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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TABLE OF CONTENT

LIST OF ABBREVIATIONS

9cRA	9-cis retinoic acid
AMP/ADP/ATP	Adenosine mono-/di-/triphosphate
AR	Adenosine receptor
ATRA	All-trans retinoic acid
Bad	BCL-2 antagonist of cell death
Bak	Bcl-2 antagonist/killer
Bax	Bcl-2‑associated X protein
Bcl-2	B‑cell lymphoma‑2
BH	Bcl-2 homology
Bid	BH3-interacting domain death agonist
Bik	BCL-2-interacting killer
Bim	BCL-2-interacting mediator of cell death
BMDM	Bone marrow-derived macrophage
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CD	Cluster of differentiation
CREB	Cyclic AMP response element binding protein
Cyclo	Cyclophilin
Cyt c	Cytochrome c
DD	Death domain
DED	Death effector domain
DISC	Death-inducing signaling complex
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DP	Double positive
DR	Death receptor
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
Epac	Exchange protein directly activated by cAMP
ERK	Extracellular signal-regulated kinase
FADD	Fas-associated death domain
FasL	Fas ligand
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FLIP	FADD-like IL-1β-converting enzyme)-inhibitory protein
Gpr65	G protein-coupled receptor 65
HPGRT	Hypoxanthine-guanine phosphoribosyltransferase
IFN	Interferon
IKK	IκB kinase
IL	Interleukin

Iono	Ionomycin
IκB	Inhibitor of kappa B
JNK	c-Jun N-terminal kinase
KC	Keratinocyte chemoattractant
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MIP-1/2	Macrophage inflammatory protein-1/2
MKK	Mitogen-activated protein kinase kinase
MOMP	Mitochondrial outer membrane permeabilization
MyD88	Myeloid differentiation primary response gene 88
NDG-1	Nur77 dependent gene-1 coded protein
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
Nor-1	Neuron-derived orphan receptor
NR	Nuclear receptor
PAMPs	Pathogen–associated molecular patterns
PBS	Phosphate-buffered saline
PdBu	Phorbol dibutyrate
PE	Phycoerythrin
PI	Propidium iodide
PKA	Protein kinase A
pMKK	Phosphorylated-mitogen-activated protein kinase kinase
Puma	p53-upregulated modulator of
RAR	Retinoic acid receptor
RPMI medium	Roswell Park Memorial Institute medium
RXR	Retinoid X receptor
STAT	Signal Transducer and Activator of Transcription
TAK	TGF-β-activated kinase
TCR	T cell receptor
TG2	Tissue transglutaminase 2
TGF-β	Transforming growth factor beta
TIR	Toll/interleukin 1 receptor
TLR	Toll–like receptor
TNF	Tumor necrosis factor-alpha
TNFR1	Tumor necrosis factor receptor 1
TRADD	TNFR1-associated death domain protein
TRAF	TNF receptor associated factor
TRAIL	TNF-related apoptosis-inducing ligand receptor
TRIF	TIR domain-containing adaptor inducing IFN-β

1. INTRODUCTION

1.1. APOPTOSIS

1.1.1. Apoptosis in general

Apoptosis is a 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 to integrate multiple physiological as well as pathological death signals (Elmore, 2007; Hassan et. al., 2014).

The study of apoptosis was initially based on the identification of various specific morphological changes occurring in dying cells by light or electron microscopy. Early during the initiation of apoptosis, cells lose contact with neighbouring cells and they round up. Apoptotic cells reorganize their cytoskeleton conferring plasticity to the whole cell promoting volume decrease as well as cell shrinkage. The cells start to show protrusions of the plasma membrane commonly referred to as blebs. The most characteristic morphological hallmarks of apoptosis in the nucleus are chromatin condensation and nuclear fragmentation. The condensation starts peripherally along the nuclear membrane, forming a crescent or ringlike structure. During later stages of apoptosis the nucleus further condenses, and finally it breaks up inside the cell with an intact cell membrane (karyorrhexis). Mitochondria and other cell organelles are largely unaffected until the late phase of apoptosis, when they swell (Kerr et al., 1972; Häcker, 2000; Elmore, 2007). Finally the blebs separate, forming apoptotic bodies which are crowded with cellular organelles and nuclear fragments. The fine structures, including membranes and mitochondria, are well retained inside the bodies (Ziegler and Groscurth, 2004). Apoptotic bodies are removed by neighbouring cells or tissue phagocytes therefore apoptosis is not associated with inflammation (Savill and Fadok, 2000; Elmore, 2007).

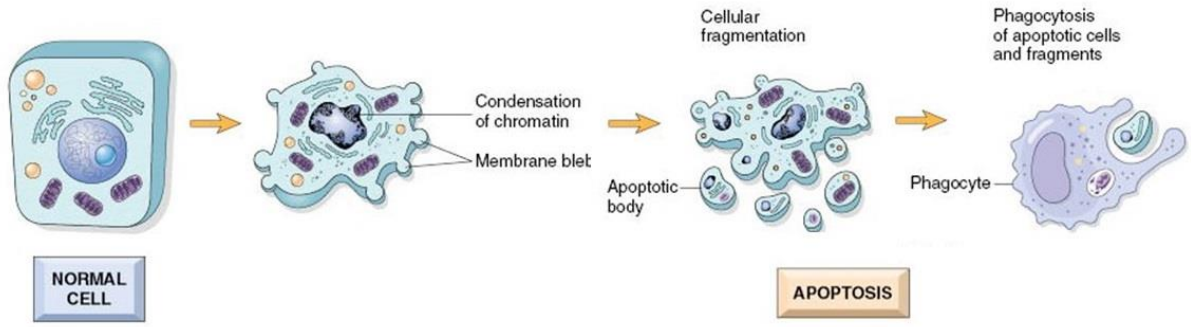

Figure 1. The major stages of apoptosis

(Adapted from www.mednotez.com/2015/09/chapter-1-cellular-responses-to-stress.html).

1.1.2. 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. Both pathways culminate in the activation of caspases, a subset of the cysteine-dependent aspartate-specific protease family and the cleavage of intracellular proteins, ultimately leading to the dismantling of the cell. Caspases are expressed as inactive zymogenes and once they are activated, initiate a proteolytic cascade (Nicholson, 1999). Apoptotic caspases fall into two major categories depending on their mode of action: initiator caspases (e.g., caspase 8, 9, and 10) that are activated on binding to specialized molecular platforms and executioner, or effector caspases (e.g., caspase 3 and 7), which can be cleaved and activated by the active initiator ones (Shi, 2002). These latter proteins can cleave hundreds of cytoskeletal proteins, DNA repair enzymes, proteins involved in cell cycle regulation or signaling pathways and can also activate DNases (Green and Llambi 2015).

1.1.2.1. Mitochondria in apoptosis: The role of Bcl-2 family

The mitochondrial or intrinsic pathway is activated in response to a variety of cellular stresses, including DNA damage, growth factor deprivation, and endoplasmatic reticulum stress. Mitochondrial outer membrane permeabilization (MOMP) is the pivotal event in the intrinsic apoptotic pathway allowing the release of pro-apoptotic factors into the cytosol (Chipuk et al., 2006). Since MOMP is an irreversible commitment point to cell death, it must

be strictly regulated. One important set of proteins involved in the regulation is the B‑cell lymphoma‑2 (Bcl-2) family (Cory and Adams, 2002).

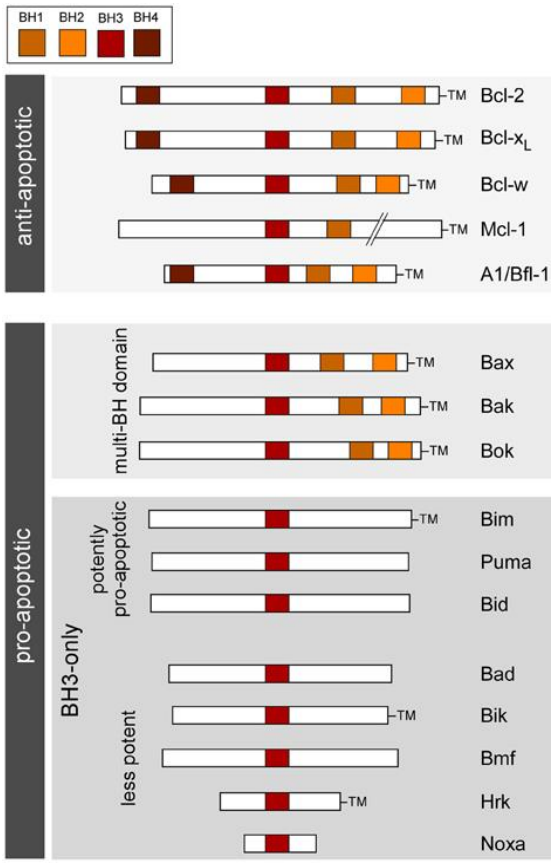

Figure 2.Bcl-2 family; organization, functions, and characteristics.
(Modified from Henshall and Engel, 2013)

The Bcl-2 family of proteins consists of three groups based on their structures and intracellular functions. The **multidomain pro-apoptotic** Bcl-2 family proteins include Bcl-2 antagonist/killer (Bak) and Bcl-2‑associated X protein (Bax). They contain 3 Bcl-2 homology (BH) domains 1–3 and can directly permeabilize outer mitochondrial membrane once activated. Bcl-2-related ovarian killer (Bok) belongs to this same subfamily as well. **Anti-apoptotic** or pro-survival family members include Bcl-2, Bcl‑X large (Bcl-x_L), Bcl-2-like protein 2 (Bcl-w), Bcl-2-like protein 10 (Bcl-b), myeloid cell leukaemia 1 (Mcl-1), and Bcl-2-related protein A1 (Bfl-1). These proteins possess strong sequence homology in four BH domains and inhibit apoptosis by binding and sequestering their pro-apoptotic counterparts (Cory and Adams, 2002; Youle and Strasser, 2008). The **BH3-only** subset of pro-apoptotic molecules, including Bad (Bcl-2 antagonist of cell death), Bid (BH3-interactingdomain death agonist), Bim (Bcl-2-interacting mediator of cell death) Noxa, Bik (Bcl-2-interacting killer), Hrk (Harakiri), Bmf (Bcl-2-modifying factor) and Puma (p53-upregulated modulator of apoptosis), are characterized by the presence of only one single BH3 domain. This BH3 domain, however, is critical for the interactions of these proteins with other members of the Bcl-2 family proteins to regulate MOMP (Lomonosova and Chinnadurai, 2008).

Studies using the pro-apoptotic Bax and Bak double-knockout cells showed that these cells fail to undergo MOMP and apoptosis in response to many different intrinsic death

stimuli, suggesting that Bax and Bak are necessary and responsible for MOMP and apoptosis as well (Wei et al., 2001). Activation of Bax and Bak during apoptosis involves multiple conformational rearrangements. In healthy cells, Bax is mainly localized as monomer in the cytosol or in a minor fraction of cells it loosely attaches to mitochondrial outer membrane (Goping et al., 1998). In the presence of apoptotic stimuli Bax undergoes conformational changes resulting in mitochondrial targeting, insertion into the outer membrane and oligomerization (Tait and Green, 2010).Unlike Bax, Bak monomers are integrated into the MOM before the induction of apoptosis and their oligomerization is inhibited by voltage-dependent anion channel 2 (Cheng et al., 2003). Bak activation leads to exposure of its BH3 domain and its insertion into the hydrophobic groove of another Bak molecule. Reciprocal BH3:groove interaction between two activated Bak monomers first produces symmetric dimers which then can be converted to the larger oligomers (Dewson et al., 2008). The intramembranous oligomerization of these proteins leads to the assembly of mitochondrial apoptosis-induced channels (MAC) in this membrane. However, the nature of these channels -whether proteinaceous versus lipidic pores- is still unclear. MAC formation correlates with release of pro-apoptotic factors from the intermembrane space (Martinez-Caballero et al., 2009; Tait and Green, 2010).

Anti-apoptotic Bcl-2 members express a characteristic hydrophobic pocket that acts as a receptor for the BH3 domain of effector or BH3-only proteins, allowing their sequestration and MOMP inhibition. Bax has high affinity for Bcl-2 and Bcl-x_L and other anti-apoptotic proteins, while the activation of Bak is mainly under the control of Mcl-1 and Bcl-x_L in healthy cells (Cheng et al., 2001; Willis et al., 2005; Llambi et al., 2011).

BH3-only molecules act as upstream sentinels of cellular damage and derangement and integrate various signals to initiate Bak/Bax activation and MOMP. BH3-only proteins can be classified into two groups: direct activators and sensitizers. Direct activators can directly interact with and induce conformational changes in Bax and Bak leading to their oligomerization and subsequent activation. Anti-apoptotic Bcl-2 proteins prevent MOMP by binding to and sequestering the activator BH3-only proteins. Another group of BH3-only proteins, called sensitizers only bind to the anti-apoptotic repertoire, thereby releasing both direct activator BH3-only proteins and Bak/Bax. (Letai et al., 2002; Chipuk et al., 2010). The first defined direct activators were Bim and truncated form of Bid (Wei et al., 2001). Later PUMA was also reported to act as a direct activator (Kim et al., 2009; Dai et al., 2014).

Moreover, Bid/Bim/Puma triple knockout mice displayed developmental abnormalities related to defective apoptosis, similar to Bak/Bax double knockouts (Ren et al., 2010). Noxa was originally described as a sensitizer BH3-only protein, but recent studies have confirmed that Noxa can directly interact with Bak and may has a role in Bak/Bax activation (Du et al., 2011; Vela et al., 2014; Chen et al., 2015). Bim, tBid and Puma BH3 domains have high affinity for all the five anti-apoptotic proteins, while Noxa specifically binds Mcl-1 and Bfl-1. Other BH3-only proteins, such as Bad, Bmf, Bik or Hrk act as sensitizers and lower the threshold for apoptosis by occupying anti-apoptotic Bcl-2 members thereby freeing apoptosis activator proteins from them to trigger Bak and Bax oligomerization. Bad and Bmf can bind to Bcl-2, Bcl-x_L, and Bcl-w, while Bik and Hrk has ability to interact with Bcl-xL, Bcl-w and Bfl-1 (Letai et al., 2002; Kim et al., 2006; Chipuk et al., 2010).

1.1.2.2. Regulation of mitochondrial pathway of apoptosis

The BH3-only proteins can be activated by many noxious stimuli and their expression level and activity can be regulated by both transcriptionally and posttranscriptionally. Activation of Noxa and Puma is under direct transcriptional regulation by p53 in response to DNA damage. Transcriptional activation of Bik by oestrogen starvation was also reported to depend on p53. Bim is transcriptionally upregulated by the forkhead transcription factor FOXO3A upon cytokine deprivation. It was shown that following growth factors withdrawal, reduced ubiquitylation and proteasomal degradation of Bim resulted in a rapid and sustained increase in the level of the protein. Bim activity is controlled by phosphorylation (Lomonosova and Chinnadurai, 2008). Bim is localized to the microtubule dynein motor complex and Bmf associates with actin filaments and dissociate in response to certain death stimuli (Chipuk et al., 2010; Green and Llambi 2015). Bad activity is inhibited through phosphorylation by several kinases in the presence of growth factors resulting cytoplasmic sequestration and inactivation. Unphosphorylated Bad is released and antagonizes anti-apoptotic Bcl-2 proteins (Zha et al., 1996). The pro-apoptotic function of the Bid BH3 domain is activated upon proteolytic cleavage by caspase 8 following death receptor ligation, therefore Bid coordinates the cross-regulation between the extrinsic and intrinsic apoptotic pathways (Luo et al., 1998).

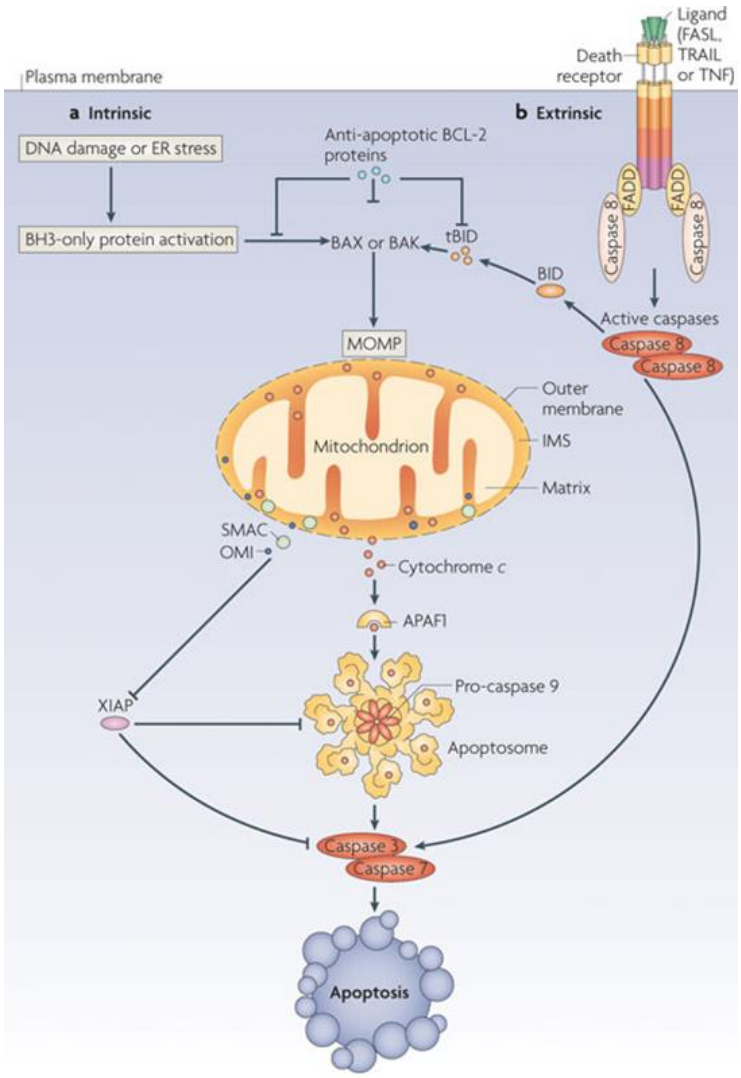

Figure 3.Extrinsic and intrinsic pathways of apoptosis (Tait and Green, 2010).

Disruption of the integrity of the mitochondrial outer membrane allows the entry of those pro-apoptotic factors into the cytosol that are normally sequestered in the intermembrane space of the mitochondria, including cytochrome c (Cyt c), Smac and Omi (Tait and Green, 2010). Once in the cytosol, Cyt c binds tightly to apoptotic protease activating factor 1 (Apaf-1) monomers leading to conformational changes. Complex of Apaf-1 and Cyt c then binds its critical cofactor, dATP or ATP, forming a heptameric wheel-like complex called the apoptosome, which then recruits and binds procaspase 9 via caspase-recruitment domain-mediated interaction (Yuan et al. 2010). The close proximity of the inactive caspase 9 monomers induces their activation and autoprocessing. Catalytically active caspase 9 stays in the holoenzyme to maintain its catalytic activity and cleaves and activates the executioner caspases 3 and 7 (Rodriguez and Lazebnik, 1999; Tait and Green, 2010). Smac (also known

as Diablo) and Omi (also known as HtrA2) potentiate the apoptosome activity by antagonizing the caspase inhibitor X-linked inhibitor of apoptosis (XIAP) protein which inhibits caspase activity by directly binding active caspases (Eckelman et al., 2006).

Following MOMP a second group of pro-apoptotic proteins are released from the intermembrane space (namely apoptosis-inducing factor (AIF) and endonuclease G (EndoG) and caspase-activated nuclease (CAD)) and translocated to the nucleus (Tait and Green, 2010; Mariño et al., 2014). AIF induces chromatin condensation and large-scale DNA fragmentation, EndoG causes oligonucleosomal DNA fragmentation via caspase-dependent and -independent manner (Arnoult et al., 2003). CAD, the main effector involved in oligonucleosomal DNA degradation, requires the caspase 3-specific cleavage of its inhibitor ICAD to be active (Sakahira et al., 1998).

1.1.2.3. Molecular mechanisms of death receptor-mediated apoptosis

The extrinsic apoptotic pathway that initiates apoptosis involves transmembrane receptor-mediated interactions. The cell surface death receptors (DRs) are a subset of the tumor necrosis factor (TNF) receptor superfamily, characterized by a ~80 amino acid cytoplasmic sequence termed the death domain (DD), which is essential for apoptosis. The most extensively studied death receptors are Fas (CD95/APO-1), TNF-receptor 1 (TNFR1/p55/CD120a), TNF-related apoptosis-inducing ligand receptor 1 (TRAILR1/D4), and receptor 2 (TRAILR2/DR5/APO-2/KILLER) (Ashkenazi and Dixit, 1998; Locksley et al., 2001).When these preassembled receptor trimers are triggered by corresponding trimeric ligands, a number of DD-containing adaptor molecules are recruited to the DD forming "death-inducing signaling complex" (DISC) (Chan et al., 2000). Death ligands also interact with decoy receptors (DcRs) which compete with specific death receptors for ligand binding but do not possess DDs and therefore cannot form signaling complexes (Ashkenazi and Dixit, 1999).

When Fas or a TRAILR is bound to its ligand, the DD of the receptor interacts with the DD of Fas-associated death domain (FADD). The interactions between the molecules at the DISC are based on homotypic contacts. FADD also contains additional protein-protein interaction modules, such as death effector domains (DEDs) that mediate the recruitment of procaspase 8, procaspase 10 and $FLIP_{L/S}$ through their association with a corresponding DED.

High local concentrations of the initiator caspases at the DISC lead to their autocatalytic cleavage resulting in formation of the active p18/p12 dimer which is released from the DISC. Finally, caspase 8 activates effector caspase 3 and 7 to drive execution of apoptosis (Lavrik et al., 2005; Dickens et al. 2012). Procaspase 10 is also activated at the DISC and active caspase 10 can serve as an initiator caspase in Fas signaling leading to Bid processing, caspase cascade activation, and apoptosis (Milhas et al., 2005). $FLIP_L$ and $FLIP_S$ inhibit activation of procaspase 8 at the DISC by blocking its processing. There is increasing evidence that $FLIP_L$ also facilitates the cleavage of procaspase 8 at the DISC by forming $FLIP_L$-procaspase 8 heterodimers (Lavrik et al., 2005).

In Type I cells, the level of DISC formation and caspase 8 activation is high enough to directly activate downstream effector caspases, while in other cell types (Type II cells) there is lower levels of DISC formation and, thus, caspase 8 interacts with the intrinsic apoptotic pathway by truncating BH3 only protein Bid, leading to the subsequent tBid-mediated release of cytochrome-c (Cyt c) from mitochondria (Barnhart et al., 2003; Ashkenazi and Salvesen, 2014; Green and Llambi, 2015).

TNFR1 stimulation is postulated to result in the formation of two signaling complexes (Micheau and Tschopp, 2003). Binding of TNF to TNFR1 induces receptor oligomerization and rapid formation of the TNFR1 signaling complex (Complex I), which is initiated by recruitment of the DD-containing adaptor proteins TRADD (TNFR-associated death domain protein) and Ser/Thr kinase RIP1 (receptor-interacting protein 1) (Ashkenazi and Dixit, 1998).This signaling complex is required for ubiquitin ligase TRAF2 (TNFR-associated factor 2) and c-IAP1 and possibly binding of other proteins. It is proposed to trigger the NF-κB signaling pathway through recruitment of the IKK complex and activates JNK through a TRAF2-dependent mechanism (Zhang et al., 2000; Micheau and Tschopp, 2003). Complex I is devoid of FADD and caspase 8. Within a short period of time TRADD, RIP1, and TRAF2 dissociate from TNFR1 and in the cytosol the DD of TRADD and RIP1 can associate via homotypic interaction with FADD, leading to the subsequent binding of procaspase 8/10 and $FLIP_{L/S}$ (traddosome or complex II) (Micheau and Tschopp, 2003). Activation of procaspase 8 takes place in the traddosome and is followed by activation of downstream death signaling. Inhibition of the pro-apoptotic activity of caspase 8 is more likely to occur through $FLIP_L$ and its availability and caspase 8 activation can determine the choice between survival and death (Kruegeret al., 2001; Micheau and Tschopp, 2003).

1.1.3. Role of apoptosis in T-lymphocyte development

Thymocytes, one of the first cell types to be characterized to undergo steady state cell death, offer valuable insight into the regulation of apoptosis and tissue homeostasis. During differentiation apoptosis ensures that only lymphocytes expressing functional antigen receptors survive. Apoptosis is essential for eliminating lymphocytes with dangerous self-reactive specificities. Impaired apoptosis induction appears to be critical for the aberrant survival of pathological cells in many chronic immune-mediated disorders, inflammation and cancer (Opferman, 2008).

The thymus is the specialized lymphoid organ responsible for the development of functional and diverse repertoire of T cells, and interactions with thymic stromal cells are thought to provide the necessary signals for thymocyte maturation. During differentiation thymocytes must go through several maturation stages based on the expression of cell surface markers (Figure 3.). Firstly, committed lymphoid progenitors from the bone marrow migrate to the thymus. These cells lack expression of T-cell receptor (TCR), CD4 and CD8, and are termed double-negative (DN) thymocytes (Hernandez et al., 2010). Then these cells rearrange their TCR-β gene locus to generate pre-TCR complex composed of a successfully rearranged β chain, a nonpolymorphic pre-α chain, and CD3. Successful pre-TCR expression leads to cell proliferation during the DN to $CD4^+CD8^+$ double positive (DP) transition and TCR-α gene rearrangements (Gameiro et al., 2010). DP cells that have appropriately rearranged their TCR α-chain, generate αβ-TCR and these αβ-$TCR^+CD4^+CD8^+$ (DP) thymocytes can interact with cortical epithelial cells that express a high density of major histocompatibility complex (MHC) class I and class II molecules associated with self-peptides.

Their fate will depend on the nature of the TCR-αβ signal. 90% of the DP thymocytes fail to generate a functional TCR and undergo a default death pathway named “death by neglect” (Szondy et al., 2012). Too intense signaling can promote activation- induced apoptosis (negative selection). Thymocytes bearing “useful” TCRs receive intermediate level of TCR signaling and undergo positive selection generating $CD4^+$ or $CD8^+$ TCR-$\alpha\beta^{high}$ single positive cells in the cortex. In the medulla they will be further exposed to self-ligands on medullary epithel cells or dendritic cells. After this second wave of negative selection the survivor cells exit the thymus and circulate in the periphery (Gameiro et al., 2010, Szondy et al., 2012).

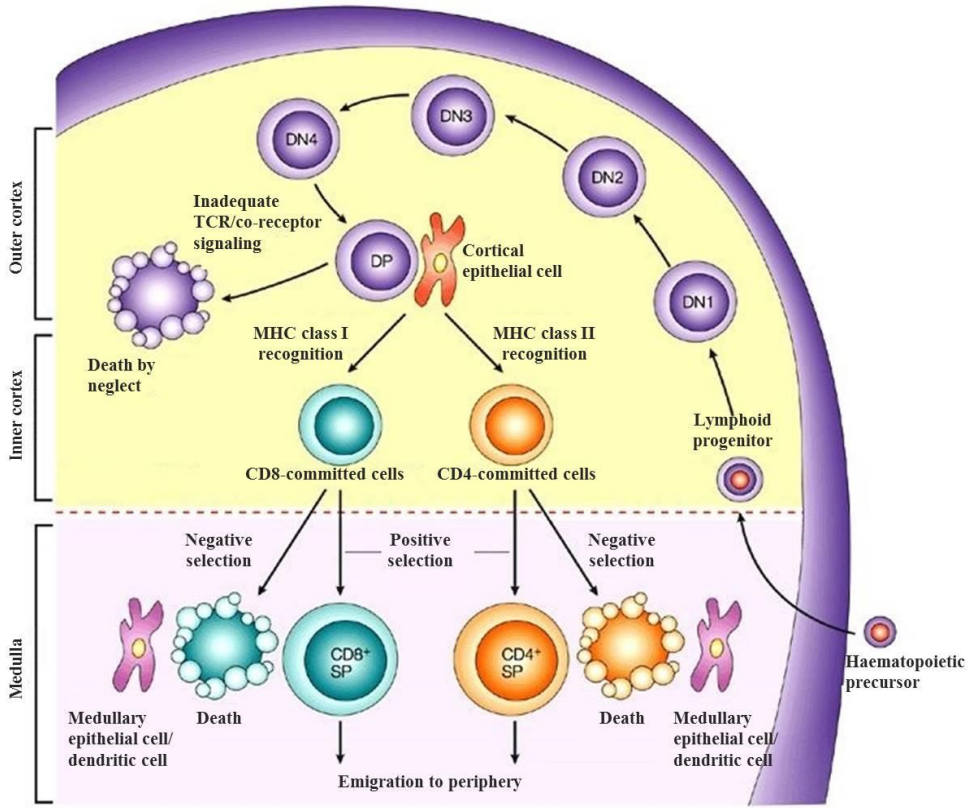

Figure 4.T-cell development in the thymus (Germain, 2002).

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 with self-peptides associated MHCI and II molecules present on epithelial cells. Lack of signaling results in "death by neglect". Too intense 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.

1.1.4. Pro-apoptotic factors involved in negative selection of thymocytes

Negative selection is primarily mediated through clonal deletion of self-reactive thymocytes, which is a TCR-induced program. Among the molecules induced by high-affinity TCR signaling, members of the orphan nuclear receptor NR4A1 family (Nur77 and Nor-1) and the Bcl-2 family member Bim have been associated with pro-apoptotic functions (Calnan et al., 1995;Bouillet et al., 2002;Palmer, 2003).

Nur77 (NGF1-B/NR4A1) is a transcription factor belonging to the steroid/thyroid hormone receptor superfamily. The Nur77 family includes three members: Nur77, neuron-

derived orphan receptor (Nor-1), and Nurr1. All of them are classified as orphan receptors, whose physiological ligands have not been identified (Ohkura et al., 1996; Winoto and Littman, 2002). They function as ligand-independent transcription factors and are immediate/early response genes, which are rapidly induced by a diverse extracellular stimulation (Maxwell and Muscat, 2006). Once their expression is induced, NR4A receptors activate gene expression either as monomers by binding to the NGFI-B-responsive element (NBRE) or as NR4A homodimers by targeting the palindromic NBRE-related sequence (Nur-responsive element, NurRE) (Philips et al 1997).Furthermore, Nur77 and Nurr1 (but not Nor-1) can heterodimerize with retinoid X receptor (RXR) and activate transcription by binding to DR-5 element containing promoters in an RXR ligand-dependent manner (Perlmann and Jansson, 1995; Maxwell and Muscat, 2006).

The role of Nur77 in T cell receptor-mediated cell death has been demonstrated, since in response to strong engagement of the TCR Nur77 was rapidly induced in T hybridoma cells, in thymocytes (negative selection) and in peripherial T-cells (Woronicz et al., 1994; Woronicz et al., 1995; Calnan et al., 1995; Zhou et al., 1996). In addition, Nur77 transcriptional activity correlates well with its apoptotic function (Kuang et al., 1999) Furthermore, transgenic mice expressing a dominant-negative version of Nur77, which blocks activity of all Nur77-related molecules, negative selection associated apoptosis of DP thymocytes is inhibited (Calnan et al., 1995; Zhou et al., 1996). Moreover, constitutive overexpression of full-length Nur77 in thymus results in enhanced cell death of thymocytes (Calnan et al., 1995; Cheng et al., 1997). These results suggest that negative selection depends on Nur77 function. Surprisingly, Nur77-deficient mice do not exhibit any defect in thymocyte negative selection (Lee et al., 1995; Cheng et al., 1997). This can be explained by the functional redundancy between Nur77 and Nor-1. Nor-1 has transcriptional transactivation activity similar to Nur77; it is rapidly induced in thymocytes in response to TCR signals, and thymocytes that express a Nor-1 transgene undergo unrestrained apoptosis (Cheng et al., 1997).

Microarray analysis using RNA isolated from Nur77 transgenic and wild type fetal thymi determined global targets of Nur77 transcription that mediate apoptosis. These studies identified several Nur77 downstream genes, including Fas ligand (FasL) and TRAIL, two known death receptor ligands, and Nur77 dependent gene 1 (NDG-1) that initiates the activation of caspase 8 by unknown mechanisms, as mediators of Nur77-dependent apoptosis (Rajpal et al., 2003).

Accumulating evidence demonstrates that translocation of Nur77 from the nucleus to mitochondria represents another mechanism through which Nur77 initiates apoptosis independently of its transcriptional activity. It has been published that in several cancer cells in response to different apoptotic stimuli and during negative selection Nur77 translocates from the nucleus and migrates to the mitochondria (Moll et al., 2006; Thompson and Winoto, 2008) where it interacts with the anti-apoptotic Bcl-2 protein. Binding to the N-terminal loop region between the BH4 and BH3 domain of Bcl-2 leads to a conformational change exposing the BH3 domain, which converts Bcl-2 from anti-apoptotic protein to pro-apoptotic one (Lin et al., 2004; Thompson and Winoto, 2008). Recent studies showed that phosphorylation regulates Nur77 subcellular trafficking (Thompson et al., 2010). Nur77 lacks classical nuclear export signal and requires heterodimerization with RXR which can serve as a carrier for nuclear export (Cao et al., 2004).

The second pathway that controls negative selection is dependent on the members of Bcl-2 family, in particular on pro-apoptotic Bim. It has been shown that Bim-deficient mice develop late onset autoimmune disease (Bouillet et al., 1999). Bim-deficiency results in defective thymocyte apoptosis in several TCR transgenic (HY, OTI, OTII) models of negative selection (Bouillet et al., 2002; Suen and Baldwin, 2012). However, this apoptotic defect does not lead to emergence of autoreactive mature T cells except in the case of negative selection to tissue-restricted antigens. In the absence of Bim large numbers of autoreactive T cells survive in the periphery (Suen and Baldwin, 2012). Additional loss of Puma, another BH3-only protein, however, enhanced the thymic deletion defect of Bim−/− mice and resulted in more severe, T cell-driven multi-organ autoimmune pathology (Gray et al., 2012). Beside the sequestration of pro-survival Bcl-2 family members that is necessary for Bax and/or Bak activation during thymocyte deletion, increasing evidence suggests that Bim can directly activate Bax and Bak via a ‘hit and run' mechanism (Kim et al. 2009).

It is possible that Nur77 and Bcl-2 family members function together at the mitochondria to cooperatively regulate cell death during negative selection.

1.2. PHAGOCYTOSIS OF APOPTOTIC CELLS AND THE RESOLUTION OF INFLAMMATION

1.2.1. Clearance of apoptotic cells

In vivo, the apoptosis program is completed by phagocytosis, with the majority of apoptotic thymocytes in mice being cleared by the thymic F4/80+ macrophages (Surh and Sprent, 1994). Not just in the thymus, but all over the body every day approximately one billion of our cells die via apoptosis, their corpses are quickly recognized and phagocytosed by engulfing cells and eliminated without initiating an inflammatory immune response (Savill and Fadok, 2000). The process of removal or clearance of apoptotic cells by phagocytes is called efferocytosis. Efferocytosis differs from classical phagocytosis in several respects including the use of distinct receptors, bridging proteins and intracellular signaling pathways.

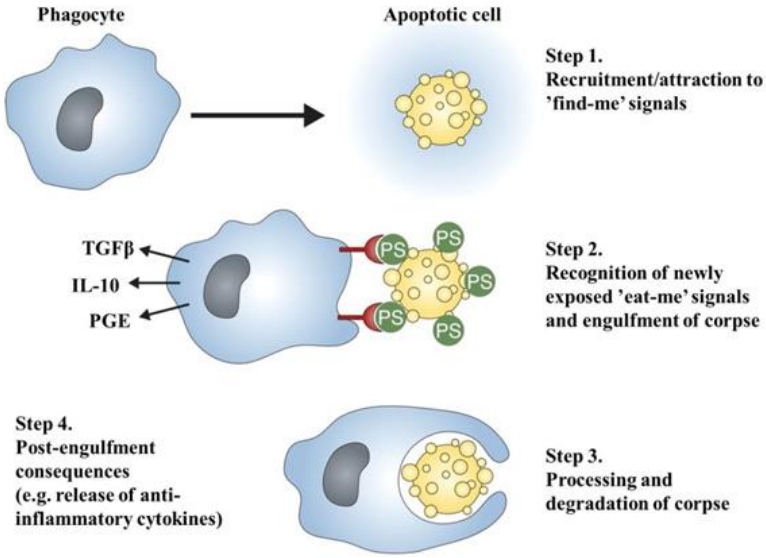

Figure 5. Different steps of efficient apoptotic cell clearance (Ravichandran, 2010).

Recruitment is mediated by 'find-me' signals released by apoptotic cells to attract phagocytes to the dying cells. The phagocytes, using engulfment receptors, engage 'eat-me' signals presented on apoptotic cells. Engagement of the engulfment receptors (linked to PtdSer recognition) has been shown to stimulate release of anti-inflammatory cytokines such as TGF-β, IL-10, and PGE2. The intracellular signaling induced within the phagocytes by the ligand–receptor interactions leads to cytoskeletal rearrangement and internalization of the dying cell. The phagocytes process the engulfed corpse through a series of steps.

The clearance of apoptotic cells is mediated by both professional engulfers (such as macrophages and dendritic cells) and by nonprofessional "neighbouring" cells (such as

epithelial cells, endothelial cells, and fibroblasts) (Elliott and Ravichandran 2010), but non-professional phagocytes show delayed kinetic of ingestion (Parnaik et al., 2000). Phagocytic clearance of apoptotic cells can be divided into four major steps (Figure 5).

At the early stage of apoptosis, dying cells secrete soluble chemoattractant factors, which are known as ‘find me’ signals to facilitate their removal by recruiting phagocytes to the sites of death within a tissue (Ravichandran, 2010). Up to now several chemotactic ‘find-me’ signals have been identified, including the lipid lysophosphatidylcholine (LPC) and sphingosine 1-phosphate (S1P), the fractalkine (CXC3CL1) and the nucleotides ATP and UTP (Lauber et al., 2003; Gude et al., 2008; Truman et al., 2008; Elliott et al., 2009).

Once in the proximity of their targets, macrophages recognize them by sensing so called ’eat-me’ signals exposed on apoptotic cell surfaces. The best-studied and universally detected ‘eat me’ signal is the exposure of phosphatidylserine (PtdSer), a membrane lipid that is kept in the inner leaflet of the plasma membrane in healthy cells (Fadok et al., 1998; Savill et al., 2002).

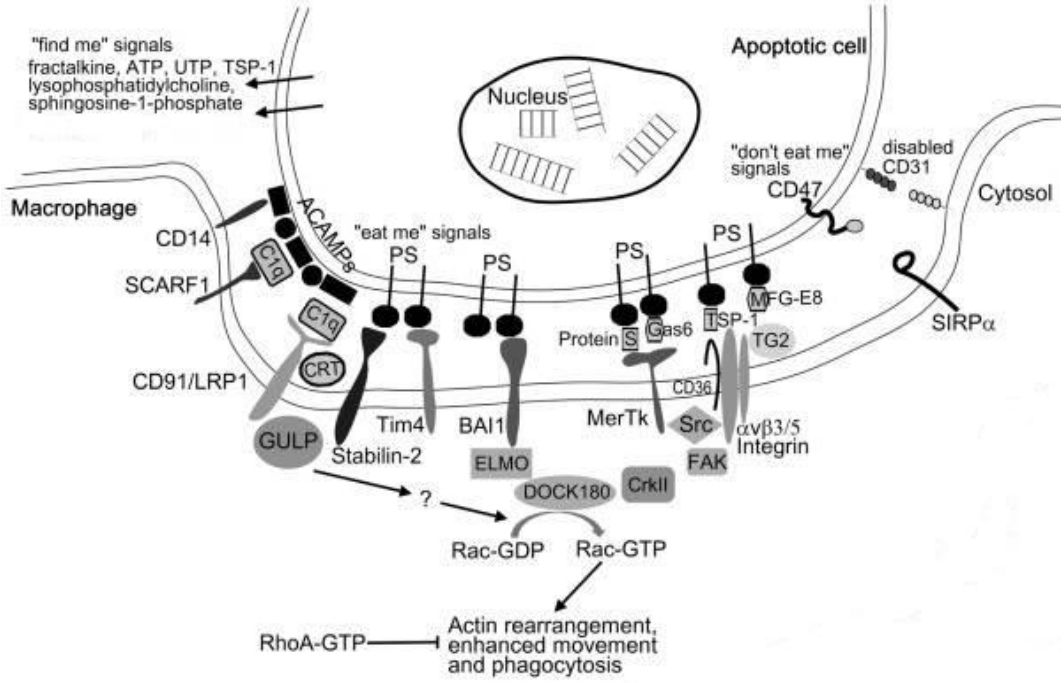

Figure 6. Apoptotic cell recognition (Modified from Szondy et al., 2014).

Phagocyte recognition of PtdSer is mediated in two ways: by direct recognition receptors and by soluble bridging molecules binding PtdSer on the apoptotic cell and a receptor on the

phagocyte, adding complexity to the process of dying cell recognition (Figure 6). Direct-binding PtdSer receptors include members of the T cell immunoglobulin and mucin (TIM) family (TIM-4, as well as TIM-1 and TIM-3) (Kobayashi et al., 2007; Nakayama et al., 2009), brain-specific angiogenesis inhibitor 1 (BAI1) (Park et al., 2007), and atypical epidermal growth factor (EGF)-motif containing stabilin-2 (Park et al., 2008).

In addition to these bona fide PtdSer receptors, bridging molecules, including milk fat globule-EGF factor 8 (MFG-E8), growth-arrest-specific 6 (GAS6) and Protein S, can bind PtdSer on the surface of apoptotic cells. MFG-E8 associates with αvβ3 or αvβ5 integrin on phagocytes via its RGD motif (Hanayama et al 2002) while Gas6 and protein S bridge PtdSer with Tyro-3-Axl-Mer family of receptors (TAM receptors) (Lemke and Rothlin, 2008; Nagata et al., 2010).

Additional ’eat-me’ signals have been also identified, which likely play tethering function e.g. altered intercellular-adhesion molecule (ICAM)-3, calreticulin, thrombospondin, oxidized low-density lipoprotein (LDL) moieties and glycosylated proteins on the surface of the apoptotic cells binding to CD14, CD91, αvβ3-integrins, scavenger receptors and lectins on phagocytes (Gregory et al., 1998; Gardai et al. 2005; Lauber et al., 2004; Hochreiter-Hufford and Ravichandran, 2013; Green et al., 2016).

Apart from the active display of ‘eat-me’ signals by apoptotic cells, viable cells express ‘don't eat-me’ signals, such as CD31 and CD47 that may need to be turned off during apoptosis to allow recognition by phagocytes (Brown et al., 2002;Gardai et al. 2005).
Therefore, exposure of a sufficient amount of ‘eat-me’ signals and loss of ‘don’t eat-me’ signals on the surface of apoptotic cells are necessary to trigger their removal by phagocytes.

Engagement of PtdSer receptors and other signaling during efferocytosis converges on the activation of evolutionarily conserved Rho family of small GTPases (RhoA, ROCK, Rac, Rab5, Cdc42) to promote actin polymerization and cytoskeletal rearrangement facilitating phagocytosis (Nakaya et al. 2006; Park et al., 2007; Hochreiter-Hufford and Ravichandran, 2013). Following internalization, the engulfed apoptotic corpses are degraded into their basic cellular components, including fats, sterols, peptides, amino acids and nucleotides within phagolysosomes (Kinchen and Ravichandran, 2008). Finally, processing of apoptotic cell proteins can be linked to establishment and maintenance of self-tolerance via cross-presentation of engulfed cell peptides by the major histocompatibility complex (MHC) class I molecules(Kinchen and Ravichandran, 2008, Hochreiter-Hufford and Ravichandran, 2013).

1.2.2. Anti-inflammatory effect of apoptotic cell clearance

Apart from the physical removal of dying cells the second homeostatic function of the clearance process is the production of anti-inflammatory signaling molecules and cytokines by phagocytes to strongly suppress inflammation and to facilitate the "immunologically silent" clearance of apoptotic cells (Gregory and Pound, 2011).

Some of the inhibitory actions are immediate, and induced by macrophage surface PtdSer-receptors. But after ingestion of apoptotic corpse, the macrophages start also releasing anti-inflammatory mediators like interleukin-10 (IL-10), transforming growth factor-β (TGF-β), prostaglandin E2 (PGE2), platelet-activating factor (PAF) in a PtdSer-dependent manner (Voll et al., 1997; Fadok et al., 1998; McDonald et al., 1999; Cvetanovic and Ucker 2004). This immunosuppressive response also exists *in vivo* (Huynh et al., 2002).

Il-10 and TGF-β production is regulated at the level of transcription in a p38 mitogen-activated protein kinase (MAPK)-dependent manner, and furthermore TGF-β is controlled translationally via activation of Rho GTPase, PI3K, mTOR, and eIF4E (Chung et al., 2007; Xiao et al., 2008).

In addition, during engulfment of apoptotic thymocytes macrophages produce **adenosine** through their cell surface 5'nucleosidase (Sándor et al., 2016) from the adenine nucleotides released by the apoptotic cells (Yamaguchi et al., 2014). Adenosine acts on macrophages as a soluble mediator, via adenosine A2A receptors, to suppress pro-inflammatory cytokine production, such as KC and MIP-2 chemokines (Köröskényi et al., 2011).

Our laboratory have also shown that following *in vivo* induction of apoptosis in the thymus similar to TGF-β and adenosine, **retinoids** are also produced by macrophages engulfing apoptotic thymocytes in a phagocytosis-dependent manner (Garabuczi et al., 2013). The lipid content of the apoptotic cells mediate retinoid production via the lipid-sensing receptor liver X receptor (LXR), which in response induces the retinaldehyde dehydrogenases, enzymes responsible for retinoid synthesis. These retinoids subsequently enhance the phagocytic capacity of macrophages by upregulating the expression of various phagocytic receptors via retinoic acid receptor signaling (Sarang et al., 2014).

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 (which by definition is vitamin A) (Blomhoff and Blomhoff, 2006). Vitamin A from food provides the retinoids necessary for a variety of biological functions including embryonic development, differentiation, vision, development of central nervous system, and apoptosis (Blomhoff and Blomhoff, 2006; Hall et al., 2011). Vitamin A deficiency is associated with defects in adaptive immunity. Countless studies have clearly demonstrated that its deficiency can cause broad immune alterations including decreased humoral and cellular responses, inadequate immune regulation, weak response to vaccines and poor lymphoid organ development (Smith et al., 1987; Bono et al., 2016). 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, conversion of retinol to retinal, is carried out by members of alcohol dehydrogenase (ADH) family. ADHs are specific to ethanol, retinoids, and other alcohols and aldehydes of physiological importance (Duester et al., 1999). Oxidation of retinol to retinal can also be catalysed by members of the short-chain dehydrogenase/reductase family of microsomal enzyme, which show a wide affinity for alcohols and aldehydes (Parés et al., 2008). More recent evidence has demonstrated that under normal physiological conditions these are the physiologically relevant enzymes in the conversion of retinol to retinal (Farjo et al., 2011). In a second reaction, retinal is irreversibly converted to retinoic acid (RA) by one of the three retinal dehydrogenase isoforms RALDH1, RALDH2 and RALDH3, which form a subfamily of class I aldehyde dehydrogenases. Each gene is expressed in a different tissue-specific pattern. RALDH2 is responsible for the production of retinoic acid in several cell types during embryonic development. RALDH1 is expressed at very high levels in the eye of embryos and in several adult tissues. RALDH3 has a more limited role during development (Kedishvili, 2013). RALDH4 was shown to recognize 9-cis-retinaldehyde but not all-trans-retinaldehyde as substrate (Lin et al., 2003). One more critical element in RA biosynthesis is the degradation of ATRA by CYP26 enzymes, which has been very well established in developmental models to disrupt action of RA by promoting RA catabolism (Ribes et al., 2007; Kedishvili, 2013).

To regulate gene expression, ATRA and 9cRA bind to their nuclear receptors, which act as ligand-induced transcription factors to bind to specific sequences in the DNA and modulate the transcription of target genes. These receptors are the retinoic acid receptors (RAR) and retinoid X receptors (RXR) that belong to the family of steroid/thyroid hormone receptors and each of these families comprises three isotypes (α, β and γ) with additional isoforms generated by alternative splicing and differential promoter usage (Germain et al., 2006a and 2006b). *In vitro* binding studies have demonstrated that ATRA and 9cRA are high affinity ligands for RARs, whereas only 9cRA binds with high affinity to RXR (Heyman et al., 1992; Soprano et al. 2004). The physiological role of 9cRA has been questioned, since it has not yet been detected *in vivo* (Rühl, 2006). However, our group has shown that in the thymus non-conventional dihydroretinoic acid derivatives are formed in an "apopto-phagocytosis-dependent" manner (Sarang et al., 2014). In addition, Rühl et al. have proved that 9-cis-13,14-dihydroretinoic acid acts as an endogenous and physiologically relevant RXR ligand in mammals (Rühl et a., 2015).

The nuclear RARs function as heterodimers with RXRs and act as ligand-dependent transcriptional regulators, binding to specific DNA sequences called RAR elements (RAREs) within the promoter of retinoid target genes. RAR agonists can activate transcription through such heterodimers, while RXRs cannot respond to RXR–selective agonist without RAR ligand (Soprano et al. 2004; Germain et al., 2006b). RXRs also can function as homodimer 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 as I mentioned above (Zhanget al., 1992; Germain et al., 2006a).

1.4. ADENOSINE

1.4.1. Adenosine metabolism and adenosine receptor signaling

Adenosine, a purine nucleoside generated by the dephosphorylation of adenine nucleotides is considered potent endogenous physiologic and pharmacologic regulator in peripheral and central nervous system and of non-neuronal cells, including immune and inflammatory cells.

Adenosine is accumulating in the locality of damaged cells in conditions of metabolic stress such as hypoxia, ischemia, tissue injury or inflammation (Thiel et al., 2003). It can also

be generated by the extracellular metabolism of adenine nucleotides. Physiological actions of adenosine are exclusively depending on its receptor ligation and the downstream receptor signaling, so those processes which determine its concentration (production, release, cellular uptake and metabolism), must be tightly regulated (Haskó and Cronstein, 2004).

Intracellular adenosine is produced from its immediate precursor, 5′-adenosine monophosphate (5′-AMP), by the action of the enzyme 5′-nucleotidase or can be derived from metabolism of S-adenosyl homocysteine (Latini and Pedat, 2001), and once it reaches high concentration it is transported into the extracellular space by specific bidirectional nucleoside transporters (Yegutkin, 2008).

Adenosine is formed extracellularly by the metabolization of released nucleotides (ATP, ADP, AMP). ATP and ADP are hydrolysed to AMP via CD39 and AMP, in turn, is rapidly converted to adenosine by CD73 ecto-5'-nucleotidase (Robson et al., 2006; Volmer et al., 2006). Another possible extracellular source of adenosine is the released cyclic adenosine monophosphate (cAMP), which can be converted to 5′-AMP by ecto-phosphodiesterase, and then to adenosine by ecto-5′-nucleotidase (Yegutkin, 2008; Latini and Pedata, 2001).

To terminate adenosine signaling, the activity and expression of adenosine deaminase is increased during inflammation and hypoxia which promotes adenosine deamination to inosine within seconds (Eltzschiget al., 2006). Although adenosine deaminase has largely been considered to be a cytosolic enzyme, there have been several reports of an extracellularly located form in a variety of tissues (Latini and Pedata, 2001; Hashikawa et al., 2004). Adenosine can also be converted back to 5′-AMP by the action of the enzyme adenosine kinase and subsequently to ADP and ATP (Lloyd and Fredholm, 1995).

Finally, after adenosine generation and receptor activation, adenosine is rapidly taken up by cells from the extracellular space mainly through equilibrate nucleoside transporters. As a result of the rapid uptake and metabolism of adenosine, levels of this mediator are maintained low in unstressed, healthy tissues. However, under pathological conditions adenosine removal cannot keep pace with its generation, resulting in markedly increased extracellular adenosine concentrations (Latini and Pedata, 2001; Helenius et al., 2012).

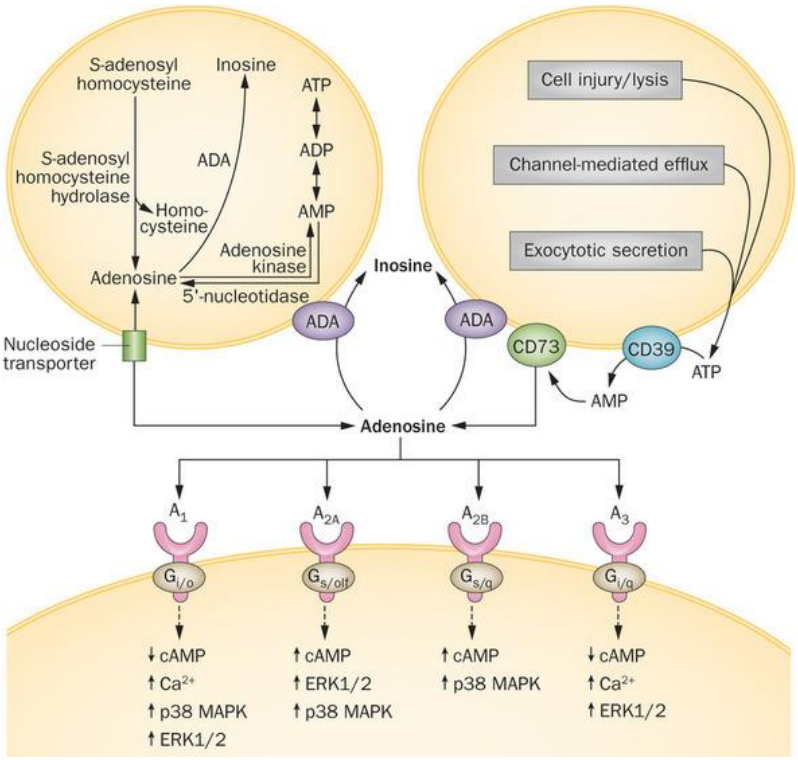

Figure 7.Adenosine metabolism and adenosine receptor signaling (Antonioli et al., 2015).

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_1, A_{2A}, A_{2B} and A_3Rs (Fredholm et al., 2011), all of them expressed on macrophages. The A_1R and A_{2A}R are capable to bind adenosine with high affinity while the A_3R and especially A_{2B}R show relatively lower affinity for adenosine (Fredholm et al., 2011). The A_1 and A_3Rs preferentially interact with members of the $G_{i/o}$ family of G proteins, decreasing adenylate cyclase activity and thereby lowering intracellular level of cAMP, protein kinase A (PKA) activity and cAMP response element binding protein (CREB) phosphorylation. A_{2A}R and A_{2B}R are coupled to $G_{s/olf}$ proteins, elevating cAMP production, resulting in activation of (PKA) and phosphorylation of CREB. A_{2B} receptors can act via both $G_{\alpha S}$ and G_q proteins(Fredholm et al., 2011, Haskó and Cronstein, 2013).

Receptor	Adenosin A1 receptor	Adenosin A2A receptor	Adenosin A2B receptor	Adenosin A3 receptor
Previous/alternative names	ADORA1; R_i; AA1R; A1R	A_{2a}, R_s; AA2AR; A2AR	A_{2b}, R_s; AA2BR;A2BR	AA3R; A3R
Affinity to adenosine	High	High	Low	Low
Selective agonists	CPA, CCPA, CHA; S-ENBA	CGS 21680, HE-NECA, CV-1808, CV-1674, ATL146e	None readily available. (LUF 5853 very potent and selective)	2-Cl-IB-MECA
Selective antagonists	DPCPX; 8-cyclopentyltheophylline, WRC0571	SCH 58261 ZM241385 (KF 17387, CSC)	MRS1754, (enprofylline)	MRS 1220, MRE 3008-F20, MRS 1191; MRS 1523; VUF 8504
G-protein coupling	G_i, G_o	G_s, G_{olf}	G_s, G_q	G_i
Some recognized physiological function(s)	Bradycardia; inhibition of lipolysis; reduced glomerular filtration; reduction of sympathetic and parasympathetic activity; presynaptic inhibition; neuronal hyperpolarization; ischemic preconditioning	Inhibition of platelet aggregation and polymorphonuclear leukocytes; vasodilatation, protection against ischemic damage; stimulation of sensory nerve activity	Relaxation of smooth muscle in vasculature and intestine; inhibition of monocyte and macrophage function; stimulation of mast cell mediator release (some species)	Enhancement of mediator release from mast cells; preconditioning (some species)
KO phenotype	Anxiety; hyperalgesia; decreased tolerance to hypoxia; altered insulin secretion; increased lipolysis; increased susceptibility to seizures; loss of preconditioning in several tissues	Anxiety; hypoalgesia; hypertension; increased tolerance to ischemia; decreased platelet aggregation	Hyperinflammation; vascular adhesion	Altered inflammatory reactions; decreased edema; altered release of inflammatory mediators

Table 1. Characterization of adenosine receptors
(Haskó and Cronstein, 2004; Fredholm, 2007)

1.4.2. The immunoregulatory effects of adenosine

Once adenosine concentration is increased in the extracellular environment, it binds to different types of adenosine receptors expressed on many cells of immune system (neutrophils, macrophages, dendritic cells and natural killer cells). Thus, depending on the ligation of different adenosine receptors, adenosine affects innate immune response during various inflammatory conditions i.e. chronic (cancer, asthma) as well as acute (sepsis, acute lung injury) inflammatory diseases (Sachdeva and Gupta, 2013).

For example 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, phagocytosis, and neutrophil death (McColl et al 2006; Cronstein et al., 1990; Barletta et al., 2012; Haskó and Cronstein, 2013). Acting through A_{2A} receptors, it can inhibit the adhesion of neutrophils to the endothelium by inhibiting integrin-mediated adhesive events (Sullivan et al., 2004). By contrast, adenosine at low concentrations, via A_1 receptors, promotes neutrophil adhesion to different adhesive molecules on the endothelium

(Cronstein et al., 1992). Furthermore adenosine promotes directed migration of neutrophils via A_1 and A_3 receptors, however A_{2A}R limits neutrophil recruitment (Cronstein et al., 1990; Chen et al., 2006).

Adenosine has been shown to be a broad inhibitor of the pro-inflammatory consequences of classical **macrophage** activation, acting via A_{2A}R (Haskó and Pacher, 2012). 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 (Haskó et al., 1996; Bouma et al. 1994; Haskó et al., 2000; Bshesh et al., 2002). Based on experiments using selective A_{2A} and A_{2B} receptor ligands and knock out macrophages it is clear now that A_{2B} receptors can also contribute to the suppressive effect of adenosine on TNF-α production (Kreckler et al 2006). Furthermore, adenosine has been shown to decrease the level of other pro-inflammatory mediators, such as MIP-1α, nitric oxide and superoxides (Szabó et al., 1998; Haskó et al., 1996; Si et al., 1997, Haskó and Pacher, 2012). In addition to this suppressor effect, adenosine can augment the formation of anti-inflammatory cytokine IL-10 (Haskó et al., 1996) via CCAAT-enhancer-binding protein β-dependent transcriptional process in response to A_{2A}R ligation and by A_{2B}R-mediated posttranscriptional mechanisms (Csóka et al., 2007; Németh et al., 2005). It has been shown that A_{2A} receptor ligation switches classically activated (inflammatory) macrophages into an alternatively activated M2 phenotype, suppressing the expression of inflammatory cytokines and inducing the production of anti-inflammatory and angiogenic factors, including IL-10 and vascular endothelial growth factor (VEGF) (Ferrante et al., 2013).

In immature **dendritic cells** adenosine promotes chemotaxis to the site of inflammation and injury via A_1 and A_3 receptors (Panther et al., 2001). However, at these sites adenosine activates A_{2A} receptors on mature dendritic cells shifting pro-inflammatory cytokine profile to an anti-inflammatory one, with reduced IL-12, IL-6, interferon-α (IFN-α) and augmented IL-10 production (Panther et al., 2001; Panther et al., 2003).

The liver damage after ischemia and reperfusion initiated by IFN-γ producing of **Natural Killer T (NKT) cells** can be eliminated by ligation of A_{2A} receptors. Furthermore, the release of IFN-γ by NKT cells was abolished by A_{2A}receptor stimulation (Lappas et al., 2006).

Apoptosis and phagocytosis of the apoptotic cells occur not only under physiological conditions, but also during inflammation in response to microbial invaders and tissue injury. Initially, neutrophils rapidly migrate to the inflamed tissue followed by monocytes, which differentiate locally into macrophages. During the normal progression of inflammation neutrophils undergo apoptosis after performing their action and macrophages ingest them. Clearance of apoptotic neutrophils prompts a switch from a pro- to an anti-inflammatory macrophage phenotype (Ortega-Gómez et al., 2013, Martin et al., 2015). TGF-β, adenosine and retinoids are produced by engulfing macrophages also under inflammatory conditions and these molecules strongly interfere with the bacterial LPS induced inflammation.

These factors released from macrophages have an anti-inflammatory effect on macrophages themselves, but in addition, they can promote immunosuppressive responses acting on other neighbouring cells. For example TGF-β plays a crucial role in the formation of regulatory T cells (Treg), a specialized lineage of CD4+ T cells that are critical players in maintaining immune system homeostasis by promoting self-tolerance and restraining excessive immune responses (Curotto de Lafaille and Lafaille, 2009). TGF-β, concomitant with TCR stimulation, is an essential factor for the induction of Foxp3 gene expression, a special transcription factor in these cells and for Treg-cell generation in the thymus (Konkel et al., 2014) and extrathymically as well (Chen et al., 2003). The presence of the pro-inflammatory cytokines leads to instability of FoxP3but recent studies have documented that in inflammatory environment ATRA prevents human Treg conversion to T effector cells and sustains their Foxp3 expression and suppressive function *in vitro* or *in vivo* (Lu et al., 2014; Liu et al., 2015).

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 recognized by soluble proteins in the blood (components of the complement system) and by membrane-bound, germline-encoded pattern-recognition receptors (PRRs) most notably on macrophages and dendritic cells. These receptors sense a wild range of evolutionarily conserved structures on pathogens (bacteria, viruses, fungi and protozoa), termed pathogen-associated molecular patterns (PAMPs) (Akira et al., 2006).

The family of Toll-like receptors (TLR) is the most extensively studied class of PRRs. Upon recognition of the PAMPs, TLRs trigger intracellular signaling cascades leading to production of a variety of pro-inflammatory mediators, which orchestrate the early host response to infection (Akira et al., 2006; Newton and Dixit, 2012). TLRs can be divided into subfamilies primarily recognizing related PAMPs, in my work I focused on TLR4 mediated downstream signaling events. TLR4 has the ability to recognize several PAMPs, including fusion (F) protein from respiratory syncytial virus and endogenous molecules, such as heat-shock proteins (Akira et al., 2006; Lu et al., 2008), but one of the most important immunostimulatory molecule of TLR4 is LPS, a structural component of the outer membrane of Gram-negative bacteria (Poltorak et al., 1998).

1.5.1. 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 form 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 (Miyake, 2007;Gioannini and Weiss, 2007). 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. There are five TIR domain-containing adaptor proteins: the firstly described MyD88 (myeloid differentiation primary response gene 88), TIRAP (TIR

domain-containing adaptor protein/Mal, MyD88-adapter-like), TRIF (TIR domain-containing adaptor inducing IFN-β), TRAM (TRIF-related adaptor molecule), and TRIF inhibitor, SARM (sterile α and HEAT-Armadillo motifs-containing protein). TLR4 is the only known TLR which applies all these adaptors (Miyake, 2007; Lu et al., 2008).

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.

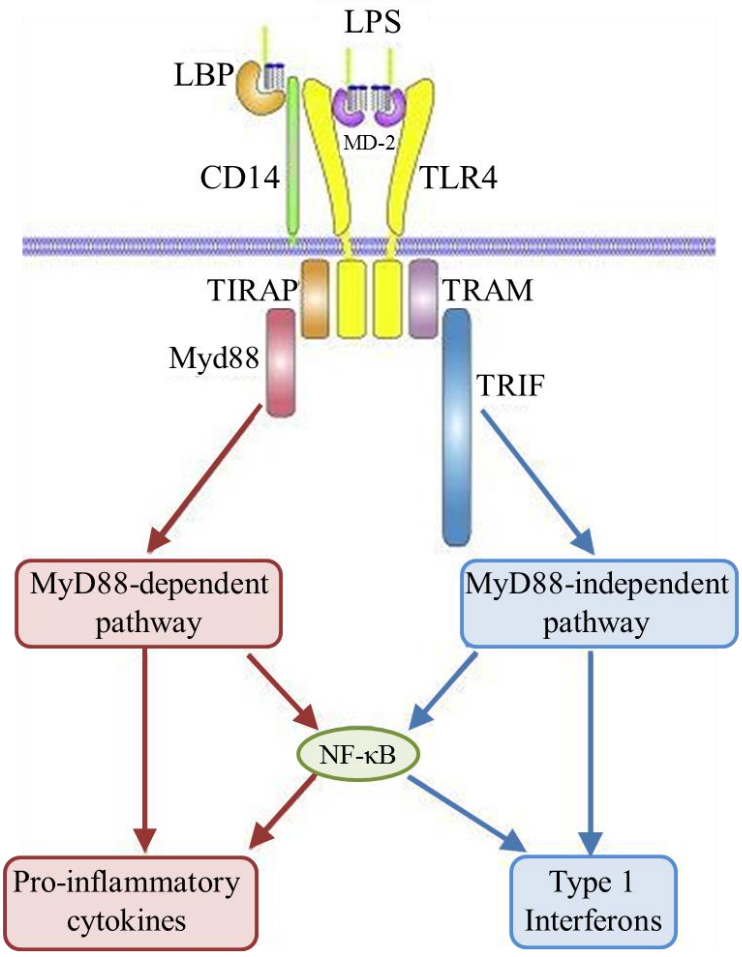

Figure 8.Overview of LPS/TLR4 signaling (Lu et al., 2008).

LBP and CD14 facilitate the LPS recognition and transfer to TLR4/MD-2 receptor complex. LPS/TRL4 signaling can be divided into Myd88-dependent and -independent pathways, which initiate the activation of pro-inflammatory cytokine and type I interferon genes.

1.5.1.1. The MyD88-dependent pathway

Following ligand binding, TLR4 dimerizes and undergoes conformational changes required for the recruitment of cytosolic TIR domain-containing downstream adaptor molecule, MyD88 (Medzhitov et al., 1998). Through death domain mediated homotypic interactions MyD88 recruits IL-1 receptor -associated kinase 4 (IRAK4) and its substrate IRAK2 or the related IRAK1 kinases in a hierarchic manner and a so-called myddosome is assembled (Motshwene et al., 2009; Lin et al., 2010). IRAK-2 plays a more central role than IRAK-1 in TLR signaling to NF-κB (Nuclear factor kappa-light-chain-enhancer of activated B cells) (Keating et al., 2007). IRAK1 and IRAK2 undergo phosphorylation, and interact with TRAF6 via their C-terminal TRAF-binding domains (Pauls et al., 2013). TRAF6 acts as an ubiquitin protein ligase (E3) and together with the ubiquitin-conjugating enzyme complex (E2), catalyzes the synthesis of K63-linked polyubiquitin chains on TRAF6 itself and other substrates, including TGF-β-activated kinase 1 (TAK1) and NF-κB essential modifier (NEMO). The polyubiquitin chains of TRAF6 can interact with the TAK1 binding protein (TAB)2 and TAB3, which induce a conformational change leading to the autophosphorylation and activation of TAK1 (Wang et al., 2001; Xia et al., 2009). TAK1 then stimulates two distinct pathways involving the IKK complex and the mitogen-activated protein kinase (MAPK) pathway, respectively.

In the first pathway, TAK 1 initiates the activation of IκB kinase (IKK) complex, which is composed of two catalytic subunits (IKKα and IKKβ) and an essential regulatory subunit, IKKγ/NEMO, resulting in site-specific phosphorylation of inhibitory IκB proteins (Rothwarf and Karin, 1999; Wang et al., 2001; Viatour et al., 2005). In unstimulated cells, NF-κB is sequestered in the cytoplasm through association with IκB family members (Ghosh et al., 1998). Once activated, IKKβ phosphorylates IκB proteins leading to proteasomal degradation. This liberates NF-κB to translocate into the nucleus and activate NF-κB-dependent gene expression by binding to κB sites in their promoter and enhancer regions (Rothwarf and Karin, 1999; Häcker and Karin, 2006).

In the second pathway, TAK1 phosphorylates members of the MAPK kinase (MKK) family, MKK3 and MKK6, which in turn phosphorylate the stress-activated protein kinase p38 (p38α, p38β, p38γ, p38δ) (Chang and Karin, 2001). The p38 signaling has been shown to regulate gene expression through phosphorylation of transcription factor CREB by mitogen-

and stress-activated protein kinase (MSK) 1/2, downstream kinase target of p38. MAP kinase-activated protein kinase 2 (MK2) is a selective substrate of p38α and it was shown to activate various substrates including small heat shock protein 27 (Hsp27), CREB and activating transcription factor ATF1. The activation of the p38 pathway plays essential role in the production of pro-inflammatory cytokines, including TNFα, IL-1β, IL10, IL12, IL-6 (Caivano and Cohen, 2000; Zarubin and Han, 2005; Newton and Dixit, 2012).

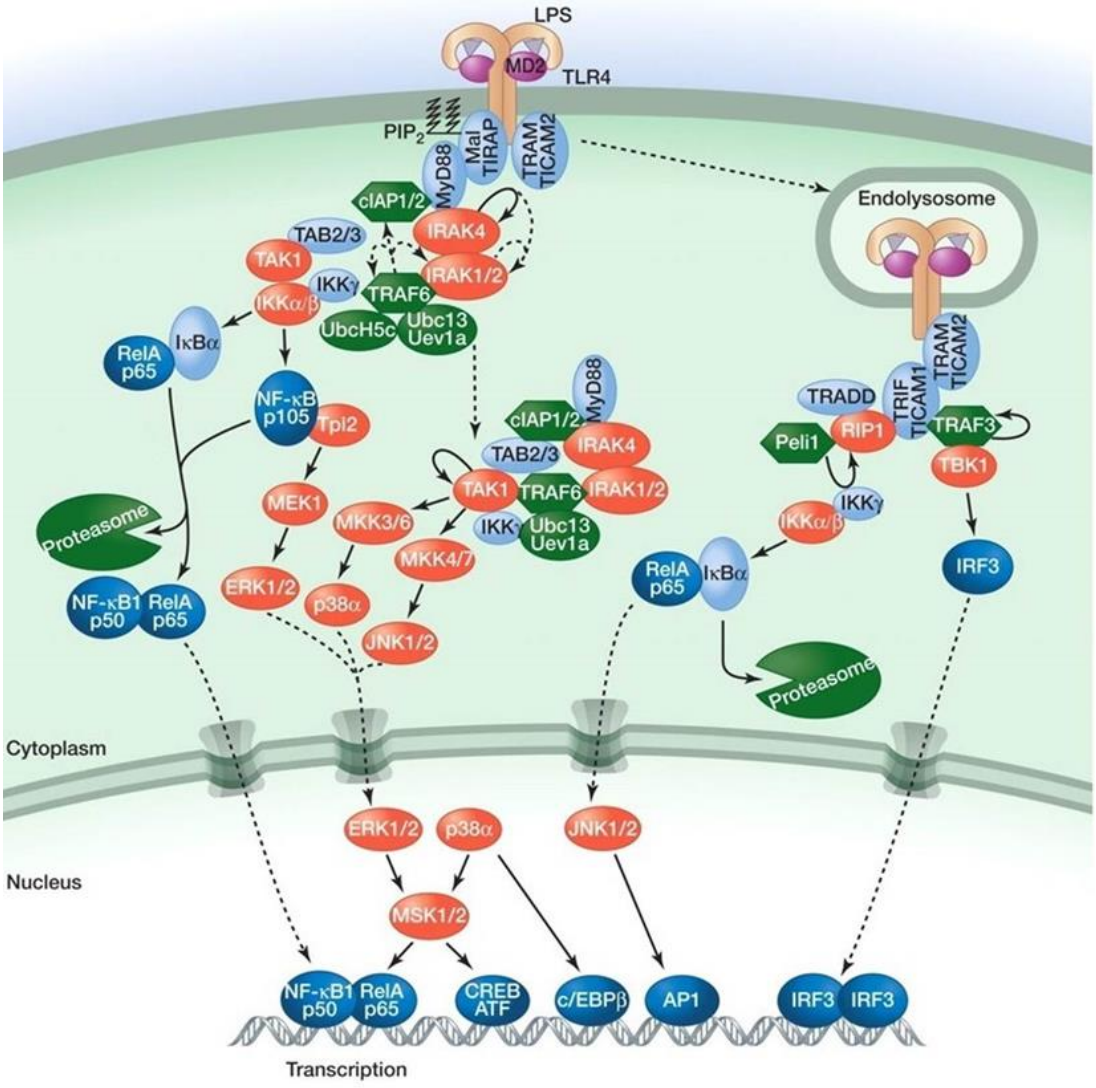

Figure 9.TLR4-activated MyD88-dependent and MyD88-independent pathways (modified from Newton and Dixit, 2012).

The MyD88-dependent pathway is responsible for early-phase NF-κB and MAPK activation, which controls the induction of pro-inflammatory cytokines. The MyD88-independent pathway activates IRF3, 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.

Upon activation, TAK1 can transmit the signals further by phosphorylating other members of MAPK kinases, namely MKK4 and MKK7 (Wang et al., 2007). In turn, these kinases activate the three c-Jun N-terminal kinases (JNK1/2/3) by direct phosphorylation. One of the best-known transcription factors regulated by JNKs, is c-Jun, which interacts with other transcription factors such as c-Fos and ATF to form "activator protein 1" (AP-1). JNK pathway is involved in the regulation of IL-2 and TNFα production (Chang and Karin, 2001; Newton and Dixit, 2012).

Moreover, IKKβ is also required for transcriptional responses downstream from the MAPK extracellular signal-regulated kinase (ERK). IKKβ is involved in the phosphorylation and degradation of the NF-κB1/p105 which complexes with tumor progression locus 2 (Tpl2) (Waterfield et al., 2004). The released Tpl2 activates ERK1/2 through a MEK1 pathway, leading to the binding of the transcriptional factor CREB to the regulatory sequences of its target genes (Waterfield et al., 2003; Newton and Dixit, 2012).

1.5.1.2. The MyD88-independent pathway

Kawai's group has revealed that Myd88-deficient macrophages failed to produce any inflammatory cytokines in response to LPS, but type I interferon production was not impaired. Notably, LPS-induced activation of NF-κB and MAPK was observed, but with delayed kinetics (Kawai et al., 1999; Kawai et al., 2001). These findings suggested a Myd88-independent TLR4 signaling pathway, in which TRIF was identified as an important adaptor in association with TRAM (Yamamoto et al., 2003). TRIF can activate the NF-κB pathway by recruiting receptor-interacting protein 1 (RIP1). RIP1 is polyubiquitinated to form a complex with TRAF6 (Chang et al., 2009) and these two molecules appear to cooperate in the recruitment of TAK1 thereby activate IKKβ and eventually NF-κB via this TRIF/TRAF6/RIP1/TAK1-TAB1-TAB2 complex (Festjens et al., 2007).

TRIF also induces type I IFN production by interacting with TRAF3, which can associate further with TRAF family member-associated NF-κB activator (TANK) and two noncanonical IKKs, TANK-binding kinase 1 (TBK1) and IKKε (Guo and Cheng, 2007). The latter two are responsible for the direct phosphorylation of interferon regulatory factor IRF3 and IRF7, which together with NF-κB and AP-1 induces the transcription of IFN-β gene (Sharma et al., 2003; Falvo et al., 2000).

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 (Sarang et al., 2014). Previous studies in our and other laboratories have shown that retinoids are also produced in the thymus *in vivo* (Kiss et al., 2008; Sitnik et al., 2012; Garabuczi et al., 2013) and they initiate the death of immature thymocytes (Szondy et al., 1997). Furthermore, we have found that retinoids induce a transcription-dependent apoptosis in these cell type via activating RARγ (Szondy et al., 1997).

- Since the action of retinoids to induce apoptosis was not investigated so far, in the first part of my thesis we aimed to identify the mechanisms 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 (Cohen et al., 2013) and increase the expression of A_{2A} receptors via NF-κB (Murphree et al., 2005). 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 regulatory mechanism.

- According to these results we aimed 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

3.1. Reagents

All reagents were obtained from (Sigma-Aldrich, Budapest) except indicated otherwise.

3.2. 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 (Lee et al., 1995). In a few experiments thymocytes were derived from STAT1-/- mice (Jackson Laboratories Durbin et al., 1996), 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 (Ledent et al., 1997). 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).

3.3. 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 (Gibco), 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 37^{o}C in a humidified incubator under an atmosphere of 5% CO_2 /95% air.

3.4. Determination of the percentage of apoptotic thymocytes

Thymocytes were cultured in 24 well plates in the presence of the indicated concentrations of retinoids for 12 hours and 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 75μM z-LEHD-fmk, a caspase 9 specific inhibitor (Calbiochem) for 1 hour. Percentage of the degraded DNA containing apoptotic cells were determined by a rapid technique using propidium iodide DNA staining. For staining, cells were fixed with 70% ice cold ethanol for 5 min, then washed and dissolved in 100 μl PBS containing 100μg/ml RNase and incubated for 10 min at room temperature. Finally 400μl PBS containing 50 μg/ml propidium iodide was added. % of cells carrying decreased amount of DNA due to apoptosis (sub G_0-G1 cells) was determined on DNA histograms by flow cytometry.

3.5. 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 labelled with anti-mouse $A_{2A}R$ antibody (BD Pharmingen) 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.

3.6. 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 (BD Pharmingen), anti-Bid (R&D Systems), anti-Bim, anti-STAT1 (Santa Cruz Biotechnology), anti-mitogen-activated kinase kinase (MKK) 3/4, pMKK3/4/6 (Cell Signaling) anti-IκB (Santa CruzBiotechnology), anti-phospho (Thr183/Tyr185)-SAPK/JNK (Cell Signaling) antibodies overnight at 4˚C. To detect antibody signals peroxidase-labelled 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, antibodies.

3.7. 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.

3.8. qRT-PCR measurement

After various treatments total RNA was extracted from thymocytes or macrophages with TRI reagent. Transcript quantitation was accomplished via quantitative real-time reverse transcriptase quantitative polymerase chain reaction (qRT-PCR) 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.

3.9. 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 re-probed with antibodies against mitochondrial

Hsp60 as loading control or the nuclear Lamin B (Santa Cruz Biotechnology) to check accidental nuclear contamination.

3.10. 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 FACS Calibur flow cytometer (BD Biosciences). Data was analyzed using WinMDI 2.9 software.

3.11. 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 TNFα cytokine was measured from the supernatant culture medium using DuoSet Mouse TNFα ELISA Kit (R&D Systems).

3.12. Determination of cytokine production

5×10^5 wild type and A2AR 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}Rspecific 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, TNFα 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.

3.13. Determination of NF-κB p50/p65 nuclear translocation

10^7wild 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 $A_{2A}R$-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). 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.

3.14. Determination of NFκB-dependent transcription

NF-κB-dependent transcription was evaluated as luciferase activity derived from the expression of an NF-κB-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.

3.15. 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 analysed 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.

3.16. 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.

3.17. 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.

3.18. 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

4.1.1. 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 (Szondy et al., 1997), 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 (Tóth et al., 2004). To figure out whether retinoids could also induce Nur77 in mouse thymocytes, thymocytes derived from 4 week old mice were exposed to ATRA and 9cRA, and Nur77 expression was determined on both mRNA (Fig. 10A) and protein levels (Fig.10B). As seen in Figure 10, 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.

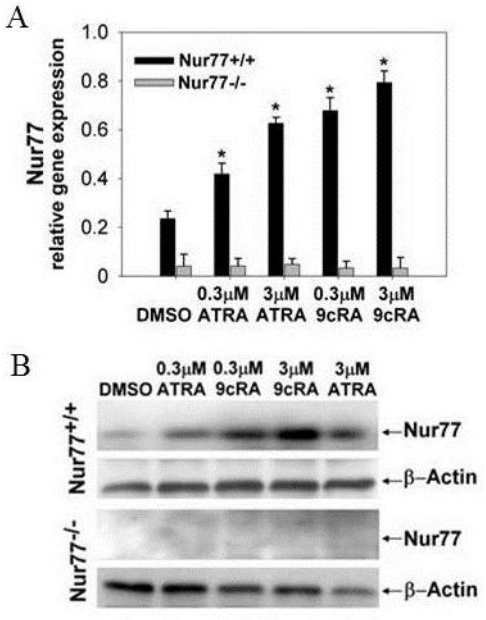

Figure 10. Retinoid can induce the Nur77 transcription factor in mouse thymocytes.

*(A) Retinoids induce the mRNA expression of Nur77 in mouse thymocytes. Thymocytes from wild type or Nur77 -/- mice were treated with the indicated concentrations of ATRA or 9cRA dissolved in DMSO. The final concentration of DMSO was 0.5% in each assay. Nur77gene expression was measured 4 h later by qRT-PCR analysis using cyclophilin as reference gene. (B) Retinoids induce the expression of Nur77 at protein level in mouse thymocytes. Wild type or Nur77 null thymocytes were exposed to the indicated concentrations of ATRA or 9cRA. Protein expression was determined 4 h later by Western blot analysis. β-actin was used for loading control. Data represent mean ± S.D. of 3independent experiments. *Significantly different from the DMSO-treated control ($p < 0.05$).*

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. As shown in Figure11A, retinoids induced cell death in the wild type thymocytes in a dose-dependent manner. 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, (Fig. 11B and C). Our observation is in line with previously published data (Lee et al., 1995).

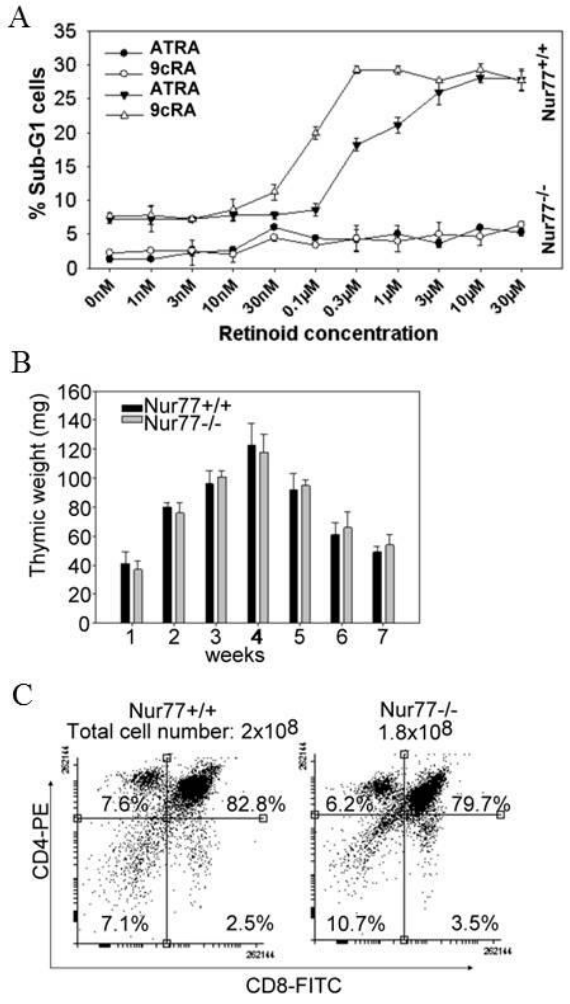

Figure 11. Nur77 is required for retinoid-induced cell death in thymocytes.

(A) Retinoids fail to induce apoptosis in Nur77-null thymocytes. Thymocytes isolated from 4 weeks old wild type mice and their Nur77 null littermates were exposed to the indicated concentrations of ATRA and 9cRA. The percentage of cells in sub-G1 population was determined in propidium iodide stained samples 12 h later. (B) Loss of Nur77 does not affect the age-dependent changes in the thymic weight in mice. (C) Unaltered total thymocyte cell number and double positive thymocyte population size in the thymus of four week old Nur77 null mice. Data represent mean ± S.D. of three independent experiments.

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

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 (Table 2). 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 (Table 2). Interestingly most of these genes were induced in a Nur77-dependent manner.

Gene description	Symbol	Nur77+/+ Fold change	Nur77-/- Fold change
Nuclear receptor subfamily 4, group A, member 1	Nur77	3.1	
Nur77 downstream gene 1	NDG-1	33.3	− 2.7
BH3 interacting domain death agonist	Bid	6.5	− 1.9
G-protein coupled receptor 65	Gpr65	3.7	− 1.9
Signal transducer and activator of transcription 1	STAT1	2.2	− 1.1
Transglutaminase 2	**TG2**	**4.8**	**4.8**
Protein tyrosine phosphatase, non-receptor type 6	Ptpn6	2.2	1.0
Myeloid differentiation primary response gene 116	**Myd116**	**2.4**	**2.4**

Table 2. 9-cis retinoic acid regulates the expression of Nur77 and several Nur77-dependent and independent apoptosis-related genes in mouse thymocytes.

Wild type and Nur77-/- thymocytes were exposed to 0.3 μM 9cRA for 4 h and the changes in their mRNA expression levels were analyzed by Affymetrix 430Av2 arrays. Data represent fold changes as compared to the DMSO-treated controls based on two independent experiments. Bold letters highlight those genes the expression of which is independent of Nur77.

NDG-1 was previously described as a Nur77-dependent gene and protein that regulates caspase 8 activity by unknown mechanisms (Rajpal et al., 2003). Surprisingly, we have found two 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 (Malone et al., 2004), and the BH3 interacting domain death agonist (Bid), which is activated by caspase 8 cleavage and initiates the mitochondrial pathway of apoptosis (Green and Llambi 2015).

To confirm 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 (Rajpal et al., 2003), 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 induction.

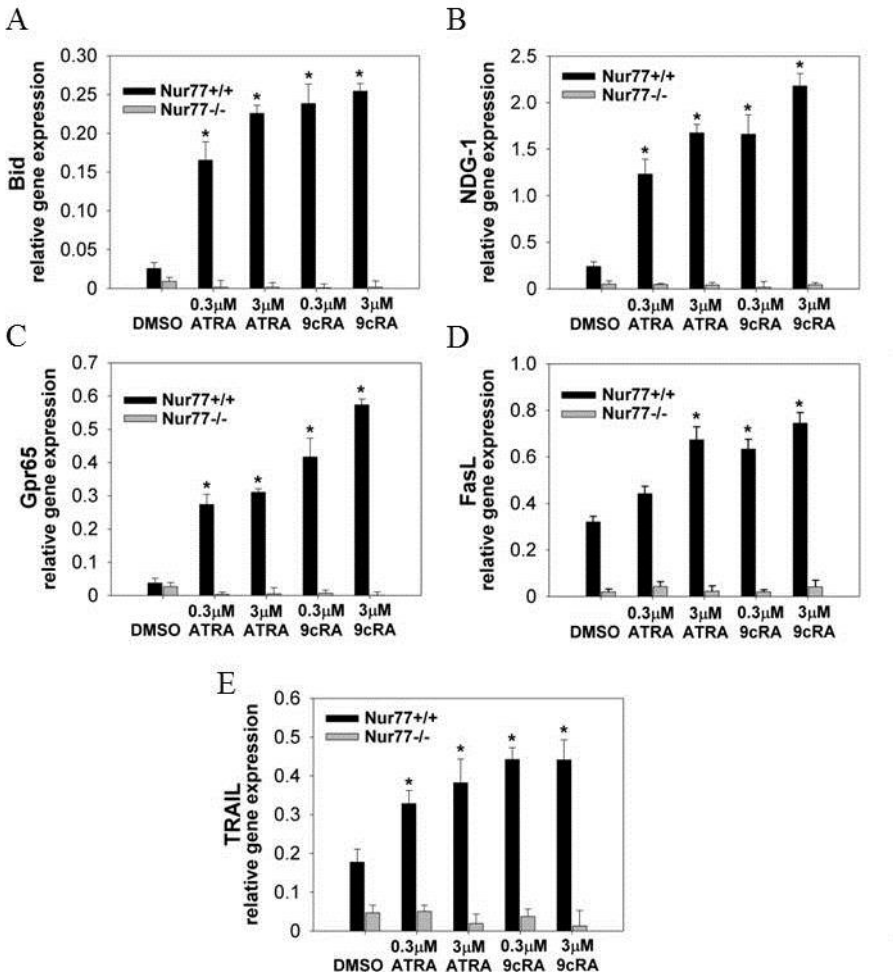

Figure 12. Retinoids induce the expression of FasL, NDG-1, TRAIL, Grp65 and Bid in a Nur77-dependent manner.

*Effect of retinoids on the mRNA expression of (A) Bid, (B) NDG-1, (C) Gpr65, (D) FasL and (E) TRAIL in wild type and Nur77 null thymocytes. Thymocytes were exposed to the indicated concentrations of retinoids and mRNA expressions were determined 4 h later by qRT-PCR analysis using cyclophilin as a reference gene. Data represent mean ± S.D. of four independent experiments. *Significantly different from the DMSO-treated control ($p < 0.05$).*

4.1.3. Caspase 8 activation contributes to the initiation of retinoid-induced apoptosis

Retinoids induced the expression of TRAIL and FasL, two cell death receptor ligands, and NDG-1, which is similar to death receptors and can also activate caspase 8 though by an unknown mechanisms (Rajpal et al., 2003). 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 in Figure 13A, cleaved caspase 8 protein can be detected in

retinoid-treated thymocytes. In DMSO-treated control cells activation of caspase 8 is not visible (data not shown). Since Bid gene was also upregulated by retinoids (Table 2), and Bid is a well-known caspase 8 substrate (Green and Llambi 2015), to demonstrate that caspase 8 is activated during retinoid-induced apoptosis we checked for Bid cleavage. As shown in Figure13B, 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.

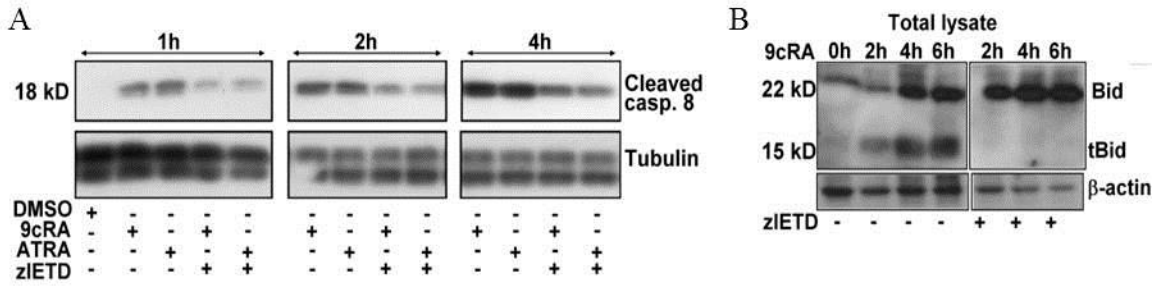

Figure 13.Activation of caspase 8 is required for retinoid-induced apoptosis.

(A) Retinoids induce the activation of caspase 8 during apoptosis. Thymocytes were exposed to 0.3 μM 9cRA or 3 μM ATRA, and the cleavage of caspase 8 was monitored in the absence or the presence of 40 μM specific caspase 8 inhibitor, z-IETD-fmk by Western blot at the indicated time points. Tubulin was used as loading control. (B) Bid is cleaved during retinoid-induced apoptosis. Thymocytes were treated with 0.3 μM 9cRA in the absence or the presence of 40 μM z-IETD-fmk for 2, 4 and 6 h. Bid expression was determined by Western blot from total cell lysate. β-actin was used as loading control.

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 (Fig. 14C). Similar effect is observed in the glucocorticoid-induced cell death, in which caspase 8 has a role, but the determining caspase is caspase 9 (Wang et al., 2006). 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.

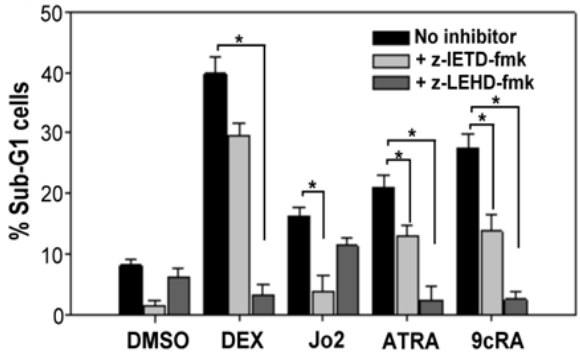

Figure14.Inhibition of both caspase 8 and 9 prevents retinoid-induced apoptosis.

*Thymocytes were exposed to 3 μM ATRA, 0.9 μM 9cRA for 12 h, to 0.1 μM dexamethasone acetate or 1 μg/ml Jo2 antibody for 6 h in the presence or absence of 40 μM zIETD-fmk, or 75 μM caspase 9 inhibitor Z-LEHD-FMK. The percentage of thymocytes in sub-G1 population was determined in propidium iodide stained samples. Data represent mean ± S.D. of three independent experiments. *Significantly different from the sample, where no caspase inhibitor was present ($p < 0.05$)*

4.1.4. 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 (Hao and Mak, 2010). Thus Fas mediated apoptosis of thymocytes is not affected by the loss of caspase 9 (Samraj et al. 2006). Though we found signs for caspase 8 activation during retinoid-induced apoptosis, we decided to investigate the requirement for caspase 9 activity (thus the mitochondrial pathway) during retinoid-induced apoptosis by inhibiting caspase 9. Fas-induced apoptosis was used as a control. As seen in Figure 14, 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 (Fig. 15). Interestingly, activation of caspase 8 occurs earlier in time than caspase 9 activation indicating that caspase 8 plays an initiator role in the retinoid-induced apoptosis.

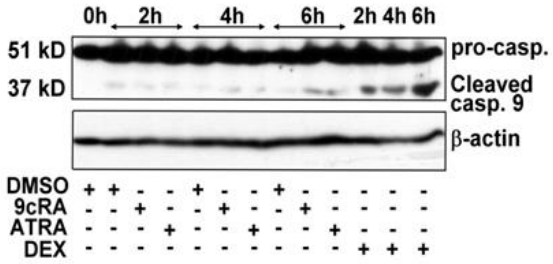

Figure 15. ***Retinoids induce the activation of caspase 9 during apoptosis.***

Thymocytes were exposed to 0.3 μM 9cRA, 3 μM ATRA or 0.1 μM dexamethasone acetate and the time-dependent cleavage of caspase 9 was detected by Western blot analysis. β-actin was used as loading control.

4.1.5. 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 (Wang et al., 2004) which might lead to apoptosis in thymocytes by enhancing the production of TNFα (Guevara Patiño et al., 2000) or by inducing Bim (Zhang and Insel, 2004). Since 9cRA induced the expression of Gpr65, we decided to test whether addition of retinoids results in TNFα 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 TNFα production (Fig.16).

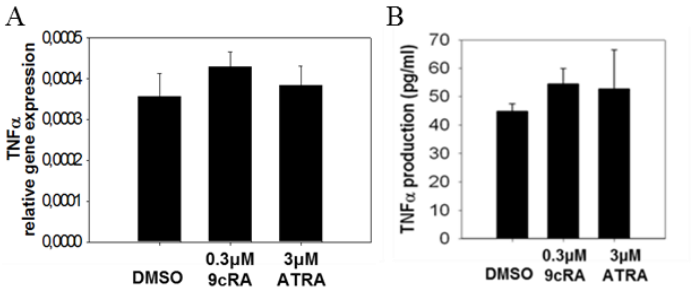

Figure 16. Retinoids failed to enhance TNF-α-production in dying thymocytes.

(A) Thymocytes were treated with 0.3 μM 9cRA or 3 μM ATRA, and the mRNA levels of TNF-α were determined by Q-PCR analysis at 4 hrs using cyclophilin as a reference gene.(B) TNF-α production was also detected by ELISA after the same treatments at 18 hrs of culture.Data represent mean ± S.D. of three independent experiments.

However, as it is illustrated in Figure 17, we could demonstrate that retinoid treatment significantly induced 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 (Van Haastert et al., 1984). Addition of PKA inhibitors had no effect on the retinoid-induced apoptosis of the thymocytes either (data not shown). Surprisingly Bim upregulation showed a Nur77-dependent fashion upon retinoid treatment. Taken together, our data indicate that though the expression of Gpr65 is increased, but under our culture conditions there is no significant acidification which would activate it and the coupled adenylate cyclase pathway.

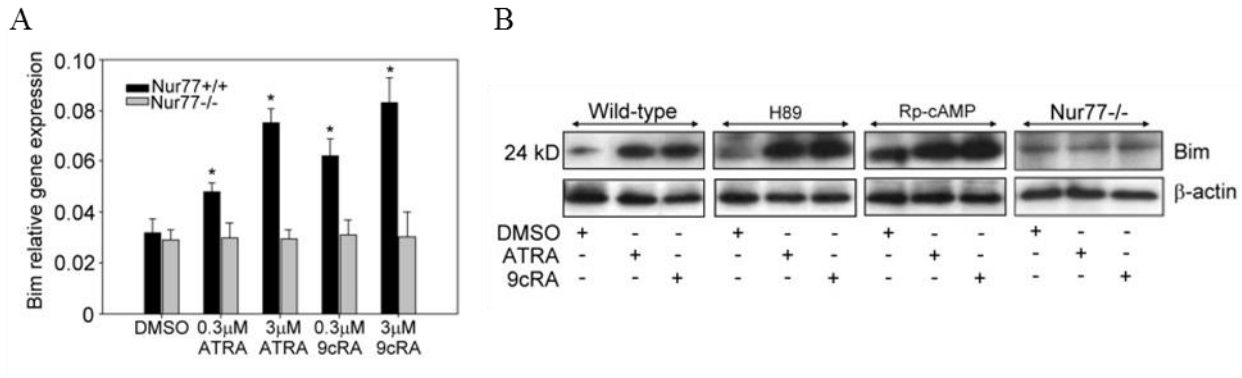

Figure 17. Retinoids induce the appearance of Bim in Nur77-dependent, but protein kinase A-independent manner.

(A) Retinoids fail to induce Bim in Nur77 deficient thymocytes. Wild type and Nur77 null thymocytes were treated with the indicated concentrations of 9cRA or ATRA for 4 h and mRNA level of Bim was measured by qRT-PCR and normalized to cyclophilin. (B) Retinoids induce the appearance of Bim protein in a protein kinase A-independent manner. Wild type thymocytes were exposed to 0.3 μM 9cRA or 3 μM ATRA in the absence or presence of protein kinase A I and II inhibitors (10 μM H89 or 50 nM Rp.cAMPS triethylamine). Thymocytes derived from Nur77 null mice were treated with 0.3 μM 9cRA or 3 μM ATRA and Bim protein was detected 6 h later by Western blot analysis. β-actin was used as loading control.

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

Previous studies have shown that STAT1 can also regulate Bim expression in thymocytes (Moro et al., 2011). The Affymetrix data revealed that STAT1 is also induced during retinoid-induced apoptosis in a Nur77-dependent manner (Table 2). We could also confirm retinoid-inducedSTAT1 upregulation on protein levels (Fig. 1A). Thus we decided to test whether the induction of Bim expression during retinoid-induced apoptosis is STAT1 dependent by using STAT1 knock out thymocytes (Durbin et al., 1996). Our results indicate that enhancement in the Bim expression during retinoid-induced apoptosis is STAT1 dependent (Fig.18B and C).

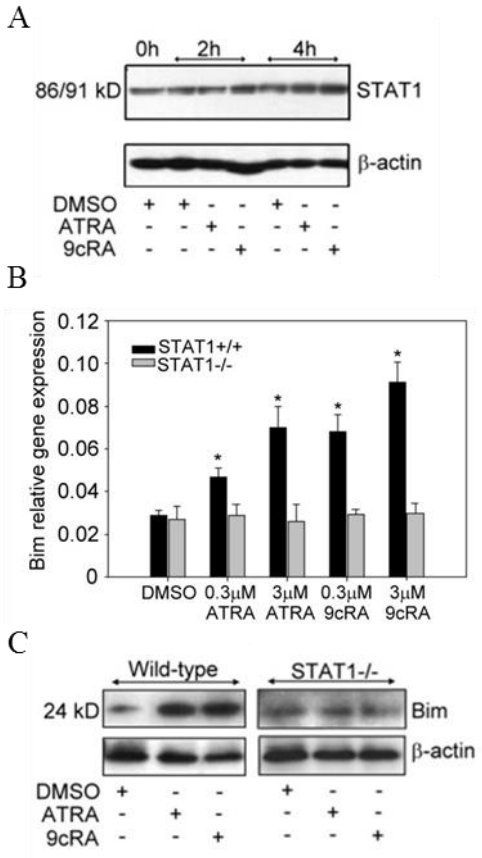

Figure 18. Bim is induced in a STAT1-dependent manner during retinoid-mediated apoptosis.

*(A) Retinoids induce the expression of STAT1 in thymocytes. Wild type thymocytes were treated with 0.3 μM 9cRA or 3 μM ATRA and STAT1 protein was detected 2 or 4 h later by Western blot analysis. β-actin was used as loading control. (B) and (C) Retinoids fail to induce the expression of Bim in STAT1 null thymocytes. Thymocytes derived from wild type or STAT1 null mice were exposed to the indicated retinoid concentrations and Bim expression was determined 6 h later by qRT-PCR analysis using cyclophilin as a reference gene. Bim protein was detected 6 h later by Western blot analysis. β-actin was used as loading control. Data represent mean ± S.D. of three independent experiments. *Significantly different from the DMSO-treated control ($p < 0.05$).*

In addition, loss of STAT1 reduced the rate of retinoid-induced apoptosis of thymocytes (Fig. 19) indicating that STAT1-induced Bim expression might contribute to the initiation of the retinoid-induced apoptosis program.

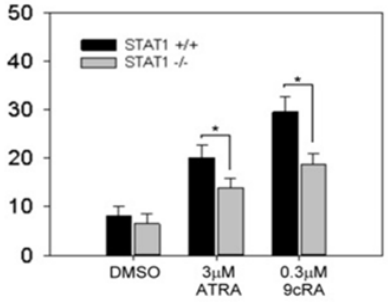

Figure 19. Loss of STAT1 attenuates retinoid induced apoptosis of thymocytes.

*Wild type and STAT1 null thymocytes were exposed to 3 μM ATRA, 0.9 μM 9cRA for 6 h. The percentage of thymocytes in sub-G1 population was determined in propidium iodide stained samples. Data represent mean ± S.D. of three independent experiments. *Significantly different from the DMSO-treated control ($p < 0.05$).*

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

It has been previously published that in addition to its transcription-dependent action, Nur77 can also contribute to apoptosis induction during negative selection in thymocytes by its translocation to the mitochondria, where it associates with Bcl-2 leading to the exposure of the BH3 domain of Bcl-2 converting the anti-apoptotic molecule to a killer protein (Thompson and Winoto, 2008). According to this finding we tested whether retinoids are capable of inducing the translocation of Nur77 into the mitochondria. As control, we used the phorbol dibutyrate/ionomycin treatment, which mimics the signaling of negative selection. As shown in Figure 20, treatment with phorbol dibutyrate/ionomycin resulted in a time dependent translocation of Nur77 into the mitochondria appearing as a wide band due to the heavy phosphorylation of the protein (Woronicz et al., 1995). 9cRA and, with a much less efficiency, ATRA also induced a time-dependent translocation of Nur77 with a peak at 2 h for each treatment. However, we could detect a much thinner protein band compared to that was found in phorbol dibutyrate/ionomycin-treated cells, indicating a less prominent or lack of phosphorylation of Nur77 during retinoid treatment. Previous studies have reported that Nur77 can form heterodimers with the retinoid X receptor (RXR) and heterodimer formation is required for the nuclear export of Nur77 (Cao et al., 2004). Interestingly, we detected mitochondrial translocation of RXR in both wild type and Nur77 null thymocytes. In phorbol

dibutyrate/ionomycin-treated cells the kinetics of the mitochondrial appearance of RXR was different from that of Nur77, and the presence or absence of Nur77 did not affect it. In retinoid treated thymocytes, however, the maximum of both Nur77 and RXR translocations was observed at 2 h after retinoid addition.

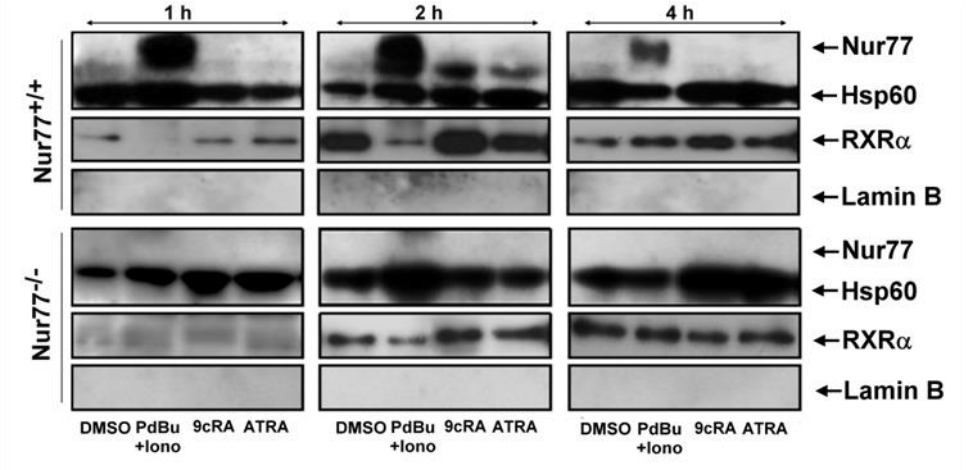

Figure 20. Nur77 translocates into the mitochondria during retinoid-induced apoptosis.

Detection of time-dependent Nur77 and RXR translocation into mitochondria in wild type and Nur77 null thymocytes following retinoid or 1 μM phorbol dibutyrate/2 μg/ml ionomycin treatment. Mitochondrial fractions of thymocytes were isolated at the indicated time points following treatment and were blotted with anti-Nur77 and -RXR antibodies. For fraction purity the blot was also probed for the nuclear Lamin B and the mitochondrial Hsp60.

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 (Thompson and Winoto, 2008). Exposure of this domain correlates with the pro-apoptotic activities of Bcl-2 (Thompson and Winoto, 2008) and can be detected by mimicking signals of negative selection by adding phorbol dibutyrate/ionomycin to the thymocytes (Thompson et al., 2010). 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 (Fig. 21). DMSO did not have an effect on the appearance 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.

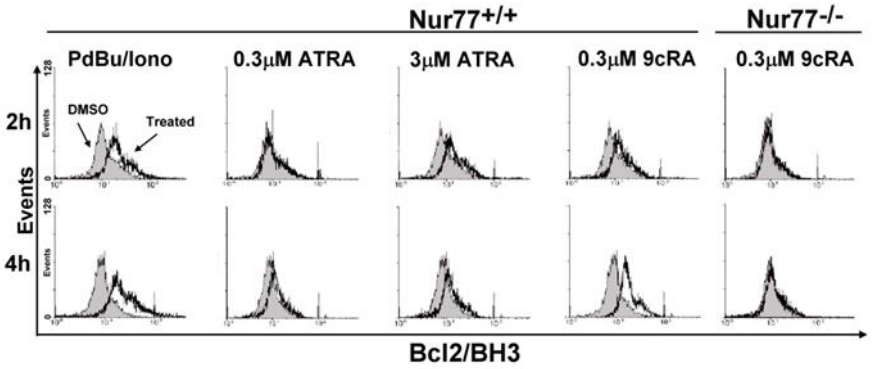

Figure 21. Time dependent Bcl-2/BH3 exposure of wild type thymocytes exposed to retinoids.

Wild type thymocytes were treated with the indicated retinoid concentrations for 2 or 4 hours and the Bcl-2/BH3 exposure was determined by flow cytometry. 1 μM phorbol dibutyrate/2 μg/ml ionomycin treatment of wild type thymocytes was used as positive control, while exposure of Nur77 null cells to the 9cRA was used as negative control.

4.1.9. 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 (Szondy et al., 1997), but the basal expression of Nur77 shows a differentiation-dependence (Moran et al., 2011). 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 (Moran et al., 2011). 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 (Fig. 22A). As shown in Figure 22B, 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. As seen in Figure 22C, the gene expression level of all these 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 labelled with anti-CD8 and anti-Bcl-2/BH3 antibodies. As it is demonstrated in Figure22D,

we could detect the exposure of Bcl-2/BH3 domain in the CD8+ population. Since the majority of CD8+ thymocytes are DP thymocytes (app 95 %), these data indicate that Nur77 can translocate into the mitochondria in the DP cells. Finally we investigated the role of caspase 8 and caspase 9 in retinoid-induced death of the sorted DP thymocytes. As it is visible in Figure 22E, both inhibitors attenuated retinoid-induced apoptosis similarly as in the unsorted thymocytes.

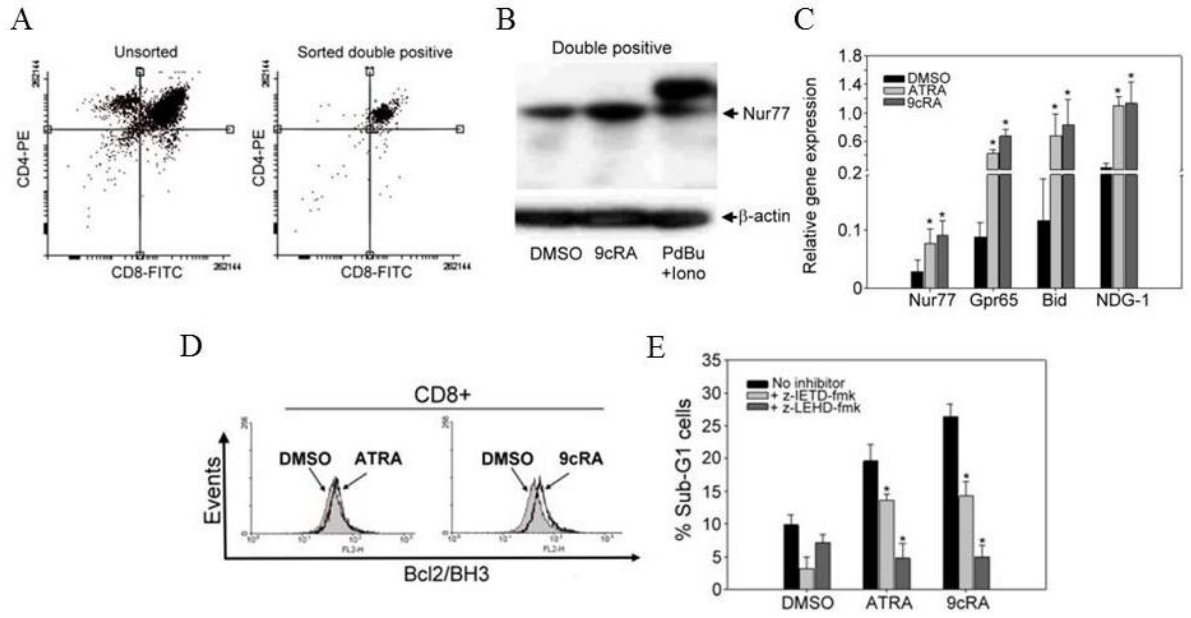

Figure22. Retinoids induce the expression of Nur77-dependent genes as well as the exposure of the Bcl-2/BH3 domain in DP thymocytes.

*(A) Result of the sorting of DP thymocytes. (B) Nur77 is induced at protein level by 0.3 μM 9cRA or by 1 μM phorbol dibutyrate/2 μg/ml ionomycin treatments at 6 hours in sorted DP thymocytes. (C) The mRNA levels of Nur77 and three Nur77-dependent genes are induced by retinoids in DP thymocytes. Thymocytes were exposed to 3 μM ATRA or 0.3 μM 9cRA and mRNA expressions were determined 4 h later by qRT-PCR analysis using cyclophilin as a reference gene. Data represent mean ± S.D. of four independent experiments. *Significantly different from the DMSO-treated control ($p < 0.05$). (D) The Bcl-2/BH3 domain is exposed following retinoid treatment in DP thymocytes. Thymocytes were exposed to 3 μM ATRA, 0.3 μM 9cRA or the vehicle control. 4 h later cells were labelled with both FITC-labelled anti-CD8 and with Cy3-labeled anti-Bcl-2/BH3 antibodies. The exposure of Bcl-2/BH3 is shown in CD8+ thymocytes, which represent mostly DP cells. (E) Inhibition of both caspase 8 and 9 prevents retinoid-induced DP thymocyte apoptosis. Thymocytes were exposed to 3 μM ATRA, 0.3 μM 9cRA, in the presence or absence of 40 μM zIETD-fmk, or 75 μM caspase 9 inhibitor Z-LEHD-fmk. The percentage of thymocytes in sub-G1 population was determined in propidium iodide stained samples 6 h later. Data represent mean ± S.D. of three independent experiments. *Significantly different from the sample, where no caspase inhibitor was present ($p < 0.05$).*

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

4.2.1. 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 (Murphree et al., 2005, Elson et al., 2013). As it is shown below, LPS indeed induced the expression of A2ARs on both on the cell surface (Fig. 23A) and on mRNA levels (Fig. 23B) 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 (Murphree et al., 2005, Elson et al., 2013) contribute to its enhanced expression.

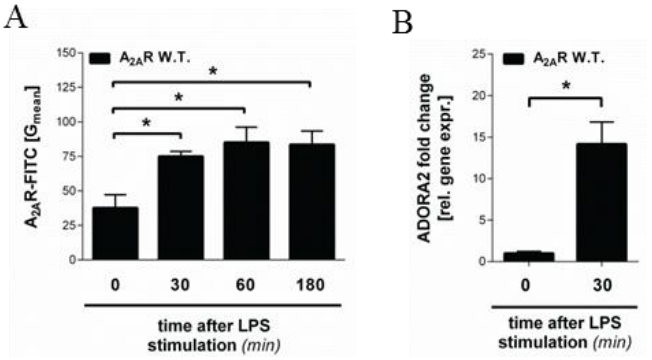

Figure 23. A_{2A}R expression is induced in response to LPS stimulation both (A) on the cell surface and (B) at mRNA expression level in BMDMs.

*Wild type BMDMs were treated with 200 ng/ml LPS for the indicated time periods, and cell surface expression of A_{2A}Rs was determined by flow cytometry, while gene expression levels were measured by qRT-PCR analysis using HGPRT as a reference gene. Data represent mean ± S.D. of five independent experiments. *Significantly different from the non-treated control ($p < 0.05$.*

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 using cytokine array. As it shown in Figure 24, 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.

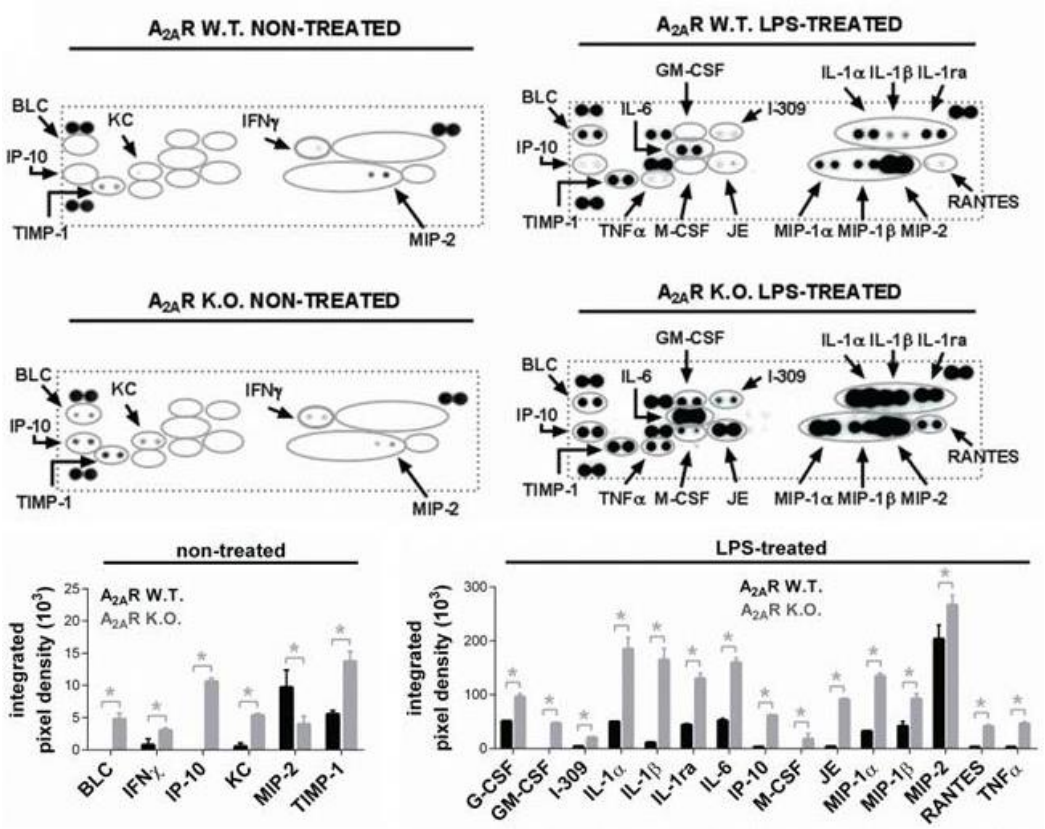

Figure 24.Loss of A_{2A}Rs results in higher pro-inflammatory cytokine formation in LPS-stimulated bone marrow derived macrophages.

*Cytokine panel of control and LPS-treated wild type and $A_{2A}R$ null BMDMs. Cells were incubated with 200 ng/ml LPS for 1 h. After the removal of LPS fresh media were added to the cells. Supernatants were collected 5 h later, and cytokine levels were determined by cytokine array. Arrows highlight those cytokines, the expression of which was significantly different in $A_{2A}R$ null BMDMs. The pixel density results are mean ± S.D. from three independent experiments. *Significantly different from wild type control ($p < 0.05$).*

We further confirmed cytokine array results by ELISA in case of TNFα, IL-6 and MIP-2 cytokines (Fig. 25A). 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 (Fig. 25B). Both macrophage type did not release these cytokines under non-stimulated conditions (data not shown).These data support previous findings that LPS-triggered macrophages produce endogenous adenosine to regulate LPS-induced pro- inflammatory cytokine formation via the adenosine $A_{2A}Rs$ (Bshesh et al., 2002, Haskó et al., 2000, Sipka et al., 2005).

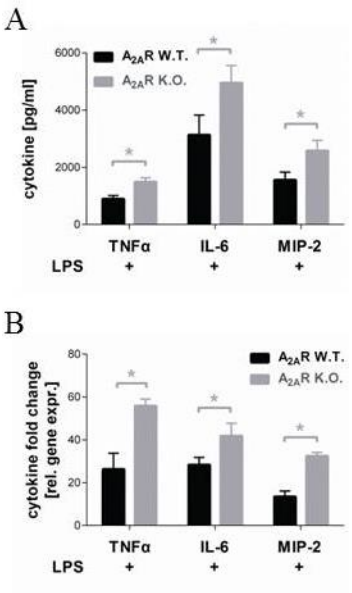

Figure 25.Effect of LPS treatment on the TNFα, IL-6 and MIP-2 production of wild type and A_{2A}R null BMDMs at (A) protein and at (B) mRNA levels.

*(A) Wild type and A_{2A}R null BMDMs were treated with 200 ng/ml LPS for 1 h. After the removal of LPS fresh media were added to the cells. 5 h later the levels of indicated cytokines in the supernatant were determined by ELISA method. (B) Total RNA was isolated after 3 h of incubation and mRNA levels were measured by qRT-PCR using HGPRT as a reference gene. Relative gene expression values are shown as fold induction normalized to the wild type control samples. Data represent mean ± S.D. of six independent experiments. *Significantly different from wild type control ($p < 0.05$).*

4.2.2. 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 (Karin and Greten, 2005), 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 Figure26A demonstrates, 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 (Fig. 26B).

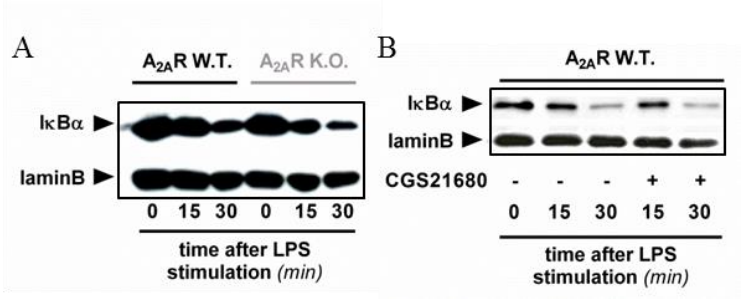

Figure 26.Western blot analysis of IκBα degradation in wild type and A_{2A}R null macrophages after LPS exposure.

Cells were treated with 200 ng/ml LPS alone (A) or in combination with A_{2A}Rspecific agonist CGS21680 (1 μM, 1 h pretreatment) (B) for the indicated time periods. Lamin-B was used as loading control.

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 (Karin and Greten, 2005). Therefore we decided to examine whether the loss of $A_{2A}R$ affects the nuclear translocation of the p65/p50 subunit of NF-κB by using the TransAM NF-κB transcription factor kits (ActiveMotif). As shown in Figure 27, no difference was detected in either the basal or in the LPS-induced nuclear appearance of these transcription factors in wild type and in $A_{2A}R$ null macrophages. In line with these observations, $A_{2A}R$ agonist CGS21680 treatment also did not alter it. The agonist treatment alone had no effect on NF-κB transcription (data not shown).

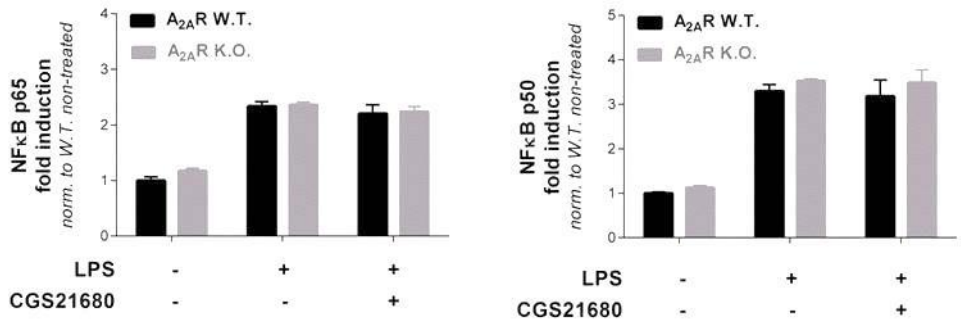

Figure 27.The enhancement in LPS-induced pro-inflammatory response of $A_{2A}R$ null macrophages is not related to an alteration in the activation of the classical, NF-κB-dependent TLR4 signaling pathway.

Nuclear levels of the p65 and p50 subunits of NF-κB transcription factor in control and LPS-stimulated macrophages. Wild type and $A_{2A}R$ null BMDMs were treated with 200 ng/ml LPS for 1 h. Nuclear levels of the 2 subunits of NF-κB were detected with TransAM NF-κB transcription factor kits. Data represent mean ± SD of three independent experiments.

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 (Cvetanovic and Ucker, 2004) either (Fig. 28). 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.

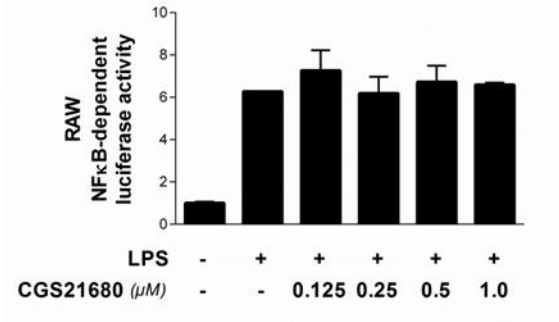

Figure 28.Activation of the $A_{2A}R$ signaling pathway does not affect the NF-κB-driven transcriptional activity in RAW264.7 macrophage cell line.

The NF-κB driven transcriptional activation was determined by measuring the luciferase activity of an NF-κB-dependent luciferase transcriptional reporter in a stably transfected RAW264.7 macrophage cell line.125 ng/ml LPS and the indicated doses of CGS21680 were added to the cells simultaneously. Cell extracts were prepared, and luciferase activities were measured after a 6 h LPS exposure. Data were normalized to the non-treated control cells. Data represent mean ± SD of three independent experiments.

4.2.3. 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 shown in Figure 29 upper panels, loss of A2ARs 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 continuously activated in non-stimulated cells. Stimulation of wild type macrophages by LPS (Fig. 29 lower panel left) resulted in activation of the MAPK pathways (Chang and Karin, 2001) 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) (Chang and Karin, 2001). Previous studies have shown that p38α can selectively activate MAPK activated protein kinase 2, which further phosphorylate CREB and heat shock protein (hsp) 27(Zarubin and Han, 2005), 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(Guha and Mackman, 2002).

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 (Fig. 29 lower middle and right panel). 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 A_{2A}R null macrophages as well (Fig. 29 lower middle panel).

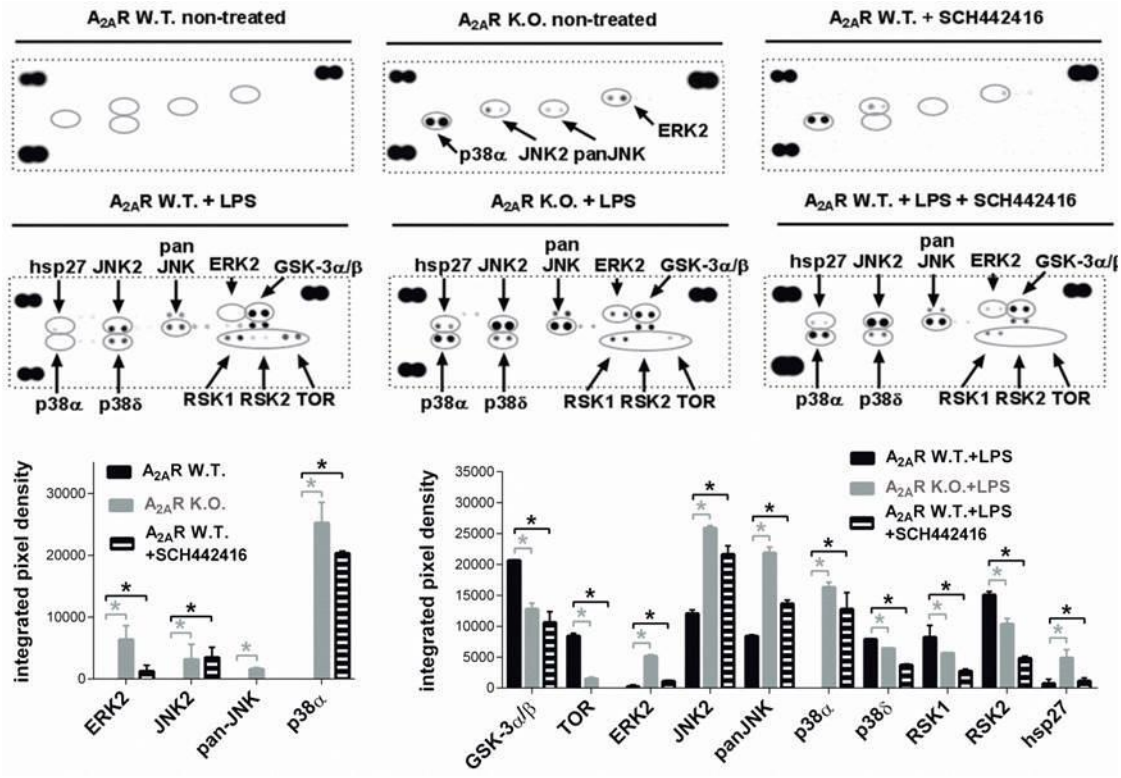

Figure 29. A_{2A}R deficiency is accompanied by enhanced MAPK phosphorylation.

Phospho-MAPK panel of wild type and A_{2A}R null BMDMs with or without LPS treatment alone or in the presence of an A_{2A}R antagonist. Wild type and A_{2A}R null cells were treated with 200 ng/ml LPS alone or in combination of t SCH442416 (10 nM, 1 h pretreatment) for 30 min. Macrophages were then lysed, and the amount of phosphorylated proteins was detected by a Phospho-MAP kinase array. Arrows highlight those proteins, the phosphorylation of which was altered in A_{2A}R null BMDMs as compared to their wild type control. One representative experiment of three is shown.

Since macrophages express JNK1 and 2 (Himes et al., 2006), but the human phospho-MAPK array kit did not detect mouse JNK1, we tested its phosphorylation byWestern blot analysis by using a mouse specific anti-JNK1 antibody. As shown in Figure 30A, 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 (Fig. 30B). 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 (Fig. 30B). 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.

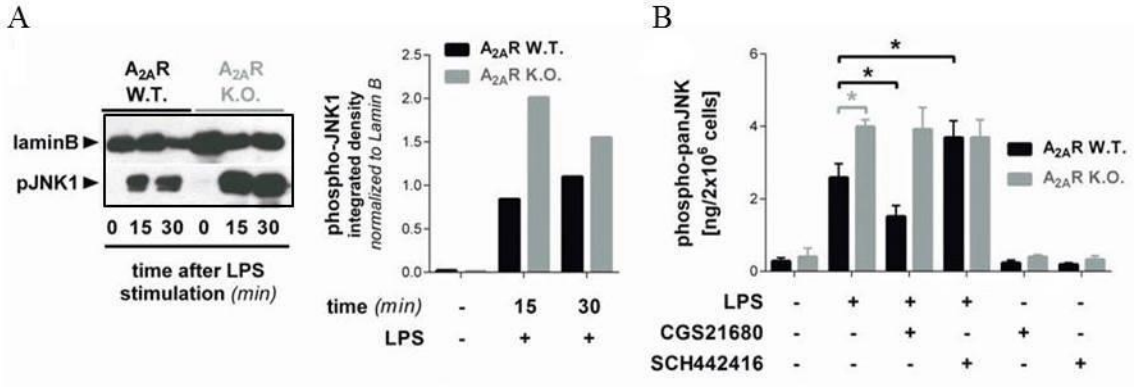

Figure 30. Analysis of JNK1 and phospho-JNK1 levels in wild type and $A_{2A}R$ null macrophages

*(A) Western blot analysis of JNK1 and phospho-JNK1 levels in wild type and $A_{2A}R$ null macrophages exposed to 200 ng/ml LPS for the indicated time periods. Lamin B was used as a loading control. Integrated density values of the blot are shown on right. (B) ELISA-based determination of phospho- JNK levels. BMDMs were treated with 200 ng/ml LPS alone or in combination with the $A_{2A}R$-specific agonist CGS21680 (1 μM, 1 h pretreatment) or the $A_{2A}R$-specific antagonist SCH442416 (10 nM, 1 h pretreatment) for 30 min. The levels of phosphorylated JNKs were determined by a phospho-panJNK-specific ELISA kit. Data represent mean ± S.D. of three independent experiments. *Significantly different from LPS-treated wild type cells ($p < 0.05$).*

4.2.4. 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. As shown in Figure 31, exposure of wild type macrophages to CGS21680 attenuated, while SCH442416, enhanced the LPS-induced production of IL-6 (Fig. 31A), MIP-2 (Fig. 31B) and TNFα (Fig. 31C). 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.

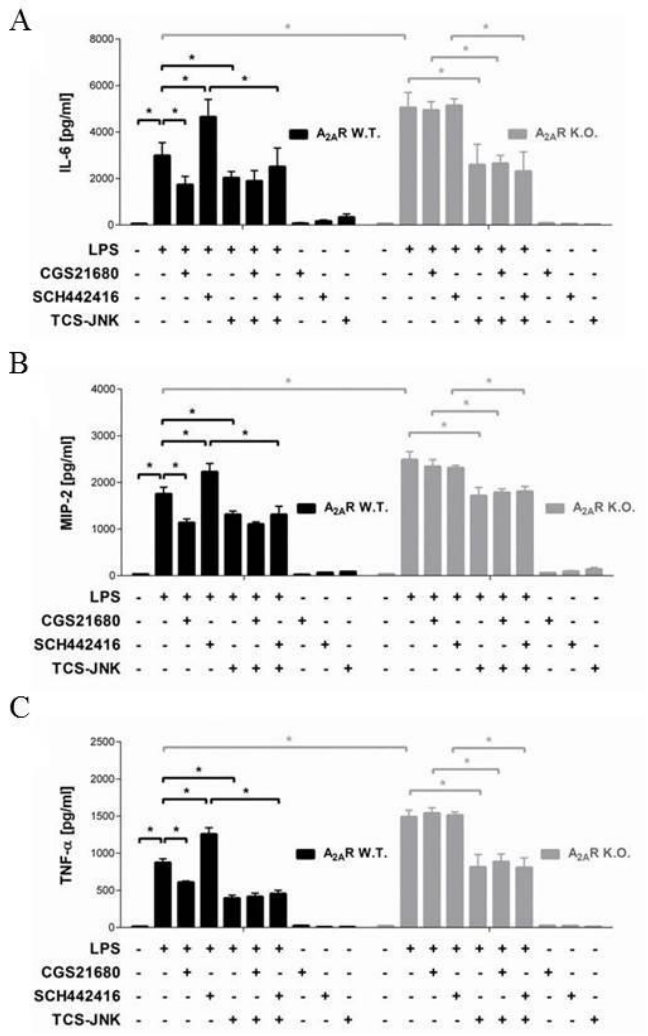

Figure 31.The A_{2A}R-mediated suppression of LPS-induced pro-inflammatory cytokine production requires JNKs.

*BMDMs were treated with 200 ng/ml LPS alone or in combination with CGS21680 (1 μM, 1 h pretreatment), SCH442416 (10 nM, 1 h pretreatment) or the JNK inhibitor TCS-JNK (100 nM, 1 h pretreatment) for 1 h. After the removal of LPS, fresh media supplemented with CGS21680, SCH442416 or TCS-JNK was added to the cells. Supernatants were collected 5 h later, and IL-6 (A), MIP-2 (B) and TNFα (C) levels were determined by ELISA. Data represent mean ± S.D. of three independent experiments. *Significantly different ($p < 0.05$).*

4.2.5. 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 (Dong et al., 2002,Zarubin and Han, 2005), we checked whether MKK4 or MKK3/6 activation is increased by LPS in the absence of A_{2A}R signaling. However, as shown in Figure32, in the case of MKK4 no alteration was found in the kinetics of 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 signaling.

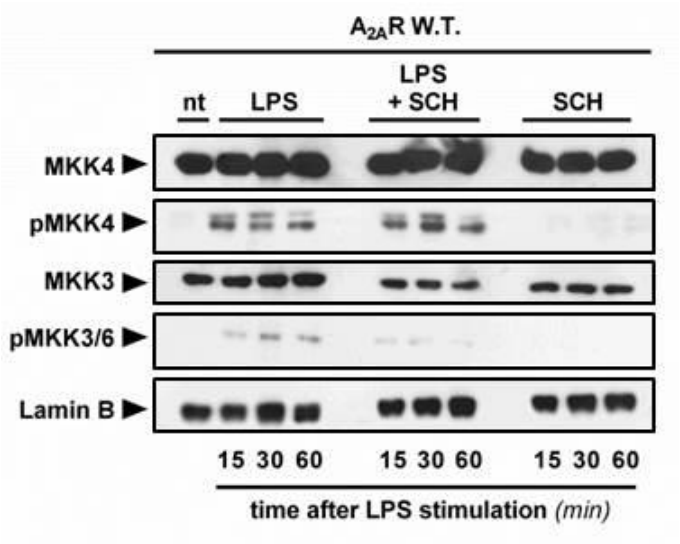

Figure 32.Analysis ofMKK4, MKK3 and phospho-MKK4, phospho-MKK3/6 levels

Wild type macrophages exposed to 200 ng/ml LPS for the indicated time periods. MKK4, MKK3 and phospho-MKK4, phospho-MKK3/6 levels were determined by Western blot. Where it is indicated, SCH442416 (10 nM, 1 h pretreatment) was also added to the cells. Lamin B was used as a loading control. nt: non-treated control sample.

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 (Chen et al., 2002,Abraham et al., 2006). 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 (Hammer et al., 2006, Zhao et al., 2006). Since already the basal phosphorylation level of both JNK and p38 was enhanced in A2AR null macrophages (Fig. 29), we decided to determine whether basal DUSP1 levels are affected by the loss of $A_{2A}R$. As shown in Figure 33, $A_{2A}R$ null macrophages express significantly lower DUSP1 at both mRNA (Fig. 33A) and protein (Fig. 33B) levels than their wild type counterparts. Exposure of wild type macrophages to SCH442416 also resulted in decreased DUSP1 expression (Fig. 33Cand D), while CGS21680 treatment increased it (Fig. 33E and F). 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 maintains basal DUSP1 expression in BMDMs.

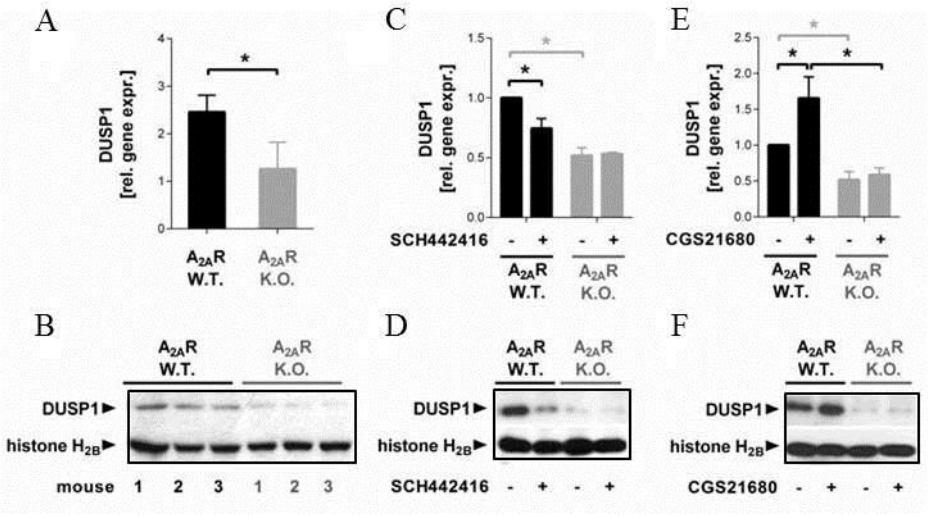

Figure 33.The mRNA and protein expression levels of DUSP1 are under the control of a continuously activated $A_{2A}R$ signaling pathway in BMDMs.

*Basal DUSP1 mRNA (A) and protein (B) levels in wild type and $A_{2A}R$ null BMDMs. Effect of an $A_{2A}R$ antagonist on the DUSP1 mRNA (C) and protein (C) levels in wild type and $A_{2A}R$ null BMDMs. Effect of an $A_{2A}R$ agonist on the DUSP1 mRNA (E) and protein (F) levels in wild type and $A_{2A}R$ null BMDMs. Total protein or total RNA of untreated BMDMs, or BMDMs exposed to either CGS21680 (1 μM, 3 h treatment) or SCH442416 (10 nM, 3 h treatment) were isolated. DUSP1 protein levels were analyzed by Western blot using histone H2B as a loading control. Alternatively, DUSP1 gene expression levels were determined by qRT-PCR using HGPRT as reference gene. Relative gene expression data represent mean ± S.D. of four independent experiments. *Significantly different ($p < 0.05$).*

Previous studies have shown that LPS stimulation itself enhances the expression of DUSP1 in macrophages, which contributes to termination of the LPS response (Chen et al., 2002). 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 (data not shown). As shown in Figure 34, 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 (Kreckler et al., 2009). 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 (Seamon et al., 1981), or Rp-cAMP, a competitive inhibitor of endogenous cAMP. As it seen in Figure 34, forskolin could enhance both the basal and the LPS-induced DUSP1 expression in both wild type and $A_{2A}R$ 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.

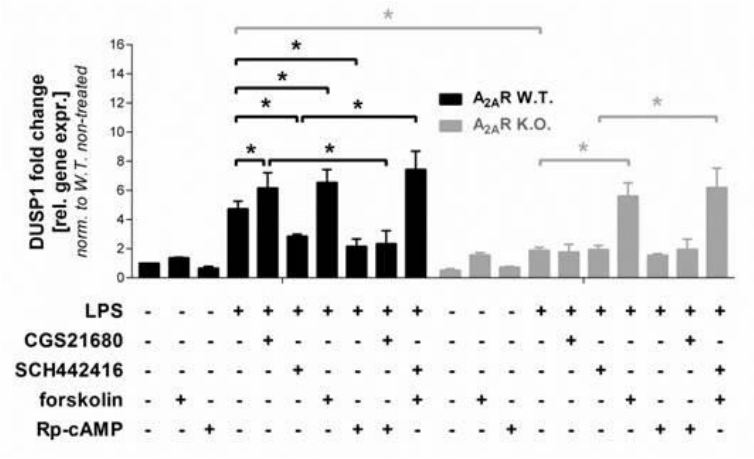

Figure 34.DUSP1 gene expression level of LPS-exposed wild type and $A_{2A}R$ null BMDMs.

*Prior to LPS exposure (200 ng/ml, 1 h treatment) cells were pretreated with CGS21680 (1 μM), SCH442416 (10 nM), Rp-cAMPS, a competitive inhibitor of cAMP (100 μM) or forskolin, an adenylyl cyclase activator (10 μM). After the LPS treatment fresh media – supplemented with CGS21680, SCH442416, Rp-cAMPS, or forskolin – were added to the cells. Total RNA was isolated 2 h later, and DUSP1 gene expression levels were determined by qRT-PCR using HPGRT as normalizing gene. Results are expressed as fold induction as compared to the wild type non-treated cells. Data represent mean ± SD of three independent experiments. *Significantly different ($p < 0.05$).*

4.2.6. $A_{2A}Rs$ 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 Figure35A demonstrates, siRNAs significantly reduced the basal DUSP1 expression at both protein and mRNA levels in wild

type and $A_{2A}R$ null macrophages. Reduction of DUSP1 level by siRNA resulted in an enhancement in both the basal and in the LPS-induced phosphorylation of JNK in wild type macrophages, and in a loss of the $A_{2A}R$ control over it (Fig. 35B). In contrast, in $A_{2A}R$ null cells the 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.

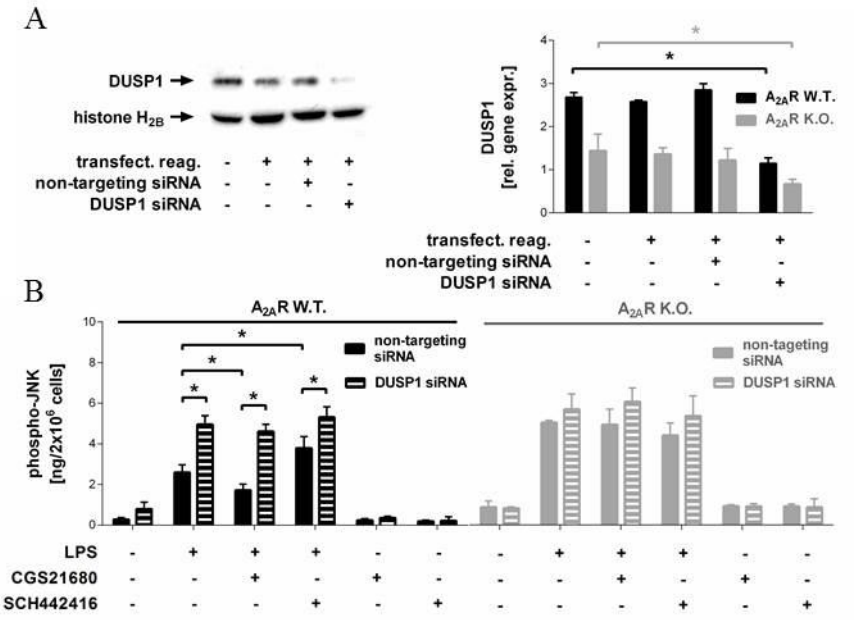

Figure 35.$A_{2A}R$ signaling is not able to regulate LPS-induced JNK activation in DUSP1-silenced BMDMs.

*(A) Transient DUSP1 silencing by siRNA. Five-day-matured BMDMs were transfected with siRNA specific for mouse DUSP1 and non-targeting siRNA. The efficiency of RNAi was determined by Western blot analysis and by qRT-PCR using HGPRT as normalizing gene. (B) Phosphorylated JNK levels of LPS-treated wild type and $A_{2A}R$ null DUSP1-silenced BMDMs in the presence of an $A_{2A}R$agonist or antagonist. Control, non-targeting siRNA and DUSP1 siRNA transfected cells were treated with 200 ng/ml LPS alone or in combination with either CGS21680 (1 μM, 1 h pretreatment)or SCH442416 (10 nM, 1 h pretreatment) for 30 min for determining pJNK levels by a phospho-panJNK-specific ELISA kit . Data represent mean ± SD of three independent experiments. *Significantly different from non-targeting siRNA ($p < 0.05$).*

4.2.7. $A_{2A}Rs$ fail to control the LPS-induced IL-6 and TNFα 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 (Fig. 36A) and TNFα (Fig. 36B) production of wild type and A2AR 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 TNFα production via controlling DUSP1 levels in macrophages.

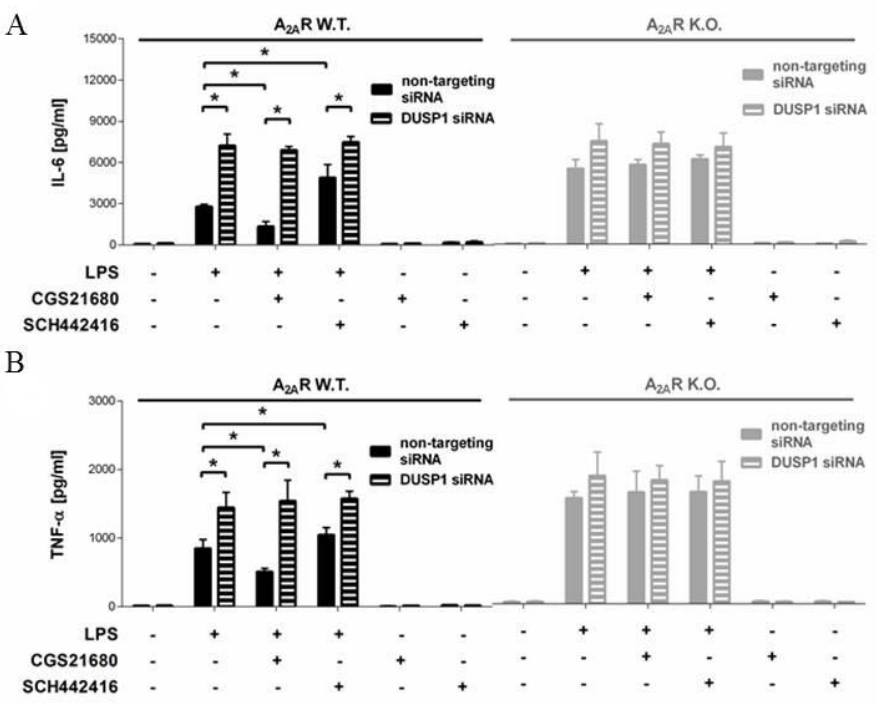

Figure36.$A_{2A}R$ signaling is not able to regulate LPS-induced pro-inflammatory cytokine production in DUSP1-silenced BMDMs.

*IL-6 and (B) TNFα levels of LPS-treated wild type and $A_{2A}R$ null DUSP1-silenced BMDMs in the presence of an $A_{2A}R$ agonist or antagonist. Control, non-targeting siRNA and DUSP1 siRNA transfected cells were treated with 200 ng/ml LPS alone or in combination with either CGS21680 (1 μM, 1 h pretreatment) or SCH442416 (10 nM, 1 h pretreatment) for the pro-inflammatory cytokine determination. After the removal of LPS fresh media supplemented with CGS21680 or SCH442416 were added to the cells. Supernatants were collected 5 h later, and IL-6 and TNFα levels were determined by ELISA. . Data represent mean ± SD of three independent experiments *Significantly different from non-targeting siRNA ($p < 0.05$).*

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 (Chaplin, 2010). 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 (Aderem and Ulevitch, 2000; Chaplin, 2010).

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 (Janeway et al., 2001). 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 (Hernandez et al., 2010). The apoptotic cells are then rapidly being cleared by thymic macrophages, which in turn release soluble molecules, such as retinoids and adenosine (Garabuczi et al., 2013; Köröskényi et al., 2011). Thus we have proposed that thymic macrophages continually engulfing apoptotic thymocytes constantly provide these efferocytosis-dependent molecules (retinoids and adenosine) 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 (Szondy et al., 1997; Kiss et al., 2006; Szondy et al., 2012). 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 (Szondy et al., 1997). 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 (Tóth et al., 2001; Tóth et al., 2004). 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 (Lee et al., 1995). 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 (Cheng et al., 1997). 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 (Rajpal et al., 2003). 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 (Hao and Mak, 2010), 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 (Shang et al., 1999) 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 (Malorni et al., 2013; Hsieh et al., 2013).

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 (Liebermann and Hoffman, 2002). Ptpn6, on the other hand, is necessary for receptor-mediated cytotoxic signaling that causes intracellular acidification and apoptosis (Thangaraju et al., 1999). While the retinoid-induced induction Ptpn6 was Nur77 dependent, the induction of Gadd34 was not (Table 2).

Independently of its transcriptional activity, Nur77 protein is known to act directly in the mitochondria to induce apoptosis (Lin et al., 2004; Thompson and Winoto, 2008). 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 (Woronicz et al., 1995). 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 (Cao et al., 2004). 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 (Thompson and Winoto, 2008).

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 (Fig. 37).

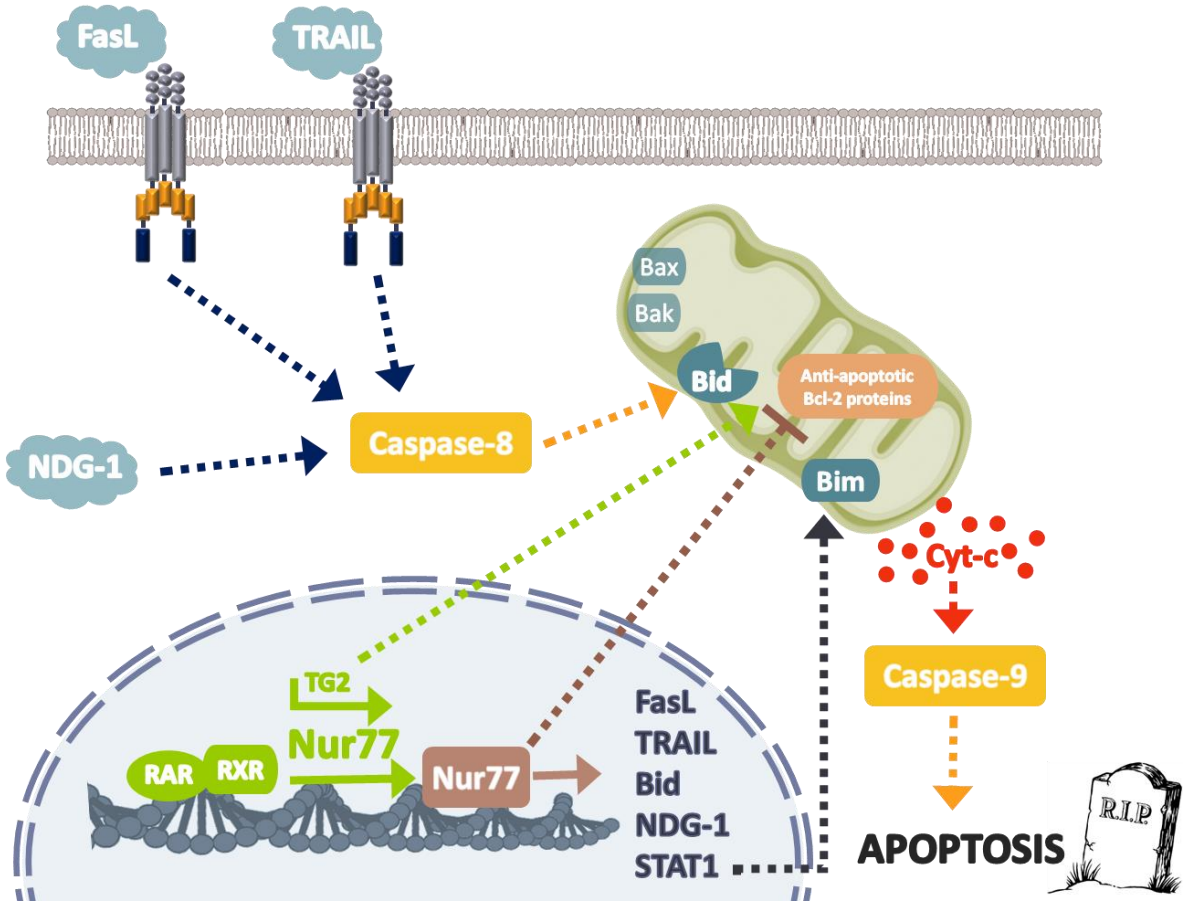

Figure 37.Proposed model for retinoid-induced apoptosis in DP mouse thymocytes.

Retinoids induce the expression of Nur77. The transcription factor Nur77 induces the expression of FasL, TRAIL, NDG-1, Gpr65 and STAT1. FasL, TRAIL and NDG-1 may contribute to Caspase 8 activation. Caspase 8 will cleave Bid, the expression of which is also enhanced during retinoid-induced apoptosis. Retinoid-induced STAT1 leads to the upregulation of Bim. Retinoids also induce the expression of transglutaminase 2. Cleaved Bid, TG2 and Nur77 can directly act on the mitochondria to initiate the mitochondrial pathway of apoptosis.

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 (Kiss et al., 2008; Sitnik et al., 2012) We also found that their production is related to apoptotic cell-engulfing macrophages (Garabuczi et al., 2013). Surprisingly, however, we could not detect the presence of any of the classical retinoids in the mouse thymus (Kiss et al., 2008), instead metabolites of the retinol saturase pathway seem be produced (Sarang et al., 2014). 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 (Chaplin, 2010) and they 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 (and DiPietro, 2011). 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 (Cohen et al., 2013).

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 (Sándor et al. 2016). 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 (Kiss és mts. 2006). Massive 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) (Köröskényi et al., 2011).

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 (Neumann et al., 1995; Lukashev et al., 2004). 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 (Kreckler et al 2006), and the NF-κB activity is not affected (Kreckler et al 2006; Haskó et al., 1996; Németh et al., 2005).

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. While JNK enhances the level of MIP-2 (Kim et al., 2003) and IL-6 (Faggioli et al., 2004) primarily via regulating transcription, expression of TNFα was shown to be regulated by JNK at the level of translation (Swantek et al., 1997).

Though the consequences of the adenosine-induced p38 MAP kinase or ERK suppression in LPS-stimulated macrophageswas not examined during our experiments, these pathways are also known to contribute to the formation of various pro-inflammatory cytokines. Thus, for example just in the context of TNFα, it was shown that A_{2A}R signaling suppresses p38 activity leading to TNFα mRNA instability in U937 cells (Fotheringham et al., 2004), while

ERK is necessary for LPS-induced TNFα mRNA production in macrophages (van der Bruggen et al., 1999).

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. ÖSSZEFOGLALÁS

A programozott sejthalál vagy apoptózis folyamata nagy jelentőséggel bír a többsejtű élőlények megfelelő egyedfejlődésében és homeosztázisának fenntartásában, valamint kulcsszerepet tölt be az immunsejtek tímuszban lezajló differenciálódásában és az autoreaktív sejtek eltávolításában. A tímuszban folymatosan keletkező apoptotikus sejtek gyors és alapos eltávolítása a környező makrofágok által történik (efferocitózis), ami általában gyulladás nélkül megy végbe. Ez részben annak köszönhető, hogy az elhaló sejteket fagocitáló makrofágok gyulladáscsökkentő mediátorokat termelnek. Emellett masszív apoptózis figyelhető meg gyulladásos környezetben is, ahol a neutrofilek feladatuk végeztével apoptózissal halnak el. Ebben az esetben a makrofágok által kibocsátott szignálok a gyulladás mielőbbi megfékezését szolgálják. Laborunkban folyó korábbi kutatások két ilyen efferocitózis-függően felszabaduló mediátort azonosítottak: az adenozint és a retinsavakat. Disszertációmban azt vizsgáltam, hogy ezek a szignálok hogyan járulnak hozzá a jól működő és hatékony apopto-fagocitózis rendszer kialakításához.

Összegezve elmondható, hogy a tímuszban a makrofágok által apoptotikus sejtfelvétel hatására termelődő retinoidok és adenozin együttesen hozzájárulnak az éretlen timociták neglekcióval történő elhalásához. Továbbá a retinoidok elősegítik az apoptotikus sejtek fagocitózisának folyamatát, míg az adenozin csökkenti a fagocitáló makrofágokban az apoptotikus sejtfelvétel által indukált kemoattraktánsok felszabadulását. Eredményeim a retinoidok által kiváltott timocita sejthalál részletes mechanizmusát írják le. Úgy véljük, hogy a retinoidok a mitokondriális útvonalat aktiválva egy Nur77-függő apoptótikus folyamatot indítanak el, hozzájárulva az éretlen timociták neglekcióval történő hatékony eltávolításához és ezáltal a megfelelően működő T-sejt populáció kialakulásához.
Gyulladási körülmények között a retinsavak azon túl, hogy elősegítik az apoptotikus sejtek efferocitózisát, hozzájárulnak a regulatorikus T-sejtek kialakulásához. Az adenozin másrészt gátolja az LPS-kiváltotta gyulladáskeltő mediátorok képződését a makrofágokban, védelmet nyújtva a túlzott, kontrollálatlan immunválasz kialakulásával szemben. Adataim azt mutatják, hogy az adenozin makrofágokra gyakorolt ezen hatásai az A_{2A} receptorokon keresztül valósulnak meg. A receptor aktiválása fokozza az LPS jelátvitel egyik negatív szabályozó fehérjéjének, a DUSP1 foszfatáznak a kifejeződését. Attól függően, hogy az efferocitózis milyen biológiai környezetben megy végbe, az apoptotikus sejtet fagocitáló makrofágok által

termelt szolubilis biomolekulák számos különböző biológiai folyamat irányításában vehetnek részt és eredményeim hozzájárulnak szerepük és hatásmechanizmusuk tisztább megértéséhez.

8. REFERENCES

Abraham SM, Clark AR. 2006. Dual-specificity phosphatase 1: a critical regulator of innate immune responses. *Biochem Soc Trans*. 34(Pt 6):1018-23.

Aderem A, Ulevitch RJ. 2000. Toll-like receptors in the induction of the innate immune response. *Nature*. 406(6797):782-7.

Akira S, Uematsu S, Takeuchi O. 2006. Pathogen recognition and innate immunity.*Cell*. 124(4):783-801.

Antonioli L, Blandizzi C, Csóka B, Pacher P, Haskó G. 2015. Adenosine signalling in diabetes mellitus--pathophysiology and therapeutic considerations.*Nat Rev Endocrinol*. 11(4):228-41.

Arnoult D, Gaume B, Karbowski M, Sharpe JC, Cecconi F, Youle RJ. 2003. Mitochondrial release of AIF and EndoG requires caspase activation downstream of Bax/Bak-mediated permeabilization. *EMBO J* 22: 4385–4399.

Ashkenazi A, Dixit V M. 1998. Death receptors: signaling and modulation. *Science.* 281:1305–1308-

Ashkenazi A, Dixit VM. 1999. Apoptosis control by death and decoy receptors. *Curr Opin Cell Biol*. 11(2):255-60.

Ashkenazi A, Salvesen G. 2014. Regulated cell death: signaling and mechanisms. *Annu Rev Cell Dev Biol*. 30:337-56.

Barletta KE, Ley K, Mehrad B. 2012.Regulation of neutrophil function by adenosine.*Arterioscler Thromb Vasc Biol*. 32(4):856-64.

Barnhart BC, Alappat EC, Peter ME. The CD95 type I/type II model. *Semin Immunol.* 2003 Jun;15(3):185-93.

Blomhoff R, Blomhoff HK. 2006. Overview of retinoid metabolism and function. *J Neurobiol*. 66(7):606-30.

Bono MR, Tejon G, Flores-Santibañez F, Fernandez D, Rosemblatt M, Sauma D. 2016. Retinoic Acid as a Modulator of T Cell Immunity. Nutrients.8(6).pii:E349.

Bouillet P, Metcalf D, Huang DC, Tarlinton DM, Kay TW, Köntgen F, Adams JM, Strasser A. Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity. *Science*. 286(5445):1735-8.

Bouillet P, Purton JF, Godfrey DI, Zhang LC, Coultas L, Puthalakath H, Pellegrini M, Cory S, Adams JM, Strasser A, et al. 2002. BH3-only Bcl-2 family member Bim is required for apoptosis of autoreactive thymocytes. *Nature* 415: 922–926

Bouma MG, Stad RK, van den Wildenberg FA, Buurman WA. 1994. Differential regulatory effects of adenosine on cytokine release by activated human monocytes. *J Immunol*. 153(9):4159-68.

Brown S., Heinisch I., Ross E., Shaw K., Buckley C.D., Savill J. 2002. Apoptosis disables CD31-mediated cell detachment from phagocytes promoting binding and engulfment. Nature. 418:200–203

Bshesh K, Zhao B, Spight D, Biaggioni I, Feokistov I, Denenberg A, Wong HR, Shanley TP. 2002. The A2A receptor mediates an endogenous regulatory pathway of cytokine expression in THP-1 cells. *J Leukoc Biol*. 72(5):1027-36.

Caivano M, Cohen P. 2000. Role of mitogen-activated protein kinase cascades in mediating lipopolysaccharide-stimulated induction of cyclooxygenase-2 and IL-1 beta in RAW264 macrophages. *J Immunol*. 164(6):3018-25.

Calnan BJ, Szychowski S, Chan FK, Cado D, Winoto A. 1995. A role for the orphan steroid receptor Nur77 in apoptosis accompanying antigen-induced negative selection.*Immunity*. 3(3):273-82.

Cao X, Liu W, Lin F, Li H, Kolluri SK, Lin B, Han YH, Dawson MI, Zhang XK. 2004. Retinoid X receptor regulates Nur77/TR3-dependent apoptosis [corrected] by modulating its nuclear export and mitochondrial targeting. *Mol Cell Biol.* 24(22):9705-25.

Chan FK, Chun HJ, Zheng L, Siegel RM, Bui KL, Lenardo MJ. 2000. A domain in TNF receptors that mediates ligand-independent receptor assembly and signaling. *Science*.288:2351–2354.

Chang L, Karin M. 2001. Mammalian MAP kinase signalling cascades.*Nature.* 410(6824):37-40.

Chang M, Jin W, Sun SC. 2009. Peli1 facilitates TRIF-dependent Toll-like receptor signaling and proinflammatory cytokine production. *Nat Immunol*. 10(10):1089-95.

Chaplin DD. 2010. Overview of the immune response.*J Allergy Clin Immunol*. 125(2 Suppl 2):S3-23.

Chen HC, Kanai M, Inoue‐Yamauchi A, Tu HC, Huang Y, Ren D, Kim H, Takeda S, Reyna DE, Chan PM et al. 2015. An interconnected hierarchical model of cell death regulation by the BCL‐2 family.*Nat Cell Biol.* 17 (10): 1270–81.

Chen P, Li J, Barnes J, Kokkonen GC, Lee JC, Liu Y. 2002. Restraint of proinflammatory cytokine biosynthesis by mitogen-activated protein kinase phosphatase-1 in lipopolysaccharide-stimulated macrophages.*J Immunol.*169(11):6408-16.

Chen W, Jin W, Hardegen N, Lei KJ, Li L, Marinos N, McGrady G, Wahl SM. 2003.Conversion of peripheral CD4 + CD25− naive T cells to CD4 + CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3.*J. Exp. Med.* 198:1875-1886.

Chen Y, Corriden R, Inoue Y, Yip L, Hashiguchi N, Zinkernagel A, Nizet V, Insel PA, Junger WG. 2006. ATP release guides neutrophil chemotaxis via P2Y2 and A3 receptors. *Science.* 314:1792–1795.

Cheng LE, Chan FK, Cado D, and Winoto A. 1997.Functional redundancy of the Nur77 and Nor-1 orphan steroid receptors in T cell apoptosis.*EMBO J.* 16:1865–1875

Chipuk JE, Bouchier-Hayes L, Green DR. 2006. Mitochondrial outer membrane permeabilization during apoptosis: the innocent bystander scenario. *Cell Death Differ.* 13:1396–402

Chipuk JE, Moldoveanu T, Llambi F, Parsons MJ, Green DR. 2010.The BCL-2 family reunion.*Mol Cell*. 37(3):299-310.

Chung EY, Liu J, Homma Y, Zhang Y, Brendolan A, Saggese M, Han J, Silverstein R, Selleri L, Ma X. 2007. Interleukin-10 expression in macrophages during phagocytosis of apoptotic cells is mediated by homeodomain proteins Pbx1 and Prep-1. *Immunity.* 27:952–964

Cohen HB, Briggs KT, Marino JP, Ravid K, Robson SC, Mosser DM. 2013. TLR stimulation initiates a CD39-based autoregulatory mechanism that limits macrophage inflammatory responses. *Blood*. 122(11): 1935–1945.

Cory S, Adams JM. 2002. The Bcl2 family: regulators of the cellular life-or-death switch. *Nat Rev Cancer*. 2(9):647-56.

Cronstein BN, Daguma L, Nichols D, Hutchison AJ, Williams M. 1990. The adenosine/neutrophil paradox resolved: human neutrophils possess both A1 and A2 receptors that promote chemotaxis and inhibit O2 generation, respectively. *J Clin Invest*. 85:1150–1157.

Cronstein BN, Levin RI, Philips M, Hirschhorn R, Abramson SB, Weissmann G. 1992. Neutrophil adherence to endothelium is enhanced via adenosine A1 receptors and inhibited via adenosine A2 receptors. *J Immunol*. 148(7):2201-6.

Curotto de Lafaille MA, Lafaille JJ. 2009. Natural and adaptive Foxp3+ regulatory t cells: More of the same or a division of labor. *Immunity.* 30: 626–635.

Cvetanovic M, Ucker DS. 2004. Innate immune discrimination of apoptotic cells: repression of proinflammatory macrophage transcription is coupled directly to specific recognition.*J Immunol*. 172(2):880-9.

Csóka B, Németh ZH, Virág L, Gergely P, Leibovich SJ, Pacher P, Sun CX, Blackburn MR, Vizi ES, Deitch EA, Haskó G. 2007. A2A adenosine receptors and C/EBPbeta are crucially required for IL-10 production by macrophages exposed to Escherichia coli. *Blood.* 110(7):2685-95.

Dai H, Pang YP, Ramirez-Alvarado M, Kaufmann SH. 2014.Evaluation of the BH3-only protein Puma as a direct Bak activator.*J Biol Chem*. 289(1):89-99

Dickens LS, Powley IR, Hughes MA, MacFarlane M. 2012. The "complexities" of life and death: Death receptor signalling platforms. *Exp Cell Res* 318: 1269–1277.

Dong C, Davis RJ, Flavell RA. 2002. MAP kinases in the immune response. *Annu Rev Immunol.* 20:55-72.

Du H, Wolf J, Schafer B, Moldoveanu T, Chipuk JE, Kuwana T. 2011. BH3 domains other than Bim and Bid can directly activate Bax/Bak. J Biol Chem. 2011; 286 (1): 491–501.

Duester G, Farrés J, Felder MR, Holmes RS, Höög JO, Parés X, Plapp BV, Yin SJ, Jörnvall H. 1999. Recommended nomenclature for the vertebrate alcohol dehydrogenase gene family.*Biochem Pharmacol*. 58(3):389-95.

Durbin JE, Hackenmiller R, Simon MC, Levy DE. 1996. Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease.*Cell*. 84(3):443-50.

Eckelman BP, Salvesen GS, Scott FL. 2006. Human inhibitor of apoptosis proteins: Why XIAP is the black sheep of the family. *EMBO Rep*. 7: 988–994.

Elliott MR, Chekeni FB, Trampont PC, Lazarowski ER, Kadl A, Walk SF, Park D, Woodson RI, Ostankovich M, Sharma P, et al. 2009. Nucleotides released by apoptotic cells act as a find-me signal to promote phagocytic clearance. *Nature.* 461:282–286.

Elliott MR, Ravichandran KS. 2010. Clearance of apoptotic cells: implications in health and disease. *J Cell Biol*. 189(7): 1059–1070.

Elmore S. 2007. Apoptosis: A Review of Programmed Cell Death. *Toxicol Pathol*. 35(4): 495–516.

Elson G, Eisenberg M, Garg C, Outram S, Ferrante CJ, Hasko G, Leibovich SJ. 2013. Induction of murine adenosine A(2A) receptor expression by LPS: analysis of the 5' upstream promoter. *Genes Immun.* 14(3):147-53.

Eltzschig HK, Faigle M, Knapp S, Karhausen J, Ibla J, Rosenberger P, Odegard KC, Laussen PC, Thompson LF, Colgan SP. 2006. Endothelial catabolism of extracellular adenosine during hypoxia: the role of surface adenosine deaminase and CD26. *Blood.* 108(5):1602-10.

Fadok VA, Bratton DL, Frasch SC, Warner ML, Henson PM. 1998. The role of phosphatidylserine in recognition of apoptotic cells by phagocytes.Cell Death Differ. 5(7):551-62.

Faggioli L, Costanzo C, Donadelli M, Palmieri M. 2004. Activation of the Interleukin-6 promoter by a dominant negative mutant of c-Jun. *Biochim Biophys Acta*. 1692(1):17-24.

Falvo JV, Parekh BS, Lin CH, Fraenkel E, Maniatis T. 2000. Assembly of a functional beta interferon enhanceosome is dependent on ATF-2-c-jun heterodimer orientation. *Mol Cell Biol*. 20(13):4814-25.

Farjo KM, Moiseyev G, Nikolaeva O, Sandell LL, Trainor PA, Ma JX. 2011. RDH10 is the primary enzyme responsible for the first step of embryonic Vitamin A metabolism and retinoic acid synthesis. *Dev Biol*. 357(2):347-55.

Ferrante CJ, Pinhal-Enfield G, Elson G, Cronstein BN, Hasko G, Outram S, Leibovich SJ. 2013. The adenosine-dependent angiogenic switch of macrophages to an M2-like phenotype is independent of interleukin-4 receptor alpha (IL-4Rα) signaling. *Inflammation*. 36(4):921-31.

Festjens N, Vanden Berghe T, Cornelis S, Vandenabeele P. 2007. RIP1, a kinase on the crossroads of a cell's decision to live or die.*Cell Death Differ*. 14(3):400-10.

Fotheringham JA, Mayne MB, Grant JA, Geiger JD. 2004. Activation of adenosine receptors inhibits tumor necrosis factor-alpha release by decreasing TNF-alpha mRNA stability and p38 activity. *Eur J Pharmacol*. 497(1):87-95.

Fredholm BB, IJzerman AP, Jacobson KA, Linden J, Müller CE. 2011. International Union of Basic and Clinical Pharmacology. LXXXI. Nomenclature and classification of adenosine receptors--an update. *Pharmacol Rev.* 63(1):1-34.

Fredholm BB. 2007. Adenosine, an endogenous distress signal, modulates tissue damage and repair. *Cell Death Differ*. 14(7):1315-23.

Gameiro J, Nagib P, Verinaud L. 2010. The thymus microenvironment in regulating thymocyte differentiation.*Cell Adh Migr*. 4(3): 382–390.

Garabuczi É, Kiss B, Felszeghy S, Tsay GJ, Fésüs L, Szondy Z. 2013. Retinoids produced by macrophages engulfing apoptotic cells contribute to the appearance of transglutaminase 2 in apoptotic thymocytes. Amino Acids. 2013 Jan;44(1):235-44.

Gardai SJ, McPhillips KA, Frasch SC, Janssen WJ, Starefeldt A, Murphy-Ullrich JE , Bratton DL, Oldenborg PA, Michalak M, Henson PM. 2005. Cell-surface calreticulin initiates clearance of viable or apoptotic cells through trans-a ctivation of LRP on the phagocyte. Cell 123: 321–334.

Germain P, Chambon P, Eichele G, Evans RM, Lazar MA, Leid M, De Lera AR, Lotan R, Mangelsdorf DJ, Gronemeyer H. 2006a. International Union of Pharmacology.LXIII. Retinoid X receptors.*Pharmacol Rev*.58(4):760-72.

Germain P, Chambon P, Eichele G, Evans RM, Lazar MA, Leid M, De Lera AR, Lotan R, Mangelsdorf DJ, Gronemeyer H. 2006b. International Union of Pharmacology. LX. Retinoic acid receptors. *Pharmacol Rev.* 58(4):712-25.

Germain RN. 2002. T-cell development and the CD4-CD8 lineage decision. *Nat Rev Immunol*. 2(5):309-22.

Ghosh S, May MJ, Kopp EB. 1998. NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. *Annu Rev Immunol*. 16:225-60.

Gioannini TL, Weiss JP. 2007. Regulation of interactions of Gram-negative bacterial endotoxins with mammalian cells. *Immunol Res*. 39(1-3):249-60.

Gray DH, Kupresanin F, Berzins SP, Herold MJ, O'Reilly LA, Bouillet P, Strasser A. 2012. The BH3-only proteins Bim and Puma cooperate to impose deletional tolerance of organ-specific antigens. *Immunity*. 37(3):451-62.

Green DR, Llambi F. 2015. Cell Death Signaling.*Cold Spring Harb Perspect Biol* 7:a006080

Green DR, OguinTH Martinez J. 2016. The clearance of dying cells: table for two. *Cell Death Differ*. 23: 915–926

Gregory CD, Devitt A, Moffatt O. 1998. Roles of ICAM-3 and CD14 in the recognition and phagocytosis of apoptotic cells by macrophages.*Biochem Soc Trans* 26: 644–649

Gregory CD, Pound JD. 2011. Cell death in the neighbourhood: direct microenvironmental effects of apoptosis in normal and neoplastic tissues.*J Pathol*. 223(2):177-94.

Gude D.R., Alvarez S.E., Paugh S.W., Mitra P., Yu J., Griffiths R., Barbour S.E., Milstien S., Spiegel S. 2008. Apoptosis induces expression of sphingosine kinase 1 to release sphingosine-1-phosphate as a "come-and-get-me" signal. *FASEB J*. 22:2629–2638

Guevara Patiño JA, Ivanov VN, Lacy E, Elkon KB, Marino MW, Nikolic-Zugić J. 2000. TNF-alpha is the critical mediator of the cyclic AMP-induced apoptosis of CD8+4+ double-positive thymocytes. *J Immunol.* 164(4):1689-94.

Guha M, Mackman N. 2002. The phosphatidylinositol 3-kinase-Akt pathway limits lipopolysaccharide activation of signaling pathways and expression of inflammatory mediators in human monocytic cells. *J Biol Chem*. 277(35):32124-32.

Guo B, Cheng G. 2007. Modulation of the interferon antiviral response by the TBK1/IKKi adaptor protein TANK.*J Biol Chem*. 282(16):11817-26.

Häcker G. 2000. The morphology of apoptosis. Cell Tissue Res. 301(1):5-17.

Häcker H, Karin M. 2006. Regulation and function of IKK and IKK-related kinases.*Sci STKE*. 2006(357):re13.

Hall JA, Grainger JR, Spencer SP, Belkaid Y. 2011. The role of retinoic acid in tolerance and immunity.*Immunity*. 35(1):13-22.

Hammer M, Mages J, Dietrich H, Servatius A, Howells N, Cato AC, Lang R, 2006. Dual specificity phosphatase 1 (DUSP1) regulates a subset of LPS-induced genes and protects mice from lethal endotoxin shock. *J Exp Med*. 203(1):15-20.

Hanayama R, Tanaka M, Miwa K, Shinohara A, Iwamatsu A, Nagata S. 2002. Identification of a factor that links apoptotic cells to phagocytes. *Nature*. 417: 182–187.

Hao Z, Mak TW. 2010. Type I and type II pathways of Fas-mediated apoptosis are differentially controlled by XIAP. *J Mol Cell Biol.* 2(2):63-4.

Hashikawa T, Hooker SW, Maj JG, Knott-Craig CJ, Takedachi M, Murakami S, Thompson LF. 2004. Regulation of adenosine receptor engagement by ecto-adenosine deaminase. FASEB J. 2004 Jan;18(1):131-3.

Haskó G, Cronstein BN. 2013. Regulation of inflammation by adenosine.*Front Immunol*. 4: 85.

Haskó G, Cronstein BN. 2004. Adenosine: an endogenous regulator of innate immunity. *Trends Immunol*. 25(1):33-9.

Haskó G, Kuhel DG, Chen JF, Schwarzschild MA, Deitch EA, Mabley JG, Marton A, Szabó C. 2000. Adenosine inhibits IL-12 and TNF-[alpha] production via adenosine A2a receptor-dependent and independent mechanisms. *FASEB J.* 14(13):2065-74.

Haskó G, Pacher P. 2012. Regulation of macrophage function by adenosine. *Arterioscler.Thromb.Vasc. Biol*. 32, 865–869.

Haskó G, Szabó C, Németh ZH, Kvetan V, Pastores SM, Vizi ES. 1996. Adenosine receptor agonists differentially regulate IL-10, TNF-alpha, and nitric oxide production in RAW 264.7 macrophages and in endotoxemic mice. *J Immunol*. 157(10):4634-40.

Hassan M, Watari H, AbuAlmaaty A, Ohba Y, Sakuragi N. 2014. Apoptosis and molecular targeting therapy in cancer.*Biomed Res Int*. 2014:150845

Helenius M, Jalkanen S, Yegutkin G. 2012. Enzyme-coupled assays for simultaneous detection of nanomolar ATP, ADP, AMP, adenosine, inosine and pyrophosphate concentrations in extracellular fluids. *Biochim Biophys Acta*. 1823(10):1967-75.

Henshall DC, Engel T. 2013. Contribution of apoptosis-associated signaling pathways to epileptogenesis: lessons from Bcl-2 family knockouts. *Front Cell Neurosci*. 7:110.

Hernandez JB, Newton RH, Walsh CM. 2010. Life and death in the thymus:cell death signaling during T cell development. *Curr Opin Cell Biol*. 22(6):865-71.

Heyman RA, Mangelsdorf DJ, Dyck JA, Stein RB, Eichele G, Evans RM, Thaller C. 1992. 9-cis retinoic acid is a high affinity ligand for the retinoid X receptor. *Cell*. 68:397–406.

Himes SR, Sester DP, Ravasi T, Cronau SL, Sasmono T, Hume DA. 2006. The JNK are important for development and survival of macrophages. *J Immunol*. 176(4):2219-28.

Hochreiter-Hufford A, Ravichandran KS. 2013. Clearing the dead: apoptotic cell sensing, recognition, engulfment, and digestion. *Cold Spring Harb Perspect Biol*. 5(1):a008748.

Hsieh YF, Liu GY, Lee YJ, Yang JJ, Sándor K, Sarang Z, Bononi A, Pinton P, Tretter L, Szondy Z et al. 2013. Transglutaminase 2 contributes to apoptosis induction in Jurkat T cells by modulating Ca2+ homeostasis via cross-linking RAP1GDS1. *PLoS One*. 8(12):e81516.

Huynh ML, Fadok VA, Henson PM. 2002. Phosphatidylserine-dependent ingestion of apoptotic cells promotes TGF-beta1 secretion and the resolution of inflammation. J Clin Invest. 2002 Jan;109(1):41-50.

Janeway CA Jr, Travers P, Walport M, et al. 2001. Immunobiology: The Immune System in Health and Disease. 5th edition.*Garland Science*; 2001.

Karin M, Greten FR. 2005. NF-kappaB: linking inflammation and immunity to cancer development and progression. *Nat Rev Immunol*. 5(10):749-59.

Kawai T, Adachi O, Ogawa T, Takeda K, Akira S. 1999. Unresponsiveness of MyD88-deficient mice to endotoxin.*Immunity*. 11(1):115-22.

Kawai T, Takeuchi O, Fujita T, Inoue J, Mühlradt PF, Sato S, Hoshino K, Akira S. 2001. Lipopolysaccharide stimulates the MyD88-independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes. *J Immunol*. 167(10):5887-94.

Keating SE, Maloney GM, Moran EM, Bowie AG. 2007. IRAK-2 participates in multiple toll-like receptor signaling pathways to NFkappaB via activation of TRAF6 ubiquitination. *J Biol Chem*. 282(46):33435-43.

Kedishvili NY. 2013. Enzymology of retinoic acid biosynthesis and degradation. *J Lipid Res*. 54(7):1744-60.

Kerr JFR, Wyllie AH, and Currie AR. 1972. Apoptosis: A Basic Biological Phenomenon with Wide-ranging Implications in Tissue Kinetics.*Br J Cancer*. 26(4): 239–257

Kim DS, Han JH, Kwon HJ. 2003. NF-kappaB and c-Jun-dependent regulation of macrophage inflammatory protein-2 gene expression in response to lipopolysaccharide in RAW 264.7 cells. *Mol Immunol*. 40(9):633-43.

Kim H, Rafiuddin-Shah M, Tu HC, Jeffers JR, Zambetti GP, Hsieh JJ, Cheng EH. 2006. Hierarchical regulation of mitochondrion-dependent apoptosis by BCL-2 subfamilies. *Nat Cell Biol*. 8(12):1348-58.

Kim H, Tu HC, Ren D, Takeuchi O, Jeffers JR, Zambetti GP, Hsieh JJ, Cheng EH. 2009. Stepwise activation of BAX and BAK by tBID, BIM, and PUMA initiates mitochondrial apoptosis. *Mol Cell*. 36(3):487-99.

Kinchen JM, Ravichandran KS. 2008. Phagosome maturation: going through the acid test. *Nat Rev Mol Cell Biol*. 9:781–795.

Kiss I, Oskolás H, Tóth R, Bouillet P, Tóth K, Fülöp A, Scholtz B, Ledent C, Fésüs L, Szondy Z. 2006. Adenosine A2A receptor-mediated cell death of mouse thymocytes involves adenylate cyclase and Bim and is negatively regulated by Nur77. *Eur J Immunol*. 36(6):1559-71.

Kiss I, Rühl R, Szegezdi E, Fritzsche B, Tóth B, Pongrácz J, Perlmann T, Fésüs L, Szondy Z. 2008. Retinoid receptor-activating ligands are produced within the mouse thymus during postnatal development. *Eur J Immunol*. 38(1):147-55.

Kobayashi N, Karisola P, Peña-Cruz V, Dorfman DM, Jinushi M, Umetsu SE, Butte MJ, Nagumo H, Chernova I, Zhu B, et al. 2007. TIM-1 and TIM-4 glycoproteins bind phosphatidylserine and mediate uptake of apoptotic cells. Immunity. 27:927–940

Koh TJ, DiPietro LA. 2011. Inflammation and wound healing: the role of the macrophage. *Expert Rev Mol Med.* 13:e23.

Konkel JE, Jin W, Abbatiello B, Grainger JR, Chen WJ. 2014. Thymocyte apoptosis drives the intrathymic generation of regulatory T cells. Proc Natl Acad Sci U S A. 111(4):E465-73.

Köröskényi K, Duró E, Pallai A, Sarang Z, Kloor D, Ucker DS, Beceiro S, Castrillo A, Chawla A, Ledent CA et al. 2011. Involvement of adenosine A2A receptors in engulfment-dependent apoptotic cell suppression of inflammation.J Immunol. 186(12):7144-55.

Kreckler LM, Gizewski E, Wan TC, Auchampach JA. 2009. Adenosine suppresses lipopolysaccharide-induced tumor necrosis factor-alpha production by murine macrophages through a protein kinase A- and exchange protein activated by cAMP-independent signaling pathway. *J Pharmacol Exp Ther.* 331(3):1051-61.

Kreckler LM, Wan TC, Ge ZD, Auchampach JA. 2006. Adenosine inhibits tumor necrosis factor-alpha release from mouse peritoneal macrophages via A2A and A2B but not the A3 adenosine receptor. *J Pharmacol Exp Ther*. 317(1):172-80.

Krueger A, Baumann S, Krammer PH, Kirchhoff S. 2001. FLICE-inhibitory proteins: regulators of death receptor-mediated apoptosis. *Mol Cell Biol*. 21(24):8247-54.

Kuang AA, Cado D, Winoto A. 1999. Nur77 transcription activity correlates with its apoptotic function in vivo. *Eur J Immunol*. 29(11):3722-8.

Lappas CM, Day YJ, Marshall MA, Engelhard VH, Linden J. 2006. Adenosine A2A receptor activation reduces hepatic ischemia reperfusion injury by inhibiting CD1d-dependent NKT cell activation. *J Exp Med*. 203(12):2639-48.

Latini S, Pedata F. 2001. Adenosine in the central nervous system: release mechanisms and extracellular concentrations. *J Neurochem*. 79(3):463-84.

Lauber K, Blumenthal SG, Waibel M, Wesselborg S. 2004. Clearance of apoptotic cells: getting rid of the corpses. *Mol Cell*. 14(3):277-87.

Lauber K, Bohn E, Kröber SM, Xiao YJ, Blumenthal SG, Lindemann RK, Marini P, Wiedig C, Zobywalski A, Baksh S, et al. 2003. Apoptotic cells induce migration of phagocytes via caspase-3-mediated release of a lipid attraction signal. *Cell.* 113:717–730

Lavrik I, Golks A, Krammer PH. 2005. Death receptor signaling.*J Cell Sci*. 118(Pt 2):265-7.

Ledent C, Vaugeois JM, Schiffmann SN, Pedrazzini T, El Yacoubi M, Vanderhaeghen JJ, Costentin J, Heath JK, Vassart G, Parmentier M. 1997. Aggressiveness, hypoalgesia and high blood pressure in mice lacking the adenosine A2a receptor.*Nature.* 388(6643):674-8.

Lee SL, Wesselschmidt RL, Linette GP, Kanagawa O, Russel JH, Milbrandt J. 1995. Unimpaired thymic and peripheral T cell death in mice lacking the nuclear receptor NGFI-B (Nur77).*Science* 269: 532-535.

Lemke G, Rothlin CV. 2008. Immunobiology of the TAM receptors.*Nat. Rev. Immunol*. 8:327–336.

Letai A, Bassik MC, Walensky LD, Sorcinelli MD, Weiler S, Korsmeyer SJ. 2002. Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics. *Cancer Cell* 2: 183–192.

Liebermann DA, Hoffman B. 2002. Myeloid differentiation (MyD)/growth arrest DNA damage (GADD) genes in tumor suppression, immunity and inflammation. *Leukemia.* 16(4):527-41.

Lin B, Kolluri SK, Lin F, Liu W, Han YH, Cao X, Dawson MI, Reed JC, Zhang XK. 2004. Conversion of Bcl-2 from protector to killer by interaction with nuclear orphan receptor Nur77/TR3. *Cell*. 116(4):527-40.

Lin M, Zhang M, Abraham M, Smith SM, Napoli JL. 2003. Mouse retinal dehydrogenase 4 (RALDH4), molecular cloning, cellular expression, and activity in 9-cis-retinoic acid biosynthesis in intact cells. *J Biol Chem*. 278(11):9856-61.

Lin SC, Lo YC, Wu H. 2010.Helical assembly in the MyD88-IRAK4-IRAK2 complex in TLR/IL-1R signalling.*Nature*. 465(7300):885-90.

Liu ZM, Wang KP, Ma J, Guo Zheng S. 2015. The role of all-trans retinoic acid in the biology of Foxp3+ regulatory T cells.*Cell Mol Immunol*. 12(5):553-7.

Lloyd HG, Fredholm BB. 1995. Involvement of adenosine deaminase and adenosine kinase in regulating extracellular adenosine concentration in rat hippocampal slices. *Neurochem Int.* 26(4):387-95.

Locksley RM, Killeen N, Lenardo MJ. 2001. The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell*. 104(4):487-501.

Lomonosova E, Chinnadurai G. 2008. BH3-only proteins in apoptosis and beyond: an overview. *Oncogene*. 27(Suppl 1):S2-19.

Lu L, Lan Q, Li Z, Zhou X, Gu J, Li Q, Wang J, Chen M, Liu Y, Shen Y, Brand DD, Ryffel B, Horwitz DAet al., 2014. Critical role of all-trans retinoic acid in stabilizing human natural regulatory T cells under inflammatory conditions.*Proc Natl Acad Sci U S A*. 111(33):E3432-40.

Lu YC, Yeh WC, Ohashi PS. 2008. LPS/TLR4 signal transduction pathway. *Cytokine.* 42(2):145-51.

Lukashev D, Ohta A, Apasov S, Chen JF, Sitkovsky M. 2004. Cutting edge: Physiologic attenuation of proinflammatory transcription by the Gs protein-coupled A2A adenosine receptor in vivo. *J Immunol*. 173(1):21-4.

Luo X, Budihardjo I, Zou H, Slaughter C, Wang X. 1998. Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. *Cell* 94:481–90.

Malone MH, Wang Z, Distelhorst CW. 2004. The glucocorticoid-induced gene tdag8 encodes a pro-apoptotic G protein-coupled receptor whose activation promotes glucocorticoid-induced apoptosis. *J Biol Chem*. 279(51):52850-9.

Malorni W, Farrace MG, Matarrese P, Tinari A, Ciarlo L, Mousavi-Shafaei P, D'Eletto M, Di Giacomo G, Melino G, Palmieri L et al. 2013. The adenine nucleotide translocator 1 acts as a type 2 transglutaminase substrate: implications for mitochondrial-dependent apoptosis. *Cell Death Differ*. 16(11):1480-92.

Mariño G, Niso-Santano M, Baehrecke EH, Kroemer G. 2014. Self-consumption: the interplay of autophagy and apoptosis. *Nat Rev Mol Cell Biol*. 15(2):81-94.

Martin KR, Ohayon D, Witko-Sarsat V. 2015. Promoting apoptosis of neutrophils and phagocytosis by macrophages: novel strategies in the resolution of inflammation.*Swiss Med Wkly*. 145:w14056.

Maxwell MA, Muscat GE. 2006. The NR4A subgroup: immediate early response genes with pleiotropic physiological roles. *Nucl Recept Signal*. 4:e002.

McColl SR, St-Onge M, Dussault AA, Laflamme C, Bouchard L, Boulanger J, Pouliot M. 2006. Immunomodulatory impact of the A2A adenosine receptor on the profile of chemokines produced by neutrophils. FASEB J. 20(1):187-9.

McDonald PP, Fadok VA, Bratton D, Henson PM. 1999. Transcriptional and translational regulation of inflammatory mediator production by endogenous TGF-beta in macrophages that have ingested apoptotic cells.*J Immunol*. 163(11):6164-72.

Medzhitov R, Preston-Hurlburt P, Kopp E, Stadlen A, Chen C, Ghosh S, Janeway CA Jr. 1998. MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways. *Mol Cell*. 2(2):253-8.

Micheau O, Tschopp J. 2003. Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes.*Cell*. 114(2):181-90.

Milhas D, Cuvillier O, Therville N, Clavé P, Thomsen M, Levade T, Benoist H, Ségui B. 2005. Caspase-10 triggers Bid cleavage and caspase cascade activation in FasL-induced apoptosis. *J Biol Chem*. 280(20):19836-42.

Miyake K. 2007. Innate immune sensing of pathogens and danger signals by cell surface Toll-like receptors.*Semin Immunol*. 19(1):3-10.

Moll UM, Marchenko N, Zhang XK. 2006. p53 and Nur77/TR3 - transcription factors that directly target mitochondria for cell death induction. *Oncogene*. 25(34):4725-43.

Moran AE, Holzapfel KL, Xing Y, Cunningham NR, Maltzman JS, Punt J, Hogquist KA. 2011. T cell receptor signal strength in Treg and iNKT cell development demonstrated by a novel fluorescent reporter mouse. *J Exp Med*.208(6):1279-89.

Moro H, Otero DC, Tanabe Y, David M. 2011. T cell-intrinsic and -extrinsic contributions of the IFNAR/STAT1-axis to thymocyte survival.*PLoS One.* 6(9):e24972.

Motshwene PG, Moncrieffe MC, Grossmann JG, Kao C, Ayaluru M, Sandercock AM, Robinson CV, Latz E, Gay NJ. 2009. An oligomeric signaling platform formed by the Toll-like receptor signal transducers MyD88 and IRAK-4. *J Biol Chem.* 284(37):25404-11.

Murphree LJ, Sullivan GW, Marshall MA, Linden J. 2005. Lipopolysaccharide rapidly modifies adenosine receptor transcripts in murine and human macrophages: role of NF-kappaB in A(2A) adenosine receptor induction. *Biochem J.* 391(Pt 3):575-80.

Nagata S, Hanayama R, Kawane K. 2010. Autoimmunity and the clearance of dead cells.*Cell*. 140(5):619-30.

Nakaya M, Tanaka M, Okabe Y, Hanayama R, Nagata S. 2006. Opposite effects of rho family GTPases on engulfment of apoptotic cells by macrophages.*J Biol Chem.* 281: 8836–8842.

Nakayama M, Akiba H, Takeda K, Kojima Y, Hashiguchi M, Azuma M, Yagita H, Okumura K. 2009. Tim-3 mediates phagocytosis of apoptotic cells and cross-presentation. *Blood*. 113: 3821–3830.

Németh ZH, Leibovich SJ, Deitch EA, Vizi ES, Szabó C, Hasko G. 2005. cDNA microarray analysis reveals a nuclear factor-kappaB-independent regulation of macrophage function by adenosine. *J Pharmacol Exp Ther*. 306(3):1042-9.

Németh ZH, Lutz CS, Csóka B, Deitch EA, Leibovich SJ, Gause WC, Tone M, Pacher P, Vizi ES, Haskó G. 2005. Adenosine augments IL-10 production by macrophages through an A2B receptor-mediated posttranscriptional mechanism. *J Immunol.* 175(12):8260-70.

Neumann M, Grieshammer T, Chuvpilo S, Kneitz B, Lohoff M, Schimpl A, Franza BR Jr, Serfling E. 1995. RelA/p65 is a molecular target for the immunosuppressive action of protein kinase A. *EMBO J.* 14(9):1991-2004.

Newton K, Dixit VM. 2012. Signaling in innate immunity and inflammation. *Cold Spring Harb Perspect Biol*. 4(3).

Nicholson DW. 1999. Caspase structure, proteolytic substrates, and function during apoptotic cell death. *Cell Death Differ*. 6(11):1028-42.

Ohkura N, Ito M, Tsukada T, Sasaki K, Yamaguchi K, Miki K. 1996. Structure, mapping and expression of a human NOR-1 gene, the third member of the Nur77/NGFI-B family.Biochim Biophys Acta. 1996 Sep 11;1308(3):205-14.

Opferman JT. 2008. Apoptosis in the development of the immune system.*Cell Death Differ*. 15(2):234-42.

Ortega-Gómez A, Perretti M, Soehnlein O. 2013 Resolution of inflammation: an integrated view.*EMBO Mol Med*. 5(5):661-74.

Palmer E. 2003. Negative selection--clearing out the bad apples from the T-cell repertoire.*Nat Rev Immunol*. 3(5):383-91.

Panther E, Corinti S, Idzko M, Herouy Y, Napp M, la Sala A, Girolomoni G, Norgauer J. 2003. Adenosine affects expression of membrane molecules, cytokine and chemokine release, and the T-cell stimulatory capacity of human dendritic cells. *Blood.* 01(10):3985-90.

Panther E, Idzko M, Herouy Y, Rheinen H, Gebicke-Haerter PJ, Mrowietz U, Dichmann S, Norgauer J. 2001. Expression and function of adenosine receptors in human dendritic cells.*FASEB J.* 15(11):1963-70.

Parés X, Farrés J, Kedishvili N, Duester G. 2008. Medium- and short-chain dehydrogenase/reductase gene and protein families : Medium-chain and short-chain dehydrogenases/reductases in retinoid metabolism. *Cell Mol Life Sci*. 65(24):3936-49.

Park D, Tosello-Trampont AC, Elliott MR, Lu M, Haney LB, Ma Z, Klibanov AL, Mandell JW, Ravichandran KS. 2007a. BAI1 is an engulfment receptor for apoptotic cells upstream of the ELMO/Dock180/Rac module. *Nature*. 450:430–434

Park S.Y., Jung M.Y., Kim H.J., Lee S.J., Kim S.Y., Lee B.H., Kwon T.H., Park R.W., Kim I.S. 2008. Rapid cell corpse clearance by stabilin-2, a membrane phosphatidylserine receptor.*Cell Death Differ*. 15:192–201

Parnaik R, Raff MC, Scholes J. 2000. Differences between the clearance of apoptotic cells by professional and non-professional phagocytes. Curr Biol.10(14):857-60.

Pauls E, Nanda SK, Smith H, Toth R, Arthur JS, Cohen P. 2013. Two phases of inflammatory mediator production defined by the study of IRAK2 and IRAK1 knock-in mice. *J Immunol*.191(5):2717-30.

Perlmann T, Jansson L. 1995. A novel pathway for vitamin A signaling mediated by RXR heterodimerization with NGFI-B and NURR1.*Genes Dev.* 9(7):769-82.

Philips A, Lesage S, Gingras R, Maira MH, Gauthier Y, Hugo P, Drouin J. Novel dimeric Nur77 signaling mechanism in endocrine and lymphoid cells. *Mol Cell Biol*. 17(10): 5946–5951.

Plotnikov A, Zehorai E, Procaccia S, Seger R. 2011. The MAPK cascades: signaling components, nuclear roles and mechanisms of nuclear translocation. *Biochim Biophys Acta*. 1813(9):1619-33.

Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, Du X, Birdwell D, Alejos E, Silva M, Galanos C, et al. 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science*. 282(5396):2085-8.

Rajpal A, Cho YA, Yelent B, Koza-Taylor PH, Li D, Chen E, Whang M, Kang C, Turi TG, Winoto A. 2003. Transcriptional activation of known and novel apoptotic pathways by Nur77 orphan steroid receptor.*EMBO J*.22(24):6526-36.

Ravichandran KS. 2010. Find-me and eat-me signals in apoptotic cell clearance: progress and conundrums. *J Exp Med*. 207(9): 1807–1817.

Ren D, Tu HC, Kim H, Wang GX, Bean GR, Takeuchi O, Jeffers JR, Zambetti GP, Hsieh JJ, Cheng EH. 2010. BID, BIM, and PUMA are essential for activation of the BAX- and BAK-dependent cell death program. *Science*. 330(6009):1390-3.

Ribes V, Otto DM, Dickmann L, Schmidt K, Schuhbaur B, Henderson C, Blomhoff R, Wolf CR, Tickle C, Dollé P. 2007. Rescue of cytochrome P450 oxidoreductase (Por) mouse mutants reveals functions in vasculogenesis, brain and limb patterning linked to retinoic acid homeostasis. *Dev Biol.* 303(1):66-81.

Robson SC, Sévigny J Zimmermann H. 2006. The E-NTPDase family of ectonucleotidases: Structure function relationships and pathophysiological significance. *Purinergic Signal*. 2(2): 409–430.

Rodriguez J, Lazebnik Y. 1999. Caspase-9 and Apaf-1 form an active holoenzyme. *Genes Dev*. 13, 3179−3184.

Rothwarf DM, Karin M. 1999. The NF-kappa B activation pathway: a paradigm in information transfer from membrane to nucleus. *Sci STKE*. 1999(5):RE1.

Rühl R. 2006. Method to determine 4-oxo-retinoic acids, retinoic acids and retinol in serum and cell extracts by liquid chromatography/diode-array detection atmospheric pressure chemical ionisation tandem mass spectrometry. *Rapid Commun Mass Spectrom*. 20(16):2497-504.

Rühl R, Krzyżosiak A, Niewiadomska-Cimicka A, Rochel N, Szeles L, Vaz B, Wietrzych-Schindler M, Álvarez S, Szklenar M, Nagy L et al. 2015. 9-cis-13,14-Dihydroretinoic Acid Is an Endogenous Retinoid Acting as RXR Ligand in Mice. *PLoS Genet*. 11(6):e1005213

Sachdeva S, Gupta M. 2013. Adenosine and its receptors as therapeutic targets: An overview. *Saudi Pharm J*. 21(3):245-53.

Sakahira H, Enari M, Nagata S. 1998. Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis.*Nature* 391: 96–99.

Samraj AK, Keil E, Ueffing N, Schulze-Osthoff K, Schmitz I. 2006. Loss of caspase-9 provides genetic evidence for the type I/II concept of CD95-mediated apoptosis. *J Biol Chem.* 281(40):29652-9.

Sándor K, Pallai A, Duró E, Legendre P, Couillin I, Sághy T, Szondy Z. 2016. Adenosine produced from adenine nucleotides through an interaction between apoptotic cells and engulfing macrophages contributes to the appearance of transglutaminase 2 in dying thymocytes. Amino Acids. 2016 May 28. [Epub ahead of print]

Sarang Z, Joós G, Garabuczi É, Rühl R, Gregory CD, Szondy Z. 2014. Macrophages engulfing apoptotic cells produce nonclassical retinoids to enhance their phagocytic capacity. *J Immunol*. 192(12):5730-8.

Savill J, Dransfield I, Gregory C, Haslett C. 2002. A blast from the past: clearance of apoptotic cells regulates immune responses. *Nat. Rev. Immunol*. 2: 965-975

Savill J, Fadok V. 2000. Corpse clearance defines the meaning of cell death. *Nature.* 407(6805):784-8.

Seamon KB, Padgett W, Daly JW. 1981. Forskolin: unique diterpene activator of adenylate cyclase in membranes and in intact cells. *Proc Natl Acad Sci U S A.* 78(6):3363-7

Shang Y, Baumrucker CR, Green MH. 1999. The induction and activation of STAT1 by all-trans-retinoic acid are mediated by RAR beta signaling pathways in breast cancer cells. *Oncogene*. 18(48):6725-32.

Sharma S, tenOever BR, Grandvaux N, Zhou GP, Lin R, Hiscott J. 2003. Triggering the interferon antiviral response through an IKK-related pathway.Science. 300(5622):1148-51.

Shi Y. 2002. Mechanisms of caspase activation and inhibition during apoptosis.*Mol Cell*. 9(3):459-70.

Si QS, Nakamura Y, Kataoka K. 1997. Adenosine inhibits superoxide production in rat peritoneal macrophages via elevation of cAMP level. Immunopharmacology. 36(1):1-7

Sipka S, Kovács I, Szántó S, Szegedi G, Brugós L, Bruckner G, József Szentmiklósi A. 2005. Adenosine inhibits the release of interleukin-1beta in activated human peripheral mononuclear cells. *Cytokine*. 31(4):258-63.

Sitnik KM, Kotarsky K, White AJ, Jenkinson WE, Anderson G, Agace WW. 2012. Mesenchymal cells regulate retinoic acid receptor-dependent cortical thymic epithelial cell homeostasis. *J Immunol.* 188(10):4801-9.

Smith SM, Levy NS, Hayes CE. 1987. Impaired immunity in vitamin A-deficient mice.

Soprano DR, Qin P, Soprano KJ. 2004. Retinoic acid receptors and cancers.*Annu Rev Nutr*. 24:201-21.

Suen AYW, BaldwinTA. 2012. Proapoptotic protein Bim is differentially required during thymic clonal deletion to ubiquitous versus tissue-restricted antigens. *Proc Natl Acad Sci U S A*. 109(3): 893–898.

Sullivan GW, Lee DD, Ross WG, DiVietro JA, Lappas CM, Lawrence MB, Linden J. 2004. Activation of A2A adenosine receptors inhibits expression of alpha 4/beta 1 integrin (very late antigen-4) on stimulated human neutrophils. *Leukoc Biol.* 75(1):127-34.

Surh CD, Sprent J. 1994. T-cell apoptosis detected in situ during positive and negative selection in the thymus.*Nature*. 372(6501):100-3.

Swantek JL, Cobb MH, Geppert TD. 1997. Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) is required for lipopolysaccharide stimulation of tumor necrosis factor alpha (TNF-alpha) translation: glucocorticoids inhibit TNF-alpha translation by blocking JNK/SAPK. *Mol Cell Biol*. 17(11):6274-82.

Szabó C, Scott GS, Virág L, Egnaczyk G, Salzman AL, Shanley TP, Haskó G. 1998. Suppression of macrophage inflammatory protein (MIP)-1α production and collagen-induced arthritis by adenosine receptor agonists.Br J Pharmacol. 125(2): 379–387.

Szondy Z, Garabuczi E, Joós G, Tsay GJ, Sarang Z. 2014. Impaired clearance of apoptotic cells in chronic inflammatory diseases: therapeutic implications. *Front Immunol*. 5:354.

Szondy Z, Garabuczi É, Tóth K, Kiss B, Köröskényi K. 2012. Thymocyte death by neglect: contribution of engulfing macrophages. *Eur J Immunol*. 42(7):1662-7.

Szondy Z, Reichert U., Bernardon JM, Michel S, Tóth R, Ancian P, Ajzner E, Fésüs L. 1997. Induction of apoptosis by retinoids and RAR gamma selective compounds in mouse thymocytes through a novel apoptosis pathway.*Mol. Pharmacol.* 51(6):972–982

Tait SW, Green DR. 2010. Mitochondria and cell death: outer membrane permeabilization and beyond.*Nat Rev Mol Cell Biol*. 11(9):621-32.

Thangaraju M, Sharma K, Leber B, Andrews DW, Shen SH, Srikant CB. 1999. Regulation of acidification and apoptosis by SHP-1 and Bcl-2. *J Biol Chem.* 274(41):29549-57.

Thiel M, Caldwell CC, Sitkovsky MV. 2003. The critical role of adenosine A2A receptors in downregulation of inflammation and immunity in the pathogenesis of infectious diseases. *Microbes Infect*.5:515–26.

Thompson J, Burger ML, Whang H, Winoto A. 2010. Protein kinase C regulates mitochondrial targeting of Nur77 and its family member Nor-1 in thymocytes undergoing apoptosis. *Eur J Immunol*. 40(7):2041-9.

Thompson J, Winoto A. 2008. During negative selection, Nur77 family proteins translocate to mitochondria where they associate with Bcl-2 and expose its proapoptotic BH3 domain. *J Exp Med.* 205(5):1029-36.

Tóth B, Ludányi K, Kiss I, Reichert U, Michel S, Fésüs L, Szondy Z. 2004. Retinoids induce Fas(CD95) ligand cell surface expression via RARgamma and nur77 in T cells. *Eur J Immunol*. 34(3):827-36.

Tóth R, Szegezdi E, Reichert U, Bernardon JM, Michel S, Ancian P, Kis-Tóth K, Macsári Z, Fésüs L, Szondy Z.Activation-induced apoptosis and cell surface expression of Fas (CD95) ligand are reciprocally regulated by retinoic acid receptor alpha and gamma and involve nur77 in T cells. *Eur J Immunol*. 31(5):1382-91.

Truman LA, Ford CA, Pasikowska M, Pound JD, Wilkinson SJ, Dumitriu IE, Melville L, Melrose LA, Ogden CA, Nibbs R, et al. 2008. CX3CL1/fractalkine is released from apoptotic lymphocytes to stimulate macrophage chemotaxis. *Blood.* 112:5026–5036

Urbach J, Rando RR. 1994. Isomerization of all-trans-retinoic acid to 9-cis-retinoic acid. *Biochem J*. 299 (Pt 2):459-65.

van der Bruggen T, Nijenhuis S, van Raaij E, Verhoef J, van Asbeck BS. 1999. Lipopolysaccharide-induced tumor necrosis factor alpha production by human monocytes involves the raf-1/MEK1-MEK2/ERK1-ERK2 pathway. *Infect Immun*. 67(8):3824-9.

Van Haastert PJ, Van Driel R, Jastorff B, Baraniak J, Stec WJ, De Wit RJ. 1984. Competitive cAMP antagonists for cAMP-receptor proteins. *J Biol Chem.* 259(16):10020-4.

Vela L, Gonzalo O, Naval J, Marzo I. 2013. Direct interaction of Bax and Bak proteins with Bcl-2 homology domain 3 (BH3)-only proteins in living cells revealed by fluorescence complementation.*J Biol Chem*288(7):4935-46.

Viatour P, Merville MP, Bours V, Chariot A. 2005. Phosphorylation of NF-kappaB and IkappaB proteins: implications in cancer and inflammation. *Trends Biochem Sci*. 30(1):43-52.

Voll RE, Herrmann M, Roth EA, Stach C, Kalden JR, Girkontaite I.1997. Immunosuppressive effects of apoptotic cells.Nature. 390(6658):350-1.

Volmer JB, Thompson LF, Blackburn MR. 2006. Ecto-5′ -nucleotidase (CD73)-mediated adenosine production is tissue protective in a model of bleomycin-induced lung injury. *J Immunol*. 176:4449–58.

Wang C, Deng L, Hong M, Akkaraju GR, Inoue J, Chen ZJ. 2001. TAK1 is a ubiquitin-dependent kinase of MKK and IKK. *Nature.* 412(6844):346-51.

Wang D, Müller N, McPherson KG, Reichardt HM. 2006. Glucocorticoids engage different signal transduction pathways to induce apoptosis in thymocytes and mature T cells. *J Immunol.* 176(3):1695-702.

Wang JQ, Kon J, Mogi C, Tobo M, Damirin A, Sato K, Komachi M, Malchinkhuu E, Murata N, Kimura T, et al. 2004. TDAG8 is a proton-sensing and psychosine-sensitive G-protein-coupled receptor. *J Biol Chem.* 279(44):45626-33.

Wang X, Destrument A, Tournier C. 2007. Physiological roles of MKK4 and MKK7: insights from animal models. *Biochim Biophys Acta*. 1773(8):1349-57.

Waterfield MR, Jin W, Reiley W, Zhang M, Sun SC.2004. IkappaB kinase is an essential component of the Tpl2 signaling pathway. *Mol Cell Biol*. 24(13):6040-8.

Waterfield MR, Zhang M, Norman LP, Sun SC. 2003. NF-kappaB1/p105 regulates lipopolysaccharide-stimulated MAP kinase signaling by governing the stability and function of the Tpl2 kinase. *Mol Cell*. 11(3):685-94.

Wei MC, Zong WX, Cheng EH, et al. Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. Science 2001;292:727–30.

Winoto A, Littman DR. 2002.Nuclear hormone receptors in T lymphocytes.*Cell*. 109 Suppl:S57-66.

Woronicz JD, Calnan B, Ngo V, Winoto A. 1994. Requirement for the orphan steroid receptor Nur77 in apoptosis of T-cell hybridomas.*Nature.* 367(6460):277-81.

Woronicz JD, Lina A, Calnan BJ, Szychowski S, Cheng L, Winoto A. 1995. Regulation of the Nur77 orphan steroid receptor in activation-induced apoptosis.*Mol Cell Biol.* 15(11):6364-76.

Xia ZP, Sun L, Chen X, Pineda G, Jiang X, Adhikari A, Zeng W, Chen ZJ. 2009. Direct activation of protein kinases by unanchored polyubiquitin chains. Nature 461: 114–119.

Xiao YQ, Freire-de-Lima CG, Schiemann WP, Bratton DL, Vandivier RW, Henson PM. 2008. Transcriptional and translational regulation of TGF-beta production in response to apoptotic cells.*J. Immunol.* 181:3575–3585

Xue Y, Chomez P, Castanos-Velez E, Biberfeld P, Perlmann T, Jondal M. 1997. Positive and negative thymic selection in T cell receptor-transgenic mice correlate with Nur77 mRNA expression. *Eur J Immunol*. 27(8):2048-56.

Yamaguchi H, Maruyama T, Urade Y, Nagata S. 2014. Immunosuppression via adenosine receptor activation by adenosine monophosphate released from apoptotic cells. *Elife*.3:e02172.

Yamamoto M, Sato S, Hemmi H, Uematsu S, Hoshino K, Kaisho T, Takeuchi O, Takeda K, Akira S. 2003. TRAM is specifically involved in the Toll-like receptor 4-mediated MyD88-independent signaling pathway. *Nat Immunol*.4(11):1144-50.

Yegutkin GG. 2008. Nucleotide- and nucleoside-converting ectoenzymes: Important modulators of purinergic signalling cascade. *Biochim Biophys Acta*. 1783(5):673-94.

Youle RJ, Strasser A. 2008. The BCL-2 protein family: opposing activities that mediate cell death. *Nat Rev Mol Cell Biol*. 9(1):47-59.

Yuan S, Yu X, Topf M, Ludtke SJ, Wang X, Akey CW. 2010. Structure of an apoptosome-procaspase-9 CARD complex.*Structure* 18: 571–583.

Zandi E, Rothwarf DM, Delhase M, Hayakawa M, Karin M. 1997. The IkappaB kinase complex (IKK) contains two kinase subunits, IKKalpha and IKKbeta, necessary for IkappaB phosphorylation and NF-kappaB activation. *Cell*. 91(2):243-52.

Zarubin T, Han J. 2005. Activation and signaling of the p38 MAP kinase pathway. *Cell Res.* 15(1):11-8.

Zha J, Harada H, Yang E, Jockel J, Korsmeyer SJ. 1996. Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L). *Cell* ;87:619–28.

Zhang L, Insel PA. 2004. The pro-apoptotic protein Bim is a convergence point for cAMP/protein kinase A- and glucocorticoid-promoted apoptosis of lymphoid cells. J Biol Chem. 279(20):20858-65.

Zhang SQ, Kovalenko A, Cantarella G, Wallach D. 2000. Recruitment of the IKK signalosome to the p55 TNF receptor: RIP and A20 bind to NEMO (IKKgamma) upon receptor stimulation. *Immunity*. 12(3):301-11.

Zhang X, Odom DT, Koo SH, Conkright MD, Canettieri G, Best J, Chen H, Jenner R, Herbolsheimer E, Jacobsen et al., 2005. Genome-wide analysis of cAMP-response element binding protein occupancy, phosphorylation, and target gene activation in human tissues.*Proc Natl Acad Sci U S A*. 102(12):4459-64.

Zhang XK, Lehmann J, Hoffmann B, Dawson MI, Cameron J, Graupner G, Hermann T, Tran P, Pfahl M. 1992. Homodimer formation of retinoid X receptor induced by 9-cis retinoic acid.*Nature*. 358(6387):587-91.

Zhao Q, Wang X, Nelin LD, Yao Y, Matta R, Manson ME, Baliga RS, Meng X, Smith CV, Bauer JA et al. 2006. MAP kinase phosphatase 1 controls innate immune responses and suppresses endotoxic shock. *J Exp Med*. 203(1):131-40.

Zhou T, Cheng J, Yang P, Wang Z, Liu C, Su X, Bluethmann H, Mountz JD. Inhibition of Nur77/Nurr1 leads to inefficient clonal deletion of self-reactive T cells. *J Exp Med.* 183(4):1879-92.

Ziegler U, Groscurth P.2004. Morphological features of cell death. *News Physiol Sci*. 19:124-8.

9. PUBLICATIONS, CONFERENCES

Registry number: DEENK/295/2016.PL
Subject: PhD Publikációs Lista

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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.
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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
IF: 3.18

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.
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IF: 4.97

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CONFERENCES

ORAL PRESENTATIONS:

Beáta Kiss. **Some new signaling aspects in the negative selection of thymocytes.** (in English) 9th Molecular Cell and Immune Biology Winter Symposium, 8-9 January, 2016, Debrecen, Hungary.

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

Beáta Kiss.**Retinoids induce a Nur77-dependent apoptosis in mouse thymocytes**. (in Hungarian) Debrecen University Graduate School of Clinical Medical Sciences (KODI) and the Graduate School of Dentistry (FODI) 2013.Annual Symposium, June 24, 2013,Debrecen, Hungary.

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

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

Beáta Kiss: **Retinoid-induced apoptosis in mouse thymocytes.** (in Hungarian) Debrecen University Graduate School of Clinical Medical Sciences (KODI) and the Graduate School of Dentistry (FODI) 2012.Annual symposium, 5 June, 2012, Debrecen, Hungary.

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

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

Beáta Kiss: **The role of Nur77 in retinoid-induced apoptosis in mouse thymocytes**. (in Hungarian) XXIX. National Student Scientific Conference, 8-10 April, 2009, Veszprém, Hungary.

Beáta Kiss: **Retinoids can induce the apoptosis of mouse thymocytes via Nur77 transcription factor**. (in Hungarian) Student Conference of the University of Debrecen, 2008, Debrecen, Hungary.

POSTERS:

Beáta Kiss, Katalin Tóth, Zsolt Sarang, Éva Garabuczi, László Fésüs, Zsuzsa Szondy: **Retinoids induce a Nur77-dependent apoptosis in mouse thymocytes.** (in English)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.** (in English) 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.** (in English)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.** (in English) 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** (in English) 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.** (in English) 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.** (in English)15th International Summer School on Immunology, 5-12 September, 2009,Hvar, Croatia.

Beáta Kiss, Katalin Tóth, Zsolt Sarang, László Fésüs, Zsuzsa Szondy: **Retinoids induce apoptosis in mouse thymocytes via inducing Nur77 expression.** (in Hungarian)Annual Meeting of Hungarian Biochemical Society, 31 August-3 September, 2008, Szeged, Hungary.

10. KEYWORDS

Apoptosis

Thymocytes

Nur77

Retinoid

Lipopolysaccharide

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Adenosine A_{2A} receptor

Dual specific phosphatase-1

11. KULCSSZAVAK

Apoptózis

Timociták

Nur77

Retinoid

Lipopoliszacharid

Makrofágok

Adenozin A_{2A} receptor

Kettős specificitású foszfatáz-1

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