

Adenosine Reduces Cell Surface Expression of Toll-Like Receptor 4 and Inflammation in Response to Lipopolysaccharide and Matrix Products

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Abstract Recent evidence suggests that Toll-like receptor 4 (TLR4) is not only involved in innate immunity but is also an important mediator of adverse left ventricular remodeling and heart failure following acute myocardial infarction (MI). TLR4 is activated by lipopolysaccharide (LPS) but also by products of matrix degradation such as hyaluronic acid and heparan sulfate. Although cardioprotective properties of adenosine (Ado) have been extensively studied, its potential to interfere with TLR4 activation is unknown. We observed that TLR4 pathway is activated in white blood cells from MI patients. TLR4 mRNA expression correlated with troponin T levels ($R^2=0.75$; $P=0.01$) but not with levels of white blood cells and C-reactive protein. Ado downregulated TLR4 expression at the surface of human macrophages (-50% , $P<0.05$). Tumor necrosis factor- α production induced by the TLR4 ligands LPS, hyaluronic acid, and heparan sulfate was potently inhibited by Ado (-75% for LPS, $P<0.005$). This effect was reproduced by the A2A Ado receptor agonist CGS21680 and the non-selective agonist NECA and was inhibited by the A2A antagonist SCH58261 and the A2A/A2B antagonist ZM241,385. In contrast, Ado induced a 3-fold increase of TLR4 mRNA expression ($P=0.008$), revealing the existence of a feedback mechanism to compensate for the loss of TLR4 expression at the cell surface. In conclusion,

the TLR4 pathway is activated after MI and correlates with infarct severity but not with the extent of inflammation. Reduction of TLR4 expression by Ado may therefore represent an important strategy to limit remodeling post-MI.

Keywords Adenosine · Inflammation · Toll-like receptor 4 · Myocardial infarction · Left ventricular remodeling

Introduction

Myocardial infarction (MI) is associated with the development of an inflammatory response in the heart which largely influences the extent of left ventricular (LV) remodeling. Toll-like receptor 4 (TLR4), a member of the pattern-recognition receptors family, constitutes a key node in the induction and regulation of this inflammatory response. TLR4 expression is enhanced in the failing heart [1], and knockdown of TLR4 has been shown to reduce the extent of LV remodeling and to preserve systolic function without affecting infarct size [2]. TLR4-deficient mice showed less interstitial fibrosis in the non-infarcted area and increased collagen in the infarcted area. This was associated with lower levels of tumor necrosis factor- α (TNF- α) and matrix metalloproteinases 2 and 9 [2]. Therefore, inhibition of TLR4 may represent a novel therapeutic approach for maladaptive LV remodeling. Of note, TLR4 binds not only to bacterial lipopolysaccharide (LPS) but also to products of extracellular matrix breakdown such as hyaluronic acid (HA) or heparan sulfate (HS) [3]. Increasing evidence implicates such endogenous TLR4 ligands as major triggers of inflammation in response to ischemia.

Adenosine (Ado) is massively released in the extracellular milieu after ischemia as a result of cell death and

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adenosine triphosphate breakdown [4]. Cardioprotective properties of Ado are known, and our previous studies suggested a role for Ado in cardiac repair and LV remodeling [5–7]. The aim of the present study was to determine the effect of Ado on TLR4. We observed that TLR4 is activated in acute MI patients and that Ado is able to limit this activation.

Materials and Methods

Patients Twelve patients with acute MI enrolled in the Luxembourg Acute Myocardial Infarction Registry and treated with primary percutaneous coronary intervention were included in this study. Acute MI was defined by the presence of chest pain <12 h with significant ST elevation and positive cardiac enzymes. Blood samples were obtained at the time of mechanical reperfusion. All patients signed an informed consent, and the registry has been approved by local ethics committee. Creatine phosphokinase, high sensitivity C-reactive protein, and troponin T (TnT) were measured using standard laboratory techniques. Demographic and clinical data are summarized in Table 1. Twelve healthy volunteers were used as controls.

Cell Culture Cell culture materials were from Lonza (Verviers, Belgium), and all other materials and reagents were from Sigma (Bornem, Belgium) unless specified. Blood samples from healthy volunteers and MI patients dedicated to RNA analysis were harvested in PAXgene[®] blood RNA tubes (Qiagen, Hilden, Germany). PBMCs were isolated by Ficoll gradient. Monocytes were purified by negative selection using the Monocyte Isolation Kit II (Myltenyi Biotec GmbH, Bergisch Gladbach, Germany) as described before [6]. Monocytes were cultured in RPMI 1640 containing L-glutamine, 1% sodium pyruvate, 1% non-essential amino acids, penicillin/streptomycin, and 1% fetal calf serum (Eurobio, Paris, France). Differentiation was achieved with 50 ng/mL M-CSF (Peprotech, Levallois-Peret, France) for 7 days. Cells from the THP-1 monocytic line cultured in the same medium were used for mechanistic investigations after differentiation to macrophages with 150 nM phorbol myristate acetate (PMA) for 24 h. Macrophages were preincubated for 15 min with Ado and erythro-9-(2-hydroxy-3-nonyl)-adenine hydrochloride to prevent Ado metabolism (10 μ mol/L each). In some experiments, agonists (CGS21680, NECA) and antagonists (SCH58261, ZM241,385) of Ado receptors were used. LPS (from *Escherichia coli* 026:B6), HS (from bovine kidney), and HA were used as TLR4 agonists. Cells and conditioned medium were harvested and stored at -80°C until use. The absence of endotoxin contamination in Ado and other drugs

Table 1 Clinical characteristics of MI patients ($n=12$)

| | | |
|---------------------------------|-----------------|--------|
| Age, years (mean \pm SD) | 58 | 18 |
| Male, n (%) | 11 | 92% |
| Body mass index (mean \pm SD) | 28 | 4 |
| Serum markers (mean \pm SD) | | |
| CPK (units/L) | 2,672 | 2,656 |
| TnT (ng/mL) | 3.9 | 3.8 |
| hsCRP (ng/mL) | 83 | 136 |
| Cardiovascular history, n (%) | | |
| Prior MI | 3 | 25% |
| CABG | 2 | 17% |
| PTCA | 2 | 17% |
| Diabetes | 2 | 17% |
| Hypertension | 3 | 25% |
| Hypercholesterolemia | 6 | 50% |
| Tobacco | 5 | 42% |
| Medications, n (%) | | |
| Beta-blockers | 9 | 75% |
| Calcium antagonists | 3 | 25% |
| Nitrates | 4 | 33% |
| ACE inhibitors | 2 | 17% |
| Statins | 2 | 17% |
| Angiotensin inhibitors | 0 | 0% |
| Blood count, % (mean \pm SD) | | Range |
| Neutrophils | 68.7 \pm 22.2 | 40–74% |
| Lymphocytes | 19.3 \pm 15.7 | 19–48% |
| Monocytes | 4.8 \pm 2.0 | 3.4–9% |
| Eosinophils | 1.2 \pm 1.5 | 0–7% |
| Basophils | 0.3 \pm 0.3 | 0–1.5% |

All patients received aspirin, clopidogrel, heparin, and abciximab
ACE angiotensin converting enzyme, *CABG* coronary artery bypass grafting, *CPK* creatine phosphokinase, *hsCRP* high-sensitive C-reactive protein, *PTCA* percutaneous transluminal coronary angioplasty, *TnT* troponin T

used in the study was verified using the E-Toxate[®] reagent from *Limulus polyphemus* (LAL assay having a detection sensitivity of 0.05 EU/mL).

Real-Time Quantitative Polymerase Chain Reaction Total RNA from cultured cells was isolated using TriReagent[®] and the RNeasy[®] mini kit (Qiagen). Total RNA from blood cells collected in PAXgene[®] blood RNA tubes was isolated using PAXgene[®] Blood RNA Kit (Qiagen). Potential contaminating genomic DNA was digested by DNase I treatment (Qiagen). One microgram of total RNA was reverse-transcribed using the Superscript[®] II Reverse Transcriptase (Invitrogen, Merelbeke, Belgium). PCR primers were designed using the Beacon Designer software (Premier Biosoft, Palo Alto, CA, USA) and were chosen to

encompass an intron. Primers were as follows: CD14 forward 5'-TAAAGCACTTCCAGAG-3' and reverse 5'-AATCTTCATCGTCCAG-3', MD-2 forward 5'-TGCCGAGGATCTGATGAC-3' and reverse 5'-ATTAGTTGGTGTAGGATGAC-3', TLR4 forward 5'-ATCCAGGTGTGAAATCCAGAC-3' and reverse 5'-AGGCTCCCAGGGCTAAAC-3', and β -actin forward 5'-AGAAAATCTGGCACCACACC-3' and reverse 5'-GGGGTGTGAAGGTCTCAA-3'. PCR was performed using the iCycler[®] and the IQ[™] SYBR[®] Green Supermix (Biorad, Nazareth, Belgium); 1/10 dilutions of cDNA were used. PCR conditions were as follows: 3 min at 95°C, 30 s at 95°C and 1 min annealing (40-fold). Optimal annealing temperature was determined for each primer pair. Melting point analysis was obtained after 80 cycles for 10 s from 55°C up to 95°C. Each run included negative reaction controls. β -actin was chosen as housekeeping gene for normalization. Expression levels were calculated by the relative quantification method ($\Delta\Delta C_t$) using the Genex software (Biorad) which takes into account primer pair efficiency.

Flow Cytometry For intracellular staining, Brefeldin A (GolgiPlug[™], BD Biosciences, Erembodegem, Belgium) was added in culture for the last 4 h of treatment in order to block the cytokine production in the Golgi apparatus prior to fixation and permeabilization (Cytofix/Cytoperm[™] Fixation/Permeabilization Solution, BD Biosciences). Cells were harvested using cell dissociation solution and washed once in phosphate-buffered saline (PBS) containing 10 mg/mL bovine serum albumin (BSA). After Fc receptor blocking (Fc blocking Reagent, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), $2 \cdot 10^5$ cells per determination were incubated at 4°C for 30 min in the dark with fluorochrome-conjugated monoclonal antibodies: anti-CD14-PE-Cy7 (clone M5E2, BD Biosciences), anti-CD45-Per-CP (clone 2D1, BD Biosciences), anti-TLR4-FITC (clone HTA125, Abcam, Cambridge, UK), anti-TNF- α -FITC (clone MAB11, eBioscience, San Diego, CA, USA), or proper isotype controls. Cells were analyzed on a BD FACSCanto[™] flow cytometer using FACSDiva[™] software (version 5.0.2); 20,000 events were acquired on FSC/SSC scatter plot gated cells and doublets were excluded on SSC-A/SSC-H scatter plot.

Immunofluorescence THP-1 monocytes were seeded onto glass coverslips and differentiated to macrophages during 2 days with 150 nM PMA. After Ado and LPS treatment, cells were fixed for 10 min in 2% paraformaldehyde, permeabilized for 10 min in PBS–1% Tween in case of intracellular staining, and blocked for 1 h in PBS–3% BSA. Cells were then incubated

with a rabbit polyclonal antibody anti-TLR4 (sc-10741, Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by incubation with Alexa Fluor[®]568 donkey anti-rabbit IgG (Molecular Probes). Pictures were collected either on a fluorescence microscope (Leica DMIL) with a $\times 100$ oil immersion objective and a Leica DFC300 FX camera using the Leica LAS V3.0 software, or on a confocal fluorescence microscope (Zeiss Laser Scanning Microscope LSM 510) with a $\times 63$ oil immersion objective using the LSM 510 META software. 4',6-Diamidino-2-phenylindole (blue) staining was used to reveal nuclei.

Immunoblotting Total proteins were extracted after homogenization of cells on ice with cell lysis buffer (Cell Signaling, Leiden, The Netherlands) added with protease inhibitor cocktail (Roche, Mannheim, Germany) and 1 mmol/L phenylmethylsulfonyl fluoride. Supernatants were collected after centrifugation at $800 \times g$ for 5 min at 4°C and were passed ten times through a 26-gauge needle. Samples were then clarified by centrifugation at $12,000 \times g$ for 15 min at 4°C. Protein concentrations were determined using BCA Protein Assay Reagent (Pierce, Aalst, Belgium). Proteins were separated on 4–8% polyacrylamide gels, transferred onto Immobilon-P membranes (Millipore, Brussels, Belgium). Membranes were incubated with a polyclonal rabbit anti-mouse TLR4 antibody (sc-10741, Santa Cruz Biotechnology) and donkey anti-mouse IgGs coupled to horseradish peroxidase (Jackson ImmunoResearch, Suffolk, UK). A mouse monoclonal anti human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (clone 0411, Santa Cruz Biotechnology) was used for normalization. Substrate used was the Super-Signal West Dura Extended Duration Substrate (Pierce). Images were captured on a Kodak Gel Logic 2200 Station (Raytest, Straubenhardt, Germany), and quantification was performed using the AIDA software.

Enzyme-Linked Immunosorbent Assay TNF- α concentration in conditioned medium was measured by ELISA using the Quantikine DTA00C kit (R&D Systems, Oxon, UK). Samples were assayed in duplicates. Detection limit of the assay was 1.6 ng/mL.

Statistical Analysis Results are expressed as mean \pm SD. Analyses were performed with SigmaPlot v11.0. Comparisons between two groups were performed with *t* test for data with a Gaussian distribution. Mann–Whitney (unpaired data) or Wilcoxon (paired data) tests were used for non-Gaussian data. One-way repeated measures ANOVA was used to compare multiple paired groups. Correlations were evaluated using the Spearman test. A *P* value < 0.05 was considered significant.

Results

Expression of TLR4 Is Enhanced After MI

We measured mRNA expression levels of TLR4 and its associated proteins CD14 and MD-2 in whole blood cells obtained from 12 patients with acute MI and 12 healthy volunteers. Gene expression was assessed using the PAXgene[®] Blood RNA system and quantitative PCR. TLR4, MD-2, and CD14 were expressed at significantly higher levels in cells isolated from patients with acute MI than in cells from healthy controls (Fig. 1A). Levels of TLR4 positively correlated with that of TnT ($R^2=0.75$; $P=0.01$) but not with the levels of white blood cells or CRP (Fig. 1B)

Adenosine Downregulates TLR4 Expression

Monocytes differentiating to macrophages during tissue infiltration are responsible for a large part of the inflammatory reaction in the infarcted heart. We isolated primary blood monocytes from healthy volunteers and differentiated the cells into macrophages by M-CSF. TLR4 expression at the surface of macrophages was analyzed by flow cytometry 24 h after activation. LPS decreased TLR4 expression at the cell surface, and this observation was in line with the notion that TLR4 is internalized after exposure to LPS. Addition of LPS and Ado further diminished the expression of TLR4, reaching a 50% inhibition. However, Ado alone had no effect on the expression of TLR4 (Fig. 2A). A kinetic of TLR4 surface expression revealed that the decrease of TLR4 after LPS exposure was maximal at 24 h and returned to baseline levels at 48 h. This effect was amplified by Ado both at 24 and 48 h (Fig. 2B). We further investigated

whether Ado alone was able to downregulate TLR4 surface expression. We observed a rapid decrease of TLR4 expression following addition of Ado, starting as early as 1 min and reaching statistical significance after 20 min (Fig. 2C). LPS also decreased TLR4 expression, but this effect was slower, reaching significance after 60 min (Fig. 2C). The loss of cell surface expression of TLR4 following Ado and LPS treatment was confirmed by immunofluorescence in macrophages derived from the human monocytic cell line THP-1 (Fig. 2D). All together, these results show that Ado downregulates cell surface expression of TLR4.

Adenosine Decreases TNF- α Production

The main consequence of the activation of the TLR4 pathway is the release of pro-inflammatory cytokines, most notably TNF- α . We tested whether the decrease of TLR4 surface expression by Ado could affect TNF- α production by macrophages from healthy volunteers. When TNF- α was measured intracellularly by flow cytometry, we observed that Ado inhibited TNF- α expression in LPS-activated macrophages (Fig. 3A).

So far, experiments were performed with LPS as prototypical TLR4 ligand. However, LPS is a component of the bacterial wall of gram negative bacteria and is not likely to be present in the heart after MI. Therefore, we also tested whether Ado regulates TNF- α production by macrophages when TLR4 is activated by endogenous ligands likely to be present in the heart after acute MI, such as HA and HS, which are products of the breakdown of the extracellular matrix. All three TLR4 agonists—LPS, HA, and HS—potently induced TNF- α secretion, and this effect was blunted by Ado (Fig. 3B). For instance, TNF- α concentration in conditioned medium of macrophages

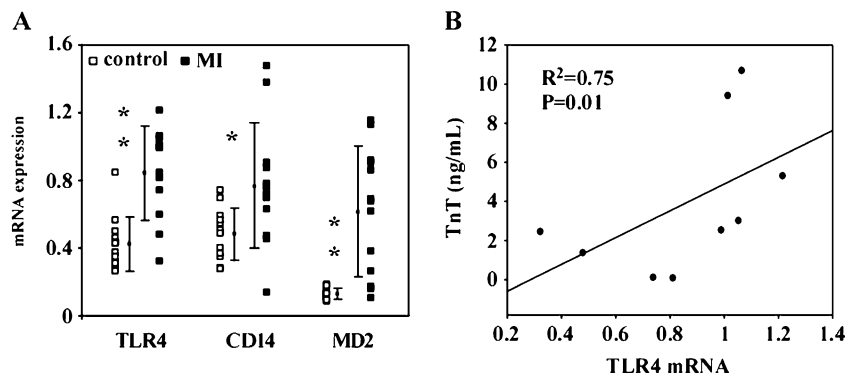


Fig. 1 Expression of TLR4 components is enhanced in MI patients compared with healthy volunteers. **A** Blood samples from healthy volunteers (controls, open square) and patients with MI (closed square) were recovered in PAXgene[®] tubes. RNA was extracted and quantitative PCR were performed on selected genes. Cells isolated from MI patients have higher levels of TLR4, CD14, and MD2 than cells isolated from

controls. Results were normalized to β -actin. Each square represents one patient and the means \pm SD ($n=12$ in each group) are displayed. $*P<0.01$ vs controls; $**P<0.0005$ vs controls. **B** Peak TnT values are correlated to TLR4 mRNA expression values. TnT values were available for nine patients, and TLR4 expression was determined by quantitative PCR. Correlation coefficient and P value are indicated

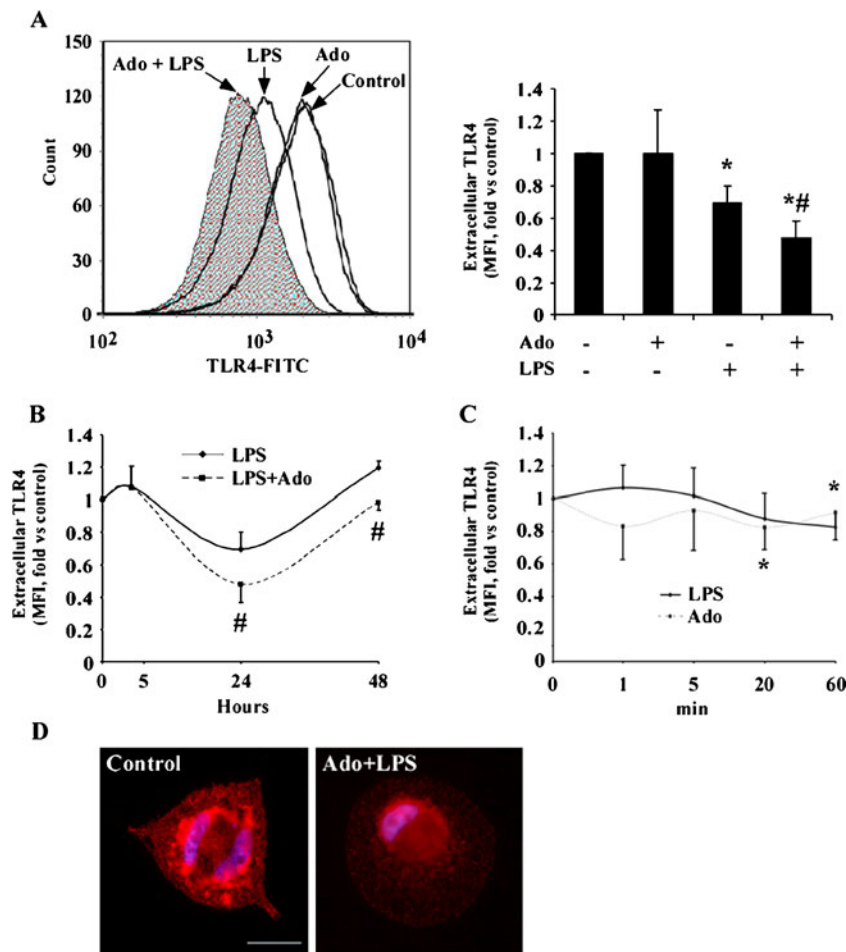


Fig. 2 Ado and LPS downregulate cell surface expression of TLR4. **A** Monocytes were isolated from PBMCs of healthy volunteers by negative selection and were differentiated to macrophages with 50 ng/mL M-CSF for 7 days. Macrophages were preincubated for 15 min with 10 μ mol/L Ado before incubation for 24 h with LPS 100 ng/mL. TLR4 expression at the cell surface was measured by FACS. LPS reduced cell surface expression of TLR4 and Ado exacerbated this effect. *Left panel* displays a representative histogram, and *right panel* shows the mean fluorescence intensities normalized to control condition. **B**, **C** Kinetics of TLR4 surface expression on primary macrophages treated by 10 μ mol/L Ado and/or 100 ng/mL LPS for different periods of time. In LPS- and Ado-treated cells, Ado was added 15 min prior to LPS. TLR4 expression was measured by FACS.

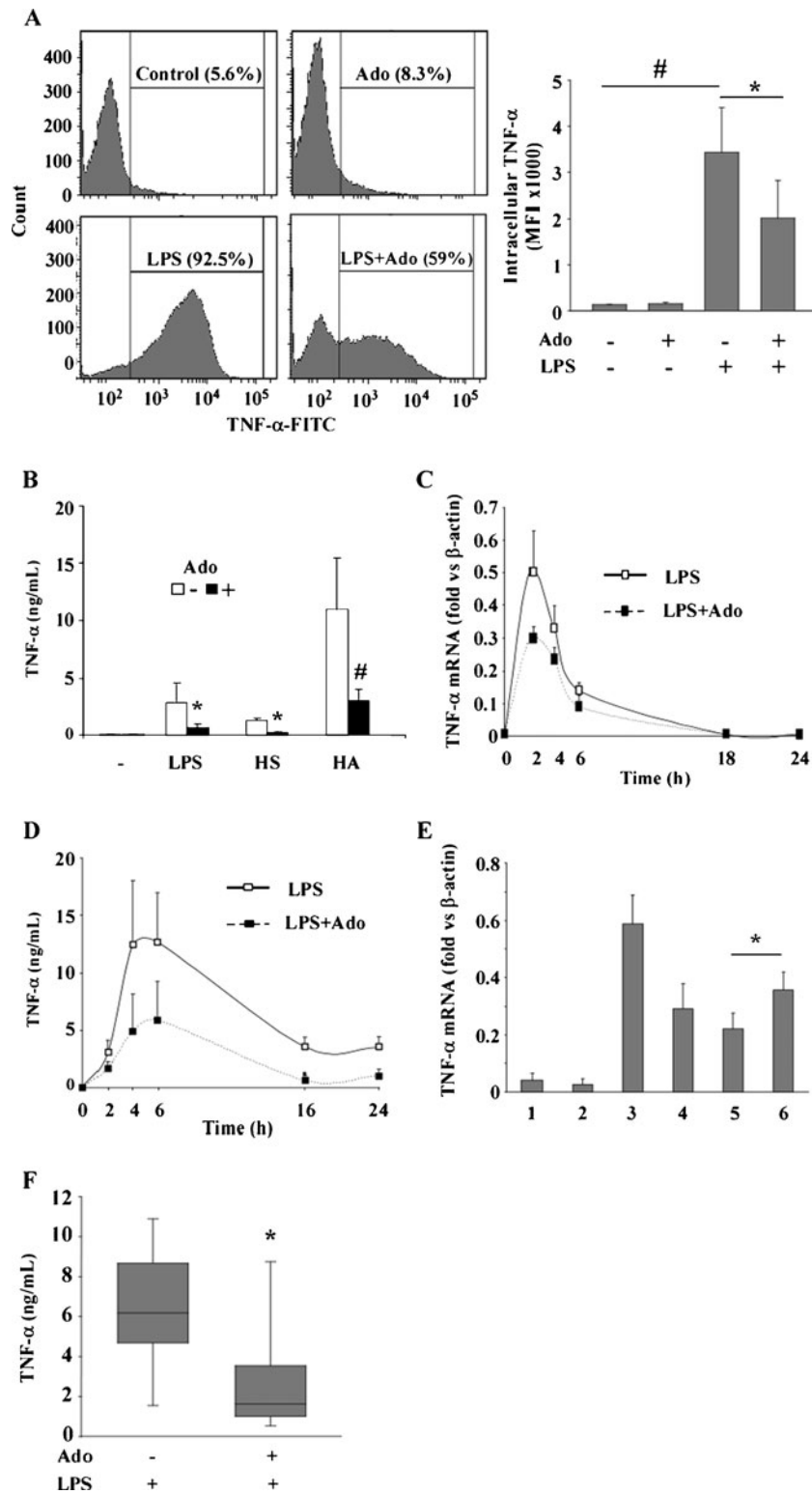
TLR4 cell surface expression was decreased 24 h after LPS administration (*full line*) and returned to basal levels at 48 h ($P=0.01$). Ado exacerbated this decrease (*dotted line*). Ado induced a rapid decrease of TLR4 expression and the decrease observed with LPS was slower. Results are mean \pm SD of at least three independent experiments. * $P<0.05$ vs control; # $P<0.05$ vs LPS. **D** Cells from the THP-1 monocytic line were differentiated in vitro to macrophages by 150 nM PMA for 24 h. Macrophages were preincubated for 15 min with 10 μ mol/L Ado before incubation with 100 ng/mL LPS for 24 h. As determined by immunofluorescence, cell surface TLR4 expression was downregulated after treatment by Ado and LPS. Representative pictures of three randomly taken pictures per condition are shown. *Bar scale*, 20 μ m

stimulated by LPS was decreased by >75% in the presence of Ado ($P<0.005$; Fig. 3B). These results demonstrate that the effect of Ado on TLR4 is not specifically related to activation by LPS but is also active in the presence of fragments of the extracellular matrix.

The effect of Ado on TNF- α production appeared to involve transcriptional mechanisms since Ado downregulated TNF- α mRNA expression induced by LPS (Fig. 3C). The peak of TNF- α mRNA (2 h after LPS) preceded the peak of TNF- α protein measured in conditioned medium (4–6 h after LPS; Fig. 3D). When added 20 min after LPS challenge, Ado less potently inhibited TNF- α expression

than when added before LPS, suggesting that Ado affects TLR4 activation (Fig. 3E).

Next, we investigated the effect of Ado in cells from MI patients. Blood monocytes from 12 MI patients were purified, differentiated to macrophages, and treated with Ado and LPS. As observed with macrophages from healthy volunteers (Fig. 3B), Ado treatment potently inhibited the accumulation of TNF- α in conditioned medium ($\sim 70\%$, $P<0.001$, Fig. 3F). It has to be noted that macrophages from MI patients secreted about 2-fold more TNF- α than the cells obtained from healthy volunteers (Fig. 3F), presumably secondary to in vivo priming.



Activation of A2A and A2B Receptors Inhibits TNF-α Production

Since the A2A is the predominant form of Ado receptors at the surface of activated macrophages [5, 7], we tested

whether specific activation or blockade of this receptor could reproduce the effect of Ado on TNF-α. In the presence of LPS, the A2A receptor agonist CGS21680 dose-dependently inhibited the release of TNF-α, and this inhibition was more robust in presence of the non-specific

Fig. 3 Adenosine inhibits TLR4-induced TNF- α production. Monocytes were isolated from PBMCs of healthy volunteers by negative selection. Macrophages were obtained by differentiation of monocytes with 50 ng/ml M-CSF for 7 days. Cells were preincubated for 15 min with Ado (10 μ mol/L) before incubation for 24 h with LPS (100 ng/mL), HS (50 μ g/mL), or HA (50 μ g/mL). Intracellular TNF- α was assessed by flow cytometry, TNF- α concentration in conditioned medium was measured by ELISA, and TNF- α mRNA expression was assessed by quantitative PCR. **A** Ado reduced the LPS-induced expression of TNF- α in macrophages. The vertical bars discriminate TNF- α -secreting cells from non-secreting cells, and the percentage of cells positive for TNF- α is indicated. Mean fluorescence intensities graphed on right panel indicate that Ado inhibits the intracellular accumulation of TNF- α induced by LPS. Mean values \pm SD from three independent experiments are shown. * P <0.05 vs LPS; # P <0.001 vs control. **B** Ado significantly inhibited TNF- α accumulation in conditioned medium induced by LPS, HS, or HA. Data are mean \pm SD ($n \geq 4$). * P <0.005 vs no Ado; # P <0.05 vs no Ado. **C** TNF- α mRNA expression was triggered as early as 2 h following LPS challenge, returned to baseline levels after 6 h, and was inhibited by Ado pretreatment. Data are mean \pm SD ($n=3$). **D** TNF- α concentration in conditioned medium was maximal after 4 h of LPS treatment and was inhibited by Ado pretreatment. Data are mean \pm SD ($n=5$). **E** TNF- α mRNA expression was measured 2 h after treatment with vehicle (1), Ado (2), LPS (3), Ado and LPS added at the same time (4), Ado added 20 min before LPS (5), and Ado added 20 min after LPS (6). Addition of Ado 20 min after LPS resulted in a lower inhibition of TNF- α expression than when Ado was added before LPS. * P <0.01. **F** Monocytes isolated from the blood of patients with acute MI were differentiated to macrophages and treated with Ado and/or LPS for 24 h as described above. Ado downregulated TNF- α concentration in conditioned medium. Results are compiled from 12 MI patients. * P <0.001 vs control

agonist NECA (Fig. 4A). The antagonists SCH58261 (A2A-specific) and ZM241,385 (A2A/A2B-specific) reversed the inhibition of TNF- α production induced by Ado (Fig. 4B). In NECA-treated cells, only high concentrations of SCH58261 and ZM241,385 were able to blunt the effect of NECA (Fig. 4C). These data demonstrate that A2-type Ado receptors are potent inhibitors of TNF- α production.

Adenosine Enhances TLR4 mRNA Expression by Macrophages

Using quantitative PCR, we measured the mRNA expression of components of the LPS recognition system in macrophages from both healthy volunteers and patients with MI treated by LPS and/or Ado for 24 h. In contrast to the effect observed at the protein level, we found that Ado increased mRNA levels of TLR4 in macrophages from healthy volunteers (3-fold, $P=0.008$; Fig. 5A). This increase was paralleled by increases of CD14 and MD2 mRNAs (Fig. 5A). Ado also reproducibly enhanced TLR4 mRNA in macrophages from MI patients (>2-fold, $P<0.001$; Fig. 5B). These data suggest that this increase of TLR4 mRNA expression is a feedback mechanism to compensate for the loss of TLR4 at the cell surface.

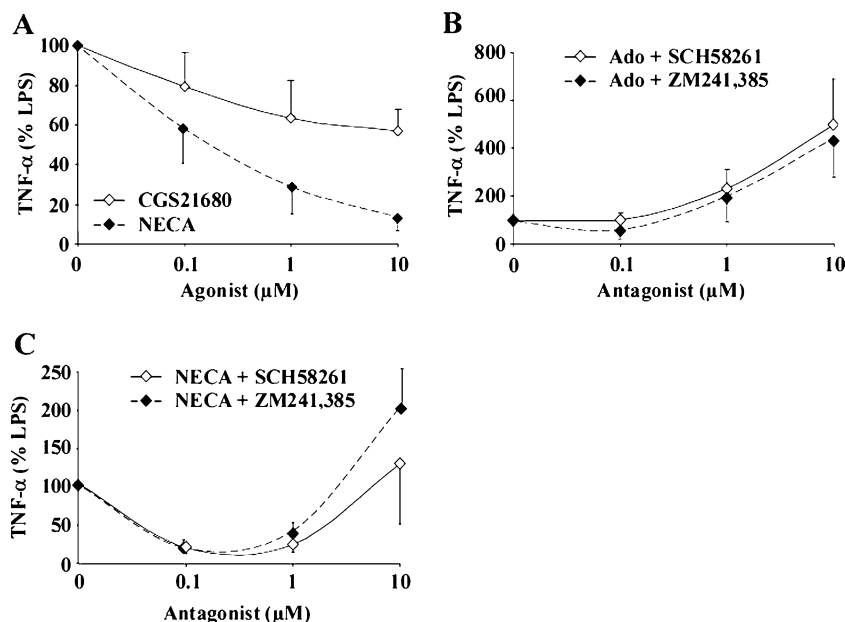


Fig. 4 The A2A Ado receptor inhibits TNF- α production. Macrophages obtained by differentiation of blood monocytes from healthy volunteers were treated with agonists and antagonists of Ado receptors. Agonists were added 15 min before LPS challenge (100 ng/mL). Antagonists were added 15 min before Ado or NECA (10 μ mol/L). Harvesting was performed 24 h after addition of LPS. TNF- α concentration in conditioned medium was assessed by ELISA.

A The Ado receptor agonists CGS21680 and NECA decreased the LPS-induced TNF- α secretion. **B** The antagonists SCH58261 and ZM241,385 reversed the effect of Ado on TNF- α production. **C** High concentrations of SCH58261 and ZM241,385 reversed the effect of NECA on TNF- α production. Results are expressed as percentage of LPS-treated cells. Data are mean \pm SD of at least three independent experiments. All effects were significant with P values <0.05

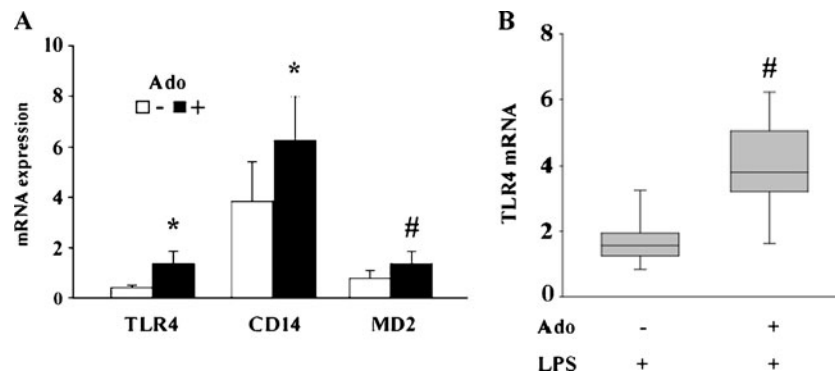


Fig. 5 Ado upregulates TLR4 mRNA expression. Macrophages isolated from PBMCs of healthy volunteers and patients with acute MI were differentiated to macrophages before treatment with 10 μmol/L Ado or vehicle for 15 min and challenge by 100 ng/mL LPS for 24 h. TLR4, CD14, and MD2 mRNA expression was measured by

quantitative PCR. **A** Ado enhanced the expression of all three mRNAs in macrophages from healthy volunteers. **B** Ado also enhanced TLR4 mRNA expression in macrophages from MI patients. Results were normalized to β-actin and are expressed as mean±SD (n=8 for **A** and n=12 for **B**). *P<0.01 vs control; #P<0.001 vs control

Adenosine Does Not Regulate Total TLR4 Protein Expression

To examine the effects of Ado on TLR4 protein levels, immunoblotting experiments were performed on whole cell extracts of THP-1 and primary macrophages treated by Ado for 15 min and then by LPS for different time periods. No significant effect was observed (Fig. 6).

Adenosine Quickly Downregulates TLR4 Surface Expression

The experiments showing an inhibition of TNF-α mRNA expression after 2 h of LPS challenge (Fig. 3C) incited us to investigate a potential rapid effect of Ado on TLR4 expression. THP-1 macrophages were treated by Ado for 15 min and imaged by a confocal fluorescence microscope. Macrophages displayed a clear expression of TLR4 at their surface under control conditions and Ado highly reduced this extracellular expression. In contrast, Ado did not modify intracellular TLR4 expression (Fig. 7). These results show that Ado is able to quickly decrease TLR4 availability at the cell surface.

Discussion

The main finding of the present study is that Ado downregulates the expression of TLR4 at the cell surface of macrophages. This effect is present in cells from patients with acute MI and leads to a reduced inflammatory response to breakdown products of the extracellular matrix.

Comparing healthy volunteers and MI patients, we found that TLR4, CD14, and MD2, the three components of the LPS-recognition system, were significantly more expressed in blood cells from MI patients than in healthy volunteers.

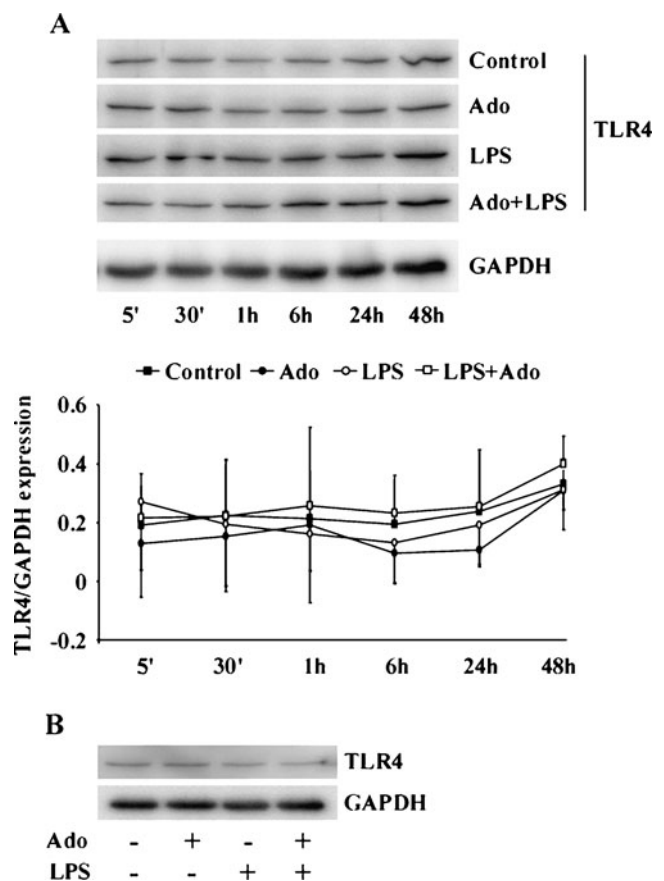


Fig. 6 Ado does not regulate total TLR4 protein expression. TLR4 protein expression was assessed in whole cellular extracts by immunoblotting. GAPDH was used as loading control. **A** THP-1 monocytes were differentiated to macrophages by 150 nM PMA for 24 h and were treated with 10 μmol/L Ado for 15 min before challenge with 100 ng/mL LPS for indicated period of times. *Upper panel* representative pictures. *Lower panel* quantification results showing a constant level of TLR4. TLR4 expression was normalized to GAPDH. Results are mean ±SD from three independent kinetics. **B** Primary human macrophages obtained from PBMCs of healthy volunteers were treated by 10 μmol/L Ado and 100 ng/mL LPS for 24 h. TLR4 expression was similar among groups. A representative experiment of two is shown

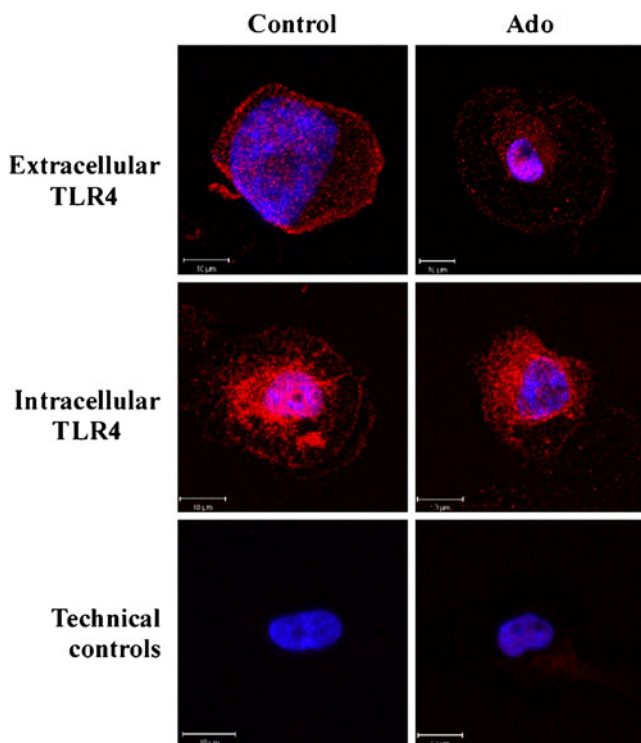


Fig. 7 Ado rapidly decreases TLR4 surface expression. THP-1 monocytes were differentiated to macrophages by 150 nM PMA for 24 h and were treated with 10 µmol/L Ado for 15 min. As determined by confocal microscopy, extracellular TLR4 expression was down-regulated by Ado. Intracellular expression remained unaffected. Technical controls performed without primary antibodies attested for the specificity of TLR4 staining. Representative pictures of six randomly taken pictures per condition are shown. Bar scale, 10 µm

These observations show that activation of TLR4 pathway after MI can be evidenced by transcriptomic profiling of blood cells, in accordance with a previous report [8]. The enhanced activation of TLR4 pathway in MI patients is not consecutive to increased number of circulating leukocytes or monocytes. Also, TLR4 expression was not correlated to blood cells numbers, and MI patients had similar monocyte frequencies (3.4% to 9%) to healthy volunteers (4% to 12%). Therefore, our data suggest that activation of TLR4 shortly after MI is not a non-specific consequence of activation of inflammation. The strong correlation between TLR4 and TnT indicates that TLR4 activation reflects the amount of myocardial injury. These results are contradictory with data from TLR4 knockout mice where infarct size remained unaffected [2].

Inflammation significantly contributes to LV remodeling after MI. Several lines of evidence indicate that Ado may be cardioprotective in the setting of MI, not only through its anti-inflammatory properties, which have been extensively characterized by several groups including ours, but also through direct interaction with regulators of extracellular matrix turnover [6, 7]. In this study, we report that Ado directly affects TLR4 activation, a major trigger of

inflammation. TLR4 is abundantly expressed in the heart [1] where it participates to the inflammatory response to ischemic injury. Genetic ablation of TLR4 [9] or administration of a TLR4 inhibitor [10] protects mice from myocardial ischemia–reperfusion injury and decreases LV remodeling after MI [2]. In studies by Oyama and colleagues [9] and Shimamoto and colleagues [10], infarct size was reduced in the absence of TLR4 activation, whereas in the study of Timmers and colleagues [2], infarct size was unaffected by TLR4 deficiency. This discrepancy most probably comes from the different experimental protocol used, i.e., ischemia/reperfusion [9] vs. permanent occlusion [2]. Together, these studies suggest that TLR4 is deleterious both during the early phase after ischemia/reperfusion and during the later phase of remodeling. Our results strengthen these findings, identify macrophages as mediators of the deleterious effects of TLR4 activation, and suggest that Ado, or better A2A agonists, may be used to circumvent this activation and prevent or limit LV remodeling.

Toll-like receptor function is tightly regulated by compartmentalization [11]. Upon ligand binding, TLR4 is endocytosed, resulting in internalization and delivery to endolysosomes where it is degraded [12]. This avoids excessive activation of the inflammatory response and links innate to adaptive immunity. Our data showing that Ado decreases cell surface expression of TLR4 is consistent with receptor internalization. Also, the observation that the inhibition of TNF-α expression by Ado is less robust when added after than before LPS is consistent with a reduction of TLR4 availability for activation at the cell surface. The absence of modifications of total TLR4 protein expression observed by immunoblotting, together with increased TLR4 mRNA expression after Ado treatment, suggest that the loss of TLR4 at the cell surface is followed by an increase in TLR4 gene transcription. Such a compensatory mechanism may limit the beneficial effect of Ado.

A synergism between Ado receptors and TLR4 has previously been reported. Indeed, stimulation of both the A2A receptor and TLR4 appear to result in increased production of vascular endothelial growth factor production and decreased production of TNF-α, a mechanism described as the angiogenic switch [13, 14]. The present study may provide an explanation for the decrease of TNF-α, namely through internalization of TLR4, which is amplified in the presence of both LPS and Ado.

To demonstrate that the effect of Ado on TLR4 reported here is relevant in the setting of MI, we also stimulated macrophages with endogenous ligands of TLR4 which are potentially present in the heart after MI, such as HS and HA. HS is an endogenous TLR4 ligand involved in endothelial barrier integrity [15] and HA is upregulated in the kidney after ischemia–reperfusion [16]. Since Ado

inhibited TNF- α production in the presence of both agonists, we conclude that the interference with TLR4 demonstrated in this study is not specific to LPS and is applicable to the inflammatory mechanism in the heart. In addition, we verified that this anti-inflammatory effect was effectively present in macrophages isolated from patients with acute MI.

We identified the A2A and A2B receptors as mediators of the anti-inflammatory effects of Ado. This is consistent with previous data showing that the A2A and A2B receptors, but not the A3 receptor, mediate the inhibition of TNF- α release by murine macrophages [17]. In contrast, several in vitro and in vivo studies reported that the A3 receptor was also able to inhibit TNF- α release [18–20]. This discrepancy is certainly attributable to cell and species specificities of Ado effects [21] and illustrates the necessity to study human primary cells. Most of the effects of Ado on TNF- α reported so far have been evidenced from murine models or human cell lines [18, 22–25]. Our present data show for the first time in human primary macrophages that Ado, through activation of A2A and A2B receptors, is a robust inhibitor of TNF- α production.

Lipopolysaccharide is known to induce A2A receptor expression in macrophages [5, 7, 26]. The inflammatory cytokines IL-1 α and TNF- α have been shown to increase A2A expression in these cells [27]. The fact that TNF- α increases A2A expression, which in turn inhibits TNF- α production, appears as a compensatory mechanism aimed at limiting potential over-activation of the inflammatory response. In addition, TNF- α has been shown to prevent A2A receptor desensitization induced by Ado, resulting in an amplification of the inhibition of TNF- α by Ado [28]. Our results showing that Ado downregulates TLR4 complicate things further and indicate that the interaction between TNF- α production and Ado is a complex phenomenon involving several regulatory loops, among which a direct limitation of TLR4 availability at the cell surface by Ado.

In conclusion, we have shown that Ado induces a reduction of TLR4 expression at the surface of human macrophages, resulting in a robust inhibition of TNF- α production. This mechanism may be important to limit the activation of TLR4 in response to injury to the extracellular cardiac matrix.

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