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

INFLUENCE OF TISSUE TRANSGLUTAMINASE AND ADENOSINE / A_{2A} RECEPTOR ON THE INFLAMMATORY RESPONSE OF MACROPHAGES

KRISZTINA KÖRÖSKÉNYI

SUPERVISOR: PROFESSOR ZSUZSA SZONDY MD, Ph.D

UNIVERSITY OF DEBRECEN

DOCTORAL SCHOOL OF DENTISTRY

DEBRECEN, 2011

INFLUENCE OF TISSUE TRANSGLUTAMINASE AND ADENOSINE/A_{2A} RECEPTOR AND ON THE INFLAMMATORY RESPONSE OF MACROPHAGES

by **KRISZTINA KÖRÖSKÉNYI**
MOLECULAR BIOLOGY/BIOCHEMISTRY M.SC.

SUPERVISOR: ZSUZSA SZONDY, M.D., D.SC.

DOCTORAL SCHOOL OF DENTISTRY, UNIVERSITY OF DEBRECEN

Head of the Examination Committee: ILDIKÓ MÁRTON, M.D., D.SC.

Members of the Examination Committee: LÁSZLÓ BUDAY, M.D., D.SC.

ÁRPÁD LÁNYI, PH.D.

The Examination takes place at Department of Restorative Dentistry, Medical and Health Science Center, University of Debrecen; December 8, 2011

THE COMMITTEE OF PUBLIC DEFENSE:

Head of the Defense Committee: PROF. ILDIKÓ MÁRTON, M.D., D.SC.

Reviewers: PROF. SÁNDOR SIPKA, M.D., D.SC

PROF. CHRISTOPHER GREGORY, PH.D.

Members of the Defense Committee: PROF. LÁSZLÓ BUDAY, M.D., D.SC.

ÁRPÁD LÁNYI, PH.D.

The Ph.D. Defense takes place at the Lecture Hall of 1st Department of Medicine, Institute for Internal Medicine, Medical and Health Science Center, University of Debrecen
December 8, 2011

1. INTRODUCTION

1.1. Apoptotic cell clearance

Cells die at different stages of life as part of embryonic development and normal tissue turnover, thus the term programmed cell death, or apoptosis. The efficient removal of apoptotic cells (ACs) not only eliminates abnormal, non-functional or harmful cells, but also helps sculpt organs and maintain the homeostasis of the host. Under normal circumstances, phagocytosis of ACs engulfed by neighboring cells or professional phagocytes, such as macrophages is fast and effective without causing inflammation and immune response. Thus beyond its role in tissue homeostasis, AC clearance is essential to avoid inflammation.

Anti-inflammatory effect of apoptotic cell uptake

Since the late 90s' it is universally accepted that in contrast to pathogen- or necrotic cell-induced inflammatory responses, AC uptake is not only immunosuppressive but also actively suppressed the Toll-like receptor (TLR)-dependent release of pro-inflammatory cytokines, such as IL-6, IL-8, IL-12, IL-1β and TNF-α. The early anti-inflammatory effect of ACs is triggered by cell surface receptors (such as MerTK and integrin $\alpha_v\beta_3$) recognizing apoptotic-cell-associated molecular patterns (ACAMPs) and primarily associated with inhibition of NF-κB-dependent gene expression changes. Subsequently, soluble mediators acting in paracrine or autocrine fashion, also take a part in the maintain and amplify the anti-inflammatory response. Thus, IL-10, transforming growth factor-β (TGF-β), platelet-activating factor (PAF), and prostaglandin E2 (PGE2) derived from macrophages exposed to ACs suppress the production of inflammatory cytokines, chemokines and lipid mediators. Moreover, phagocytosis of ACs brings in large amounts of cellular lipids into the phagocytes. Recognition of oxidized fatty acids and cholesterol by lipid-sensing nuclear receptors (peroxisome proliferator-activated receptors: PPARδ and PPARγ; liver X receptors: LXRs), is also involved in the clearance and anti-inflammatory effect of cells dying by apoptosis.

1.2. Adenosine

Adenosine (ADO) is an endogenous purine which plays an important role in various biochemical processes, such as energy transfer as well as in signal transduction. It is also an inhibitory neurotransmitter, and potent anti-inflammatory substance.

There are several important producer cell types of extracellular ADO. Neutrophils and endothelial cells release large amounts of ADO at sites of metabolic distress, inflammation and infection. Activated macrophages (e.g. LPS-treated) can also serve as a major source of extracellular ADO via ATP release and degradation. The cellular response to ADO strictly depends on the expression of the different adenosine receptor (AR) subtypes, which can be co-expressed by the same cell and serve as active modulators in signal transduction. Further factor that determine the net effect of ADO on macrophage function is the coupling efficacy of ARs to intracellular signaling.

Adenosine receptors

ARs belong to the class A G protein-coupled receptor (GPCR) family. On the basis of their affinity ARs can divide into four subtypes: A1 (A_1R), A2A ($A_{2A}R$), A2B ($A_{2B}R$) and A3 (A_3R). The A_1R (G_i-coupled) and A_2Rs (G_s-coupled) were initially subdivided on the basis of their ability to inhibit and stimulate adenylyl cyclase, respectively. The more recently discovered A_3R is also G_i coupled. The common feature of all ARs, is the coupling to the classical cAMP/protein kinase A (PKA) pathway, which might be both activated (A_2Rs) and inhibited (A_1R/A_3R) by ARs.

Immunemodulatory role of adenosine

ADO and its analogues can alter the course of a variety of immune-mediated/inflammatory diseases such as endotoxin shock, rheumatoid arthritis, pleural inflammation and nephritis. On the basis of genetic studies, it is becoming increasingly recognized that $A_{2A}Rs$ represent the major immunoregulatory arm of the ADO-AR system, and there is also general agreement that $A_{2A}Rs$ serve to down-regulate inflammation and immunity. For example, $A_{2A}R$ activation suppresses the migration of neutrophils, the oxidative burst and NO production of leukocytes, as well as the LPS-induced TNF-α and IL-12 production of monocytes/macrophages. These inhibitory effects are mediated by the p38 MAPK, the NF-κB or the AP-1-dependent signalling pathways. In addition, $A_{2A}R$ stimulation also triggers the release of anti-inflammatory cytokine IL-10, hence many anti-inflammatory effects of ADO is mediated via this cytokine.

1.3. Tissue transglutaminase

Transglutaminases (TGs) are a family of intracellular and extracellular enzymes that catalyze Ca^{2+}-dependent posttranslational modification of proteins. Via this function TGs participate in many biological processes such as cell adhesion and migration, wound healing, mineralization and apop-

tosis. Besides its classical protein cross-linking activity, tissue transglutaminase (TG2) can function also a G protein (G_h), protein disulfide isomerase (PDI), protein kinase or DNA nuclease. TG2 is predominantly a cytoplasmic protein, but it is also found in the nucleus and in the mitochondria, as well as on the plasmamembrane and the extracellular cell surface, and in the extracellular matrix (ECM). Extracellular TG2 interacting directly with extracellular molecules participates not only in the formation of cell-ECM interactions, but also in the inflammatory responses by the regulation of cytokines' availability (i.e. activation of latent TGF-β) in the ECM. TG2 is also involved in inflammation by participating in an inflammatory loop with NF-κB the "master switch" for inflammation. In addition, TG2 also contributes to neutrophil migration into areas of inflammation.

The consequences of TG2 deficiency
–with special regard to the clearance of apoptotic cells and inflammation

TG2 deficiency fundamentally impairs the capacity of macrophages to ingest ACs and promotes dysregulated macrophage release of TGF-β and other cytokines (i.e. IL-12), and defective autoinhibition of inflammation modulated by AC uptake *in vivo*. Although TG2 null mice are developmentally normal, they develop splenomegaly and immune complex glomerulonephritis beyond 12 months, putatively via dysregulated AC removal. Moreover, the lack of TG2 favours to the development of chronic inflammatory disorders such as atherosclerosis.

TG2 contributes to the uptake of ACs by binding to both integrin β_3, a known phagocytic receptor, and its bridging molecule, MFG-E8 during the formation of phagocytic portals. In the absence of TG2 the AC-induced integrin β_3 signaling leads to impaired RhoG and Rac1 activation and consequently to failed cytoskeletal rearrangement. As a compensatory response, TG2 null macrophages elevate the expression levels of both integrin β_3 and RhoG, and the cell adhesion-induced integrin β_3 signaling is enhanced. This is in line with the defective AC cell clearance of $TG2^{-/-}$ macrophages.

In addition to impaired phagocytotic activity, $TG2^{-/-}$ mice as compared to wilde type ones respond to inflammatory stimuli with reduced superoxide anion generation, but increased neutrophil phagocytic activity compared to wild type mice. In contrast, inflammatory responses mediated by monocytes/macrophages (such as inflammatory cytokine production) are more pronounced in TG2 deficient mice than in their wild type counterparts.

1.4. Recognition of pathogenes

Recognition of pathogens implicates a wide variety of pattern recognition receptors (PRRs) specific for pathogen-associated molecular patterns (PAMPs), including Toll-like receptors (TLRs), lectins, scavenger receptors, and integrins. Among them, TLRs are critical for the development of innate immune responses.

TLR4 signalling cascade

TLR4 (also designated as CD284) is the signalling receptor for LPS – a component of the cell wall of Gram-negative bacteria −, and is thus play an important role in the activation of the innate immune system. Potent TLR4-dependent cell activation by LPS depends on sequential endotoxin-protein and protein-protein interactions with LBP, CD14, MD-2 and TLR4.

The activation of TLR signaling pathways originates from the cytoplasmic TIR domains of TLR4, which interact with MyD88 adaptor molecule. Upon stimulation, MyD88 recruits IL-1 receptor-associated kinase (IRAK) to TLRs. IRAK is activated by phosphorylation and then associates with TRAF6/TAK1/TAB1/TAB2 complex, leading to the activation of IKKα,/IKKβ/IKKγ complex. The IκBα protein inactivates the NF-κB transcription factor by masking the nuclear localization signals of NF-κB proteins and keeping them sequestered in an inactive state in the cytoplasm. IKK complex phosphorylates the inhibitory IκBα protein. This phosphorylation results in the dissociation of IκBα from NF-κB and thereby activates NF-κB, which migrates into the nucleus, where it induces the transcription of pro-inlammatory genes (i.e. IL-1β, IL-6, IL-12β, TNF-α, iNOS). In parallel, IKK also activates JNK and MAPK pathways via the phosphorylation of NF-κB1 p105. Recent evidence indicates that TLR signaling involves also a a MyD88-independent pathway also, which induces the expression of IFN-inducible genes through activation of the transcription factor, IFN regulatory factor 3 and 7 (IRF3/7).

2. AIMS OF THE STUDY

We have previously reported that the both the clearance of apoptotic cells clearance, and the LPS-induced pro-inflammatory cytokine production is disturbed in $TG2^{-/-}$ macrophages. Hence, the age-dependent autoimmunity of TG2 null mice is probably caused by these defective processes. Since the activation of TGF-β − which not only mediates the anti-inflammatory effects of ACs but also takes a part in the resolution of LPS-induced inflammatory responses − is defective in the absence of TG2, it is very likely that the lack of TGF-β is responsible for the enhanced inflammatory responses of TG2 null macrophages. With our present study we wanted to test this hypothesis. Our questions were:

1. *Is the lack of active* TGF-β responsible for *the enhanced pro-inflammatory cytokine production of TG2 null macrophages?*
2. *Are there any other TGF*-β-independent mechanism involved in the increased LPS-sensitivity of macrophages lacking TG2?

Adenosine has been known for a long time to mediate anti-inflammatory effects on macrophages via its A_{2A}R subtype. Since our previous results have challenged the central and exclusive role of TGF-β in the immunosuppressive effect of AC uptake, we concentrated to the following issues:

3. *Is it possible that adenosine is one of the soluble anti-inflammatory mediators released by macrophages in response to apoptotic cell exposure?*
4. *If so, does A_{2A}R mediate the anti-inflammatory signal of adenosine during apoptotic cell clearance in macrophages?*

3. MATERIALS AND METHODS

Animals

Most of the experiments were done using 3 months old male wild type (C57B/6 and FVB), $A_{2A}R$ deficient mice generated on an FVB background; and TG2 deficient mice generated on C57B/6 background. Some of the experiments were also carried out on PPARδ or LXR deficient mice generated on 129 SvJ, or on a mixed background of C57Bl/6 and 129Sv, re-spectively. These studies have been reviewed and approved by the review committee of the University of Debrecen (DEMÁB).

Macrophage isolation and culturing

Macrophages were obtained by peritoneal lavage with sterile physiological saline. Cells were cultured for 2 days before use. After 3-4 hrs. 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 macrophages, wild type and PPARδ null bone marrow was isolated from femurs, and cultured in media supplemented with 10% L929 conditioned media for 10 days. The non adherent cells were washed away from the 3rd day daily.

Adenoviral gene delivery system

Recombinant, replication-deficient adenoviral vectors encoding either LacZ and the murine TG2 gene or the secretion deficient, guanine nucleotide binding deficient or crosslinking function deficient TG2 mutants were produced using the AdEasy XL system according to the manufacturer's instruction. Virus titers were determined by plaque assay in 293 cells after exposing them to virus for 48 hrs. For gene delivery, macrophages were exposed to virus particles for 48 hrs. LacZ expression was determined with X-gal staining, while TG2 expression by Western blot analysis using anti-TG2 specific antibodies.

Thymocyte apoptosis induction in vitro

ACs were prepared from wild-type mice in all cases. Thymocytes isolated from 4 weeks old mice were cultured for 24 hrs. in the absence of serum. This method typically resulted in >80% ACs. In case of NB4 cells the apoptosis was induced by 10 μM As_2O_3–treatment for 12hrs. ACs were used at a 10:1 (AC: macrophage) ratio.

Preparation of the cell culture medium for adenosine measurement

The experiments were performed by co-incubating $A_{2A}R^{+/+}$ macrophages with ACs in 1:10 ratio. For the respective experiments, macrophages were pre-treated with 50 mM cytochalasin D for 1 hr. to block the phagocytic activity of macrophages. After 2 hrs. of phagocytosis the supernatants were replaced with fresh culture media. After 5 hrs. of incubation (at 37°C) the supernatants were collected, deproteinized with 5 ml ice cold 0.6 N $HClO_4$ and stored on -80°C. The determination of ADO concentration was carried out with a reverse phase HPLC method as described.

Flow cytometry

Wild type and $A_{2A}R$ null peritoneal macrophages were coincubated with apoptotic thymocytes for one hr. in 1:10 ratio. After replacing media and washing away the ACs, macrophages were incubated for additional one, three or five hrs. For some experiments, all these treatments were carried out in the presence of 5 µg / ml actinomycin D, or 0.1 µg / ml cycloheximide. For characterizing the regulation of the expression of the receptor macrophages were treated with various concentrations of 22-R(OH)-cholesterol, an LXR agonist, or GW501516, a PPARδ agonist, for 3 hrs. After the treatments macrophages were washed, collected, blocked with 50% FBS for 30 min. and labeled with anti-mouse $A_{2A}R$ antibody or goat IgG isotype control. For the detection cells were stained with FITC-conjugated anti-goat IgG. In case of TLR4 and CD14 determination peritoneal macrophages were labeled with FITC conjugated anti-CD14 antibody or rabbit-anti mouse TLR4 antibody washed and incubated further with FITC-anti-rabbit antibody. Stained cells were analyzed on a FACSCalibur. The results were analyzed by WinMDI 2.9 software.

Determination of cytokine production

Wild-type and $A_{2A}R$ null peritoneal macrophages were exposed to ACs for one hr in the presence or absence of an $A_{2A}R$-selective antagonist SCH442416 (10 nM) or $A_{2A}R$-selective agonist CGS21680 (10 nM). ACs than were washed away, SCH442416 or CHS21680 were re-added and the macrophages were cultured for an additional five hrs. At the end of culture cell culture media were analyzed by Mouse Cytokine Array. The pixel density in each spot of the array was determined by Image J software. Alternatively, cytokine-induced neutrophil-attracting chemokine (KC), TGF-β, macrophage inflammatory protein-2 (MIP-2) and IL-10 cytokine levels were measured with ELISA kits. In case of TG2 experiments wild-type and TG2 null peritoneal macrophages were treated with 100 ng/ml LPS for one hour in the presence or absence of increasing concentrations of recombinant TGF-β as indicated in the results section. After one hour LPS was re-moved and fresh medium was added to the cells containing either recombinant TGF-β, 4 ng/ml neutralizing anti-pan TGF-β anti-

body, isotype control antibody or vehicle. Supernatants were collected and frozen at the indicated time points. IL-6, TNF-α and active TGF-β cytokine levels were measured with ELISA kits.

Determination of gene expressions by real-time quantitative PCR

Wild type, $A_{2A}R$ null, TG2 null and LXR null peritoneal, or PPARδ wild type and knock out bone mar-row derived macrophages were coincubated with LPS or various target cell types (apoptotic, living, heat killed (45 min, 55°C) or anti-CD3-pretreated (10 μg/ml, 20 min) thymocytes for 1 hr in 1:10 ratio. After washing away the ACs and replacing media mRNA was collected 2 hours later by Tri-reagent. cDNA was synthesized with High-Capacity cDNA Archive Kit on ABI 2720 Thermal Cycler. Cyclophilin, MIP-2, ADORA2, iNOS, eNOS, IL-6, TNF-α, ArgI and ArgII levels were determined with Taq-Man PCR using FAM-GMB-labelled primers. Samples were run in triplicates on an ABI Prism 7900 using ABM Prism SDS2.1 software for evaluation. Gene expression was normalized to cyclophilin expression.

mRNA stability determination

Wild-type and TG2 null peritoneal macrophages were pre-treated with 100 ng/ml LPS for 1 h followed by addition of 1 μg/ml Actinomycin D. Total RNA was isolated at the indicated time points and TNF-α mRNA was measured by quantitative RT-PCR.

Phagocytosis assay

For phagocytosis assays, macrophages were stained overnight with 10 μM 5-(and 6-)-(((4-chloromethyl)benzoyl)amino) tetramethylrhodamine (CMTMR), while thymocytes were labeled overnight with 6 μM 6-carboxy-3 ,6 -diacetylfluorescein (CFDA). Macrophages were incubated with apoptotic thymocytes in 10:1 target/macrophage ratio for one hr. Cells incubated with apoptotic thymocytes incubated at 4°C were used as controls. After washing, cells were analyzed for each point by flow cytometry. For visualizing ACs in macrophages, macrophages were cocultured with CFDA labeled ACs for 30 min, 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. 500 cells were counted for AC uptake in each individual experiment.

Determination of NO production of macrophages engulfing apoptotic cells

$A_{2A}R^{+/+}$ or $A_{2A}R^{-/-}$ macrophages were exposed to ACs for 1 hr. Media were replaced and macrophages were incubated for an additional 1 hr. Cell culture supernatants were analyzed for NO. Nitrite concentration in the samples was then measured by the Griess by adding 100 μl of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamide in 5% phosphoric acid) to 100 μl

samples of medium. The optical density (OD550) at 550 nm was measured using a Spectramax 250 microplate reader. Nitrate concentrations were calculated by comparison with OD550 of standard solutions of sodium nitrate prepared in culture medium.

***In vivo* neutrophil migration assay**

$A_{2A}R^{+/+}$ and $A_{2A}R^{-/-}$ mice were injected with 2 ml of 4% thioglycollate intraperitoneally. 4 days later they were injected intraperitoneally with 2×10^6 of ACs suspended in 2 ml of physiological saline or 2 ml physiological saline. After 3 hrs. the peritoneal cells were col-lected, washed, blocked with 50% FBS and stained with FITC-conjugated rat anti-mouse Gr-1, or V450 rat IG2bκ isotype control for 30 min. The detection was carried out using FITC conjugated anti-rat IgG. Cells were then washed, fixed and analyzed by flow cytometry to determine the percent-ages of neutrophils in the total cell population. In some experiments rat anti–mouse KC, rat anti–mouse MIP-2 or their isotype controls IgG2a, and IgG2b were injected together with the ACs into mice.

Western blot

Wild-type and TG2 null peritoneal macrophages were treated with 100 ng/ml LPS for the indicated time periods, pretreated with 1μg/ml soluble vitronectin for 30 minutes or pretreated with 2 μM PP2 for 24 hours. Cells were harvested at the indicated time points and boiled 2x sample buffer and loaded onto SDS PAGE gels. PVDF membranes were probed with anti-IκKβα, anti-cSrc, anti-phospho(Tyr^{416})-Src, anti-integrin β_3, anti-phospho (Tyr^{474}) integrin β_3, anti-TG2 and β-actin anti-bodies.

Determination of NK-κB p65 nuclear translocation

Wild-type and TG2 null peritoneal macrophages were treated with 100 ng/ml LPS for 30, 60 and 120 minutes. Cell were rinsed with ice cold PBS and nuclei were isolated with Nuclei EZ kit according to manufacturer's instruction. Nuclear p65 subunit was detected with TransAM p65 kit according to manufacturer's instruction.

Statistical analyses

All the data are representative of at least three independent experiments carried out on three different days. Values are expressed as mean ± S.D. P values were calculated by using two-tailed Student's t-test for two samples of unequal variance. The analysis of cytokine array ex-periments was carried out by ANOVA test. Statistical significance is indicated by a single asterisk ($P < 0.05$).

4. RESULTS

4.1. ALTERED $\alpha_v\beta_3$ INTEGRIN-MEDIATED SRC SIGNALING IN TG2 NULL MACROPHAGES HAS AN EFFECT ON THE INFLAMMATORY RESPONSE OF MACROPHAGES

TG2 null macrophages respond by elevated pro-inflammatory cytokine production to LPS treatment

To determine the time course of LPS-stimulated pro-inflammatory cytokine production of wild type resident peritoneal macrophages, macrophages were exposed to 100 ng/ml LPS, and the LPS-induced IL-6 and TNF-α production was determined by ELISA at various time points. To avoid detection of *in vivo* induced pro-inflammatory cytokine production, macrophages were plated for 2 days, by a time when no more endogenous cytokine production was found, before addition of the LPS. The IL-6 and TNF-α production of wild-type peritoneal macrophages reached their maximum at 6h following LPS stimulation, and these levels remained with no significant alterations during the following 20 hours. While IL-6 production started to raise only after one hour of LPS stimulation, a significant amount of TNF-α was detected already at one hour of LPS stimulation indicating that some TNF-α is stored within the macrophages, which is released upon LPS-stimulation. Peritoneal macrophages responded with more IL-6 than TNF-α production to LPS stimulation. Loss of TG2 did not affect the kinetics of IL-6 or TNF-α production , but the levels of pro-inflammatory cytokines produced by TG2 null macrophages were higher at each time points as compared to that produced by the wild-type ones. These data indicate that in the absence of TG2 macrophages are more sensitive to LPS stimulation than their wild type counterparts.

Elevated pro-inflammatory cytokine production by TG2 null macrophages is not related to the lack of TGF-β activation

Since TG2 is required for the activation of latent TGF-β produced by macrophages, and active TGF-β has been proposed to act as an autocrine feed back regulator of pro-inflammatory cytokine production of LPS-stimulated macrophages, we decided to test whether the enhanced pro-inflammatory cytokine production is related to the lack of TGF-β activation by TG2 null macrophages. In line with the previously reported data about the requirement of TG2 for the activation of latent TGF-β, TG2 null macrophages indeed were unable to produce detectable amounts of active TGF-β.

However, a pan-TGF-β neutralizing antibody failed to enhance the LPS-induced early pro-inflammatory cytokine production. The neutralizing antibody concentration used was sufficient to block all the active TGF-β, because after addition of the neutralising antibody no active TGF-β was detectable in the supernatants by ELISA. In addition, recombinant TGF-β, in the concentrations we detected active TGF-β in the cell culture medium, could not significantly affect LPS-induced pro-inflammatory cytokine production of TG2 null macrophages, indicating that TGF-β has no effect on the short-term LPS-induced cytokine formation of macrophages, and not the lack TGF-β production is responsible for the enhanced pro-inflammatory cytokine formation by TG2 null macrophages. So we decided to test further the characteristics of LPS signaling in TG2 null peritoneal macrophages.

Basal levels of IκBα are decreased in TG2 null macrophages

LPS is recognized by macrophages via TLR4 together with the accessory molecule CD14. To test, whether the expression of the LPS sensing receptors have changed in TG2 null macrophages, the cell surface expression levels of CD14 and TLR4 were detected by flow cytometry. However, no change in the cell surface expression of these receptors was found in TG2 null macrophages, indicating that not an altered expression of these receptors is responsible for the enhanced LPS sensitivity.

There are several levels, at which LPS-induced production of TNF-α and IL-6 can be controlled. First we decided to test whether their transcription is altered. Using Q-PCR technique we could not detect basal levels of mRNA for the two pro-inflammatory cytokines. Exposure to LPS enhanced the expression of TNF-α and IL6 in both types of macrophages, but the TG2 null macrophages showed about two and four fold higher mRNA productions, respectively. To differentiate whether the transcription or the stability of the mRNA was altered in the absence of TG2, LPS-stimulated macrophages were exposed to actinomycin D, a transcription inhibitor and the time dependent decrease in the mRNA expression of TNF-α was followed in both wild-type and TG2 null macrophages. Since no change in the kinetics of the mRNA degradation was found, it is likely that the loss of TG2 alters the pro-inflammatory cytokine production at transcriptional level.

Since the signaling pathways induced by LPS transduce their effect on pro-inflammatory cytokine production partly via activating NF-κB, and these pathways regulate the degradation of the inhibitory subunit IκBα, a negative regulator of NF-κB, we decided to determine the IκBα levels in wild-type and TG2 null macrophages following LPS stimulation. There was no change in the kinetics of the IκBα degradation induced by LPS stimulation, but the basal levels of IκBα in TG2 null macrophages were significantly lower than that of the wild type cells.

IκBα is commonly associated with the NF-κB dimer p50(NF-κB1)/p65(RelA). Following proteolytic degradation of IκBα by the proteasome, the NF-κB dimer becomes free to enter the nucleus and to activate transcription of target genes. While NF-κB p65 is transcriptionally active, NF-κB p50 does not possess a transactivation domain. Thus, though p65 and p50 can synergistically activate for example the TNF-α promoter, the presence of p65 is crucial for the initiation of transcription. That is why we decided to test the nuclear translocation of the p65 subunit of NF-κB by the TransAM p65 kit (ActiveMotif). LPS stimulation induced the nuclear translocation of p65 in both types of macrophages. However, in accordance with the lower IκBα levels in TG2 null cells, both the basal and the LPS-induced levels of p65 were higher in the nucleus of TG2 null macrophages than in that of the wild-type macrophages at each time point tested. These data indicate that the loss of TG2 alters a signaling pathway that is coupled to the control of IκBα levels.

TG2 is required on the cell surface to decrease LPS-induced pro-inflammatory cytokine production in TG2 null macrophages

Previous studies have already shown association between NF-κB activation and TG2. In LPS-stimulated microglial cells, TG2 activates NF-κB via a novel pathway. Rather than stimulating phosphorylation and degradation of IκBα, TG2 interferes with its action by protein polymerization. On the other hand, in cancer cells over expression of TG2 enhances NF-κB activation by promoting integrin signaling. However, if these mechanisms exist in macrophages, loss of TG2 should lead to a decreased, not to an enhanced LPS signaling. To answer which biological functions of TG2 are required to downregulate LPS-induced pro-inflammatory cytokine production, adenoviral gene delivery system was used to transfect primary peritoneal macrophages with various mutants of TG2. The following TG2 mutants were tested: a crosslinking activity mutant (TG2-X) by replacement of catalytic *Cys*^{277} by Ser, two guanine nucleotide binding mutants by replacement of Lys^{172} and Phe^{173} by Asn and Asp (TG2-G1), and of Glu^{578}and Arg^{579} by Gln and Glu (TG2-G2), and a secretion mutant (TG2-S) by replacement of Tyr^{274} by Ala. Using IL-6 production as a read out, only the wild type and the crosslinking mutant were able to fully revert the LPS sensitive phenotype, while the secretion mutant and the two guanine nucleotide binding mutants were ineffective. Our data indicate that cell surface TG2 regulates negatively the LPS-induced pro-inflammatory cytokine production, and the crosslinking activity is not required for this effect. In addition, these data confirm those findings, which suggested that the enhanced LPS-induced pro-inflammatory cytokine production of TG2 null macrophages is not related to the lack of TGF-β activation, as it would require the crosslinking activity of TG2.

Altered $\alpha_v\beta_3$ signaling is responsible for the enhanced LPS-induced pro-inflammatory cytokine production in macrophages

On the cell surface TG2 has been shown to act as an integrin-binding adhesion coreceptor and acting so to suppress Src kinase activity. Since it has been reported that $\alpha_v\beta_3$ integrin (ITG$\alpha_v\beta_3$) signaling can lead to NF-κB activation and enhance LPS-induced NF-κB signaling via activating Src kinase, we decided to test the potential role of an altered $\alpha_v\beta_3$ integrin signaling in the enhanced LPS-induced pro-inflammatory cytokine production of TG2 null macrophages. Preincubation of TG2 null macrophages with soluble vitronectin, an inhibitor of the ITG$\alpha_v\beta_3$ signaling, decreased the LPS-induced pro-inflammatory cytokine production on mRNA levels, indicating that ITG$\alpha_v\beta_3$ signaling promotes the LPS-induced pro-inflammatory cytokine production in TG2 null macrophages. Interestingly, the same treatment enhanced the pro-inflammatory cytokine production by wild-type macrophages. In line with these observations, LPS-induced IκBα levels were further decreased following vitronectin treatment in wild-type cells, but remained more elevated in knock out cells. Since cell surface TG2 was reported to crosslink soluble vitronectin, and crosslinked soluble vitronectin might enhance instead of inhibiting ITG$\alpha_v\beta_3$ signaling, for wild-type macrophages we repeated the experiments in the presence of R294, a non-permeable TG2 inhibitor, which blocks the crosslinking activity of cell surface TG2.

In the presence of the TG2 inhibitor and soluble vitronectin wild-type cells responded to LPS with nearly the same amount of cytokine mRNA expression as in the absence of them. In control experiments, addition of the TG2 inhibitor did not influence the response of TG2 null cells to soluble vitronectin. Alterations in the IκB levels mirrored these changes in the cytokine mRNA expression. Altogether these data indicate that under our experimental conditions in wild type cells ligand-activated ITG$\alpha_v\beta_3$ signaling, which can be inhibited by soluble vitronectin, does not play a determining role in influencing LPS signaling (though stimulation of it by crosslinked vitonectin is capable of its enhancement), while in TG2 null cells it does.

In TG2 null macrophages enhanced $\alpha_v\beta_3$ integrin-induced src family tyrosine kinase activation is responsible for the enhanced NF-κB signalling

Next we decided to test the activation state of Src family tyrosine kinases in TG2 null macrophages. The Src-family tyrosine kinases are highly conserved allosteric enzymes playing a key role in in-

tegrin cellular signaling. Phosphorylation of Tyr^{416} plays a central role in their activation. In line with the report, which suggested that TG2 might negatively control ITG$\alpha_v\beta_3$-regulated Src kinase activity, an enhanced phosphorylation of c-src family kinases was detected at Tyr^{416} in TG2 null cells without a detectable change in the c-src protein levels as compared to the wild type cells. LPS stimulation enhanced the amount of phosphorylated Src family tyrosine kinases in both types of macrophages, in line with previous publications, which showed that Src kinase is also involved in LPS signaling.

The cytoplasmic domain of ITGβ_3 contains tyrosines at positions 747 and 759 in domains that have been implicated in regulation of $\alpha_v\beta_3$ function and that serve as potential substrates for Src family kinases. Phosphorylation of Tyr at residue 747 was reported to be required for optimal post-ligand binding effects, as well as for proper binding of the integrin ligands thus participating in both in the "outside in" and "inside out" integrin signaling. To test the activation state of ITGβ_3 in TG2 null cells both the level of ITGβ_3 and the phosphorylation state of its Tyr^{747} residue were determined by Western blot analysis. In accordance with our previous findings the levels of ITGβ_3 were elevated in TG2 null macrophages. While in wild-type cells ITGβ_3 was only slightly phosphorylated at the Tyr^{747} site, in TG2 null cells the phosphorylation level was much more pronounced indicating an enhanced activation of ITGβ_3 in the absence of TG2. However, phosphorylation of ITGβ_3 and Src tyrosine kinases seems to be related to each other in reciprocal way, as inhibition of ITG$\alpha_v\beta_3$ by soluble vitronectin decreased the phosphorylation level of Src tyrosine kinase, and similarly inhibition of Src tyrosine kinase with 2 μM PP2 [(4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-***d***]pyrimidine)], an Src tyrosine kinase family inhibitor, inhibited the phosphorylation state of ITG$\alpha_v\beta_3$.

Then we tested whether the decreased IκBα levels could be related to the enhanced activation of Src. Preincubation of macrophages with 2 μM PP2 for 24 hours did not affect their viability, but equalized the basal levels of IκBα detected in the wild-type and TG2 null macrophages indicating that the enhanced activity of Src kinase is responsible for the altered IκBα levels in TG2 null cells. In addition, inhibition of Src kinase delayed LPS-induced degradation of IκBα in both types of cells, and in the presence of PP2 no difference was found in the IκBα levels following addition of LPS. Finally, addition of PP2 decreased, but at the same equalized the LPS-induced mRNA production of the two pro-inflammatory cytokines within the wild-type and TG2 null macrophages. All together these data provide evidence for the involvement of the ITG$\alpha_v\beta_3$ regulated Src family tyrosine kinases in the altered LPS signaling in TG2 null macrophages.

4.2. INVOLVEMENT OF ADENOSINE A_{2A} RECEPTORS IN APOPTOTIC CELL INDUCED SUPPRESSION OF INFLAMMATION

Apoptotic cell uptake leads to adenosine release and increased gene and cell surface expression of A_{2A}R in macrophages

At the beginning of our study, the first and most important point was the determination of possible ADO production by macrophages during the clearance of ACs. ADO was found in the culture medium of macrophages exposed to ACs. The release of ADO was not specific for thymocytes, because the uptake of As_2O_3-treated apoptotic NB4 acute promyelocytic leukemia cells also triggered ADO release. In parallel, the ADO concentration in the culture medium alone, or in the culture medium of macrophages or thymocytes cultured alsone was below the detection limit. The amount of ADO produced by macrophages engulfing ACs was in the range of the A_{2A}R receptor affinity range. When we blocked the phagocytosis with actin polymerization inhibitor cytochalasin D, the ADO disappeared from cell culture media, suggesting that ADO production of AC-treated macrophages is engulfment-dependent.Moreover, AC exposure not only triggered ADO release in macrophages, but also enhanced the cell surface level of A_{2A}R in time-dependent manner. We made four important observations in relation to A_{2A}R upregulation. First, AC-treatment modulates the expression of A_{2A}R on transcriptional level, because the induction disappeared in the presence of transcription inhibitor actinomycin D, or protein synthesis inhibitor cycloheximide. Indeed, induction on the level of mRNA was evident following the engulfment of ACs. Secondly, when we prevented the uptake of ACs with cytochalasin D pretreatment, or blocked the PS-mediated recognition process by masking the surface of ACs with recombinant annexin V (which binds to PS) the A_{2A}R inducing effect of ACs was inhibited. These findings demonstrate that engulfment of ACs, rather than their recognition triggers enhanced A_{2A}R expression. Third, the induction in A_{2A}R expression during phagocytosis is specific for the engulfment of ACs, as uptake of neither necrotic nor antibody-coated cells triggered it.

Finally, we tested the possible involvement of two lipid-sensing nuclear receptors (LXR and PPARδ) in the A_{2A}R upregulating effect of ACs. Both 22-(R)OH-cholesterol, an LXR agonist, and GW501516, a PPARδ agonist, promoted the mRNA expression of A_{2A}R in peritoneal macrophages indicating that LXR and PPARδ might mediate the effect of AC engulfment on A_{2A}R expression. Since the effect of these agonists might be not be fully specific, to prove further the involvement of these receptors in the A_{2A}R upregulation LXR knock out and PPARδ knock out macrophages were

also tested for their response. While in case of PPARδ$^{-/-}$ macrophages the upregulation of $A_{2A}R$ was attenuated as compared to their wild-type controls, we could not draw a definite conclusion from the LXR KO mice, as their wild type control did not show an up-regulation. We have no explanation why induction of the expression of $A_{2A}R$ was seen in macrophages on FVB and 129/SvJ backgrounds, but was not seen in mice on a mixed background of C57Bl/6 and 129Sv. These data imply that lipid sensing receptors might mediate the effect of AC on the $A_{2A}Rs$, but since the biological activity of these receptors overlap, only testing double knock out cells would give a full answer.

Loss of adenosine $A_{2A}R$ influences the pro-inflammatory cytokine production of macrophages engulfing apoptotic cells

In the next step we studied the effect of $A_{2A}R$-deficiency on the cytokine profile of resting and AC-treated peritoneal macrophages 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 results show that 85% of all available cytokines on the filters were detectable, even though some were at a very low levels. The loss of the $A_{2A}R$ did not affect significantly the cytokine profile of resting macrophages. In contrast, we found nine cytokines in AC-treated samples whose levels were increased in the lack of $A_{2A}R$. These cytokines include IP-10, KC, and MIP-2, which act as chemoattractants for neutrophils and/or other cell types. The pro-inflammatory cytokines IL-17 and IL-1α and IL-3, which stimulates the differentiation of multipotent hematopoietic stem cells into the myeloid direction and proliferation of all cells in the myeloid lineage, as well as the anti-inflammatory cytokine IL-1 receptor antagonist (IL-1ra) also were produced in an enhanced amount. In addition, release of MIP-1α, and -1β were increased also. Since wild type and $A_{2A}R$ null macrophages have similar phagocytotic activity and capacity, the detected alterations are not a consequence of phagocytosis-deficiency of cells lacking $A_{2A}R$. Among the nine cytokines overproduced by $A_{2A}R^{-/-}$ macrophages, MIP-2 showed the most dynamic change in response to AC exposure, and MIP-2 and KC levels were detected in the highest amounts. No cytokines were detected in the supernatants when thymocytes (viable or apoptotic) were incubated alone, demonstrating that the secreted cytokines were macrophage-related.

To exclude the possibility, that alterations in the cytokine profile are a result of $A_{2A}R$-related developmental effects in the knock out cells, we repeated the cytokine profiling experiment in the presence of the $A_{2A}R$ specific antagonist, SCH442416, and the $A_{2A}R$ agonist CGS21680. Using wild type macrophages we could confirm the enhanced expression of KC and MIP-2 in the presence of an $A_{2A}R$ antagonist as well, indicating that the altered pattern of KC and MIP-2 secretion observed in

$A_{2A}R^{-/-}$ macrophages is indeed a consequence of the lack of the $A_{2A}R$ signaling during phagocytosis of ACs. In addition, the enhanced expression of MIP-1α and MIP-1β two further neutrophil chemoattractants, was more clearly seen. Administration of CGS 21680 on the other hand, further decreased the amount of KC and MIP-2 produced by wild-type macrophages engulfing ACs, while had no significant effect on that by $A_{2A}R$ null macrophages engulfing ACs. Since the cytokine array is a semi-quantitative method, we confirmed the results by the usage of specific MIP-2 and KC ELISA. In harmony with the array results, the protein levels of both chemoattractants were highly increased in the conditioned media of AC treated $A_{2A}R$ null macrophages compared to the wild type ones.

Since previous studies indicated that in long term (one day) experiments TGF-β and IL-10 might mediate the anti-inflammatory effects of ACs, we checked whether TGF-β or IL-10 release is altered in macrophages lacking $A_{2A}R$. However, in such short term experiments we could not detect the release of IL-10 neither with the cytokine array nor by the ELISA technique. Active TGF-β was detectable, but its production was not altered in the $A_{2A}R^{-/-}$ macrophages. These data indicate that not an altered IL-10 or TGF-β production regulates the altered MIP-2 production in $A_{2A}R^{-/-}$ macrophages.

Macrophages not expressing adenosine $A_{2A}R$ induce migration of neutrophils when exposed to apoptotic cells in a sterile peritonitis model

To prove the biological significance of enhanced neutrophil chemoattractant production of $A_{2A}R$ null macrophages uptaking ACs, we decided to investigate whether injection of ACs affects the migration of neutrophils in $A_{2A}R^{-/-}$ mice. For this purpose we used a sterile peritonitis model, in which we injected mice intraperitoneally, first with thioglycollate and then, 4 days later, with 2×10^6 ACs. Injection of apoptotic thymocytes did not induce a significant neutrophil migration into the peritoneum in wild-type mice, while in the $A_{2A}R^{-/-}$ mice a significant neutrophil migration was detected in this model. This was accompanied by enhanced levels of MIP-2 and KC in the peritoneal fluid of $A_{2A}R^{-/-}$ mice. To confirm, that the enhanced KC and MIP-2 production is responsible for the phenomenon, blocking antibodies - anti-KC (50 μg) and anti–MIP-2 (50 μg) - or their isotype controls were injected together with the ACs. The addition of blocking antibodies to both KC and MIP-2 completely prevented the migration of neutrophils, while their isotype controls had no effect. Addition of blocking antibodies to MIP-2 alone did not fully block the migration of neutrophils. These data indicate that the loss of $A_{2A}R$ leads to sufficient neutrophil chemoattractant production by macrophages engulfing ACs to affect migration of neutrophils in an *in vivo* peritonitis model.

Production of MIP-2 by $A_{2A}R$ null macrophages exposed to apoptotic cells is related to lack of protein kinase A-mediated inhibition of NO production

Since MIP-2 showed the most dramatic changes during phagocytosis of ACs in $A_{2A}R$ null macrophages , we decided to investigate further the alterations in the regulation of this cytokine. First we tested the role of the adenylate cyclase pathway, since many of the anti-inflammatory effects of the $A_{2A}R$ were reported to be mediated via this signaling pathway. On one hand we mimicked the action of ADO-$A_{2A}R$ system in $A_{2A}R$ null macrophages by the elevation of cAMP levels with cholera toxin; by the activation of adenylate cyclase with forskolin; or by the addition of a membrane permeable cAMP analogue dibutyryl-cAMP. Preincubation of $A_{2A}R^{-/-}$ macrophages with these compounds for 30 min. prevented the increase in MIP-2 levels when exposed to ACs. On the other hand, the inhibition of PKA-mediated pathway by the usage of non-specific PKA inhibitor Rp-cAMPS triethylamine, resulted in increased MIP-2 production in wild-type macrophages when they were exposed to ACs. In addition we observed the same effect when we eliminated the ADO from cell culture media by adenosine deaminase (ADA) treatment. Overall, these data indicate that MIP-2 production of macrophages exposed to ACs is actively suppressed by the $A_{2A}R$ stimulated by ADO in an autocrine way using the adenylate cyclase/PKA signaling pathway. Inhibiting the *de novo* MIP-2 production by pretreatment with actinomycin D, or cycloheximide we were able to prevent the MIP-2-inducing effect of ACs, which suggests that the regulation occurs at the transcriptional level. Indeed, engulfment of ACs induced the mRNA levels of MIP-2 in $A_{2A}R^{-/-}$ macrophages, but not in their wild-type ones.

According to previous studies AC-exposure triggers NO-release in macrophages, which can affect the MIP-2 production. An interesting aspect of the biochemistry of NO is that both concentration and location are key determinants of its ability to activate different cell signaling pathways. We investigated the potential role of NO in three different ways. On one hand macrophages were exposed to nitric oxide synthase (NOS) inhibitor L-(G)-Nitro-L-arginine methyl ester (L-NAME) before the addition of ACs. The inhibition of NO production resulted in decreased MIP-2 protein and mRNA expression in both wild-type and $A_{2A}R^{-/-}$ macrophages, indicating that NO production contributes to the effect. On the other hand, we determined the NO release of wild-type and $A_{2A}R$ null macrophages. The AC-induced NO production is significantly higher in $A_{2A}R$ null macrophages, than in the wild-type ones. Finally, we investigated the effect of exogenously added NO on MIP-2 production of AC-treated macrophages. The results are in line with our previously shown data: the addition of sodium nitropusside (SNP), a potent NO donor, enhanced AC-induced MIP-2

production in wild type macrophages. Similarly to the induction of MIP-2, production of NO by $A_{2A}R$ null macrophages engulfing ACs was inhibited by the adenylate cyclase activator forskolin, while it was enhanced in wild type macrophages by the PKA A inhibitor Rp-cAMP triethylamine suggesting that $A_{2A}R$-mediated adenylate cyclase signaling inhibits primarily NO production.

Taking together, our data indicate that NO contributes to the AC-induced MIP-2 production. However, addition of sodium nitropusside to macrophages alone was not able to induce MIP-2 production, implying that AC-derived signals contribute to the induction of MIP-2.

Enhanced NO production in $A_{2A}R$ null macrophages is accompanied by altered expressions of iNOS and arginase II

NO is synthesized intracellularly through the action of NOS enzymes, which catalyze the oxidation of L-arginine to L-citrulline and NO. Endothelial NOS (eNOS) generates the lowest levels of NO and it is found in neurons, epithelial cells, and cardiomyocytes. Its activity are controlled by Ca^{2+} and calmodulin, and post-translational modifications. Neuronal NOS (nNOS) is constitutively present in neurons, skeletal muscle, and epithelial cells. It is also a Ca^{2+}/calmodulin-dependent form. Inducible NOS (iNOS) has the highest capacity to generate NO. This isoform is expressed in multiple cell types in response to inflammatory stimuli.

The L-arginine pool is also used by arginases (Arg), which catalyze the hydrolysis of L-arginine to ornithine and urea, and exists in two isoforms. *ArgI* participates in the urea cycle, and is expressed at high levels in the liver. *ArgII* is highly expressed in the prostate and kidney, and thought to be involved in the synthesis of proline and polyamines, which control cell prolifera-tion and collagen production. Hence the NO production is affec-ted by the balance between NOS- and Arg-mediated metabolic ways.

In the next step we investigated the expression of the the above-mentioned enzymes –except nNOS. eNOS was not detectable neither in resting, nor in AC-engulfing macrophages, suggesting that iNOS is responsible for the AC-associated NO production. In line with the higher NO production, the expression of iNOS was significantly higher in $A_{2A}R^{-/-}$ macrophages than in their wild type counterparts. Exposure to ACs did not alter the levels of Arg I, but induced a downregulation in iNOS and an upregulation in the Arg II, favoring the utilization of arginine in the production of polyamines. However, both the upregulation of Arg II and the downregulation of iNOS by ACs were delayed in $A_{2A}R^{-/-}$ macrophages. All these data indicate that exposure to ACs induces a modification

in the arginine metabolism that favors polyamine production. But this shift is delayed in $A_{2A}R$ null macrophages.

Since the $A_{2A}R$-induced adenylate cyclase pathway suppresses NO production, we tested whether influencing the adenylate cyclase pathway alters arginase II or iNOS expression The inhibition of PKA by Rp-cAMP triethylamine in wild type macrophages enhances the expression of iNOS, while the addition of forskolin to $A_{2A}R^{-/-}$ macrophages inhibits it. Similar manipulations altered the expression of arginase II conversely.

5. DISCUSSION

Today it is believed that inflammation is part of the non-specific immune response that occurs in reaction to harmful stimuli, such as pathogens or damaged cells and serves a protective attempt by the organism to remove the injurious stimuli and to initiate the healing process.

Inflammation is stimulated by chemical factors released by injured cells and initiated by cells already present in all tissues, mainly by resident macrophages. Chemical factors produced during inflammation (such as IL-8, MCP-1, MIP-2 and fractalkine) attract phagocytes. The first cells attracted to the site of infection are generally neutrophils. They are followed by monocytes, which differentiate into further tissue macrophages. Neutrophils and macrophages not only phagocytose pathogens and infected cells, but also release variety of inflammatory mediators. These include the lipid mediators (prostaglandins, leukotrienes, and platelet-activating factor), pro-inflammatory cytokines (such as IL-1, IL-6, TNF-α) and chemoattractants, which trigger other parts of the immune system. In parallel, coordinated program of resolution initiates in the first few hours after an inflammatory response begins. Resolution of inflammation occurs by different mechanisms in different tissues. These mechanisms include: the short half-life of inflammatory mediators, the production and release of TGF-β and IL-10 from macrophages, the down-regulation of pro-inflammatory molecules, the upregulation of anti-inflammatory molecules such as the IL-1ra or the soluble TNF receptor (sTNFR), the desensitization of receptors. In the resolution phase, neutrophil recruitment breaks off and the pro-inflammatory cells die by apoptosis. Consequently, apoptotic neutrophils undergo phagocytosis by macrophages, leading to neutrophil clearance and release of anti-inflammatory and reparative cytokines such as TGF-β.

In the present study, on one hand we investigated the potential role of TG2 as a regulatory molecule in the inflammatory responsiveness of macrophages. We found that in the absence of TG2 macrophages become more sensitive to LPS treatment and respond by enhanced pro-inflammatory cytokine production as compared to their wild-type counterparts. Increasing evidence suggests that TGF-β released by macrophages exposed to LPS or ACs play a key role in the control or termination of the pro-inflammatory response. However, to act so macrophages have to be exposed prior or for a longer time period to TGF-β. As a result, though we confirmed previous suggestions that TG2 is required for TGF-β activation by murine macrophages, testing by addition of neutralizing anti-TGF-β antibodies, lack of active TGF-β production did not significantly affect the LPS-induced pro-inflammatory cytokine production of TG2 null macrophages in short term cultures.

Instead we found that loss of TG2 altered the ITG$\alpha_v\beta_3$ signaling in macrophages leading to an enhanced basal Src tyrosine kinase activity. The cross-linking activity of TG2 was not required for proper ITG$\alpha_v\beta_3$ signaling and LPS-induced cytokine production, but TG2 had to be expressed on the cell surface and bind guanine nucleotides. These results are in agreement with previous findings, which demonstrated that TG2 modifies integrin signaling in guanine nucleotide bound form. Though TG2 can act as a G protein in many physiological settings, in the context of regulating integrin signaling proper guanine nucleotide binding of TG2 was suggested to be required for stabilizing the protein in a conformation state that can facilitate physical interactions with other proteins, such as integrins. Our findings seem to confirm that of others, which showed a synergism between ITG$\alpha_v\beta_3$ signaling and LPS sensitivity.

Interestingly, though loss of TG2 sensitized macrophages to LPS, loss of TG2 prevented mice from the endotoxic shock induced by LPS. The pathogenesis of the endotoxic shock, however, is very complex, and the various effects of the multifunctional protein TG2 in various tissues, such as heart, kidney or neutrophils, explain the controversy between our findings and the *in vivo* results. Our data, however, demonstrate that TG2 null macrophages might be more sensitive to all stimuli that lead to pro-inflammatory cytokine production via activation of the NF-κB pathway. This pro-inflammatory phenotype of TG2 null macrophages might contribute to the development of autoimmunity in these mice and their increased sensitivity to develop atherosclerosis.

In the other part of our work, we have shown that macrophages engulfing ACs produce ADO at levels that can trigger A_{2A}Rs and, at the same time, elevate the expression of the receptor itself. Thus, ADO can act in an autocrine manner during phagocytosis. Loss of A_{2A}Rs did not affect the rate of phagocytosis. This was a surprise for us, as increases in cAMP levels were reported to inhibit engulfment of ACs. However, when exposed to ACs, A_{2A}R null macrophages notably produced increased amounts of MIP-2 and KC acting as chemoattractants for various cell types, especially for neutrophils. We could confirm these data using a specific A_{2A}R agonist and an A_{2A}R antagonist indicating that lack of actual A_{2A}R signaling rather than altered macrophage differentiation in the absence of A_{2A}R explains the phenomenon. These data suggest that during engulfment of ACs, especially when macrophages participate in the resolution of inflammation, where they clear large numbers of apoptotic neutrophils, A_{2A}Rs might participate in the negative feedback control of neutrophil transmigration to the inflammation site. Since inflammatory cytokines were shown to sensitize A_{2A}Rs, the role of ADO mediating the inhibitory effect of ACs might be more significant under inflammatory conditions, than it was observed in our in vitro model, which lacked inflamma-

tion. In support of this hypothesis, enhanced production of MIP-2 and KC by A_{2A}R null macrophages engulfing ACs was shown in an *in vivo* peritonitis model, and this was accompanied by MIP-2- and KC-dependent neutrophil migration which was not seen in wild-type mice.

In our further experiments, MIP-2 production by A_{2A}R null macrophages was studied in details. Though previous studies have shown that AC-induced IL-10 production in macrophages can negatively regulate the production of pro-inflammatory cytokines, and A_{2A}Rs were reported in certain inflammatory contexts to promote IL-10 formation, we found no detectable IL-10 production in our experimental system. Instead, we found that MIP-2 synthesis was partially related to an enhanced NO production by A_{2A}R null macrophages engulfing ACs that regulated MIP-2 production on transcriptional level. Enhanced NO production of A_{2A}R null macrophages as compared to the wild types seems to be related to higher levels of iNOS, which produces NO, and lower levels of arginase II, which normally degrades arginine, the substrate of NO synthesis, prior and following AC exposure. However, mRNA levels alone might not reflect the real activities or activity ratio of these enzymes, as just iNOS activity alone was shown to be regulated by various signals on transcriptional, mRNA, translational and posttranslational levels. In accordance with the existence of these additional regulation levels, we observed that though iNOS is already expressed on mRNA level by resting macrophages, NO production is seen only following the AC uptake indicating that ACs must induce an early signaling pathway that triggers NO production. Our attempts to identify this signaling pathway have failed so far. Since both wild type and A_{2A}R null cells produce NO following engulfment, our current hypothesis is that A_{2A}Rs might not affect this pathway. Instead the inverse levels of iNOS and the arginase II affected by the A_{2A}Rs will determine the magnitude of the NO response. In support of our hypothesis, alterations in the arginine metabolism (favouring the arginase pathway leading to polyamine synthesis and inhibiting the synthesis of NO) following engulfment of ACs have already been reported. Interestingly, both TGF-β released by macrophages engulfing ACs and compounds known to activate PKA were shown to increase arginase activity and decrease NO production in macrophages. This indicates that both TGF-β andA_{2A}Rs, that activate PKA, might mediate or support the effect of ACs on the arginine metabolism in macrophages engulfing ACs. The role of TGF-β was proven previously, while our data indicate the additional involvement of A_{2A}Rs.

All together, our data demonstrate for the first time that macrophages exposed to ACs release ADO in addition to TGF-β and IL-10. Beside its anti-inflammatory effect on different immune cell types (macrophages, neutrophils, dendritic cells, mast cells, iNKT cells, naïve, cytotoxic, regulatory and

helper T cells), ADO is a general regulatory molecule both in the innate and in the adaptive immune systems. ADO regulates the release of allergy-promoting cytokine IL-13 and histamine in mast cells, suppresses the migration of neutrophils and immature dendritic cells to sites of inflammation and injury, and drives the T-cell responses towards a Th2 profile. Since the ADO release of macrophages is induced by both apoptotic thymocytes, and apop-totic neutrophil-like NB4 cells, this phenomenon seems to be general among the different target cells. Hereby both the anti-inflammatory and immunomodulatory effects of ADO might be expected in any part of the organism, where apoptosis is going on.

6. SUMMARY

Multicellular organisms respond to bacterial and fungal infection with the complex biological program of inflammation. In the initial phase of inflammation, macrophages and neutrophil granulocytes migrate to the site of infection, where they not only phagocytose pathogens, but also recruit further immune cells and induce systemic inflammatory response by the production and release of pro-inflammatory cytokines. In the resolution phase of inflammation, macrophages remove neutrohils, which died during their action. The uptake of these apoptotic cells induces phenotype shift: macrophages pass into deactivated phase, in which they release anti-inflammatory cytokine TGF-β and IL-10 instead of pro-inflammatory mediators.

In the present study we report, that the lack of TG2 alters the inflammatory response of macrophages, as TG2 null macrophages release elevated levels of IL-6 and TNF-α upon LPA treatment-production. Though TGF-β has been proposed to act as a negative feed back regulator of pro-inflammatory cytokine production in LPS-stimulated macrophages, this phenomenon is not related to the lack of active TGF-β production. Instead, in the absence of TG2 $ITG\beta_3$ maintains an elevated basal Src family kinase activity in macrophages, which leads to enhanced phosphorylation and degradation of the IκBα. Low basal levels of IκBα explain the enhanced sensitivity of TG2 null macrophages to signals that regulate NF-κB-mediated pro-inflammatory cytokine production. Our data suggest that TG2 null macrophages bear a pro-inflammatory phenotype, which might contribute to the enhanced susceptibility of these mice to develop autoimmunity and atherosclerosis.

In addition to TGF-β, macrophages engulfing apoptotic cells release adenosine in sufficient amount to trigger A_{2A}Rs, and simultaneously increase the expression of A_{2A}Rs, as a result of possible activation of LXR and PPARδ. In macrophages engulfing apoptotic cells, stimulation of A_{2A}Rs suppresses the NO-dependent formation of neutrophil migration factors, such as MIP-2, using the adenylate cyclase/PKA pathway. As a result, loss of A_{2A}Rs results in elevated chemoattractant secretion. This was evident as pronounced neutrophil migration upon exposure of macrophages to apoptotic cells in an in vivo peritonitis model. Altogether, our data indicate that adenosine is one of the soluble mediators released by macrophages that mediate engulfment-dependent apoptotic cell suppression of inflammation, and TG2 is anti-inflammatory by both promoting active TGF-β formation and regulating integrin signaling.

7. PUBLICATIONS

CONFERENCES / ORAL PRESENTATIONS:

Köröskényi K. **Adenosine acting via A_{2A} receptor takes a part in the anti-inflammatory effect of apoptotic cell uptake and in this way in the termination of inflammatory response.** (in Hungarian) Annual meeting of the Hungarian Immunological Society 2010, Harkány, Hungary; October 29.−November 10, 2010

CONFERENCES / POSTER PRESENTATIONS

Tóth B, Sarang Z, Köröskényi K, Garabuczi E, Aeschlimann D, Vereb G, Fésüs L, Szondy Z: **Roles of tissue transglutaminase in the phagocytosis of apoptotic cells.** 5th Euroconference on Apoptosis, Portoroz, Slovenia; October 26-31, 2007,

Köröskényi K, Sarang Z, Duró E, Fésüs L, Szondy Z: **Adenosine A_{2A} receptors mediate the anti-inflammatory phenotype of macrophages exposed to apoptotic cells.** 16th Euroconference on Apoptosis, Bern, Switzerland; September 6-9, 2008

Köröskényi K, Sarang Z, Fésüs L, Szondy Z: **Studies on anti-inflammatory effect of apoptotic cells in wild type and tissue transglutaminase (TG2) deficient macrophages.** (in Hungarian) Annual meeting of the Hungarian Biochemical Society 2006, Pécs, Hungary; August 30−September 02, 2006

Köröskényi K, Sarang Zs, Fésüs L, Szondy Zs: **The regulatory role of tissue transglutaminase (TG2) in the pro-inflammatory cytokine production of macrophages.** (in Hungarian) Annual meeting of the Hungarian Biochemical Society 2007, Debrecen, Hungary; August 26−29, 2007

Köröskényi K, Sarang Z, Duró E, Fésüs L, Szondy Z: **Adenosine A2A receptors mediate partially the anti-inflammatory phenotype of macrophages exposed to apoptotic cells.** (in English) Annual meeting of the Hungarian Biochemical Society 2008, Szeged, Hungary; August 31−September 03, 2008

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.** (in English) Annual meeting of the Hungarian Biochemical Society 2009, Budapest, Hungary, 2009.08.23−26.

Köröskényi K, Sándor K, Sarang Zs, Szondy Zs: **The role of adenosine A_{2A} receptor in the anti-inflammatory effect of apoptotic cell engulfment.** (in Hungarian) Annual meeting of the Hungarian Biochemical Society 2010, Budapest, Hungary; August 25−28, 2010 / Annual meeting of the Hungarian Immunological Society 2010, Szeged, Hungary; November 03-05, 2010

Köröskényi K, Sándor K, Pallai A, Duró E, Sarang Z, Fésüs L, Szondy Z **Involvement of Adenosine A_{2A} Receptors in Apoptotic Cell Induced Suppression of Inflammation.** (in English) Gordon Research Conference on Apoptotic Cell Recognition & Clearance, Lewiston, USA; July 17-22, 2011

UNIVERSITY AND NATIONAL LIBRARY UNIVERSITY OF DEBRECEN
KENÉZY LIFE SCIENCES LIBRARY

Register Number: DEENKÉTK /204/2011.
Item Number:
Subject: Ph.D. List of Publications

Candidate: Krisztina Köröskényi
Neptun ID: NSPXVL
Doctoral School: Doctoral School of Dental Sciences

List of publications related to the dissertation

1. **Köröskényi, K.**, Duró, E., Pallai, A., Sarang, Z., Kloor, D., Ucker, D.S., Beceiro, S., Castrillo, A., Chawla, A., Ledent, C.A., Fésüs, L., Szondy, Z.: Involvement of adenosine A2A receptors in engulfment-dependent apoptotic cell suppression of inflammation. *J. Immunol. 186* (12), 7144-7155, 2011.
DOI: http://dx.doi.org/10.4049/jimmunol.1002284
IF:5.745 (2010)

2. Sarang, Z., **Köröskényi, K.**, Pallai, A., Duró, E., Melino, G., Griffin, M., Fésüs, L., Szondy, Z.: Transglutaminase 2 null macrophages respond to lipopolysaccharide stimulation by elevated proinflammatory cytokine production due to an enhanced [alfa](v)[béta](3) integrin-induced Src tyrosine kinase signaling.
Immunol. Lett. 138 (1), 71-78, 2011.
DOI: http://dx.doi.org/10.1016/j.imlet.2011.03.004
IF:2.511 (2010)

List of other publications

3. Sarang, Z., Tóth, B., Balajthy, Z., **Köröskényi, K.**, Garabuczi, É., Fésüs, L., Szondy, Z.: Some lessons from the tissue transglutaminase knockout mouse.
Amino Acids. 36 (4), 625-631, 2009.
DOI: http://dx.doi.org/10.1007/s00726-008-0130-x
IF:3.877

H-4032 Debrecen, Egyetem tér 1. e-mail: publikaciok@lib.unideb.hu