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

THE ROLE OF MOLECULES PRODUCED IN AN EFFEROCYTOSIS-DEPENDENT MANNER BY MACROPHAGES IN THE REGULATION OF THE APOPTO-PHAGOCYTOSIS PROGRAM

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1. INTRODUCTION

1.1. APOPTOSIS

In tissues apoptosis is a very tightly programmed cell death process with distinct biochemical and genetic pathways that play a crucial role in the development and homeostasis. Apoptosis is a physiological response that eliminates unnecessary and potentially dangerous cells to maintain the healthy balance between cell survival and cell death in metazoan. It is critical to animals especially long-lived mammals that must integrate multiple physiological as well as pathological death signals.

Mechanisms of apoptosis

The mechanism of apoptosis is an exceedingly complex and refined process which is initiated by two main apoptotic pathways: the intrinsic or mitochondrial and the extrinsic or death receptor mediated pathway. The pathway is activated in response to a variety of cellular stresses. In the intrinsic pathway, mitochondrial outer membrane permeabilization (MOMP) is the crucial event, which leads to the release of pro-apoptotic proteins, including Cyt c, Smac, and Omi from the mitochondrial intermembrane space. In the cytosol Cyt c binds to apoptotic protease-activating factor 1 (APAF1) triggering its oligomerization and leading to the formation of a caspase activation platform, namely apoptosome, which then recruit and activate procaspase-9 which than cleaves and activates the executioner caspases-3 and -7. MOMP is a highly regulated process controlled by interactions between pro- and anti-apoptotic members of the B cell lymphoma 2 (Bcl-2) family. Upon activation, pro-apoptotic Bcl-2-associated X protein (Bax) and/or Bcl-2 antagonist or killer (Bak) oligomerization and insertion into the outer mitochondrial membrane, thereby forming supramolecular channels is essential for MOMP. BH3 (Bcl-2 homology 3)-only proteins, Bim and truncated Bid (BH3-interacting domain death agonist) can activate Bax and Bak through transient interaction while anti-apoptotic Bcl-2 proteins inhibit MOMP by sequestering the direct activator proteins and/or the effectors. Following MOMP apoptosis-inducing factor (AIF) and endonuclease G (EndoG) are also released and contribute to apoptosis in caspase-independent manner.

The extrinsic apoptotic pathway is initiated with the ligation of cell surface death receptors (DRs), a subset of the tumor necrosis factor receptor (TNFR) superfamily, containing a so-called death domain (DD) in their cytosolic tail. DRs include such as TNFR1 (DR1), Fas (CD95/APO-1), TNF-related apoptosis-inducing ligand receptor 1/2 (TRAILR1/TRAILR2). After ligand binding, a number of molecules are recruited to the DD forming “death-inducing signaling complex” (DISC). The DISC contains the adaptor protein Fas-associated death domain protein (FADD) and caspases 8 and, in some cases, caspase-10 and leads to the autoproteolytic activation of caspase 8, which in turn can activate effector caspase-3 and -7 to drive execution of apoptosis (type I cells), while in other

cell types caspase 8 interacts with the intrinsic apoptotic pathway by truncating Bid, leading to the subsequent tBid-mediated release of cytochrome-c (Cyt c) from mitochondria.

Role of apoptosis in T-lymphocyte development

Thymocytes, one of the first cell types to be characterized as undergoing steady state cell death, offer valuable insight into the regulation of apoptosis and tissue homeostasis. The thymus is the specialized lymphoid organ responsible for the development of functional and diverse repertoire of T cells. Dysfunctional induction of apoptosis appears to be critical for the aberrant survival of pathological cells in many chronic immune-mediated disorders, inflammation and cancer. During differentiation thymocytes must go through several maturation stages based on the expression of cell surface markers. Bone marrow-derived progenitors migrate to the thymus. In the thymus cortex they go through the double negative (DN; CD4-CD8-) stages of T cell differentiation and become double positive, complete TCR expressing (DP; e.g., CD4+CD8+) cell, then interact self-peptides associated MHC I and II molecules presenting on epithelial cells. Lack of signaling results in death by neglect. Too much signaling can promote apoptosis (negative selection); Lower level of TCR signaling initiates differentiation into CD4+ or CD8+ single positive (SP) thymocytes (positive selection). SP thymocytes are exported from the thymus and populate the peripheral lymphoid organs.

Pro-apoptotic factors involved in negative selection of thymocytes

During negative selection Nur77is rapidly induced by high-affinity TCR signaling and it plays a critical role in the process. Nur77 is a ligand-independent transcription factors. NR4A receptors activate gene expression either as monomers, homodimers and Nur77 can heterodimerize with retinoid X receptor (RXR). Furthermore, transgenic mice expressing a dominant-negative version of Nur77 can inhibit apoptosis associated with negative selection of DP thymocytes. Moreover, constitutive overexpression of full-length Nur77 in thymus results in enhanced cell death of thymocytes. These results suggest that negative selection depends on Nur77 function. Winoto et al. identified several Nur77 downstream genes, including Fas ligand (FasL) and TRAIL and Nur77 dependent gene 1 (NDG-1) that initiate the activation of caspase 8 by unknown mechanisms, as mediators of Nur77-dependent apoptosis. Nur77 initiates apoptosis independently of its transcriptional activity. It has been published that during negative selection Nur77 translocate from the nucleus and migrate to the mitochondria where Nur77 interacts with the anti-apoptotic Bcl-2 protein and converts it from a protector to a killer protein by exposing its BH3 domain. The second pathway that controls negative selection is dependent on the pro-apoptotic Bim. Bim-deficiency

results in defective thymocyte apoptosis in several TCR transgenic models of negative selection. In the absence of Bim large numbers of autoreactive T cells survive in the periphery.

1.2. PHAGOCYTOSIS OF APOPTOTIC CELLS AND THE RESOLUTION OF INFLAMMATION

Clearance of apoptotic cells

A approximately one billion of our cells die daily via apoptosis, their corpses are quickly recognized and phagocytosed by engulfing cells eliminated without initiating an inflammatory immune response. The process of engulfing apoptotic cells is called efferocytosis which is mediated by both professional engulfers such as macrophages and dendritic cells and by nonprofessional "neighbouring" cells. Phagocytic clearance of apoptotic cells can be divided into four major steps: recruitment is mediated by find-me signals released by apoptotic cells to attract phagocytes to the dying cells. The phagocytes then using engulfment receptors engage eat-me signals on apoptotic cells. The intracellular signaling induced by the ligand–receptor interactions leads to cytoskeletal rearrangements and internalization of the dying cell. The phagocyte processes the engulfed corpse through a series of steps. After ingestion of apoptotic corpse, macrophages start to release soluble anti-inflammatory regulators such as interleukin-10 (IL-10), transforming growth factor-β (TGF-β), prostaglandin E2 (PGE2). Previous studies in our laboratory have identified two efferocytosis-dependent dependent mediator released by engulfing macrophages: the retinoids and adenosine.

1.3. RETINOIDS AND THEIR MECHANISMS OF ACTION

By definition, retinoids include natural and synthetic compounds that have a structural resemblance to all-trans-retinol. Most of these immunological functions associated with vitamin A are mediated by its derivatives, all-trans-retinoic acid (ATRA) and 9-cis-retinoic acid (9cRA).

The synthesis of all-trans-retinoic acid from all-trans retinol occurs in a two-step reaction. The first step is the conversion of retinol to retinal and is carried out by members of alcohol dehydrogenase (ADH) family or by members of the short-chain dehydrogenase/reductase family of microsomal enzyme. In a second reaction, retinal is irreversibly converted to RA.

To regulate gene expression, all-trans- and 9-cis-RA bind to their nuclear receptors. These receptors are the retinoic acid receptors (RAR) and retinoid X receptors (RXR) that belong to the family of steroid/thyroid hormone receptors. ATRA and 9-cis retinoic acid are high affinity ligands for RARs, whereas only 9-cis retinoic acid binds with high affinity to RXR. The nuclear RARs function as heterodimers with RXRs and act as ligand-dependent transcriptional regulators. RXRs also can function in homodimer form binding to retinoid X response elements and it is well known

that RXRs are common heterodimerization partners of the steroid/thyroid receptor family, including Nur77.

1.4. ADENOSINE

Adenosine metabolism and adenosine receptor signaling

Adenosine -a purine nucleoside generated by the dephosphorylation of adenine nucleotides- is considered potent regulator in peripheral and central nervous and immune system. Adenosine is released in the locality of damaged cells in conditions of metabolic stress such as hypoxia, ischemia, tissue injury or inflammation. Intracellular adenosine is produced from its immediate precursor, 5′-adenosine monophosphate (5′-AMP) and once it reaches high concentration it is transported into the extracellular space. Adenosine is formed extracellularly by the metabolism of released nucleotides (ATP, ADP, AMP) via CD39 and CD73 enzymes. To terminate adenosine signaling, the adenosine deaminase promotes adenosine deamination to inosine within seconds.

Adenosine initiates its biological effects through ligation of adenosine receptors, which consist of a family of four cell surface 7-transmembrane, G-protein coupled receptors, namely the A_1R, $A_{2A}R$, $A_{2B}R$ and A_3Rs and all of them are expressed on macrophages. The A_1R and A_3Rs preferentially interact with members of the $G_{i/o}$ proteins, decreasing adenylate cyclase activity and thereby lowering intracellular level of cyclic adenosine monophosphate (cAMP), protein kinase A (PKA) activity and cyclic AMP response element binding protein (CREB) phosphorylation. While $A_{2A}R$ and $A_{2B}R$ are coupled to $G_{s/olf}$ proteins, elevating cyclic AMP production, resulting in activation of (PKA) and phosphorylation of CREB. A_{2B} receptors can act via both $G_{\alpha S}$ and G_q proteins.

The immunoregulatory effects of adenosine

Adenosine receptors are expressed on many of the cell of immune system. Adenosine affects innate immune response during various inflammatory conditions i.e. chronic (asthma) as well as acute (sepsis) inflammatory diseases. In neutrophils adenosine at higher concentration, acting at A_{2A} receptors, suppresses the production of a range of pro-inflammatory cytokines, inhibits production of oxygen free radicals, the adhesion of neutrophils to the endothelium. By contrast, adenosine via A1 receptors promotes neutrophil adhesion to the endothelium and promotes directed migration. Adenosine has been shown to be a broad inhibitor of the pro-inflammatory consequences of classical **macrophage** activation, acting via $A_{2A}R$. It has been shown that adenosine inhibits pro-inflammatory cytokine production, such as TNF-α, IL-6 and IL-12 release by lipopolysaccharide (LPS)- or bacteria-activated macrophages mostly through A_{2A} receptors. Adenosine has been shown to decrease the level of other pro-inflammatory mediators, like MIP-1α, nitric oxide and superoxides. In addition to this suppressor effect, adenosine can augment the formation of anti-

inflammatory cytokine IL-10 in response to $A_{2A}R$ ligation and by $A_{2B}R$-mediated posttranscriptional mechanisms.

1.5. PATHOGEN RECOGNITION AND TOLL-LIKE RECEPTOR MEDIATED SIGNALING PATHWAY

The innate immune system is the first line of defences against invading microbial pathogens which are initially recognized partially by germline-encoded pattern-recognition receptors (PRRs). The family of Toll-like receptors (TLR) is the most extensively studied class of PRRs. TLRs can be divided into subfamilies primarily recognizing related pathogen-associated molecular patterns, but in my work I focused on TLR4 mediated downstream signaling events. TLR4 has the ability to recognize several PAMPs, but one of the most important immunostimulatory molecules of TLR4 is lipopolysaccharide (LPS), a structural component of the outer membrane of Gram-negative bacteria.

LPS-induced TLR4 signal transduction

LPS recognition is mediated by interactions with several proteins, including LPS binding protein (LBP), CD14 and MD-2 and TLR4. LBP and CD14 can directly bind LPS and they are involved in the transfer of it to the TLR4/MD-2 receptor complex. LBP is a shuttle protein catalyzing the association between LPS and CD14. CD14 has a role in loading LPS to the receptor complex. MD-2 is a soluble protein that non-covalently forms a complex with TLR4 and can bind LPS directly without TLR4, therefore MD-2 serve as a ligand-binding component of the receptor complex TLR4/MD-2.

Ligand recognition induces oligomerization of TLR4, leading to activation of downstream signaling pathways mediated by a conserved region of the receptor, called cytoplasmic Toll/interleukin 1 receptor (TIR) domain that serves as the docking site for TIR-containing cytoplasmic adaptor proteins such as MyD88 (myeloid differentiation primary response gene 88), and TRIF (TIR domain-containing adaptor inducing IFN-β). TLR4 induced signaling can be separated into 2 distinct pathways: a MyD88-dependent pathway, which is responsible for pro-inflammatory cytokine expression and a MyD88-independent (TRIF-dependent) pathway, which mediates the induction of Type I interferons and interferon-inducible genes. The MyD88-dependent pathway is responsible for early-phase NF-κB and MAPK activation, which control the induction of pro-inflammatory cytokines. The MyD88-independent pathway activates IRF3/7, which is required for the induction of IFN-β- and IFN-inducible genes and mediates late-phase NF-κB as well as MAPK activation, also contributing to inflammatory responses.

2. AIMS OF THE STUDY

Apoptosis and the consequent engulfment of apoptotic cells occur permanently in the thymus and molecules released continuously by engulfing macrophages contribute to the formation of a thymic milieu, that regulates apoptosis and differentiation of thymocytes. Previous studies in our and other laboratories have shown that retinoids are also produced in the thymus in vivo and they initiate the death of immature thymocytes. Furthermore, we have found that retinoids induce a transcription-dependent apoptosis in these cells via activating RARγ.

- Since the action of retinoids to induce apoptosis was not investigated so far, in the first part of my thesis we wanted to identify the mechanism by which retinoids induce apoptosis program of thymocytes

It has been previously described that adenosine released by macrophages engulfing apoptotic cells react on macrophages as a soluble mediator, via A_{2A}Rs, to suppress pro-inflammatory cytokine production (KC and MIP-2) (Köröskényi et al., 2011). It is also known that LPS exposure in macrophages triggers ATP release leading to CD39-dependent ATP degradation and adenosine production and increase the expression of A_{2A} receptors via NF-κB. Since adenosine has been shown to inhibit the LPS-induced pro-inflammatory cytokine production of macrophages via A_{2A} receptors, upregulation of these receptors by LPS stimulation provides a delayed feedback mechanism.

- According to these results we wanted to characterize the detailed downstream signaling pathway through which adenosine via A_{2A} receptors suppress LPS-stimulated pro-inflammatory cytokine formation by studying A_{2A} receptor null macrophages.

3. MATERIALS AND METHODS

Experimental animals

Most of the cell death experiments with thymocytes were carried out in thymocytes isolated from 4-week-old Nur77$^{+/+}$ mice and Nur77deficient littermates. In a few experiments thymocytes were derived from STAT1-/- mice, as well. Bone marrow-derived macrophages were isolated from 3 to 6 months old wild type and A_{2A} receptor null mice generated on FVB background. Mice were maintained in specific pathogen-free condition in the Central Animal Facility of our university and all animal experiments were approved by the Animal Care and Use Committee of University of Debrecen (DEMÁB).

Cell culturing

Thymocyte suspensions were prepared from thymus glands of 4 week-old mice by mincing the glands in RPMI 1640 media supplemented with 10% charcoal-treated FBS, 1mM Na-pyruvate, 2 mM glutamine, and 100 U/ml penicillin and 100 μg/ml streptomycin. Thymocytes were washed and diluted to a final concentration of 5 x10^6 cells/ml.

For bone marrow-derived macrophages, bone marrow progenitors were isolated from the femurs of mice and cells were differentiated for 10 days in DMEM containing 10% FBS, 1mM Na-pyruvate, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin and 10% L929 cell supernatant. Non-adherent cell were removed by washing away after 3 days.

Both cell types were incubated at 37oC in a humidified incubator under an atmosphere of 5% CO2 /95% air.

Determination of the percentage of apoptotic thymocytes

Thymocytes were cultured in 24 well plates in the presence of the indicated concentrations of retinoids, dexamethasone-acetate (0.1 μM) or Jo2 antibody (1 μg/ml) for 6 hours. To test the contribution of caspases during cell death thymocytes were pretreated with 40 μM z-IETD.fmk, specific caspase 8 inhibitor (BD Pharmingen) or 75 μM z-LEHD.fmk, a caspase 9 specific inhibitor (Calbiochem) for 1 hour. Percentage of the degraded DNA containing apoptotic cells were determined using propidium iodide DNA staining. For staining cells were fixed with 70% ice cold ethanol for 5 min, then washed and redissolved in 100 μl PBS containing 100μg/ml RNase and incubated for 10 min at room temperature. Finally 400 μl PBS containing 50 μg/ml propidium iodide was added. % of cells carrying decreased amount of DNA due to apoptosis (sub G0-G1 cells) was determined on DNA histograms by flow cytofluorometry.

Flow cytometry

5×10^5 BMDMs were treated with 200 ng/ml LPS for the indicated time periods. After the incubation, macrophages were washed with PBS, collected, blocked with 50% FBS for 30 min, and labeled with anti-mouse $A_{2A}R$ antibody or goat IgG isotype control. For detection, cells were stained with FITC-conjugated anti-goat IgG. Stained cells were analyzed on a FACSCalibur (BD Biosciences). The results were analyzed by WinMDI 2.9 software.

Western blot analysis

Whole cell homogenates from thymocytes or BMDMs were used. Protein samples were diluted to 1mg/ml with Laemmli buffer and were fractionated on polyacrylamide gel, and transferred onto polyvinylidene difluoride membranes using the Bio-Rad electrophoresis and transfer system. After blocking with 5% non fat dry milk in TTBS membranes were probed with anti-Nur77, anti-Bid, anti-Bim, anti-STAT1, anti-mitogen-activated kinase kinase (MKK) 3/4, pMKK3/4/6 (Cell Signaling) anti-IκB (Santa Cruz Biotechnology), anti-phospho (Thr183/Tyr185)-SAPK/JNK (Cell Signaling) antibodies overnight at 4˚C. To detect antibody signals peroxidase-labeled anti-mouse IgG (1:1000), anti-rat IgG (1:10000) or anti-rabbit IgG (1:10000) were used and the enhanced chemiluminescence was visualized using the Immobilon Western Chemiluminescent HRP Substrate (ECL)(Merck Millipore). Equal loading of protein was demonstrated with probing the membranes with anti-α-tubulin, anti-lamin-B (Santa Cruz Biotechnology) or anti-β-actin (Sigma-Aldrich), antibodies.

Affymetrix analysis

To identify retinoid-regulated apoptosis genes in mouse thymocytes Mouse Affymetrix 430Av2 arrays carrying probes for 22690 transcripts were used. Wild type and Nur77 knock out DMSO exposed control and 0.3 μM 9cRA (4 h) treated thymocyte samples were run in duplicates. Hybridization was carried out in Genomics Core Facility in Heidelberg. Raw intensity data was scaled and normalized in Affymetrix Microarray suite 5.0. Changed transcripts were filtered in Microsoft Access and Excel software. Increased transcripts were judged as “Present” and increased +2 fold in both duplicates. Decreased transcripts had minimum of -1.6 fold change value in both duplicates.

qRT-PCR for detecting changes in the mRNA expression of various genes

After various treatments total RNA was extracted from thymocytes or macrophages with TRI reagent. Transcript quantitation was accomplished via quantitative real-time RT (reverse transcriptase) PCR (polymerase chain reaction) using Taqman gene expression assay. RNA samples

were reverse transcribed to cDNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to manufacturer's guidelines. Real-time monitoring was carried out using an ABI Prism 7900 or a Roche LightCycler LC 480 instrument performing 40 cycles of 94 ˚C for 12 sec and 60 ˚C for 1 min with pre-designed FAM-MGB-labelled specific probes (Applied Biosystems). All samples were measured in triplicates and the gene expression was calculated relative to cyclophilin in the case of thymocytes and to hypoxanthine-guanine phosphoribosyltransferase (HGPRT) in the case of macrophages.

Preparation of mitochondria and Western blot to detect mitochondrial translocation of Nur77/RXR

Mitochondrial proteins were isolated from thymocytes with the help of the ProteoExtract Cytosol/Mitochondria Fractionation Kit from Calbiochem according to the manufacturer's instructions, using 10^7 cells per sample. At the end of the procedure mitochondrial samples were denatured in 5x Laemmli buffer. The presence of Nur77 and RXR were analysed by Western blotting using the anti-Nur77 (BD Pharmingen) and anti-RXRα (Santa Cruz Biotechnology) antibodies. The blots were reprobed with antibodies against mitochondrial Hsp60 as loading control or the nuclear Lamin B (Santa Cruz Biotechnology) to check accidental nuclear contamination.

Intracellular staining for Bcl-2/BH3

For the detection of the intracellular levels of Bcl-2 BH3 domain thymocytes (2×10^6/ sample) were washed twice with PBS, were fixed and permeabilized using 250 µl of Cytofix/Cytoperm solution (BD Biosciences) for 20 min at 4°C. After fixation and blocking steps the samples were washed twice using perm/wash solution (200 µl/sample) and incubated with anti-Bcl-2 BH3 domain antibody (Abgent, San Diego, USA 1:100) for overnight at 4°C. Cells were further labelled with FITC- or Cy3-conjugated anti-rabbit IgG secondary antibody and were examined by FACSCalibur flow cytometer (BD Biosciences). Data was analyzed using WinMDI 2.9 software.

Detection of TNF alpha content in thymocyte supernatant

Wild type thymocytes were seeded onto 24-well plates at a density of 5 x 10^6 per 0,5ml and were treated with retinoids and the combination of phorbol dibutyrate and ionomycin for 18 hours. The collected samples were than centrifuged at 4000 g for 5 min. The concentration of TNF-α cytokine was measured from the supernatant culture medium using DuoSet Mouse TNF alpha ELISA Kit (R&D Systems).

Determination of cytokine production

5×10^5 wild type and $A_{2A}R$ null BMDMs were treated with 200 ng/ml crude LPS for 1 h. In some experiments, cells were pretreated with the $A_{2A}R$-specific agonist CGS21680 (1 μM, Tocris), the $A_{2A}R$ specific antagonist SCH442416 (10 nM, Tocris), the JNK inhibitor TCS JNK 60 (100 nM), Rp-cAMPs (100 μM) or forskolin (10 μM) for 1 h. After 1 h LPS was removed and fresh medium was added to the cells. Where it is indicated, media was supplemented with the compounds used in the pretreatments. 5 h later supernatants were collected, centrifuged and analyzed by Mouse Cytokine Array (Proteome Profile Array from R&D Systems) or by interleukin (IL)-6, macrophage inflammatory protein (MIP)2, tumor necrosis factor (TNF)-α ELISA kits (R&D Systems) according to the manufacturer's instructions. In case of cytokine array, the pixel density in each spot of the array was determined by Image J software.

Determination of NF-κB p50/p65 nuclear translocation

10^7 wild-type and $A_{2A}R$ null BMDMs were treated with 200 ng/ml LPS for 60 min. Where it is indicated, cells were pretreated with A2AR-specific agonist 1 μM CGS21680 for 1 h. Cells were rinsed with ice cold PBS and nuclei were isolated with Nuclei EZ kit (Sigma) according to manufacturer's instruction. Nuclear p65 and p50 subunits were detected with TransAM p65 and p50 kits (ActiveMotif) according to manufacturer's instruction. TransAM NF-κB Kits contains a 96-well plate to which an oligonucleotide containing the NF-κB consensus site has been immobilized. 20 μl diluted nuclear extract lysate was added to each well for 1 hour. After washing, samples were incubated for 1 hour with primary antibodies against an epitope on p65 and p50, which is accessible only when NF-κB is activated and bound to its target DNA. HRP-conjugated secondary antibody was used. After adding the developing solution the colorimetric reaction was detected by spectrophotometry.

Determination of NFκB-dependent transcription

NFκB-dependent transcription was evaluated as luciferase activity derived from the expression of an NFκB-dependent luciferase transcriptional reporter in a stably transfected macrophage cell line, H2. H2 cells were plated (4×10^5 cells/well) in a 24-well plate. The next day, 125 ng/ml LPS and the indicated doses of CGS21680 were added simultaneously. After incubation for 6 h, cell extracts were prepared, and luciferase activity was measured by the Luciferase Assay System (Promega) in an FB12 Luminometer (Zylux). Each condition was repeated in duplicate wells, and the luciferase activities in cells from each well were determined independently.

Determination of MAPK phosphorylation

1×10^6 cells/well BMDMs treated with 200 ng/ml LPS for 1 h. Where it is indicated, cells were pretreated with 10 nM SCH442416 (10 nM, Tocris) for 1 h. After 30 min LPS treatment, cells were rinsed with PBS. Total cell lysates were analyzed by Human Phospho-MAPK Array Kit (R&D Systems) according to manufacturer's instruction. The pixel density in each spot of the array was determined by ImageJ software.

DUSP1 siRNA transfection

Five-day-matured BMDMs were transfected with ON TARGETPLUS SMARTpool siRNA specific for mouse DUSP1 and ON-TARGETPLUS Non-targeting Control Pool (Dharmacon) using the DharmaFECT 1 Transfection Reagent (Dharmacon) according to the DharmaFECT's Transfection Protocol. siGLO Green (6-FAM) Transfection Indicator was used to monitor the transfection efficiency. Transfected cells were used 5 days after the transfection. The efficiency of RNAi was determined by Western blot and quantitative PCR against DUSP1.

Isolation of CD4+CD8+ double positive thymocytes by flow cytometry sorting

Thymocyte suspension of 4 week-old Nur77+/+ mice was prepared freshly and the cells were labelled in PBS with phycoerythrin-anti-CD4 and fluorescein isothiocyanate–anti-CD8 antibodies (BD Biosciences) at room temperature for 10 min. Double-positive (DP) thymocytes were separated as CD4+CD8+ cells from the cell suspension with FACS Aria III instrument (BD Biosciences). Cell sorting was performed at 4˚C to reduce cell death. Data analysis was carried out by using BD FACSDiva Version 6.1.3 software.

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 and MAPK array experiments was carried out by two-way ANOVA test. Statistical calculations were run on GraphPad Prism6 software. Statistical significance is indicated by a single asterisk ($P < 0.05$).

4. RESULTS

4.1. RETINOIDS INDUCE NUR77-DEPENDENT APOPTOSIS IN MOUSE THYMOCYTES

Nur77 is upregulated and essential in retinoid induced apoptosis of mouse thymocytes

Previously our research group have shown that retinoids can induce the apoptosis of mouse thymocytes in a transcription-dependent manner via activating RARγ, and subsequent studies on mouse T cell hybridoma cell line have revealed that ligation of RARγ can induce the expression of Nur77 and its target FasL. To figure out whether retinoids could also induce Nur77 in mouse thymocytes, so thymocytes derived from 4 week old mice were exposed to ATRA and 9cRA, and Nur77 expression was determined on both mRNA and protein levels. Both ATRA and 9cRA were able to induce the expression of Nur77 at concentrations where they saturate RARs, however, 9cRA was proved to be more potent indicating that ligation of RXR might contribute to the induction of Nur77.

To test whether Nur77 is required for retinoid-induced apoptosis, thymocytes isolated from both wild-type and Nur77 null mice were exposed to increasing amount of retinoids. Retinoids induced a dose dependent cell death in the wild-type thymocytes. ATRA was much less effective in apoptosis induction than 9cRA at each used concentrations, in accordance with the lower expression of Nur77 in the presence of ATRA. However, none of these compounds could induce cell death in Nur77 null thymocytes indicating an essential role of Nur77 in mediating retinoid-induced apoptosis of thymocytes. Interestingly, thymocytes lacking Nur77 showed slighter background rate of apoptosis indicating that the spontaneous cell death involves Nur77-dependent elements. We did not detect any change in the age-dependent size or thymocyte composition of the Nur77 null thymuses suggesting that these results were not related to a Nur77-dependent developmental defect of thymocytes. Our observation is in line with previously published data.

FasL, TRAIL, NDG-1, Gpr65 and Bid are induced by retinoids to initiate thymocyte death

To identify genes mediating retinoid-induced apoptosis, mRNA expression profile of control (DMSO-treated) and retinoid-treated thymocytes were compared by Affymetrix 430Av2 arrays carrying probes for 22,690 transcripts. As an effective retinoid, 0.3 μM 9cRA was selected for these studies. We confirmed that Nur77 is one of those genes which are significantly upregulated upon retinoid treatment. The Nur77 family consists of Nur77, Nor-1, and Nurr1 with partially overlapping biological activity, however, only Nur77 was upregulated by 9cRA in mouse thymocytes. To test whether these apoptosis-related genes are regulated in a Nur77-dependent manner during retinoid-induced apoptosis, 9cRA was added to both wild-type and Nur77 null thymocytes for 4 h and the

changes in their mRNA levels were monitored by Affymetrix 430Av2 arrays. We found eight apoptosis related genes to be induced by retinoids. Interestingly most of these genes were induced in a Nur77-dependent manner. NDG-1 was previously described as a Nur77-dependent gene and protein regulates caspase 8 activity by unknown mechanisms. Surprisingly, we have found 2 novel, unexpected apoptosis related genes among the Nur77-regulated genes: G protein-coupled receptor 65 (Gpr65), also known as T-cell death-associated gene 8, which is one of the proton-sensing G protein-coupled receptors (GPCRs) coupled to the adenylate cyclase, and the BH3 interacting domain death agonist (Bid), which is activated by caspase 8 cleavage and initiates the mitochondrial pathway of apoptosis.

To affirm the induction of these 3 genes by retinoids, their mRNA expression was also measured by qRT-PCR analysis 4 h after ATRA or 9cRA exposure. In addition, we also determined the expression of FasL and TRAIL, two death receptor ligands, which are already known Nur77-regulated apoptosis related genes, but were not detectable by the Affymetrix analysis. As shown Figure 12, all these genes were induced by retinoids in wild-type thymocytes, but not in Nur77 null thymocytes confirming that these genes are indeed retinoid-induced Nur77 dependent genes. All the Nur77-dependent genes were induced more effectively by 9cRA treatment than ATRA, in accordance with the higher levels of Nur77 induced by it.

Caspase 8 activation contributes to the initiation of retinoid-induced cell death

Since retinoids induced the expression of TRAIL and FasL, two cell death receptor ligands, and NDG-1, which similar to death receptors, can also activate caspase 8 though by unknown mechanisms, we decided to check whether caspase 8 plays a role in retinoid-induced apoptosis. First we tested caspase 8 cleavage during retinoid induced apoptosis. As it is demonstrated, cleaved caspase 8 protein can be detected in retinoid-treated thymocytes. Since Bid was also induced by retinoids, and Bid is a well-known caspase 8 substrate, to demonstrate that caspase 8 is activated during retinoid-induced apoptosis we checked, whether Bid is also cleaved. We could detect the truncated form of Bid in thymocytes treated by 9cRA, but not in thymocytes treated by 9cRA in the presence of z-IETD-fmk, a specific caspase 8 inhibitor.

Next we inhibited caspase 8 activity to test the role of its activation in retinoid-induced apoptosis. Preincubation of thymocytes with z-IETD-fmk significantly reduced retinoid-induced cell death, but did not inhibit it completely. Similar effect is observed in the glucocorticoid-induced cell death, in which caspase 8 has a role, but the determining caspase is caspase 9. At the same time z-IETDK completely inhibited Fas-induced death, which requires caspase 8 for mediating apoptosis. These data indicate that caspase 8 is the initiator caspase in retinoid-induced apoptosis, but the death is not entirely dependent on its activity.

Caspase 9 also plays a determining role in retinoid-induced apoptosis

Thymocytes are considered to be Type I cells, in which cell death receptor activated caspase 8 drive the killing directly by cleaving and thereby activating sufficient amount of executioner caspases, without the involvement of the intrinsic pathway. Thus Fas mediated apoptosis of thymocytes is not affected by the loss of caspase 9. Though we found signs for caspase 8 activation during retinoid-induced apoptosis, we decided to investigate the requirement of caspase 9 activity (thus the mitochondrial pathway) during retinoid-induced apoptosis by inhibiting caspase 9. Fas-induced apoptosis was used as a control. While the caspase 9 inhibitor z-LEHD.fmk had only slight effect on the apoptosis induced by the Jo2 anti-Fas antibody, it absolutely prevented retinoid-induced and dexamethasone-induced apoptosis. These data indicate that though caspase 8 is activated during retinoid-induced apoptosis, its apoptosis-inducing action requires the mitochondrial pathway of apoptosis involving caspase 9. In line with this observation we also detected the activation of caspase 9. Interestingly, activation of caspase 8 occurs earlier in time than caspase 9 activation in Caspase 9 also plays a determining role in retinoid-induced apoptosis dicating that caspase 8 plays an initiator role in the retinoid-induced apoptosis.

Gpr65-mediated signals do not seem to be critical in retinoid-induced apoptosis of thymocytes

Previous studies have shown that Gpr65 can increase the cytosolic cAMP levels via activating adenylate cyclase pathway which might lead to apoptosis in thymocytes by enhancing the production of TNF-α or by inducing Bim. Since 9cRA induced the expression of Gpr65, we decided to test whether addition of retinoids results in TNF-α production by checking its mRNA expression by qRT-PCR and by detecting its protein level by ELISA. Exposure of thymocytes to retinoids did not result in detectable TNF-α production. However, we could demonstrate that retinoid treatment significantly induce the gene expression and protein level of Bim, but this increase was not inhibited by Rp-cAMPS triethylamine or H89, specific membrane-permeable inhibitors of cAMP dependent protein kinase I and II. Addition of PKA inhibitors had no effect on the retinoid-induced apoptosis of the thymocytes either. Surprisingly Bim upregulation showed a Nur77-dependent fashion upon retinoid treatment. Taken together, our data indicate that though the expression of Gpr65 is increased, under our culture conditions there is no significant acidification which would activate it and the coupled adenylate cyclase pathway.

Bim is induced in a STAT1-dependent manner during retinoid-mediated apoptosis of thymocytes and contributes to cell death

Previous studies have shown that STAT1 can also regulate Bim expression in thymocytes. The Affymetrix data revealed that STAT1 is also induced during retinoid-induced apoptosis in a Nur77-

dependent manner. We could also confirm retinoid-induced STAT1 upregulation on protein levels. Thus we decided to test by using STAT1 knock out thymocytes whether the induction of Bim expression during retinoid-induced apoptosis is STAT1 dependent. Our results indicate that enhancement in the Bim expression during retinoid-induced apoptosis is STAT1 dependent. In addition, loss of STAT1 reduced the rate of retinoid-induced apoptosis of thymocytes indicating that STAT1-induced Bim expression might contribute to the initiation of the retinoid-induced apoptosis program.

Nur77 translocates into the mitochondria during retinoid-induced apoptosis and leads to the exposure of the Bcl-2/BH3

Previous studies have shown that Gpr65 can increase the cytosolic cAMP levels via activating adenylate cyclase pathway which might lead to apoptosis in thymocytes by enhancing the production of TNF-α or by inducing Bim. Since 9cRA induced the expression of Gpr65, we decided to test whether addition of retinoids results in TNF-α production by checking its mRNA expression by qRT-PCR and by detecting its protein level by ELISA. Exposure of thymocytes to retinoids did not result in detectable TNF-α production. However, we could demonstrate that retinoid treatment significantly induce the gene expression and protein level of Bim, but this increase was not inhibited by Rp-cAMPS triethylamine or H89, specific membrane-permeable inhibitors of cAMP dependent protein kinase I and II. Addition of PKA inhibitors had no effect on the retinoid-induced apoptosis of the thymocytes either. Surprisingly Bim upregulation showed a Nur77-dependent fashion upon retinoid treatment. Taken together, our data indicate that though the expression of Gpr65 is increased under our culture conditions there is no significant acidification which would activate it and the coupled adenylate cyclase pathway.

Next we studied whether translocation of Nur77 into the mitochondria leads to Bcl-2 conformational change and exposure of its BH3 domain. In the anti-apoptotic Bcl-2 protein the BH3 domain remains hidden or buried inside the molecule and is undetectable by the Bcl-2/BH3-specific antibodies. Exposure of this domain correlates with the pro-apoptotic activities of Bcl-2 and can be detected by mimicking signals of negative selection by adding phorbol dibutyrate/ionomycin to the thymocytes. For this reason we checked the Bcl-2/BH3 exposure during retinoid-induced apoptosis as well using specific antibody against this domain, and found a time dependent exposure of the BH3 domain. The exposure of the domain was related to Nur77, as the appearance of this domain was not detected in Nur77 null thymocytes exposed to 9cRA.

Retinoids induce both Nur77-dependent transcription and the appearance of Bcl-2/BH3 domain in the DP thymocytes

Previous publications have reported that retinoids induce apoptosis primarily in double positive (DP) thymocytes, but the basal expression of Nur77 shows a differentiation-dependence. While in DP thymocytes the expression of Nur77 is low, thymocytes undergoing both positive and negative selection induce Nur77, the expression correlating with the strength of the TCR signal. As a result, in the thymus of transgenic mice expressing GFP under the control of the Nur77 promoter, mostly medullary (positively selected) thymocytes were GFP-positive. Accordingly we used DP thymocytes received by sorting to investigate whether the Nur77-dependent events that we observed in non-separated thymocytes are related to DP thymocytes. DP thymocytes expressed Nur77 and reacted to both 9cRA and the PdBu/ionomycin treatment by upregulating Nur77. Since we could generate only small amounts of sorted cells we decided to check the retinoid-induction of three Nur77-regulated genes: Gpr65, NDG-1 and Bid. We have proved that the gene expression level of all these three genes were induced by retinoids. Next we tested whether Bcl-2/BH3 exposure can be detected in DP thymocytes. For this purpose thymocytes were exposed to retinoids and then labeled with anti-CD8 and anti-Bcl-2/BH3 antibodies. As it is demonstrated, we could detect the exposure of Bcl-2/BH3 domain in the CD8+ population. Since the majority of CD8+ thymocytes are DP thymocytes, these data indicate that Nur77 can translocate into the mitochondria in these cells. Finally we investigated the role of caspase 8 and caspase 9 in retinoid-induced death of the sorted DP thymocytes. and we have found that both inhibitors attenuated retinoid-induced apoptosis similarly as in the unsorted thymocytes.

4.2. ADENOSINE A_{2A} RECEPTOR SIGNALING ATTENUATES LPS-INDUCED PRO-INFLAMMATORY CYTOKINE FORMATION OF MOUSE MACROPHAGES BY INDUCING THE EXPRESSION OF DUSP1

Adenosine A_{2A} receptors mediate a feedback regulatory mechanism to decrease the LPS-induced pro-inflammatory cytokine formation in bone marrow derived macrophages

Previous studies have revealed that exposure of macrophages to LPS leads to increased expression of A_{2A}Rs on macrophages. As it is shown, LPS indeed induced the expression of A_{2A}Rs on both on the cell surface and on mRNA levels in BMDMs. Since the increased cell surface appearance of A_{2A}Rs could be detected already within 30 min, it is very likely that not only the reported transcriptional mechanisms contribute to its enhanced expression.

To study the involvement of A_{2A}Rs in the regulation of LPS-induced pro-inflammatory cytokine production, both wild type and A_{2A}R null macrophages were exposed to LPS for 1 h, and the pro-inflammatory cytokine production was determined after an additional 5 hours by using cytokine

array. We have found that the lack of A_{2A}Rs influenced already the basal pro-inflammatory cytokine production of BMDMs, A_{2A}R null macrophages produced more B-lymphocyte chemoattractant (BLC), interferon (IFN)-γ, IFN-γ-inducible cytokine (IP-10), keratinocyte chemoattractant (KC) and tissue inhibitor of metalloproteinases (TIMP)-1. After LPS exposure macrophages started to release a number of pro-inflammatory cytokines and the production of all of these was found to be enhanced by A_{2A}R null cells. We further confirmed cytokine array results by ELISA in case of TNF-α, IL-6 and MIP-2 cytokines. Not only the protein, but also the gene expression level of these cytokines was higher in LPS-treated A_{2A}R null cells as compared to the wild type cells. These data support previous findings that LPS-triggered macrophages produce endogenous adenosine to regulate LPS-induced pro- inflammatory cytokine formation via the adenosine A_{2A}Rs.

Loss of adenosine A_{2A} receptors does not affect the LPS-induced NF-κB signaling pathway in macrophages

Since the LPS-induced signaling pathways mediate the 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 A_{2A}R null macrophages following LPS challenge. As we demonstrated, neither the amount, nor kinetics of the IκBα degradation induced by LPS stimulation showed dissimilarities in the two cell types. Similar was the finding, if wild type macrophages were pretreated for 1 h with CGS21680, an A_{2A}R agonist.

NF-κB is a dimeric transcription factor that belongs to the Rel homology domain-containing protein family, which includes p65/RelA, p50/NF-κB1, p52/NF-κB2, RelB and c-Rel. The prototypical NF-κB form exists as a p50/p65 heterodimer in most types of cells. Therefore we decided to examine whether the loss of A2AR affects the nuclear translocation of the p65/p55 subunit of NF-κB by using the TransAM NF-κB transcription factor kits (ActiveMotif). As we have shown, no difference was detected in either the basal or in the LPS-induced nuclear appearance of these transcription factors in wild type and in A2AR null macrophages. In line with these observations, A_{2A}R agonist CGS21680 treatment also did not alter it. Administration of CGS21680 did not alter the LPS-induced NF-κB driven transcriptional activation of a luciferase construct in a stably transfected RAW264.7 mouse macrophage cell line either. All together these data indicate that NF-κB is properly activated in the presence of adenosine, and not the NF-κB pathway is the main target of A_{2A}R signaling through which it attenuates the LPS-induced pro-inflammatory cytokine formation. Thus we decided to study the MAPK signaling pathways.

Adenosine suppresses both basal and LPS-induced JNK activities acting via A_{2A}Rs

To test the basal and LPS-induced MAPK activation in the absence of A_{2A}R signaling in macrophages, we applied a human phospho-MAPK array kit, which enables the parallel determination of relative levels of phosphorylation of MAPKs and several related proteins. As we have shown, loss of A_{2A}Rs resulted in enhanced basal phosphorylation of p38α, c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) 2. This phenomenon could be partially mimicked by a 1 h administration of the A_{2A}R antagonist SCH442416 indicating that alteration in the basal phosphorylation pattern is not a result of a developmental defect of macrophages in the absence of A_{2A}Rs. This observation also indicated that A_{2A}Rs must be continually activated in non-stimulated cells. Stimulation of wild type macrophages by LPS resulted in activation of the MAPK pathways leading to enhanced phosphorylation of p38, ERK2 and JNK and that of the downstream proteins of the p38 MAP kinase pathway such as ribosomal S6 kinase (RSK) 1, 2, mitogen and stress activated kinase 2 (MSK2). Previous studies have shown that p38α can selectively activate MAPK activated protein kinase 2, which further phosphorylate CREB and heat shock protein (hsp) 27, the activation of which was also detected. In addition, we could also demonstrate enhanced phosphorylation of proteins of the phosphatidylinositol-3 kinase pathway, such as target of rapamycin (TOR) and glycogen synthetase kinase 3. This latter pathway has been suggested to limit the LPS-induced signaling pathways by inhibiting the LPS-induced nuclear translocation of NF-κB.

While loss or inhibition of A_{2A}Rs resulted in enhanced basal phosphorylation of the p38α and ERK2, the phosphorylation of these kinases did not increase further following LPS stimulation in the absence of A_{2A}R signaling. However, despite of the lack of further phosphorylation, following LPS exposure the phosphorylation of the p38α MAP kinase was still higher in A_{2A}R null macrophages than in wild type cells. In contrast, after LPS stimulation the phosphorylation of JNKs increased significantly further in A2AR null macrophages as well.

Since macrophages express JNK1 and 2, but the human phospho-MAPK array kit did not detect mouse JNK1, we tested its phosphorylation by Western blot analysis by using a mouse specific anti-JNK1 antibody. Phosphorylation of JNK1 was also enhanced following LPS stimulation in A_{2A}R null macrophages as compared to their wild counterparts. The findings were further confirmed by using a phospho-panJNK- specific ELISA kit. In accordance, LPS induced phosphorylation of JNKs in wild type cells was further enhanced when these cells were exposed to SCH442416, while was inhibited when exposed to CGS21680. These data indicated that A_{2A}R signaling might negatively influence JNK activity. Thus, we decided to test the potential involvement of JNKs in the A_{2A}R-mediated attenuation of LPS signaling in macrophages.

JNK is a downstream target in the control of LPS-induced pro-inflammatory cytokine formation by adenosine

If $A_{2A}R$ signaling affects LPS-induced pro-inflammatory cytokine formation via decreasing the LPS-induced phosphorylation of JNK, then inhibition of JNK should affect the ability of adenosine to control LPS-induced pro-inflammatory cytokine formation. We have found that exposure of wild type macrophages to CGS21680 attenuated, while that of SCH442416 enhanced the LPS-induced production of IL-6, MIP-2 and TNF-α. The same compounds did not affect the LPS-induced pro-inflammatory cytokine production of $A_{2A}R$ null macrophages confirming the $A_{2A}R$ selectivity of these compounds. In the presence of the $A_{2A}R$ antagonist a similarly high LPS-induced pro-inflammatory cytokine production was found by wild type macrophages, as by $A_{2A}R$ null cells.

Inhibition of JNK activity by TCS-JNK attenuated LPS-induced pro-inflammatory cytokine production to a similar degree in wild type macrophages, as in their $A_{2A}R$ null counterparts. In addition, those wild type macrophages, in which JNK activity was inhibited, failed to respond to $A_{2A}R$ signaling in the regulation of LPS-induced pro-inflammatory cytokine production. All together these data indicate that JNK is a downstream target of adenosine signaling.

$A_{2A}R$ signaling enhances both basal and LPS-induced DUSP1 expression via the adenylate cyclase pathway

Enhanced phosphorylation of MAPKs in $A_{2A}R$ null macrophages might be the result of either an enhanced activation of their upstream kinases, or a decreased removal of their phosphate groups by their specific phosphatases. Since we detected activation of both p38α and JNKs, downstream elements of LPS-induced MKK4 or MKK3/6 activation, we checked whether MKK4 or MKK3/6 activation is increased by LPS in the absence of $A_{2A}R$ signaling. However, in the case of MKK4 no alteration was found in the kinetics phosphorylation. In addition, following LPS exposure both the levels of MKK3 and the phosphorylation of MKK3/6 decreased in the presence of the $A_{2A}R$ antagonist. These observations indicate that not an enhanced activation of the upstream kinases in the LPS signaling pathway is responsible for the enhanced MAP kinase phosphorylation observed in the absence of $A_{2A}R$.

During the last decade, a family of dual specificity phosphatases was identified that acts as MAPK phosphatases by dephosphorylating them at threonine and tyrosine residues. The prototypic member of this family is DUSP1, which is expressed by macrophages, and the expression of which is enhanced during LPS stimulation. The enzyme is an important negative-feedback regulator of macrophage function and the inflammatory response to TLR signaling, and plays key regulatory roles in innate immune responses via inactivation of p38 and JNK. Since already the basal phosphorylation level of both JNK and p38 was enhanced in $A_{2A}R$ null macrophages, we decided to

determine whether basal DUSP1 levels are affected by the loss of $A_{2A}R$.We have shown that $A_{2A}R$ null macrophages express significantly lower DUSP1 at both mRNA and protein levels than their wild type counterparts. Exposure of wild type macrophages to SCH442416 also resulted in a decrease in DUSP1 expression, while that to CGS21680 increased it. These compounds did not affect DUSP1 levels in $A_{2A}R$ null cells indicating that they acted indeed via $A_{2A}Rs$. All together these data indicate that $A_{2A}R$ signaling continuously enhances the basal DUSP1 expression in BMDMs.

Previous studies have shown that LPS stimulation itself enhances the expression of DUSP1 in macrophages, which contributes to termination of the LPS response. Thus we decided to investigate whether $A_{2A}R$ signaling affects LPS-regulated DUSP1 expression. For this purpose macrophages were pretreated for 1 h with either the $A_{2A}R$ agonist or the $A_{2A}R$ antagonist, and DUSP1 mRNA expressions were determined. The time point was set for 2 h following LPS treatment, as preliminary studies revealed that the LPS-induced DUSP1 mRNA expression is the highest at this time point. Not only the basal, but also the LPS-induced DUSP1 mRNA expressions were higher in wild type macrophages as compared to that of the $A_{2A}R$ null cells. In line with this finding, in wild type cells addition of the $A_{2A}R$ agonist enhanced, while that of the $A_{2A}R$ antagonist decreased the LPS induced DUSP1 mRNA expression.

Previous studies have shown that $A_{2A}Rs$ mediate their effect on the LPS signaling in macrophages by elevating intracellular cAMP levels. To investigate whether alterations in intracellular cAMP levels affect DUSP1 mRNA levels, macrophages were pretreated for 1 h with either forskolin, an adenylate cyclase activator, or Rp-cAMP, a competitive inhibitor of endogenous cAMP. Forskolin could enhance both the basal and the LPS-induced DUSP1 expression in both wild type and A2AR null macrophages, while Rp-cAMP decreased both the basal and the LPS-induced DUSP1 mRNA levels, but only in wild type cells. In addition, Rp-cAMP prevented the enhancing effect of the $A_{2A}R$ agonist on DUSP1 levels, while forskolin the decreasing effect of the $A_{2A}R$ antagonist on DUSP1 levels in wild type cells. All together these data indicate that $A_{2A}Rs$ upregulate the mRNA levels of DUSP1 in both resting and in LPS-induced macrophages acting via the adenylate cyclase pathway.

$A_{2A}Rs$ fail to control the LPS-induced JNK phosphorylation in DUSP1-silenced macrophages

To prove the involvement of DUSP1 in $A_{2A}R$ signaling, DUSP1 levels were silenced in both wild type and in $A_{2A}R$ null macrophages. As we demonstrated, siRNAs significantly reduced the basal DUSP1 expression at both protein and mRNA levels in wild type and $A_{2A}R$ null macrophages. Reduction in DUSP1 levels by siRNA resulted in an enhancement in both the basal and in the LPS-induced phosphorylation levels of JNK in wild type macrophages, and in a loss of the $A_{2A}R$ control

over it. In contrast, in $A_{2A}R$ null cells reduction in DUSP1 levels had only slight effect on the enhanced basal and LPS-induced JNK phosphorylation. These data indicate that $A_{2A}Rs$ regulate JNK phosphorylation via modifying the expression of DUSP1.

$A_{2A}Rs$ fail to control the LPS-induced IL-6 and TNF-α production in DUSP1-silenced macrophages

To investigate further the involvement of DUSP1 in the $A_{2A}R$mediated control of LPS-induced pro-inflammatory cytokine formation, the effect of DUSP1 silencing was studied on the LPS-induced IL-6 and TNF-α production of wild type and $A_{2A}R$ null macrophages. In wild type macrophages, silencing of DUSP1 enhanced the LPS-induced production of each of these three pro-inflammatory cytokines. DUSP1 silenced cells became resistant to $A_{2A}R$ signaling, since neither CGS21680, nor SCH442416 affected their LPS-induced pro-inflammatory cytokine formation. In contrast, silencing of DUSP1 only slightly affected the LPS-induced enhanced production of these pro-inflammatory cytokines in $A_{2A}R$ null cells. These data indicate that $A_{2A}Rs$ regulate LPS-induced IL-6 and TNF-α production via controlling DUSP1 levels in macrophages.

5. DISCUSSION

The function of the immune system is to eliminate the pathological microbes and toxic, tumors or allergic proteins, but at the same time to avoid responses that lead to self-tissue damage. In vertebrates, the immune response to microbial pathogens consists of innate and adaptive components. Innate and adaptive immunity usually act together: the first line of the host defence is mediated by the innate system sensing the danger, and then the adaptive immune responses are build up which require the innate signals for the activation, provide important protection against invading microbes and permit the development of immune memory.

The key biological function of the T-cell arm of the adaptive immune response is to identify and destroy infected cells and also to recognize pathogen-derived peptide fragments together with self-structures. For this purpose the generation of the adequate T-cell repertoire in the thymus must be properly regulated. During their thymic differentiation fewer than 5% of the developing T cells survive due to strict selection processes. 90% of the DP cells bear TCRs that are not able to recognize peptide loaded MHC molecules and they undergo death by neglect. The apoptotic cells are then rapidly being cleared by thymic macrophages, which in turn release soluble molecules, such as retinoids and adenosine. Thus we have proposed that thymic macrophages continually engulfing apoptotic thymocytes constantly provide these efferocytosis-dependent molecules therefore contribute to establishment of a thymic environment for the developing thymocytes, which ensures the fast death by neglect in the absence of TCR signaling. Previous results from our laboratory have revealed that thymocytes can express RARγ and retinoids, via ligating this receptor can induce transcription-dependent cell death of immature double positive thymocytes. On the basis of these results in the first part of my PhD work I investigated the possible mechanism through which retinoids can induce the apoptosis in the immature thymocytes.

Previous studies carried out on the Jurkat T cell line revealed that similar to T-cell receptor signaling, retinoids are able to induce the upregulation of the Nur77 transcription factor. In our experiments we could demonstrate that similar to Jurkat cells, retinoids potently induce the expression of Nur77 in mouse thymocytes and retinoid-induced apoptosis was fully dependent on Nur77. T cell receptor (TCR)-mediated apoptosis, a model for negative selection of self-reactive thymocytes was also described to depend on Nur77, however, Nur77-deficiency does not affect the TCR-induced cell death. This observation was explained by the simultaneous induction of its family member Nor1 by TCR signals, and the functional redundancy between Nur77 and Nor-1 gene products. Surprisingly, unlike TCR signaling, retinoids were not able to induce other members of the Nur77 family in dying thymocytes. These data indicate that though Nur77 and Nor1 show overlapping biological activities, they might be regulated by divergent signals.

Our results also demonstrated that both retinoids, 9cRA and ATRA were able to induce the expression of Nur77 in mouse thymocytes, but 9cRA was more potent indicating that ligation of RXR might contribute to the Nur77 induction in mouse thymocytes. In line with the higher induction of Nur77 by 9cRA, 9cRA also induced a higher rate of apoptosis in thymocytes.

The orphan nuclear receptor Nur77 functions as transcription factor. Microarray analysis in retinoid-exposed mouse thymocytes revealed the induction of nine retinoid-induced apoptosis genes, the majority of which was induced in a Nur77-dependent manner. FasL, TRAIL and NDG-1 have already been reported to be targets of Nur77 transcription that mediate apoptosis in thymocytes. In addition, we identified four novel Nur77-dependent genes, Bid, a BH3-only protein, Gpr65, a pH sensitive receptor, Bim, another BH3 only protein, and Ptpn6, a protein phosphatase, during retinoid-induced apoptosis.

In addition, we have detected the activation of caspase 8 and cleavage of Bid during retinoid-induced apoptosis. Since FasL, TRAIL and NDG-1 all could contribute to caspase 8 activation, we examined the role of caspase 8 in retinoid-induced apoptosis by using a specific caspase 8 inhibitor, in a concentration at which it only slightly affected dexamethasone-induced cell death. This concentration of the inhibitor, however, completely blocked the cleavage of Bid and significantly diminished the rate of retinoid-induced cell death indicating a central role of caspase 8 in retinoid induced apoptosis of thymocytes. Though thymocytes are considered to be type I cells in the context of Fas-induced cell death, our data, demonstrating that caspase 9 inhibition also prevents retinoid-induced apoptosis, indicate that during retinoid-induced apoptosis caspase 8 drives the mitochondrial pathway of apoptosis in thymocytes, and caspase 8-induced Bid cleavage and activation might play a mediator event in this.

The importance of the mitochondrial pathway was further proved by the fact that retinoid-induced STAT1 leads to the upregulation of Bim, another BH3-only protein. The absence of STAT1 prevented Bim upregulation and significantly decreased retinoid-induced apoptosis. Interestingly, retinoid-induced STAT1 expression and phosphorylation was observed in other types of cells as well indicating that STAT1 is not a unique mediator of retinoid signaling in the thymocytes. In addition, we also found the retinoid-dependent, but Nur77-independent upregulation transglutaminase 2 which is a multifunctional protein crosslinking enzyme and it was previously demonstrated to act on the mitochondria.

In addition, Affymetrix results showed increased expression of two proteins that negatively regulate protein phosphorylation, Gadd34 and Protein tyrosine phosphatase, non-receptor type 6 (Ptpn6). Gadd34 (also known as MyD116) was originally characterized as a growth arrest and DNA damage-inducible gene. It facilitates protein phosphatase type 1 activity through both direct binding to the protein, as well as through binding to other proteins that also modulate phosphatase activity.

Increased expression of Gadd34 was subsequently found to correlate with apoptosis, and forced overexpression of the protein leads to apoptosis. Ptpn6, on the other hand, is necessary for receptor-mediated cytotoxic signaling that causes intracellular acidification and apoptosis. While the retinoid-induced induction Ptpn6 was Nur77 dependent, the induction of Gadd34 was not.

Independently of its transcriptional activity, Nur77 protein is known to act directly in the mitochondria to induce apoptosis. We could observe the mitochondrial translocation of Nur77 also during retinoid-induced apoptosis of thymocytes. This mitochondrial targeting was showing similar time kinetics as during T cell receptor mediated death, but the translocated Nur77 lacked heavy phosphorylation, which characterizes the protein during negative selection. Interestingly, 9cRA was more effective in inducing Nur77 translocation than ATRA, in contrast to a previous finding which suggested that ligation of RXR might interfere with Nur77 translocation. However, those studies were carried out in the presence of another apoptosis-inducing signal, which leads to a protein kinase C-dependent phosphorylation of Nur77. Thus mitochondrial targeting of Nur77 might be simultaneously influenced by both its phosphorylation status and the presence of the RXR ligand. The translocation of Nur77 bears a relation with the exposure of the pro-apoptotic BH3 domain of Bcl-2.

Though the majority of our studies were conducted on unsorted thymocytes, we repeated some key experiments on sorted DP thymocytes as well. We could demonstrate the induction of Nur77, the transcription of the Nur77-dependent genes and the appearance of Bcl-2/BH3 domain in DP cells; furthermore the death was proved to be Caspase 8 and 9 dependent. Based on our findings we propose that retinoids induce a Nur77-dependent cell death program in mouse DP thymocytes.

Data coming from our laboratory and that of Agace indicate that cells located in the thymus are capable of producing RAs generating an endogenous RA milieu within the tissue. We also found that their production is related to apoptotic cell-engulfing macrophages. Surprisingly, however, we could not detect the presence of any of the classical retinoids in the mouse thymus instead metabolites of the retinol saturase pathway seem be produced. Thus, our data indicate that *in vivo* novel retinoids might contribute to the regulation of thymocyte apoptosis, which are produced in the thymic cortex during the course of the constant apoptotic cell engulfment.

The macrophages of the innate immune system provide a first line of defence against many common microorganisms and contribute to the defence mechanisms by recognizing and engulfing the pathogens and also by secreting of biologically active molecules, cytokines and chemokines in response to bacterial constituents, initiating inflammation and recruiting neutrophils and monocytes into the inflamed, infected tissue in large numbers. Although, the effective host immune response requires a certain degree of local inflammation against invading microbes to survive, failure in

controlling inflammatory responses can lead to tissue destruction, organ failure, shock and death. To avoid the overproduction of macrophage derived inflammatory cytokines, the immune system developed several mechanisms to keep inflammation under control, including negative feedback regulators, such as production of adenosine in response to LPS.

Our group has recently shown that apoptotic cell engulfing macrophages are capable of producing adenosine extracellularly in the thymus. Dying thymocytes release adenine nucleotides which are quickly degraded to adenosine by cell surface enzymes of the macrophages. In the thymic cortex, macrophages are continuously exposed to dying cells and produce adenosine which contributes to the death by neglect process of immature thymocytes similar to retinoids. Massisve apoptosis occurs under inflammatory conditions as well, when neutrophils undergo apoptosis and are rapidly phagocytosed by the surrounding macrophages. We have demonstrated that adenosine released by efferocytosing macrophages react on macrophages as a soluble mediator, via A_{2A}Rs, to suppress pro-inflammatory cytokine production, such as neutrophil-attracting chemokine (KC) and macrophage inflammatory protein-2 (MIP-2). On the other hand adenosine also suppresses pro-inflammatory cytokine formation in LPS-stimulated macrophages. Despite of the fact that the suppressive effect of adenosine on LPS-induced pro-inflammatory cytokine production has been known for a long time, the underlying signaling mechanism has not been described so far. In the second part of my thesis I investigated the molecular signaling pathway by which adenosine interferes with the LPS signaling in macrophages. Our data confirmed previous reports that the suppressive effect of adenosine is mediated via the adenosine A_{2A} receptors.

…..It was previously believed that A_{2A}R activation inhibits the LPS-induced expression of pro-inflammatory mediators by increasing the intracellular concentration of cAMP, which subsequently represses NF-κB activity and thereby gene transcription through a signaling mechanism involving cAMP dependent protein kinase A. However, it was published later that although inhibition of LPS-induced pro-inflammatory cytokine production by adenosine is mediated by cAMP, it is independent of protein kinase A or Epac, and the NF-κB activity is not affected.

In line with other findings, we also found that LPS-regulated NF-κB activation is not affected by A_{2A}R signaling in macrophages. Instead, we demonstrated that A_{2A}R signaling is continuously activated in macrophages and suppresses MAPK activities. Consequently, in macrophages lacking A_{2A} receptors the basal phosphorylation levels of p38α, ERK2 and JNK MAP kinases were found to be higher than in their wild type counterparts. Following LPS stimulation the A_{2A} receptor levels are increased in macrophages, and adenosine signaling suppressed also the LPS-induced MAP kinase activation. As a result, JNK phosphorylation and signaling were attenuated and the LPS-induced expression of JNK-dependent pro-inflammatory cytokines, such as MIP-2, IL-6 and TNFα, was found to be attenuated.

MAP kinase activity can be regulated by upstream kinases that phosphorylate MAP kinases at both serine and threonine residues, and by dual specific phosphatases, which dephosphorylate and inactivate these enzymes. While we have not found an increase in the activation of MKK4 and MKK3/6, upstream activators of p38 MAP kinases and JNKs, we have demonstrated that the expression of at least one of the MAPK phosphatases, DUSP1, is positively regulated by the A_{2A}R-induced adenylate cyclase signaling pathway. The mechanism of DUSP1 upregulation was not investigated in our studies.

Our data indicate that macrophages continuously produce adenosine to activate A_{2A}Rs, which trigger a cAMP-mediated pathway in macrophages to maintain the expression of DUSP1 and consequently to suppress both the basal and the LPS-induced MAP kinase activities.

6. SUMMARY

Programmed cell death or apoptosis is one of the major contributors to the proper development and homeostasis of metazoans and also plays a key and essential role in the differentiation of immune cells in the thymus, where autoreactive cells are eliminated. In the thymus continuously generated apoptotic cells are rapidly cleared by neighbouring macrophages without inflammatory consequences. This is partially due to that fact that the clearance of dying cells by macrophages leads to the production of several soluble anti-inflammatory mediators. Massive apoptosis occurs during acute inflammation when the emigrated neutrophils - following elimination of pathogens - undergo apoptosis. Under inflammatory conditions the released signals are necessary for the fast and effective resolution of inflammation. Our laboratory has identified two efferocytosis-dependent mediators released by macrophages: the retinoids and adenosine. In my thesis I examined how these molecules can contribute to the development of effective apopto-phagocytosis program.

Overall, in the postnatal thymus retinoids and adenosine derived from macrophages engulfing apoptotic cells accelerate the "death by neglect" of immature thymocytes. In addition, retinoids promote efferocytosis, while adenosine suppresses apoptotic cell-induced chemoattractant formation of engulfing macrophages. My results describe the mechanisms through which retinoids induce thymocyte death. Based on our findings we propose that retinoids induce a Nur77-dependent cell death program in mouse DP thymocytes, involving already known and new Nur77-dependent elements, which all together contribute to the induction of the mitochondrial pathway of apoptosis.
Under inflammatory conditions retinoids, besides promoting efferocytosis, were described to contribute to Treg formation (Lu et al., 2014; Liu et al., 2015). Adenosine, on the other hand, inhibits LPS-induced pro-inflammatory cytokine formation, thus prevents the overshooting of the immune response. My data demonstrate that these effects of adenosine on macrophages are mediated via A_{2A} receptors, which upregulate DUSP1, a negative regulator of LPS signaling in a cAMP-dependent manner. Biomolecules released by engulfing macrophages can orchestrate various biological processes depending on the biological context, where the engulfment of apoptotic cells occurs and my data contribute to our understanding how they act.

7. PUBLICATIONS, CONFERENCES

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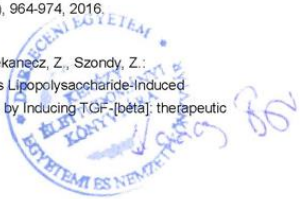
Candidate: Beáta Kiss
Neptun ID: E0C2ZZ
Doctoral School: Doctoral School of Dental Sciences

List of publications related to the dissertation

1. Köröskényi, K., **Kiss, B.**, Szondy, Z.: Adenosine A2A receptor signaling attenuates LPS-induced pro-inflammatory cytokine formation of mouse macrophages by inducing the expression of DUSP1.
   Biochim. Biophys. Acta-Mol. Cell Res. 1863 (7), 1461-1471, 2016.
   DOI: http://dx.doi.org/10.1016/j.bbamcr.2016.04.003
   IF: 5.128 (2015)
2. **Kiss, B.**, Tóth, K. Á., Sarang, Z., Garabuczi, É., Szondy, Z.: Retinoids induce Nur77-dependent apoptosis in mouse thymocytes.
   Biochim. Biophys. Acta-Mol. Cell Res. 1853 (3), 660-670, 2015.
   DOI: http://dx.doi.org/10.1016/j.bbamcr.2014.12.035
   IF: 5.128

List of other publications

3. Sándor, K., Dániel, B., **Kiss, B.**, Kovács, F., Szondy, Z.: Transcriptional control of transglutaminase 2 expression in mouse apoptotic thymocytes.
   Biochim. Biophys. Acta, Gene Reg. Mechan. 1859 (8), 964-974, 2016.
   IF: 5.373 (2015)
4. 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.
   DOI: http://dx.doi.org/10.4049/jimmunol.1501573
   IF: 4.985 (2015)



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5. Sarang, Z., Garabuczi, É., Joós, G., **Kiss, B.**, Tóth, K. Á., Rühl, R., Szondy, Z.: Macrophages engulfing apoptotic thymocytes produce retinoids to promote selection, differentiation, removal and replacement of double positive thymocytes.
Immunobiology. 218 (11), 1354-1360, 2013.
DOI: http://dx.doi.org/10.1016/j.imbio.2013.06.009
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6. Garabuczi, É., **Kiss, B.**, Felszeghy, S. B., Tsay, G. J., Fésüs, L., Szondy, Z.: Retinoids produced by macrophages engulfing apoptotic cells contribute to the appearance of transglutaminase 2 in apoptotic thymocytes.
Amino Acids. 44 (1), 235-244, 2013.
DOI: http://dx.doi.org/10.1007/s00726-011-1119-4
IF: 3.653

7. Szondy, Z., Garabuczi, É., Tóth, K. Á., **Kiss, B.**, Köröskényi, K.: Thymocyte death by neglect: contribution of engulfing macrophages.
Eur. J. Immunol. 42 (7), 1662-1667, 2012.
DOI: http://dx.doi.org/10.1002/eji.201142338
IF: 4.97

Total IF of journals (all publications): 32,417
Total IF of journals (publications related to the dissertation): 10,256

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

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CONFERENCE PRESENTATIONS RELATED TO THE THESIS

ORAL PRESENTATIONS:

Beáta Kiss. **Retinoids induce a Nur77-dependent apoptosis in mouse thymocytes.** 8th Molecular Cell and Immune Biology Winter Symposium, 8-10 January, 2015, Debrecen, Hungary.

Beáta Kiss: **The role of Nur77 in retinoid-induced apoptosis of mouse thymocytes.** Collaboration with Prof. Gregory J. Tsay, Chung Shan Medical University, 1 April, 2013, Taichung, Taiwan.

Beáta Kiss**: Retinoid-induced apoptosis in mouse thymocytes**. 6th Molecular Cell and Immune Biology Winter Symposium, January 8-11, 2013, Galyatető, Hungary.

Beáta Kiss: **Retinoids induce a Nur77-dependent apoptosis in mouse thymocytes**. 5th Molecular Cell and Immune Biology Winter School, 4-7 January, 2012, Galyatető, Hungary

Beáta Kiss: **Regulation of retinoid induced apoptosis of thymocytes.** 4th Molecular Cell and Immune Biology Winter School, 11-14 January, 2011, Galyatető, Hungary.

POSTERS:

Beáta Kiss, Katalin Tóth, Zsolt Sarang, Éva Garabuczi, László Fésüs, Zsuzsa Szondy: **Retinoids induce a Nur77-dependent apoptosis in mouse thymocytes.** 3rd Defense-net (Véd-Elem) Scientific meeting, 13 June, 2014, Debrecen, Hungary.

Beáta Kiss, Katalin Tóth, Zsolt Sarang, Éva Garabuczi, László Fésüs, Zsuzsa Szondy: **Retinoids induce a Nur77-dependent apoptosis in mouse thymocytes.** 21st ECDO Euroconference on Apoptosis, 25-28 September, 2013, Paris, France.

Beáta Kiss, Katalin Tóth, Zsolt Sarang, Éva Garabuczi, László Fésüs, Zsuzsa Szondy: **Retinoids induce a Nur77-dependent apoptosis in mouse thymocytes.** FEBS 3+ Meeting, 13-16 June, 2012, Opatija, Croatia.

Beáta Kiss, Katalin Tóth, Zsolt Sarang, László Fésüs, Zsuzsa Szondy: **Regulation of retinoid induced apoptosis of thymocytes.** 18th Euroconference on Apoptosis, 1-4 September, 2010, Ghent, Belgium.

Beáta Kiss, Katalin Tóth, Zsolt Sarang, László Fésüs, Zsuzsa Szondy: **Regulation of retinoid induced apoptosis of thymocytes.** Annual Meeting of Hungarian Biochemical Society, 23-26 August 2010, Budapest, Hungary.

Beáta Kiss, Katalin Tóth, Zsolt Sarang, László Fésüs, Zsuzsa Szondy: **Retinoids induce apoptosis in mouse thymocytes via inducing Nur77 expression.** MAC09' EMBO Workshop on Mitochondria, Apoptosis, Cancer, 1-3 October, 2009, Prague, Czech Republic.

Beáta Kiss, Katalin Tóth, Zsolt Sarang, László Fésüs, Zsuzsa Szondy: **Retinoids induce apoptosis in mouse thymocytes via inducing Nur77 expression.** 15th International Summer School on Immunology, 5-12 September, 2009, Hvar, Croatia.