \(^{3}H\)-ZM 241385 to their PMN membranes compared to control patients: \(K_D\) values were elevated from 1.02 ± 0.22 nM to 1.86 ± 0.32 nM and \(B_{\text{max}}\) values from 64.2 ± 17.5 fmol/mg protein to 70.2 ± 15.5 fmol/mg protein (Fig. 4c, Table 3), indicating that sepsis patients exhibited an increased receptor density and a decreased receptor affinity compared to healthy volunteers.

Changes in the affinity of the A2A receptor are not due to differential expression of splice variants

The human \(A_2\)R gene consists of a complex organized 5′-UTR with five non-coding exons followed by two coding exons which
are separated by a single intron leading to the translation of a 43-kD protein. We analysed whether so far undetected splice variants may lead to changes in the A2A receptor protein sequence which may account for the observed changes in receptor affinity. Therefore, we performed RT-PCR experiments with primer sets spanning the exon–intron boundaries to check for splice variants (primer pairs are listed in Table 4). All RT-PCR experiments did not result in differences between native and LPS-stimulated cells, and PMN of sepsis patients (Fig. 5).

### Table 4 RT-PCR Primer Sequences to check for A2AR splice variants: sequences, annealing temperatures, expected amplification product lengths and accession number

| Primer name       | Sequence                             | Tm [°C] | Product size [bp] |
|-------------------|--------------------------------------|---------|------------------|
| A2A-splice 3'-For | 5'-GACCGGTACATGGCCATCC-3'            | 58.8    | 298              |
| A2A-splice-3'-Rev | 5'-GCAAATAGACACCCAGATGA-3'           | 57.9    |                  |
| A2A-splice 5'-For | 5'-GTTGGTCAACAGCAACCT-3'             | 59.4    | 301              |
| A2A-splice 5'-Rev | 5'-ACCAGCAGATGCCAATGAT-3             | 57.3    |                  |

Accession no. A2AR: NM_000675.

**Discussion**

In view of the multitude of immunomodulatory effects of adenosine via occupancy of adenosine receptors [20, 21], expression and regulation of adenosine receptors in PMN of sepsis patients is an important topic with high clinical relevance which has not been analysed conclusively so far. In our previous studies, we have reported observations concerning the adenosine receptors in sepsis patients that, at the first sight, seem to be contradictory: We found a PMN dysfunction syndrome with enhanced tissue toxic functions going along with diminished anti-inflammatory potency of adenosine [8]. Although one would expect a down-regulation of anti-inflammatory A2A receptor expression or an up-regulation of pro-inflammatory A1-receptor expression to account for these phenomena, we found, in fact, an A2AR up-regulation and no changes in A1R expression [13]. The present study was accomplished in order to dissolve these apparently contradictory findings and to characterize the underlying mechanisms. First, by stimulating PMN with LPS, we established an appropriate in vitro model that mimics the diminished anti-inflammatory effects of adenosine found in PMN of sepsis patients. LPS stimulation is a widely used and accepted model to investigate some important aspects of the multitude of mediators being engaged in the pathomechanisms of sepsis as activation of TLR4 (a member of IL-1/Toll receptor family) by its ligand LPS has been shown to be critical for the regulation of innate immune response [22, 23]. Challenge of PMN with LPS activates several mitogen-activated protein kinase signalling pathways, the extracellular signal-regulated kinase, c-Jun NH2-terminal kinase and p38 mitogen-activated protein kinase, which in turn induce gene transcription [24]. LPS also induces the activation of nuclear transcription factors, nuclear factor κB (NF-κB), activator protein-1 (AP-1), PU.1 and interferon regulatory factor-1 in PMN [25, 26].

In the current study, all in vitro stimulation experiments have been performed with LPS concentrations of 10 μg/ml. A saturable dose–response relationship of LPS on the PMN answer has already been pointed out in one of our previously published studies (stable maximum effects from 10 ng/ml up to 10 μg/ml with no adverse effects of high LPS concentrations) [27]. Because LPS physically adheres to the polystyrene material of reaction tubes [28], the μg/ml concentration range was used here in order to guarantee maximum stimulation conditions within the saturation range for all reactions. Moreover, even in vivo a wide range of endotoxin concentrations occur: While average plasma concentrations of endotoxin reported for sepsis patients are within the pg-ng/ml range [29], several clinical reports have documented
endotoxinemia within the range of μg/ml for selected patients with systemic meningococcal disease [29, 30].

We show that LPS incubation of human PMN for 6 hrs leads to a 4.4-fold increase of the IC50 of adenosine on the H2O2 production; in other words: 4.4-fold higher concentrations of adenosine are needed to achieve a comparable inhibition of H2O2 production in LPS-treated versus native human PMN. These results, together with the determined adenosine levels being present in the cell culture supernatants after stimulation, were completely in line with our previously published data for PMN of patients with septic shock [8] which – regarding our question – proved the LPS-in vitro model as suitable.

Recent studies have shown that treatment with TNF-α and other inflammatory mediators enhances the function and expression of adenosine receptors, and specially A2A receptors, by increasing the receptor number and by modulating mechanisms of desensitization [31–34]. The changes in A2AR signalling we investigated in this study could not be provoked by those pro-inflammatory cytokines: Incubation with TNF-α, IL-8, IFN-γ and IL-1 did not significantly influence the anti-inflammatory effects of adenosine which indicates that the observed effects of LPS incubation are not due to the secondary induction of cytokines but may be related to one of the signalling pathways described above.

Because A2A receptors are well known to ‘put the brake on inflammation’ [35], which means they inhibit overactive immune cells during acute inflammation thereby limiting inflammatory collateral tissue damage, it is reasonable to assume that the here reported adenosine effects were mainly A2A mediated. This is further supported by our recently reported findings providing evidence that A2A receptor mRNA expression in human PMN increases upon LPS stimulation whereas the expression of the other adenosine receptors does not change significantly [13]. In the current study, we performed cAMP-assays with both adenosine and the specific A2A receptor agonist CGS 21680 which exhibited a good concordance thereby giving an experimental prove for A2A receptors to be the key players in the here examined scenario. Furthermore, these experiments showed that the attenuating effects of adenosine on H2O2 production were mediated by the signal transduction system typically associated to the A2AR as a prototypical Gs-coupled receptor: Treating PMN with LPS was followed by an impaired potency to stimulate cAMP accumulation, as indicated by a significant increase in the EC50 value.

In the presence of continued agonist activation of the A2AR, signalling is attenuated by a coordinated process of desensitization [33, 36, 37]. In sepsis, mechanisms of desensitization may also play a role in the network of A2AR regulatory processes. However, it seems unlikely that the here reported observations of a reduced anti-inflammatory potency of adenosine and A2A receptors are due to desensitization processes because (i) treatment times with LPS and/or with agonist were identical in all reactions tested and (ii) adenosine concentrations in control and LPS-treated PMN were comparable after a culturing time of 6 hrs. Therefore, the applied experimental setting excluded most influences of potentially occurring desensitization processes. However, desensitization from endogenous adenosine exposure occurring in the first minutes of exposure to LPS that may also influence the characteristics of A2A receptors even after 6 hrs of incubation cannot completely be ruled out.

To determine whether the changes in functional responses of A2A receptors observed in PMN of sepsis patients correlated with changes in binding parameters, we performed ligand-binding studies with membranes from native and LPS-incubated PMN, and from PMN of sepsis patients. A2A receptor density (Bmax) and dissociation constant (Kd) determined with [3H] ZM 241385 for native PMN were in good concordance with those previously described by Varani et al. [15, 38, 39]. After LPS incubation, we detected significant changes in ligand-binding parameters: Receptor number increased 1.4-fold which definitely excluded a reduced A2AR number being responsible for the observed diminished anti-inflammatory potency of adenosine. Surprisingly, although an increase in Bmax is expected to go along with a decrease in Kd [40], Kd actually increased (2.5-fold), thereby proving a significantly impaired ligand binding. In addition, saturation-binding experiments with the specific A1 receptor antagonist [3H]DPCPX showed that LPS did neither alter the affinity nor the expression of A1 receptors in PMN. In PMN of sepsis patients, ligand binding of the A2AR exhibited identical alterations: Receptor number increased 1.1-fold (the sample size did not allow reliable statistical analysis in the current study. Notwithstanding that, results exhibited a clear tendency towards an increase in Bmax in all of the 6 conducted experiments), whereas receptor affinity decreased by almost 50% (Kd increased 1.9-fold) as compared to PMN of healthy volunteers. Consequently, although more receptor molecules are available, higher concentrations of adenosine are needed to inhibit tissue-toxic H2O2 production. Up-regulation of A2A receptors during inflammatory processes is a useful physiological mechanism to protect own tissues from an overwhelming immune response which obviously in sepsis is overcompensated by a reduced receptor affinity thereby promoting further development of the septic disease.

Changes in the protein primary structure as a result of alternative splicing or conformational changes in the receptor protein caused by allosteric modulation may account for the reduced affinity of the A2AR in sepsis. The human A2AR gene consists of two coding exons separated by a single intron leading to the translation of a 45-kD protein [41]. In view of this genetic organization, the existence of splice variants leading to the differential expression of receptor protein with varying functions appears to be unlikely. Nevertheless, we performed RT-PCR analyses to definitely exclude the existence of such receptor isoforms that are typically generated by alternative splicing at the exon–intron boundaries or by usage of alternative startcodons. The latter is not likely because an in-frame stop codon 21 bp upstream of the start ATG avoids 5′ extension of the open reading frame. RT-PCR fragments amplified across the exon–intron boundaries did not exhibit any length differences in native versus LPS incubated PMN which excluded splice variants being responsible for the altered receptor affinity.
GPCRs are per se allosteric, in that they possess binding sites for the endogenous ligand and the G protein. Additionally, binding sites for allosteric ligands exist, that act by inducing conformational changes that are transmitted from the allosteric site to the orthosteric site and/or to effector coupling sites [42]. It therefore is widely accepted that also the A2A receptor has more than one binding site [43–45]. Ligands binding to the allosteric site can alter the binding of ligands to the orthosteric site. Additionally, dimerization with other receptors (i.e. D1 receptor) [46] is discussed. The effects on the receptor affinity we found in sepsis can be explained by allosteric effects provoked by so far unknown mediators arising in the inflammatory environment: A negative allosteric regulator decreases the binding of the orthosteric ligand such that either the orthosteric agonist concentration–response curve will be shifted to higher agonist concentrations (i.e. rightwards) and/or the maximum response will be decreased [47].

In view of the multiplicity of mediators being involved in the activation of human PMN, the identification of the allosteric modulators giving rise to the phenomena reported here appears to be rather complex and may be subject of further studies.

In summary, the present study provides evidence that the cellular environment in which it is expressed and thus further exacerbation of inflammation and ensuing tissue damage in the course of sepsis.

It now appears that A2AR function is highly dependent upon the cellular environment in which it is expressed and thus further functional characterization of A2AR responses in sepsis may be a promising approach to develop new adenosine or A2A receptor agonists based therapeutic strategies.

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Conflict of interest

The authors declare no competing financial interests.

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