SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

INVESTIGATION OF POTENTIAL MECHANISMS INVOLVED IN THE ANTI-INFLAMMATORY EFFECTS OF APOPTOTIC CELLS

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The PhD Defense takes place at the Lecture Hall of Building A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, at 1:00 p.m. on 10th of Januar, 2017.

1. INTRODUCTION

1.1. APOPTOTIC CELL CLEARANCE

Most cell types have a limited life span, which ends physiologically through the process of apoptosis, or programmed cell death. Apoptotic cell death and the clearance of dying cells play an important and physiological role not only in the embryonic development but also in normal tissue turnover, tissue remodelling, maintenance of immune homeostasis, and resolution of inflammation. Under normal circumstances, phagocytosis of apoptotic cells engulfed by phagocytes that are professional engulfers (such as macrophages and immature dendritic cells) or neighboring cells (such as fibroblasts and epithelial cells) is fast and effective without causing inflammation and immune response.

The anti-inflammatory effects of apoptotic cell clearance

Whereas the phagocytosis of a variety of pathogenic targets, especially bacteria and virally-infected cells, normally triggers a pro-inflammatory response in macrophages, ingestion of apoptotic cells by macrophages usually induces an anti-inflammatory phenotype. Apoptotic cells do not simply fail to provide pro-inflammatory signals; rather, they can initiate signaling pathways in macrophages which can actively interfere with the inflammatory program. Preincubation of macrophages with apoptotic cells strongly suppresses the inflammatory response induced *via* Toll-like receptor 4 (TLR4) by lipopolysaccharide (LPS), a component of the cell wall of Gram-negative bacteria.

Subsequently, soluble mediators are released from macrophages, which act in a paracrine or autocrine fashion to amplify and sustain the anti-inflammatory response. An inverse relationship were observed between the secretion of the pro-inflammatory cytokines TNF-α, IL-1β, and IL-12 and the transient release of IL-10 from LPS-stimulated macrophages after their interaction with apoptotic cells and a similarly between the release of TGF-β, platelet-activating factor, and PGE2 and the secretion of TNF-α, IL-1β, and several other inflammatory cytokines by macrophages after engulfment of apoptotic targets.

Lipids derived from the engulfed apoptotic cells serve as source of endogenous ligands (fatty acids, oxysterols) to activate lipid sensing receptors (LXRα and β, PPARγ and δ, PPARs). Nuclear receptor activation upon apoptotic cell phagocytosis leads to the upregulation of phagocytic receptors (e.g., MerTK, CD36), TG2, and opsonins (e.g., MFG-E8, C1qb, Gas6 and thrombospondin), and phagocyte metabolism.

1.2. ADENOSINE

Adenosine is an endogenous purine nucleoside that, following its release from cells or after being formed extracellularly, diffuses to the cell membrane of surrounding cells, where it binds to its receptors.

There are several important producer cell types of extracellular adenosine. Neutrophils and endothelial cells release large amounts of adenosine at sites of metabolic distress, inflammation and infection. Activated macrophages (e.g. LPS-treated) can also serve as a major source of extracellular adenosine *via* ATP release and degradation.

Adenosine receptors and signaling pathways

There are four adenosine receptors, all of which are G protein-coupled receptors (GPCR) and are abundantly expressed by macrophages. The genes for these receptors have been analyzed in detail and are designated A_1 (A_1R), A_{2A} ($A_{2A}R$), A_{2B} ($A_{2B}R$) and A_3 (A_3R).

The Gi-coupled adenosine A_1 receptors are stimulated by 10^{-10}–10^{-8} M concentrations of adenosine and mediate decreases in intracellular cyclic AMP (cAMP) levels, Gs-coupled adenosine A_{2A} and A_{2B} receptors are stimulated by higher (5×10^{-7} M and 1×10^{-5} M, respectively) concentrations of adenosine and mediate increases in cAMP levels, while Gi-coupled adenosine A_3 receptors are stimulated by 10^{-6} M concentrations of adenosine and mediate adenylate-cyclase inhibition. Lower concentrations of adenosine activate the high affinity A_1, A_{2A}, and A_3 receptors, and high adenosine concentrations stimulate the low affinity A_{2B} receptors.

The roles of adenosine with special regard to its immune modulatory effects

The general extracellular signalling molecule adenosine acts as a modulator of various biological processes in many cell types, tissues and organs. It's effect is critical in the normal functions of the cardiovascular (CV) system, gastrointestinal tract, central nervous system (CNS) and immune system. In the CNS adenosine is an inhibitory neurotransmitter. Under physiological conditions, it is involved in the development and maturation of neuronal networks, in the process of cognition and memory, as well as in the regulation of sleep and arousal. In the CV adenosine exerts vasodilatatory, antithrombotic, blood pressure and heart rate lowering effects.
In the immune system, adenosine acting *via* different types of it's receptors expressed by various immune cells modulates wide spectra of innate immune responses e.g.:

- Adenosine receptor ligation on monocytes or macrophages inhibits production of IL-12 upon binding of lipopolysaccharide (LPS) through Toll-like receptor 4 (TLR-4). The stimulation of adenosine A_{2A} receptor leads to a decrease in TLR-4 induced release of TNF-α, MIP-1α, and nitric oxide (NO) and augments production of anti-inflammatory cytokine IL-10. Broussas et al. (2002) investigated that adenosine also inhibited the release of tissue factor (TF) from LPS stimulated human monocytes *via* binding to adenosine A_3 receptors. Adenosine increases vascular endothelial growth factor (VEGF) production from macrophages, thus facilitates the process of angiogenesis.
- During metabolic stress, without TLR activation, adenosine acts as a chemotactic molecule and promotes chemotaxis of immature human dendritic cells *via* binding to adenosine A_1 and A_3 receptors and increases intracellular calcium concentration and actin reorganization. However, mature plasmacytoid dendritic cells express adenosine A_{2A} receptors and binding of adenosine to these receptors leads to a decrease in cytokine (IL-6, IL-12 and IFN-α) production in response to CpG oligodeoxynucleotides (ODN).
- In chronic inflammatory conditions like asthma and chronic obstructive pulmonary disorder (COPD), adenosine mediated stimulation of adenosine A_{2B} and A_3 receptors expressed on human mast cells causes degranulation of mast cells and release of histamine, prostaglandins, leukotrienes, serotonin, pro-inflammatory cytokines (IL-4 IL-13, IL-8, IL-1β), angiogenic factors (IL-8 and VEGF), chemokines and various tissue damaging enzymes, proteases.
- Adenosine A_1 receptor activation induces chemotaxis and increases adherence of neutrophils to endothelial cells. While adenosine A_{2A} receptor activation decreases neutrophil adherence to endothelium, uncouples chemoattractant receptors from their stimulus transduction proteins and inhibits the synthesis of toxic free radicals.
- Adenosine *via* binding to adenosine A_3 receptor increases the antitumor activity of NK cells.

1.3. GENERAL ASPECTS OF TNF BIOLOGY

About 30 years ago, a soluble cytokine termed tumor necrosis factor (TNF) has been identified that is produced upon activation by the immune system, able to exert significant cytotoxicity on many tumor cell lines and to cause tumor necrosis in certain animal model systems.

Structure and synthesis of TNF-α

Human TNF-α is expressed as a 27-kDa (233 amino acid) protein. It is primarily produced as a type II transmembrane protein arranged in stable homotrimers. From this membrane-integrated form 27-kDa TNF-α (mTNF-α) the soluble homotrimeric cytokine (sTNF) 17-kDa is released *via* proteolytic cleavage by the metalloprotease TNF alpha converting enzyme (TACE).

Though TNF-α is a pleiotropic cytokine produced by many different types of cells in the body, cells of the monocytic lineage - such as macrophages, astroglia, microglia, Langerhans cells, Kupffer cells, and alveolar macrophages - are the primary synthesizers of it.

TNF receptors

The members of the TNF ligand family exert their biological functions *via* interaction with their cognate membrane receptors, comprising the TNF receptor (TNF-R) family, whose members include TNF receptor 1 (TNF-R1), also known as CD120a, p55 or p60), TNF receptor 2 (TNF-R2), also known as CD120b, p75 or p80, Fas antigen, CD27, CD30, CD40, 4-1BB and several other receptors.

TNF-R1 is constitutively expressed in most tissues, whereas expression of TNF-R2 is highly regulated and is typically found in cells of the immune system. In the vast majority of cells, TNF-R1 appears to be the key mediator of TNF signalling, whereas in the lymphoid system TNF-R2 seems to play a major role.

TNF-α signal transduction and biological role

TNF family members can generate bidirectional signals. While on one side, TNF-TNFR interactions initiate multiple signaling pathways promoting cell survival, death, differentiation, or inflammation in TNFR-expressing cells, TNF-R family members (TNF) also serve as ligands to initiate reverse signaling, regulating cell proliferation, cytokine secretion, oxidative burst and T cell maturation.

Transmembrane TNF-α acts as a ligand by binding to TNF-α receptors as well as functioning as a receptor that transmits outside-to-inside (reverse) signals back into the transmembrane TNF-α-bearing cells.

Owing to its strong pro-inflammatory and immunostimulatory activities, TNF is, in general, an important mediator of progression of many autoimmune diseases. Important examples are

rheumatoid arthritis and inflammatory bowel disease (Crohn's disease), where significant clinical improvement can be achieved when patients are treated with TNF neutralizing agents.

The anti-TNF-α molecules used in therapy

Even though TNF-α is important for normal homeostatic mechanisms including host defense, dysregulated production of TNF-α has been found in several inflammatory diseases. In addition, TNF-α-activated macrophages are the principal components of the immunopathology of many autoimmune diseases, including rheumatoid arthritis, inflammatory bowel disease, psoriatic arthritis, ankylosing spondylitis, juvenile chronic arthritis, atherosclerosis, and sepsis.

Nowadays five drugs (TNF-α blocking agents) are currently licensed for the treating of some diseases. These five drugs are: 1) etanercept is a recombinant human soluble fusion protein of TNF-R2 coupled to the Fc portion of IgG; 2) infliximab is an anti-TNF human-murine chimeric IgG1 monoclonal antibody; 3) adalimumab is a human anti-human TNF-α antibody; 4) certolizumab pegol is a PEGylated TNF-α antibody; and 5) golimumab is a human anti-TNF-α IgG1κ monoclonal antibody that can be administered by the patient. These drugs are almost equally neutralize the TNF-α, however significant differences can be found as to the disease they are effective. This suggests that in addition to the neutralization of TNF-α, other effects may dictate the therapeutic results.

2. AIMS OF THE STUDY

In my thesis I decided to investigate the potential involvement of two macrophage cell surface receptors (adenosine A_3 receptor, mTNF-α) in the apoptotic cell-mediated anti-inflammatory effects on macrophages. Our main objectives were:

1. It is known, that adenosine is produced by macrophages in from the adenine nucleotide released by apoptotic cells and exerts anti-inflammatory effects in an adenosine A_{2A}R-dependent manner. In the present study, I investigated the involvement adenosine A_3R in the regulation of inflammatory responses of macrophages engulfing apoptotic cells.

2. Since both apoptotic cell uptake and mTNF-α signaling have been shown to inhibit pro-inflammatory cytokine production of LPS-treated macrophages, I decided to investigate whether apoptotic cells trigger mTNF-α to inhibit LPS-induced pro-inflammatory production of macrophages. Since mTNF-α signaling was not known I decided also to identify its signaling elements.

3. MATERIALS AND METHODS

Experimental animals

The experiments were done using 3-month-old wild type, adenosine A_3 receptor deficient mice (generated on a C57BL/6 background), and adenosine A_{2A} receptor deficient mice (generated on a FVB background).

In second part of the experiments 2-mo-old NMRI male mice were used, whereas in some experiments TNF-α –deficient male mice and their C57BL/6 wild-type littermates were used after being killed by ether anesthesia. Mice were maintained in a specific pathogen-free condition in the Central Animal Facility and all animal experiments were approved by the Animal Care and Use Committee of the University of Debrecen (DEMÁB).

Cell culture procedures

Macrophages were obtained by peritoneal lavage with sterile physiological saline. Cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 2 mM glutamine, 1 mM Na-pyruvate, 50 μM 2-mercaptoethanol, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in 5% CO_2 for 2 days before use. After 3–4 h incubation, the non-adherent cells were washed away. Before the experiments the cells were cultured for 2 days replacing media daily.

For bone marrow–derived macrophage (BMDMs), bone marrow was isolated from femurs with sterile physiological saline and cultured in DMEM supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% L929 conditioned media at 37°C in 5% CO_2 for 7 days before use. RAW264.7 cells were obtained from the American Type Culture Collection and were maintained in a DMEM culture medium containing glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% FBS. Peripheral human blood mononuclear cells were isolated by density gradient centrifugation with Ficoll-Paque Plus from buffy coats obtained from the blood collected from healthy volunteers by the Blood Donation Center, Debrecen. Buffy coats not used up therapeutically were provided to us with the permit (RKEB 3582-2012) from the Regional Research Ethical Committee. $CD14^+$ cells were separated by magnetic sorting with MACS, followed by washing with PBS containing 0.5% BSA and 2 mM EDTA. Freshly isolated monocytes were cultured for a period of 5 days in IMDM supplemented with 10% AB serum and 5 ng/ml M-CSF to differentiate them from macrophages.

Thymocyte apoptosis induction *in vitro*

Thymocytes were prepared from 4-week-old wild-type or pannexin null mice. The isolated thymocytes were cultured for 18 or 24 h (10^{6-7} cells/ml) in RPMI 1640 medium supplemented with penicillin/streptomycin in the absence of serum. This method typically resulted in >80% apoptotic cells (as assessed by propidium iodide/annexin V–FITC staining). In some experiments apoptosis was induced by 0.1 μM dexamethasone or by a combination of 5 ng/ml phorbol dibutyrate and 1 μg/ml ionomycin. Apoptotic cells that died spontaneously were used in the immune downregulation assays at a 10:1 (apoptotic cell:macrophage) ratio.

Determination of adenosine A_3 receptor expression on the cell surface

Wild type and A_3R null peritoneal macrophages were co-incubated with apoptotic thymocytes for 1 h in 1:10 ratio. After replacing the media and washing away the apoptotic cells, macrophages were incubated for additional 1, 3 or 5 h. After the treatments the macrophages were washed (1× PBS), collected, blocked with 50% FBS for 30 min and labeled with the anti-mouse A_3R antibody or goat IgG isotype control. For detection the cells were stained with FITC-conjugated anti-goat IgG. The stained cells were analyzed on a FACSCalibur. The results were analyzed by using the WinMDI 2.9 software.

Determination of adenosine A_3 receptor mRNA expression

Wild-type peritoneal macrophages were co-incubated with various target cell types: apoptotic, living, heat killed (45 min, 55°C) or anti-CD3-pretreated (10 μg/ml, 20 min) adenosine A_3 null thymocytes for 1 h in the 1:10 ratio. After washing away the apoptotic cells and replacing the media, mRNA was collected 2 h later.

Determination of adenosine A_3 receptor protein levels

Peritoneal macrophages were homogenized in an ice-cold lysis buffer containing 0.5% Triton X-100. The protein concentration of samples was diluted to 2 mg/ml, then the samples were boiled with an equal volume of Laemmli buffer. Electrophoresis was performed in a 10% SDS-polyacrylamide gel. The separated proteins were transferred to an Immobilion-P transfer membrane and were probed with an anti-mouse A_3R antibody. To detect A_3R signals, HRP-labeled anti-goat antibodies were used. Protein bands were visualized by the Immobilon Western Chemiluminescent HRP substrate.

Determination of cytokine production

Wild-type and A_3R null peritoneal macrophages were plated onto 24-well plates at a density of 1×10^6 cells/well. To determine cytokine production by macrophages exposed to apoptotic cells, the macrophages ($A_3R^{+/+}$or $A_3R^{-/-}$) were exposed to apoptotic cells for 1 h in the presence or absence of the A_3R-selective agonist IB-MECA (10 μM) or the A_3R-selective inhibitor MRS1523 (10 μM). Then the apoptotic cells were washed away, the compounds were readded and the macrophages were cultured for an additional 5 h.

The cytokine content of the cell culture media in various experiments was analyzed by a mouse cytokine array. The pixel density in each spot of the array was determined by ImageJ software. Alternatively, cytokine-induced neutrophil-attracting chemokine (KC), macrophage inflammatory protein-2 (MIP-2), TNF-α, IL-6 and TGF-β cytokine levels were measured with ELISA kits. For TGF-β, the supernatants were acid-activated before the assay according to the manufacturer's instructions.

Phagocytosis assay

For visualizing apoptotic cells in macrophages, the macrophages were plated in 2-well chamber slides in the concentration of 5×10^5/well and cultured for 48 h before staining overnight with 10 μM CMTMR ((5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethyl-rhodamine), while thymocytes were labeled overnight with 6 μM CFDA (6-carboxy-3',6'-diacetylfluorescein). After coculturing the macrophages with CFDA-labeled apoptotic cells for 30 min, the cells were washed and fixed in ethanol/acetone (1:1) for 10 min at −20°C. Images were taken with an Olympus FV1000 confocal laser scanning microscope. Five hundred cells were counted for apoptotic cell uptake in each individual experiment.

Determination of NO production of macrophages engulfing apoptotic cells

Wild-type or A_3R null macrophages were exposed to apoptotic cells for 1 h. The media were replaced and macrophages were incubated for an additional 1 h. Cell culture supernatants were analyzed for NO production by measuring nitrite, a stable oxidation product of NO, using the Griess–Ilosvay method.

Immunohistochemistry

BMDMs and RAW264.7 macrophages (1×10^6) were washed in PBS, after which fixation in acetone (15 min at -20°C) was performed just before the immunolabeling. After rinsing in PBS, the samples were incubated at 4°C overnight with FITC-labeled anti-mouse TNF-α or FITC-labeled rat

IgG1 isotype Abs. Then the samples were washed in PBS and incubated for 1 h at room temperature with DAPI (1:600). After the rinse and cover, PBS-glycerol samples were examined using an Olympus microscope and analyzed by a computer image analysis (magnification, x600).

Determination of TNFR I and II expression on the apoptotic thymocyte cell surface

Control and apoptotic thymocytes (1 x 10^6) were washed with PBS, incubated with PE-labeled anti-mouse TNFR I and II Abs or their isotype controls for 30 min at room temperature, then washed and fixed in 1% paraformaldehyde. Stained cells were analyzed on a FACSCalibur. The results were analyzed by WinMDI 2.9 software.

Immunofluorescence staining and confocal microscopy

BMDMs were plated in eight-well chamber slides (2.5 x 10^5/well) and cultured for 7 days before use. The cells were treated with 100 ng/ml LPS for 1 hour. After the exposure of macrophages to apoptotic cells stained overnight with 10 μM CMTMR (1:5) for 30 min, the cells were washed with HEPES buffer and fixed in methanol/acetone (1:1) for 10 min at -20°C. The macrophages were blocked with the 10% normal donkey serum in HEPES for 2 hours at 4°C, then washed with ice-cold HEPES buffer twice and stained with goat anti-mouse TNF-α primary Ab overnight at 4°C. After three washes, samples were incubated with Alexa Fluor 488–conjugated donkey anti-goat secondary Ab for 4 hours. The cells were then stained with DAPI for 10 min, washed four times, left in the HEPES buffer, and imaged immediately using a Zeiss LSM 510 confocal laser scanning microscope. For visualizing the distribution of mTNF-α, overview images and three-dimensional stacks were acquired at 1.5-mm optical thickness. Red fluorescence of CMTMR–stained apoptotic cells and blue fluorescence of nuclei were imaged for the same fields in the multitrack mode to avoid spectral overlap.

Determination of mTNF-α expresssion on the cell surface

The BMDM and RAW264.7 cells were stimulated with 100 ng/ml bacterial LPS for the indicated time points after preincubation with galardin (5 μg/ml) for 4 hours or alone. After the treatment, macrophages were washed (1x PBS), collected, blocked with 50% FBS for 30 min, stained with FITC-labeled anti-mouse TNF-α or FITC-labeled rat IgG1 isotype control Abs, and fixed in 1% paraformaldehyde. The stained cells were analyzed on a FACSCalibur. The results were analyzed by WinMDI 2.9 software.

Isolation of total RNA and quantitative RT-qPCR

Total RNA was isolated from control and bacterial LPS-treated BMDMs and RAW264.7 macrophages (3 x 10^6 cells/sample) by TRI reagent. After various treatments, the macrophages were washed with ice-cold PBS. RNA was extracted with TRI reagent. cDNA was synthesized with a high-capacity cDNA archive kit according to the manufacturer's instructions. TNF-α, TGF-β1, and IFN-α mRNA levels were determined with TaqMan PCR using FAM-MGB–labeled probes. Samples were run in triplicates on a Roche (LC480) platform using SDS2.1 software for evaluation. The gene expression was normalized to the GAPDH expression.

Phosphorylation changes in the MAPK signaling pathway following triggering mTNF-α

Anti–mTNF-α Ab or its isotype control was coated to six-well plates to which BMDMs were added (3 x 10^6 cells/well) alone or in the presence of neutralizing TGF-β Ab (5 μg/ml). After 2 hours of incubation the relative levels of phosphorylation of MAPKs and other serine/threonine kinases were determined by using a human phospho-MAPK array. The pixel density in each spot of the array was determined by ImageJ software.

Western blot analysis to detect phosphorylation levels of MKK3/MKK4/MKK6/MKK7

The whole cell homogenate was used. 40 μg protein was run on a 12% polyacrylamide gel, and blotted onto polyvinylidene difluoride membranes using the Bio-Rad electrophoresis and transfer system. The free binding sites of membranes were blocked with a 5% non fat dry milk powder in 20 mM Tris, 0.1 M NaCl buffer with 0.1% Tween for 1 hour at room temperature. The blots were then incubated overnight with primary Abs (MKK3/4/7, p-MKK4/7 and 3/6) at a 1:500 dilution. To detect these signals, peroxidase-labeled anti-rabbit IgGs (1:10,000) were used and the ECL was visualized using an ECL system. An equal loading of proteins was demonstrated by probing the membranes with β-actin Ab.

Statistical analysis

All data are representative of at least three independent experiments carried out on three different days. The values are expressed as mean ± SD. The p values were calculated by using a two-tailed Student t test for two samples of unequal variance. The analysis of cytokine array experiments was carried out by using the ANOVA test. A p value < 0.05 was considered to be statistically significant.

4. RESULTS

4.1. ADENOSINE A_3 RECEPTORS NEGATIVELY REGULATE THE ENGULFMENT-DEPENDENT APOPTOTIC CELL SUPPRESSION OF INFLAMMATION

The expression of adenosine A_3 receptors is decreased on the cell surface of macrophages during the engulfment of apoptotic cells

Previous studies have shown that adenosine A_3Rs are expressed by macrophages and contribute to the directed migration of these cells. Since our studies have shown that macrophages engulfing apoptotic cells produce adenosine and express increasing levels of A_{2A}Rs, we decided to test whether the expression levels of the A_3R are altered during the phagocytosis of apoptotic cells. We can confirm the expression of the cell surface adenosine A_3 receptors in mouse peritoneal macrophages, but this expression is significantly decreased following the incubation with apoptotic cells. The inhibition partially involved transcriptional regulation, since not only the cell surface expression of A_3R, but its mRNA and total protein levels also decreased during long term phagocytosis. However, the decrease in the cell surface expression could be detected much earlier than that of the mRNA and the protein, indicating that fast posttranslational mechanisms might also be involved in the downregulation of A_3R.

Cytochalasin D does inhibit the engulfment process, but it does not influence the recognition of apoptotic cells. The binding of phosphatidylserine on the surface of apoptotic cells plays a key role in their recognition and subsequent uptake by the macrophages, and this recognition can be inhibited by the preincubation of apoptotic cells with recombinant annexin V (which binds to phosphatidylserine). While recombinant annexin V prevented the downregulation of A_3R mRNA and protein expression by apoptotic cells, cytochalasin D had no effect on it, suggesting that not the engulfment, but the recognition *per se* triggers the decrease in the A_3R mRNA and protein expression. The inhibition of the A_3R expression during phagocytosis was specific for the engulfment of apoptotic cells, as the uptake of neither necrotic nor antibody-coated cells affected it.

Loss of adenosine A_3 receptor influences the pro-inflammatory cytokine production by macrophages engulfing apoptotic cells

The evaluation of the cytokine secretion profile of unstimulated macrophages was performed using a highly sensitive cytokine antibody array method, enabling the simultaneous detection of low concentrations of multiple cytokines in one assay (picogram per milliliter range). The map of the 40

cytokines detected on the membranes. The cytokines in our experimental systems were first evaluated by the experiments using untreated wild-type and $A_3R^{-/-}$ macrophages *in vitro*. The results show that the loss of the A_3R did not affect significantly the composition of the cytokines released. In the levels of the cytokines we found individual animal variations, but when the MIP-2 and KC levels were determined in many samples by ELISA, no significant difference could be detected in their basal levels when we compared the wild-type and the $A_3R^{-/-}$. However, when macrophages were exposed to apoptotic cells, we found four cytokines whose production was increased by the wild-type macrophages engulfing apoptotic cells compared to their non-engulfing counterparts, but decreased in $A_3R^{-/-}$ macrophages. These cytokines include the cytokine-induced neutrophil-attracting chemokine (KC), and the macrophage inflammatory protein-2 (MIP-2), which act as chemoattractants for neutrophils and/or other cell types, the pro-inflammatory cytokine IL-1α and the antagonist of the IL-1 receptor (IL-1ra). Among the cytokines released in altered amounts in the supernatant by cultured $A_3R^{-/-}$ macrophages, MIP-2 showed the most dynamic change in response to the apoptotic cell exposure, and MIP-2 and KC levels were detected in the highest amounts. These observations correlate with those found in the $A_{2A}R$ null macrophages, in which the loss of the $A_{2A}R$ affected the production of the same cytokines in the presence of the apoptotic cells, but the direction of the alteration was the opposite: the loss of $A_{2A}Rs$ lead to an increase in the production of these cytokines. To confirm further the effect of A_3Rs on the expression of the above cytokines during the apoptotic cell engulfment, we decided to test the effect of A_3R on the pro-inflammatory cytokine production by exposing wild-type macrophages either to the highly specific A_3R agonist IB-MECA or to the A_3R antagonist MRS1523.

Using wild-type macrophages we could confirm the decreased expression of the pro-inflammatory cytokines in the presence of the A_3R antagonist, indicating that altered cytokine response of engulfing A_3R null macrophages is not due to an altered differentiation of the cells. In addition, the presence of the A_3R agonist enhanced the expression of these cytokines indicating that A_3R signaling indeed negatively regulates the engulfment-dependent apoptotic cell suppression of inflammation, at least if these cytokines are concerned. The same compounds had no effect on the apoptotic cell-induced pro-inflammatory cytokine production, when they were administered to $A_3R^{-/-}$ macrophages (data not shown) indicating that they indeed act *via* the A_3Rs. To confirm the results, MIP-2 and KC protein levels were also assessed by ELISA. In harmony with the cytokine array results, while the production of MIP-2 and KC was enhanced by wild-type macrophages exposed to apoptotic cells as compared to non-engulfing macrophages, the release of both MIP-2 and KC by $A_3R^{-/-}$ macrophages was decreased under the same conditions.

The apoptotic cell-mediated decrease in MIP-2 and KC production by $A_3R^{-/-}$ macrophages is related to an enhanced protein kinase A signaling by A_{2A}Rs

Since among the neutrophil chemoattractants, the release of which was simultaneously altered by the loss of both the A_{2A}R and the A_3R, the production of MIP-2 and KC was studied in our previous experiments in detail, we decided to investigate further the regulation of the expression of these two cytokines. Previously we have shown that the production of these two cytokines would be triggered by the uptake of apoptotic cells, but the simultaneous activation of the adenylate-cyclase pathway triggered by the A_{2A}Rs attenuated the response. Since A_3Rs are known to inhibit the adenylate-cyclase activity *via* stimulating Gi, we speculated that if adenosine is produced by engulfing macrophages in an amount that can trigger A_3Rs, the loss of A_3R signaling would lead to the enhanced adenylate-cyclase signaling by A_{2A}Rs in A_3R null macrophages. If it is so, the enhanced adenylate-cyclase signaling could explain the more effective inhibition of KC and MIP-2 production by engulfing A_3R null macrophages. Indeed, the addition of SCH442416, a selective A_{2A}R antagonist, H89, a protein kinase A inhibitor, or Rp-cAMPS triethylamine, a specific membrane-permeable inhibitor of activation by cAMP of cAMP dependent protein kinase I and II, all significantly enhanced the production of MIP-2 and KC in apoptotic cell-triggered A_3R null macrophages. In contrast, the addition of forskolin, an adenylate-cyclase activator decreased the MIP-2 and KC production in wild-type macrophages. The same concentration of forskolin, however, was ineffective in A_3R null cells indicating that adenylate-cyclase is fully activated in these cells. When the adenylate-cyclase pathway inhibitor Rp-cAMPS triethylamine was applied to A_{2A}R or A_3R null macrophages and their wild-type counterparts, a similar MIP-2 and KC production was found in the paired macrophages indicating that in the absence of the adenylate-cyclase signaling, the difference in apoptotic cell triggered-MIP-2 and KC production disappears. At the same time a significant strain difference was detected in the apoptotic cell-induced pro-inflammatory cell response. However, as we do not know at the moment how apoptotic cells induce the adenosine-controlled pro-inflammatory response, we cannot identify the molecular determinants for the observed difference between the mouse strains. These data indicate that in wild-type macrophages the adenylate-cyclase pathway is not fully activated, and a balance in the A_{2A}R and A_3R signaling, which regulates the activity of the adenylate-cyclase pathway in an opposite way, will decide the outcome of MIP-2 and KC production in the presence of apoptotic cells.

Altered MIP-2 and KC production by A_3R null macrophages is a consequence of altered NO production

Our previous studies have shown that the upregulation of NO production in macrophages exposed to apoptotic cells contributes to MIP-2 release. To confirm that the regulation of NO production is involved in the altered MIP-2 and KC response of A_3R null macrophages as well, the macrophages were exposed to the nitric oxide synthase (NOS) inhibitor L-(G)-nitro-L-arginine methyl ester(L-NAME) before the addition of apoptotic cells., As we have previously reported, the addition of L-NAME attenuated apoptotic cell-induced the MIP-2 protein expression by wild-type macrophages, L-NAME had no effect on the MIP-2 production generated by A_3R null macrophages. In accordance with these results, the wild-type macrophages produced detectable NO levels upon the apoptotic cell exposure while the A_3R null macrophages did not. In the presence of Rp-cAMP, however, the apoptotic cell exposed to the $A_3R^{-/-}$ macrophages were capable of producing similar amounts of NO as wild-type macrophages indicating that their NO production is suppressed by the adenylate-cyclase pathway. Previous studies have shown that the addition of sodium nitropusside, a potent NO donor, alone did not induce the MIP-2 production, but significantly enhanced the apoptotic cell-induced MIP-2 production in wild-type macrophages. Interestingly, sodium nitropusside had no effect on the MIP-2 production in the A_3R null engulfing macrophages. When, however, sodium nitropusside was applied to them together with Rp-cAMP, an enhanced MIP-2 production could be detected by $A_3R^{-/-}$ engulfing macrophages as well. These data indicate that the enhanced adenylate-cyclase signaling in A_3R null macrophages must inhibit simultaneously not only the NO production, but also other signaling element(s) which contribute to the apoptotic cell-induced MIP-2 production.

4.2. TRANSMEMBRANE TNF-α REVERSE SIGNALING INHIBITS LIPOPOLYSACCHARIDE-INDUCED PRO-INFLAMMATORY CYTOKINE FORMATION IN MACROPHAGES BY INDUCING TGF-β

Stimulation of mouse BMDMs or RAW264.7 macrophages with LPS enhances the cell surface expression of mTNF-α

First, we investigated with confocal microscopy whether TNF-α is detectable in non-stimulated BMDMs or RAW264.7 macrophages. TNF-α is detectable in both BMDMs and RAW264.7 macrophages in intracellular vesicles. Although the protein could be readily seen, only a minimal amount of TNF-α mRNA was detected in non-stimulated cells.

When, however, macrophages were exposed to LPS (100 ng/ml), a significant increase in TNF-α mRNA levels was detected in both cell types, reaching its maximum following a 2-hour stimulation. Then the mRNA levels gradually decreased. In line with these observations, the cell surface levels of mTNF-α of both BMDMs and RAW264.7 macrophages, which were not detectable in non-stimulated cells, started to rise after the LPS stimulation and became more readily detectable when the cell surface cleavage of mTNF-α was inhibited by galardin, a synthetic metalloproteinase inhibitor.

The galardin treatment tested in RAW264.7 cells alone also increased the cell surface expression of mTNF-α, indicating that 1) the metalloprotease responsible for the cleavage of mTNF-α must be expressed in non-stimulated cells as well, and 2) although we could not detect the presence of mTNF-α without galardin, there must also be some basal level of mTNF-α production in non-stimulated cells. The cell surface expression of mTNF-α of LPS-treated BMDMs and RAW264.7 cells reached its maximum in 1 hour, and then it started to gradually decrease. In galardin-treated RAW264.7 cells, the increase in the cell surface mTNF-α expression could be detected as soon as 15 min after the LPS stimulation, indicating that mTNF-α at the beginning must come from the already existing intracellular TNF-α pool. The sharp decrease of LPS-induced mTNF-α expression following 1 hour of stimulation despite the increase in TNF-α mRNA expression indicates that the expression of the metalloprotease, which is responsible for the mTNF-α cleavage, must also be induced by the LPS stimulation in both types of macrophages. The galardin treatment considerably delayed the decrease in the mTNF-α expression of macrophages and also delayed and decreased the appearance of soluble TNF-α in the cell culture medium.

mTNF-α signaling activates the MKK3/6-, Jun kinase–, and PI3K-regulated pathways

Since our preliminary data indicated that mTNF-α must be present on the surface of non-stimulated macrophages as well, we decided to investigate mTNF-α signaling in BMDMs stimulated with the help of a phospho-MAPK array kit, which enables the parallel determination of relative levels of phosphorylation of MAPKs and those of several related proteins. The data received in the presence of coated anti-mTNF-α Abs were compared with those that resulted in the presence of isotype control Abs. The stimulation of mTNF-α by coated anti–mTNF-α Abs led to the activation of the MKK3/6-regulated MAPK, the JNK, the ERK kinase, and the PI3K pathways. Since preliminary experiments, which we used as a read-out to detect the activation of mTNF-α signaling, demonstrated that soluble anti–TNF-α Abs are not able to inhibit LPS-induced IL-6 and MIP-2 production, we applied coated Abs to trigger mTNF-α signaling.

MKK3/6, Jun kinase, ERK kinase, and PI3K pathways are activated by TGF-β induced *via* the mTNF-α signaling pathway

Because these alterations in the phosphorylation status of various proteins were detected 2 hours after the macrophages were seeded onto the coated Abs, we could not exclude the possibility that mTNF-α signaling induced the expression of a cytokine that drives the above-mentioned three signaling pathways. Since TNF-α, IFN-α, and TGF-β were previously shown to activate the MKK3/6, Jun kinase, and PI3K pathways, we decided to test whether the expression of these cytokines is induced by triggering mTNF-α. Although we could not detect the induction of IFN-α or TNF-α at mRNA levels (data not shown), the induction of TGF-β was readily detected by a pan anti–TGF-β Ab at protein levels and that of TGF-β1 at mRNA levels. To determine whether TGF-β is indeed responsible for the mTNF-α triggering–induced MKK3/6, JNK, ERK kinase, and PI3K pathway activation, the experiments were repeated in the presence of neutralizing anti–TGF-β Abs as well. mTNF-α–induced phosphorylation of those proteins, which are known to be activated by the TGF-β signaling pathway, was completely or partially prevented by the administration of neutralizing TGF-β Abs. Indeed, these data confirm that the mTNF-α signaling–induced TGF-β is responsible for their phosphorylation.

mTNF-α signaling induces the MKK4 signaling pathway

The same blots were then analyzed further to determine the kinases that belong directly to mTNF-α signaling. Those kinases were selected to belong to the mTNF-α signaling pathway, the activation of which was not or was only partially affected by the administration of neutralizing TGF-

β Abs. The elements of the PI3K (TOR, Akt1, 2, and 3), the p38α/β, MAPK, and JNK pathways were found to be activated directly by mTNF-α. The MAPK pathways are evolutionally conserved, and it was shown that the p38α/β pathways can be activated by either MKK3/6 or MKK4/MKK7. Since MKK3 and MKK6 were not activated by the mTNF-α signaling pathway, whereas MKK4 and MKK7 can also activate Jun kinases, we decided to determine their phosphorylation levels following mTNF-α triggering.

To avoid interference with the TGF-β signaling, the experiments were performed in the presence of neutralizing TGF-β Abs. The phosphorylation of MKK4 transiently increased, whereas that of MKK7 was not detectable during mTNF-α signaling. These data indicate that MKK4 mediates the effect of mTNF-α. In the absence of neutralizing TGF-β Abs, the induced phosphorylation levels of MKK4 remained high even at 2 hours, in line with the fact that TGF-β also triggers the activation of MKK4. The phosphorylation of MKK3/6 induced by TGF-β appeared with a delay as compared to that of MKK4 without alterations in MKK3 levels. The protein levels of MKK7, alternatively, gradually decreased, indicating that TGF-β downregulates MKK7 signaling.

Next, we decided to investigate which of the mTNF-α activated signaling pathways contribute to the induction of TGF-β. The phosphorylation levels of both mitogen and stress-activated kinase 2 and ribosomal S6 kinase 2 known to be activated by p38 MAPKs were induced by mTNF-α signaling. These kinases can phosphorylate CREB at Ser133 and activate CREB-dependent gene expression. Because the promoter of both TGF-β2 and –β3 contains cAMP response element, we decided to investigate whether selective inhibition of p38 MAPKs by SB203580 (10 μM) could interfere with the mTNF-α–induced TGF-β production. The inhibition of p38 MAPKs attenuated the mTNF-α–induced TGF-β production. The TGF-β1 promoter, alternatively, was reported to be regulated principally by AP-1 sites. In line with this observation, inhibition of Jun kinases by TCS JNK 60 also attenuated mTNF-α–induced TGF-β production and completely prevented the mTNF-α–induced TGF-β1 mRNA formation. The inhibition of p38 α/β kinases by SB203580, alternatively, had no effect on the TGF-β1 mRNA production. However, when the p38 kinase α/β inhibitor was added together with the Jun kinase inhibitor, the mTNF-α–induced TGF-β production was completely prevented.

These data indicate that the Jun kinase pathway regulates primarily TGF-β1, whereas the p38 kinase α/β pathway regulates the production of other TGF-βs. In striking contrast, inhibition of PI3K (which is known to activate the Akt pathway) by wortmannin (a PI3K inhibitor) resulted in the increased TGF-β formation. This observation indicates that this pathway might initiate a negative

autoregulatory loop in the regulation of the TGF-β production. The mechanism of Akt activation was not investigated in our studies, but in other studies it was found that the MKK4 pathway can down-regulate the levels of PIP3 (phosphatidylinositol-3,4,5-trisphosphate) 3-phosphatase, leading to the activation of Akt.

mTNF-α signaling inhibits the production of a subset of LPS-induced pro-inflammatory cytokines

Because our preliminary experiments indicated that mTNF-α signaling inhibits LPS-induced IL-6 and MIP-2 formation to a different degree, we decided to examine the effect of mTNF-α signaling on the LPS-induced pro-inflammatory cytokine formation by simultaneously detecting the production of pro-inflammatory cytokines using a cytokine array. BMDMs generated from NMRI animals generally produced TIMP-1, M-CSF-1, and JE pro-inflammatory cytokines at basal levels. Exposure to LPS did not affect the production of these cytokines. Although we always exposed the cells to the same amount of LPS, their response to LPS varied from isolation to isolation. A 2-hour exposure of BMDMs to coated anti–mTNF-α always attenuated the LPS-induced pro-inflammatory cytokine formation, but the effect was more dramatic when the cells showed a lower LPS response. However, not each cytokine demonstrated the same sensitivity to mTNF-α inhibition. The inhibition of LPS-induced G-CSF, I-309, IL-10, IL-16, IL-23, IL-1α, and IL-1β production was nearly complete, IL-6, IP-10, MIP-1β, IL-1ra, M-CSF, and RANTES production was significantly inhibited, but MIP-1α, MIP-2, TIMP-1, JE, or KC productions were also reduced. To prove that coated anti–mTNF-α Abs indeed act *via* mTNF-α, we also exposed TNF-α–null macrophages to LPS and checked whether anti–mTNF-α Abs affect their cytokine production. Coated anti–mTNF-α Abs did not significantly alter the LPS-induced pro-inflammatory cytokine production of macrophages isolated from TNF-α–null mice.

mTNF-α signaling inhibits LPS-induced pro-inflammatory cytokine formation by upregulating TGF-β

Next we decided to examine whether TGF-β is involved in the suppression of the LPS-induced pro-inflammatory cytokine formation by mTNF-α by applying neutralizing anti–TGF-β Abs. Neutralizing anti–TGF-β Abs did not affect the LPS induced pro-inflammatory cytokine production in the presence of isotype control Abs with the exception of IL-23, IL-1α, and IL-1β. However, they strongly interfered with the mTNF-α – mediated inhibition of the LPS-induced pro-inflammatory cytokine formation with the exception of those three cytokines, which were affected by the neutralizing anti–TGF-β Abs in the isotype control Ab–treated samples as well. A summary of the

inhibitory effect of mTNF-α signaling on the LPS-induced IL-6 and MIP-2 production detected by ELISA and the effect of neutralizing TGF-β Abs from 10 independent experiments. Additionally, preincubation with recombinant TGF-β for 2 hours also attenuated the LPS-induced pro-inflammatory cytokine formation in BMDMs in a cytokine-specific manner.

Apoptotic thymocytes do not use the mTNF-α signaling pathway to downregulate the LPS-induced pro-inflammatory response of macrophages

Whereas the phagocytosis of a variety of pathogenic targets, especially bacteria and virally infected cells, normally triggers a pro-inflammatory response in macrophages, the ingestion of apoptotic cells by macrophages usually induces an anti-inflammatory phenotype. Apoptotic cells do not simply fail to provide pro-inflammatory signals; rather, they actively interfere with the inflammatory program. For example, the preincubation of macrophages with apoptotic cells strongly suppresses the inflammatory response induced by LPS, and the mechanism involves the release of TGF-β. Since most mammalian cells express TNFRs, and thymocytes express both TNFR I and II, we decided to research, by using the macrophages of TNF-α –null mice, whether mTNF-α could contribute to the immune silencing effects of apoptotic cells. The loss of TNF-α did not affect the basal pro-inflammatory cytokine formation of macrophages and the downregulation of them when they were engulfing apoptotic cells. The exposure of apoptotic cells to macrophages resulted in the down-regulation of the LPS-induced response of a number of pro-inflammatory cytokines in a TGF-β –dependent manner, but the loss of mTNF-α tested on TNF-α –null macrophages did not affect their capability of responding to apoptotic cells.

Additionally, we did not observe a correlation between the location of TNF-α –containing vesicles of LPS-treated macrophages and the position of phagocytic portals for apoptotic cells. These data indicate that mTNF-α signaling does not play a role in the apoptotic cell-induced anti-inflammatory response. Thus, we checked whether apoptotic thymocytes still express TNFRs. Thymocytes express both TNFR I and II, but as they enter apoptosis following the serum withdrawal, they downregulate the expression of both TNFRs. The down-regulation was not apoptotic signal specific, as thymocytes exposed to dexamethasone or phorbol dibutyrate/ionomycin also responded with full down-regulation of their TNFRs within 5 hours. Interestingly, apoptotic neutrophils known to encounter LPS-activated macrophages in an inflammatory situation also downregulate their TNFRs during apoptosis. The data indicate that apoptotic cells do not trigger an mTNF-α response for immune silencing.

TNF-α –targeting molecules selectively trigger TGF-β production in human macrophages

TNF-α –targeting molecules are widely used in the therapy of various inflammatory diseases such as rheumatoid arthritis, psoriatic arthritis, juvenile arthritis, Crohn's colitis, ankylosing spondylitis, and psoriasis. Etanercept is a recombinant human soluble fusion protein of TNFR2 coupled to the Fc portion of IgG, infliximab is a chimeric mAb with murine variable regions and human Ig G1 constant regions, and golimumab is a human anti-TNF-α IgG1κ mAb that can be administered by the patient. Although all compounds neutralize TNF-α with the same efficiency and are similarly effective in the treatment of rheumatoid arthritis, golimumab and infliximab are effective, whereas etanercept failed in the trials of Crohn's disease, Wegener's granulomatosis, and sarcoidosis. Thus. we decided to study whether these compounds can trigger the TGF-β production in human macrophages. Human macrophages generated from buffy coats in three separate experiments were exposed to either etanercept, infliximab, or golimumab all in 50 μg/ml concentration for 4 hours, and then the TGF-β levels were determined in the cell culture fluid. In the case of nontreated macrophages, 39 ± 5 pg/ml TGF-β could be detected. Exposure to etanercept (TGF-β, 40 ± 4 pg/ml) did not affect the basal levels of TGF-β, whereas in the cultures of infliximab (78 ± 15 pg/ml) and in those of golimumab (96 ± 12 pg/ml) TGF-β could be detected, the levels of which were significantly higher ($p < 0.05$) than basal levels.

5. DISCUSSION

Macrophages, as a primary sentinels of the immune system, play a critical role in the recognition of potentially dangerous „non self" (e.g. pathogens) and „altered/damaged self" (e.g. dying cells, tumor cells) cells, as well as in the initiation of appropriate, immediate and efficient immune response against these structures. These macrophage functions are essential in the maintenance of immune homeostasis and the disturbance of them may lead to the development of autoimmune and chronic inflammatory diseases.

The recognition of so called PAMPs (pathogen-associated molecular patterns, like Gram negative bacterial cell wall component LPS) and DAMPs (damage-associated molecular patterns, like HMGB1 released by necrotic cells) shift macrophages to M1 inflammatory phenotype. M1 macrophages express not only pro-inflammatory mediators (e.g. prostaglandins, leukotrienes, IL-6, TNF-α, iNOS), rather than phagocytosis and antigen presentation associated cell surface molecules, growth factors and chemoattractants (e.g. MIP-2, MIP-1). By these mechanisms macrophages take a part in the removal of the potentially harmful cells, and in the activation/recruitment of other cell types of the immune system.

In addition, macrophages are exposed to the continuously generated and present dying apoptotic cells. In contrast to PAMPs and DAMPs, the recognition of ACAMPs (apoptotic-cell-associated molecular patterns, eg. cell surface PS) does not trigger M1 phenotype switch and inflammatory responses in the macrophages. Moreover, ACAMPs represent active anti-inflammatory signals for macrophages. This phenomenon is indispensable for the immune tolerance against to the harmless apoptotic cells.

In the present study, on one hand we investigated whether the A_3R could be involved in the regulation of the anti-inflammatory response induced in macrophages by apoptotic cells. We found that in contrast to $A_{2A}Rs$, engulfing macrophages downregulate A_3Rs during phagocytosis. While upregulation of $A_{2A}Rs$ involves activation of the nuclear lipid sensing receptors following phagocytosis, expression of A_3Rs seems to be downregulated *via* cell surface macrophage receptors recognizing phosphatidylserine on the surface of apoptotic cells.

Similar to $A_{2A}Rs$, loss of adenosine A_3Rs did not affect the rate of phagocytosis. However, when exposed to apoptotic cells, $A_3R^{-/-}$macrophages notably produced decreased amounts of MIP-2 and KC acting as chemoattractants for various cell types, especially for neutrophils. We could confirm these data by using a specific A_3R antagonist indicating that lack of the actual A_3R signaling rather

than altered macrophage differentiation in the absence of A_3R explains the phenomenon. Interestingly, the production of a similar set of cytokines, namely the neutrophil chemoattractants, was affected by the loss of A_3Rs as that of the loss of $A_{2A}Rs$, but in an opposite direction indicating that A_3R receptor signaling in wild-type macrophages might inhibit the $A_{2A}R$ signaling.

The data presented indicate that in A_3R null macrophages the loss of A_3Rs, which would normally negatively regulate the adenylate-cyclase activity during engulfment, results in an enhanced adenylate-cyclase signaling by $A_{2A}Rs$ leading to full suppression of the apoptotic cell-induced NO production and the consequent neutrophil chemoattractant formation. As a result, neither addition of the forskolin, which could further activate adenylate-cyclase, nor inhibition of NO synthesis, since no NO production was detected, had an effect on the amount of MIP-2 produced by A_3R null macrophages engulfing apoptotic cells.

Previous studies have shown that ingestion of apoptotic cells is accompanied also by a release of PGE2 which acting *via* EP2–4 receptors could also activate adenylate-cyclase. However, when we inhibited adenylate-cyclase signaling by Rp-cAMPS triethylamine, we found no difference in the apoptotic cell-induced neutrophil chemoattractant formation between A_3R or $A_{2A}R$ null macrophages and their wild-type counterparts indicating that the main receptor that triggers adenylate-cyclase activity and inhibits neutrophil chemoattractant formation in the absence of A_3R signaling must be the $A_{2A}R$. This observation is further supported by the finding that inhibition of $A_{2A}R$ by signaling in A_3R null engulfing cells results in similar amount of pro-inflammatory cytokine formation as the inhibition of the adenylate-cyclase pathway by Rp-cAMPS. Our data also indicate that the main receptor that inhibits the adenylate-cyclase pathway is the A_3R.

Previously we have shown that exogenously added NO alone is not sufficient to trigger neutrophil chemoattractant formation in macrophages, but enhances it, when it is administered to engulfing wild-type macrophages. This observation indicated that apoptotic cells induce neutrophil chemoattractant formation not only *via* inducing NO formation, but also simultaneously triggering additional signaling pathway(s). Since in A_3R null engulfing macrophages, in which the adenylate-cyclase signaling pathway is strongly activated, exogenous NO could enhance neutrophil chemoattractant formation only when it was added together with Rp-cAMPS triethylamine, our data indicate that the enhanced adenylate-cyclase signaling must interfere with the additional pathway(s) as well.

Our data demonstrate that in wild-type macrophages engulfing apoptotic cells initially both $A_{2A}Rs$ and A_3Rs are activated by adenosine, and a balance between the activities of the two receptors

decides the strength of the adenylate-cyclase signaling and the consequent degree of suppression of apoptotic cell-induced chemoattractant formation. But because A_{2A}Rs are upregulated, while A_3Rs disappear from the cell surface of engulfing macrophages, adenosine on long term will mediate suppression of inflammation when macrophages engulf apoptotic cells.

Besides studying the involvement of adenosine A_3 receptor in the apoptotic cell-mediated immune silencing, we also investigated the potential involvement of mTNF-α signaling in the inhibition of LPS-triggered inflammatory responses of macrophages by apoptotic cells. We found that TNF-α is expressed, although at low levels, in unstimulated macrophages, and some of it appears also on the cell surface as mTNF-α. Exposure to LPS increased both the synthesis of TNF-α and its cell surface expression, but this increase was only transiently due to the activation of the metalloprotease, which is responsible for its cleavage. Already the basal cell surface levels of mTNF-α allowed an efficient mTNF-α signaling, and we found that the signaling pathway led to the production of TGF-β. The induction of TGF-β production, however, is not an universal response of cells to mTNF-α signaling, because monocytes were shown to produce TNF-α and not TGF-β when their mTNF-α was triggered by the TNF-Rs of activated T cells.

We have also identified the signaling pathway that regulates mTNF-α–induced TGF-β production in macrophages. This pathway involves the MKK4-initiated MAPK pathway, which leads the activation of both p38 MAPKs and JNKs. Whereas the Jun kinase pathway regulates the production of TGF-β 1, the p38 MAPKs are responsible for the production of the rest of TGF-βs. In contrast, the Akt pathway also activated by mTNF-α seems to act as a negative autoregulatory loop in the control of mTNF-α–induced TGF-β production. Although previous studies have suggested that mTNF-α triggers the ERK kinase pathway, our data indicate that the activation of these kinases is only secondary and a consequence of TGF-β signaling. We have also demonstrated that mTNF-α signaling alone induces only a transient MKK4 and p38 MAPK activation when TGF-β is neutralized; however, the appearance of TGF-β sustains MKK4 and the consequent p38 MAPK signaling.

We have confirmed that mTNF-α signaling indeed inhibits the LPS-induced pro-inflammatory cytokine formation, but we found that different subsets of cytokines show different susceptibility for this inhibition. We have also demonstrated that mTNF-α signaling interferes with the LPS signaling *via* the autocrine production of TGF-β. Because previous studies have shown that different elements of the LPS induced signaling pathway are involved in the induction of individual cytokines, our data indicate that TGF-β signaling might selectively interfere with the various LPS-induced signaling

pathways. Our data do confirm previous findings, in which it was demonstrated that in the presence of TGF-β the balance between the LPS-induced ERK and p38 MAPK activities is dysregulated owing to the simultaneous TGF-β–stimulated ERK activation. As a result, the NF-κB–driven transcription was shown to be attenuated, whereas the AP-1–driven transcription was shown to be enhanced.

We have also tested whether three TNF-α–targeting molecules introduced already in the therapy of various inflammatory diseases could also trigger the mTNF-α–driven TGF-β production in human macrophages. We found that etanercept is not able to trigger such a mTNF-α–driven TGF-β production, whereas infliximab and golimumab are able to do so, indicating that infliximab and golimumab trigger mTNF-α signaling. In support of our observation, infliximab in patients was reported to induce the transient phosphorylation of p38 MAPKs and Hsp70, the elements of the mTNF-α signaling pathway. Previous studies have suggested that the therapeutic efficacy of TNF-α –targeting molecules might be related not only to their capability of neutralizing TNF-α, but it might also be affected by their biological nature or whether they could also trigger mTNF-α signaling. Indeed, although all three compounds neutralize TNF-α with the same efficiency and are similarly effective in the treatment of rheumatoid arthritis, etanercept, which in our assay, failed to upregulate TGF-β, and it also failed in the trials of Crohn's disease, Wegener's granulomatosis, and sarcoidosis. Because in other biological assays etanercept responded similarly to infliximab and golimumab, our data indicate that its failure in the treatment of these above-mentioned diseases might be related to the lack of TGF-β induction. Interestingly, Crohn's disease, Wegener's granulomatosis, and sarcoidosis are all characterized by altered TGF-β signaling.

Every day billions of cells die by apoptosis as part of the normal tissue homeostasis. Anti-inflammatory effects of apoptotic cells result in a silent removal of dying cells as well as strongly contribute to the resolution of inflammation. We have studied whether apoptotic cells known to interfere with the LPS signaling of macrophages also by upregulating TGF-β do use mTNF-α signaling to act in this manner. Our data indicate that apoptotic cells cannot trigger the mTNF-α signaling pathway in macrophages, because they downregulate their TNF-Rs.

Collectively, our data indicate that the induction of TGF-β, a strong anti-inflammatory cytokine, might be an essential contributing factor in determining the therapeutic efficacy of TNF-α–targeting molecules in chronic inflammatory diseases, such as Crohn's disease. Because in Crohn's disease patients, who do not respond to infliximab therapy, phosphorylation of both ATF-2 and Hsp70 was

found to be impaired, it is very likely that in these patients the mTNF-α–signaling pathway is suppressed.

As a new result we demonstrated that adenosine A_3 receptor in contrast to A_{2A} receptor enhances the production of MIP-2 and KC chemoattractants in the uptake of apoptotic cells, but the expression disappears during phagocytosis. The mTNF-α is not involved in the apoptotic cell-mediated anti-inflammatory effect, since the apoptotic cells downregulate their TNF receptors. Triggering of mTNF-α signaling pathway in macrophages leads to production of TGF-β. Certain molecules used in TNF-α therapy are able to acivate the mTNF-α signaling pathway which can be effective in the treatment of several chronic inflammatory diseases.

Taken together, the present studies revealed the involvement of adenosine/A_3R and mTNF-α signaling in the regulation of the inflammatory responses of macrophages. Our results not only improve our knowledge about the macrophage-associated immune functions, but they also could serve potential therapeutic targets for ‘personalized medicine’ in the treatment of various autoimmune and chronic inflammatory diseases.

6. SUMMARY

Macrophages have three major functions in the immune system: phagocytosis of invading pathogens as well as damaged or death cells within the tissues; antigen presentation and immunomodulation through the release of various cytokines and growth factors. The composition of the released mediators depends on the target cell type recognized by the macrophages and determines the responses of the surrounding tissue and the immune system.

It is well known that the apoptotic cell uptake induces a strong anti-inflammatory response in macrophages. Previously it has been thought that TGF-β released upon apoptotic cell uptake is the most important, moreover the only one anti-inflammatory cytokine mediating this inhibitory effect. However, results coming from the last few years contest this obligate function of TGF-β. Our group has found that apoptotic cell-exposed macrophages produce also adenosine, which inhibits the apoptotic cell-induced pro-inflammatory response in a cytokine dependent manner by triggering macrophage adenosine A_{2A} receptors (A_{2A}Rs). In the present study we showed that the expression of adenosine A_3 receptors (A_3R) is decreased on the cell surface of macrophages during engulfment of apoptotic cells. The pro-inflammatory behavior of this receptor is confirmed by the fact that A_3R deficiency is accompanied by decreased production of neutrophil chemoattractant MIP-2 and KC during engulfment of apoptotic cells. The decreased MIP-2 and KC production of A_3R null macrophages is a consequence of an attenuated apoptotic cell-induced NO production, which is inversely regulated by the adenosine $A_{2/3}$ receptor/adenylate-cyclase/protein kinase A signaling pathway.

Apoptotic cells do not simply fail to provide pro-inflammatory signals; rather, they actively interfere with the inflammatory program. For example, preincubation of macrophages with apoptotic cells strongly suppresses the inflammatory response induced *via* Toll-like receptor 4 by lipopolysaccharide (LPS), a component of the cell wall of Gram-negative bacteria. mTNF-α was shown to act both as a ligand by binding to TNF-α receptors, as well as a receptor that transmits outside-to-inside (reverse) signals back into the mTNF-α bearing cells. Since the reverse mTNF-α signaling, similar to apoptotic cells, was shown to suppress LPS-induced pro-inflammatory cytokine formation, we tested the potential involvement of mTNF-α in the apoptotic cell-induced immunosuppression. We found that even non-activated macrophages express basal levels of mTNF-α and triggering mTNF-α induces an MKK4-dependent signaling pathway, which leads to TGF-β production. The production of TGF-β 1 is regulated *via* Jun kinases, while that of other TGF-βs *via* p38 MAP kinases. Exposure to LPS further induced the expression of mTNF-α, and triggering of

mTNF-α strongly suppressed the LPS-induced pro-inflammatory response in a TGF-β–dependent manner. Apoptotic cells, however, do not use this signaling pathway to achieve immune silencing, because they downregulate their TNF receptors.

Our main findings that in phagocytosis of apoptotic cells near TGF-β, also adenosine and its receptors play a role in the anti-inflammatory macrophage-activating processes, but mTNF-α doesn't. In contrast, LPS-induced macrophage activation is delayed with the involvement of mTNF-α induced TGF-β.

Taken together, our results highlight the involvement of adenosine A_3 receptor and mTNF-α in the regulation of inflammatory responses exerted by macrophages. Agents, targeting these molecules – A_3R antagonists or mTNF-α inducers – represent potential therapeutic tools for the treatment of various inflammatory diseases.

7. PUBLICATIONS

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Candidate: Anna Pallai
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List of publications related to the dissertation

1. **Pallai, A.**, Kiss, B., Vereb, G., Armaka, M., Kollias, G., Szekanecz, Z., Szondy, Z.: Transmembrane TNF-[alfa] Reverse Signaling Inhibits Lipopolysaccharide-Induced Proinflammatory Cytokine Formation in Macrophages by Inducing TGF-[béta]: therapeutic Implications.
   J. Immunol. 196 (3), 1146-1157, 2016.
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ORAL PRESENTATIONS:

Anna Pallai. **mTNF alpha signaling inhibits LPS-induced pro-inflammatory cytokine formation by upregulating TGF-β in macrophages.** 8th Molecular Cell and Immune Biology Winter Symposium, Debrecen, Hungary, January 8-10, 2015

Anna Pallai. **mTNF alpha signaling inhibits-LPS-induced pro-inflammatory cytokine formation by upregulating TGF-beta in macrophages.** 7th Molecular Cell and Immune Biology Winter Symposium, Galyatető, Hungary, January 7-10, 2014

Anna Pallai. **The role of mTNF-α signaling on LPS stimulated macrophages.** Debrecen University Graduate School of Clinical Medical Sciences (KODI) and the Graduate School of Dentistry (FODI) 2013. Annual Symposium, Debrecen, Hungary, June 24, 2013

Anna Pallai. **mTNFalpha and inhibition of pro-inflammatory cytokine formation.** 6th Molecular Cell and Immune Biology Winter Symposium, Galyatető, Hungary, January 8-11, 2013

Anna Pallai. **The production and function of TNF-α on LPS stimulated macrophages.** Debrecen University Graduate School of Clinical Medical Sciences (KODI) and the Graduate School of Dentistry (FODI) 2012. Annual symposium, Debrecen, Hungary, June 5, 2012

Anna Pallai. **GluLys dipeptide produced by transglutaminase inhibits the pro-inflammatory cytokine production of LPS stimulated macrophages.** 5th Molecular Cell and Immune Biology Winter School, Galyatető, Hungary, January 4-7, 2012

Anna Pallai. **Investigation of the anti-inflammatory effect of Glutamyl-Lysine isodipeptide produced by transglutaminase.** Debrecen University Graduate School of Clinical Medical Sciences (KODI) and the Graduate School of Dentistry (FODI) 2011. Annual symposium, Debrecen, Hungary, June 27, 2011

POSTER PRESENTATIONS:

Anna Pallai, Aliz Bozó, Réka Tóth, Zsolt Sarang, Zsuzsa Szondy: **mTNF-α signaling inhibits the pro-inflammatory cytokine production of LPS-stimulated macrophages by upregulating TGF-β.** ECDO 2014, 22nd Euroconference on Apoptosis Cell Death & Rejuvenation, Hersonissos, Crete, Greece, October 1-4, 2014

Anna Pallai, Aliz Bozó, Réka Tóth, Zsolt Sarang, Zsuzsa Szondy: **mTNF-α signaling inhibits the pro-inflammatory cytokine production of LPS-stimulated macrophages by upregulating TGF-β.** Semmelweis Symposium 2013, Budapest, Hungary, November 7-9, 2013

Anna Pallai, Zsolt Sarang, Zsuzsa Szondy: **The role of mTNF-α signaling on LPS stimulated macrophages**. 17th International Summer School on Immunology, Rabac, Croatia, September 14-21, 2013

Anna Pallai, Aliz Bozó, Réka Tóth, Zsolt Sarang, Zsuzsa Szondy: **The role of mTNF-α signaling on LPS stimulated macrophages**. EMDS European Macrophage and Dendritic Cell Society 2012 Meeting, Debrecen, Hungary, September 1-3, 2012

Anna Pallai, Zsolt Sarang, Zsuzsa Szondy: **Glutamyl-lysine isodipeptide produced by transglutaminase inhibits the pro-inflammatory cytokine production of LPS stimulated macrophages.** FEBS3+ Meeting "From molecules to life and back", Opatija, Croatia, June 13-16, 2012

Krisztina Köröskényi, Katalin Sándor, Anna Pallai, Edina Duró, Zsolt Sarang, László Fésüs, Zsuzsa Szondy: **Involvement of adenosine A_{2A} receptors in engulfment-dependent apoptotic cell suppression of inflammation.** FEBS3+ Meeting "From molecules to life and back", Opatija, Croatia, June 13-16, 2012

Köröskényi K, Sándor K, Pallai A, Duró E, Sarang Z, Fésüs L, Szondy Z: **Involvement of Adenosine A2A Receptors in Apoptotic Cell Induced Suppression of Inflammation.** Gordon Research Conference on Apoptotic Cell Recognition & Clearance, Lewiston, USA, July 17-22, 2011

Köröskényi K, Pallai A, Fésüs L, Szondy Z: **Adenosine acting *via* A_{2A} receptors of macrophages takes a part in the anti-inflammatory effect of apoptotic cell uptake and in this way in the termination of inflammatory response.** Annual Meeting of the Hungarian Society of Biochemistry, Budapest, Hungary, August 23- 26, 2009

Krisztina Köröskényi, Zsolt Sarang, Anna Pallai, Edina Duró, László Fésüs, Zsuzsa Szondy: **Adenosine is a soluble mediator of immune downregulation induced by apoptotic cells.** 2nd Molecular Cell and Immune Biology Winter School Krompachy, Slovakia, January 5- 8, 2009

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