

THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

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

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LIST OF ABBREVIATIONS

AR	Adenosine receptor
Ab	Antibody
ABCA1	ATP-binding cassette transporter protein, sub-family A member 1
APO	Apoptotic cell
ACAMPs	Apoptotic cell-associated molecular patterns
ADO	Adenosine
AK	Adenosine kinase
Akt	Serine/threonine-specific protein kinase
AMP/ADP/ATP	Adenosine mono-/di-/triphosphate
AMPK	AMP kinase
AP-1	Activator protein 1
BAI1	Brain-specific angiogenesis inhibitor 1
BMDM	Bone marrow-derived macrophage
BSA	Bovine serum albumin
C1q	Complement 1q
cAMP	Cyclic adenosine monophosphate
CD	Cluster of differentiation
CFDA	6-carboxy-3',6'-diacetylfluorescein
CKI	Casein kinase 1
CMTMR	5-(and-6)-(((4-chloromethyl)-benzoyl)amino)tetramethyl-rhodamine
CREB	cAMP response element-binding protein
CRT	Calreticulin
DAMPs	Damage-associated molecular patterns
DAPI	4',6-diamidino-2-phenylindole
DEX	Dexamethasone
DMEM	Dulbecco's Modified Eagle Medium
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EPAC	Exchange factor directly activated by cAMP
ERK 1/2	Extracellular signal-regulated kinases 1/2
FAK	Focal Adhesion Kinase
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
G-CSF	Granulocyte-colony stimulating factor
GAPDH	Glycerinaldehyde-3-phosphate dehydrogenase
Gas6	Growth arrest-specific 6
GC-BP	GC-binding protein
GDP/GTP	Guanosine di-/triphosphate
GPCR	G protein-coupled receptor

GSK-3 α/β	Glycogen synthase kinase 3 alpha/beta
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMGB1	High-mobility group box 1
HRP	Horseradish peroxidase
HSP-27	Heat shock protein 27
IB-MECA	N6-(3-Iodobenzyl)adenosine-5'-N-methylcarboxamide
ICAM-1	Intercellular Adhesion Molecule 1
IFN	Interferon
IgG	Immunoglobulin G
IL	Interleukin
IL-1 ra	Interleukin-1 receptor antagonist
IL-1 α	Interleukin 1-alpha
IL-1 β	Interleukin 1-beta
IMDM	Iscove's modified Dulbecco's medium
iNOS	Inducible nitric oxide synthase
IP-10	Interferon-gamma induced protein 10
I-309	Chemokine (C-C motif) ligand 1 (CCL1)
JE/MCP-1	C-C chemokine monocyte chemotactic protein 1
JNK	c-Jun NH2-terminal kinase
KC	Cytokine-induced neutrophil-attracting chemokine (CXCL1)
L-NAME	L-(G)-nitro-L-arginine methyl ester
LPS	Lipopolysaccharide
LT/LT- α	Lymphotoxin-alpha
LXR	Liver X receptor
MAPK	Mitogen-activated protein kinase
M-CSF	Macrophage colony-stimulating factor
MerTk	Mer tyrosine kinase
MFG-E8	Milk-fat globule epidermal growth factor 8
MFI	Mean fluorescence intensity
MIP-1 α/β	Macrophage Inflammatory Protein-1 alpha/beta (CCL3/CCL4)
MIP-2	Macrophage inflammatory protein-2 (CXCL2)
MKK	Mitogen-activated protein kinase kinase
M ϕ	Macrophages
MSK2	Mitogen- and stress-activated protein kinase 2
mTNF	Membrane-bound tumor necrosis factor alpha
mTOR	Mammalian target of rapamycin
NF-AT	Nuclear factor activated T cells
NF κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer cell
NO	Nitric oxide
NOS	Nitric oxide synthase

nTGF	Neutralizing transforming growth factor beta
NTPDase	Nucleoside triphosphate diphosphohydrolase
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate-buffered saline
PdBu/Iono	Phorbol dibutyrate ionomycin
PE	Phycoerythrin
PGE2	Prostaglandin E2
PI	Propidium iodide
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PKA	Protein kinase A
p-MKK	Phosphorylated-mitogen-activated protein kinase kinase
PPAR	Peroxisome proliferator-activated receptor
PS	Phosphatidylserine
p70S6K	Ribosomal protein S6 kinase
RALDH	Retinaldehyde dehydrogenase
RANTES	Chemokine (C-C motif) ligand 5 (CCL5)
RARE	Retinoic acid response element
RPMI medium	Roswell Park Memorial Institute medium
RSK 1/2	Ribosomal s6 kinase 1/2
rTGF- β	Recombinant transforming growth factor beta
RXR	Retinoid X receptor
RXRE	RXR response element
SCARF1	Scavenger Receptor Class F Member 1
SIRP- α	Signal Regulatory Protein α
SNP	Sodium nitroprusside
SREBP	Sterol response element binding protein
STAT	Signal Transducer and Activator of Transcription
sTNF- α	Soluble tumor necrosis factor alpha
TACE	TNF-alpha converting enzyme
TG2	Tissue transglutaminase
TGF- β	Transforming growth factor beta
Th2	T helper cells
TIM	T-cell immunoglobulin- and mucin-domain-containing molecule
TIMP-1	Tissue inhibitor of metalloproteinase 1
TLR	Toll-like receptor
TNF/TNF- α	Tumor necrosis factor-alpha
TNFR 1/I	Tumor necrosis factor receptor 1
TNFR 2/II	Tumor necrosis factor receptor 2
TRADD	TNF-R1-associated death domain protein
TRAF2	TNF receptor associated factor 2
TRAIL	TNF-related apoptosis-inducing ligand receptor
TRAPS	TNF-RI-associated periodic-syndromes
TSP1	Thrombospondin 1

UTP	Uridine-5'-triphosphate
VEGF	Vascular endothelial growth factor
WT	Wild-type

1. INTRODUCTION

1.1. APOPTOTIC CELL CLEARANCE

Most cell types have a limited life span, which ends physiologically through the process of apoptosis, or programmed cell death. A key feature of apoptosis is that cells undergoing this programmed form of death are recognized by phagocytes and ingested while still intact, protecting tissues from the potentially harmful consequences of exposure to the contents of the dying cells. Apoptotic cell death and the clearance of dying cells play an important and physiological role not only in the embryonic development but also in normal tissue turnover, tissue remodeling, maintenance of immune homeostasis, and resolution of inflammation (Gregory and Devitt, 2004; Munoz et al., 2005).

Under normal circumstances, phagocytosis of apoptotic cells engulfed by phagocytes that are professional engulfers (such as macrophages and immature dendritic cells; Aderem and Underhill, 1999) or neighboring cells (such as fibroblasts and epithelial cells; Monks et al., 2008) is fast and effective without causing inflammation and immune response (Savill et al. 1993). The immunologically silent removal of apoptotic cells is orchestrated by the timed release and/or exposure of so-called 'find-me', 'eat me' and 'tolerate me' signals. Mononuclear phagocytes are attracted by various 'find-me' signals, including proteins, nucleotides, and phospholipids released by the dying cell, whereas the involvement of granulocytes is prevented *via* 'stay away' signals (Biermann et al., 2013).

The clearance process is rapid such that even in tissues with high cellular turnover, very few apoptotic cells are seen (Gardai et al., 2006). However, there are many disease states where uncleared corpses/debris are seen, including systemic lupus erythematosus, atherosclerosis, Alzheimer's disease, and Parkinson's disease (Camins et al., 2008; Gorman, 2008; Calissano et al., 2009; Nagata et al., 2010). Therefore, defining the specific steps by which apoptotic cells are recognized and removed quickly is important for understanding diseases associated with defective clearance and for potentially manipulating the engulfment machinery for future therapeutic benefits (Ravichandran, 2010).

1.1.1. Steps of apoptotic cell engulfment

The engulfment process can be broadly broken down into three major steps (Figure 1).

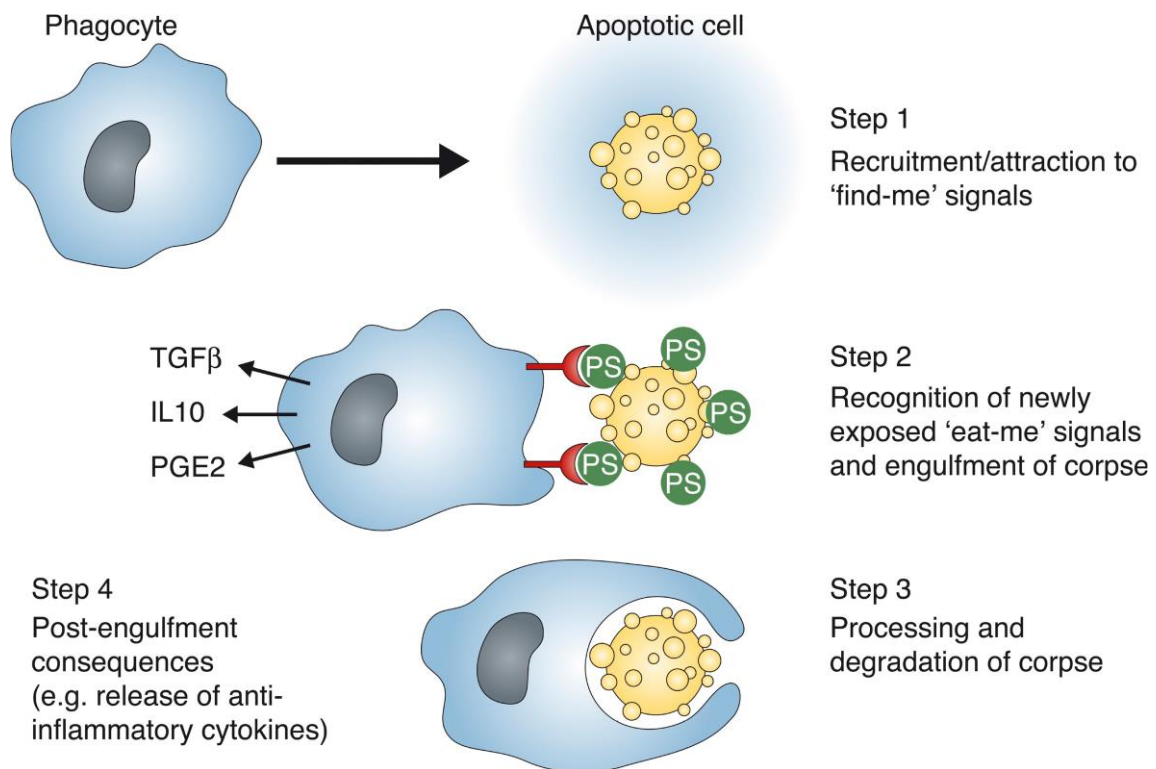


Figure 1. The steps of efficient apoptotic cell clearance

(Ravichandran, 2010)

1. Recruitment of phagocytes

The first step involves the release of so called „find-me” signals - such as triphosphate nucleotides (ATP/UTP), fractalkine, lysophosphatidylcholine, or sphingosine 1-phosphate (Lauber et al., 2003; Gude et al., 2008; Truman et al., 2008; Elliott et al., 2009)-, from apoptotic cells which help to attract phagocytes to the sites of death within a tissue (Gregory C, 2009).

2. Recognition of apoptotic cells by phagocytes

The second step is the exposure of „eat-me” signals on the apoptotic cell surface, which promotes the specific recognition by the phagocyte and subsequent internalization of the corpse (Grimsley and Ravichandran, 2003).

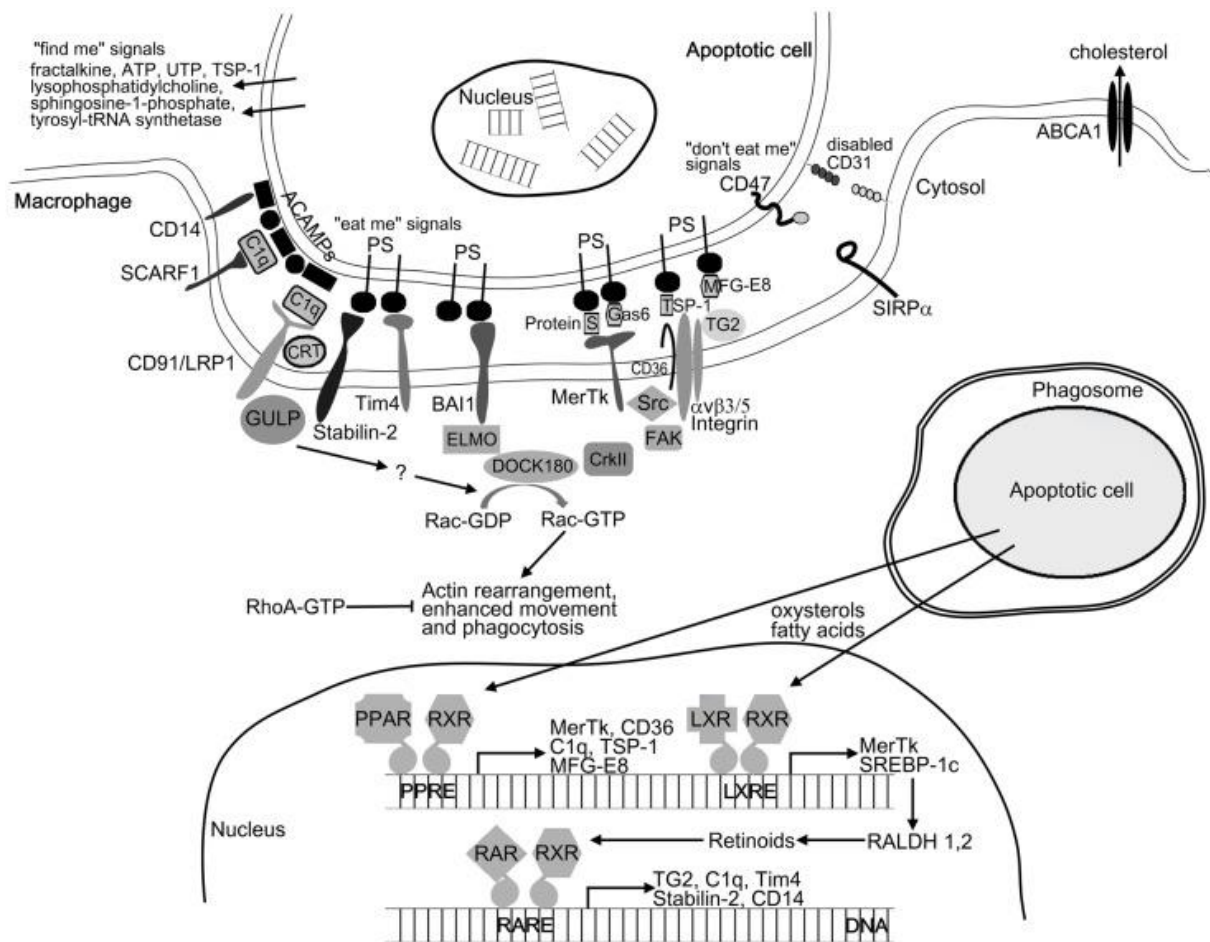


Figure 2. Mechanism of apoptotic cell clearance

(Szondy et al., 2014)

Although find-me signals can get phagocytes to the proximity of apoptotic cells within a tissue, the specific recognition of the dying cell among the neighboring live cells depends on eat-me signals exposed by the apoptotic cells (Lauber et al., 2004; Gardai et al., 2006). To date, multiple eat-me signals have been identified (Gardai et al., 2006). These include exposure of phosphatidylserine (PS), changes in charge and glycosylation patterns on the cell surface, alteration of ICAM-1 epitopes on the cell surface, and exposure of calreticulin.

Multiple distinct receptors on phagocytes can engage PS exposed on apoptotic cells (Bratton and Henson, 2008). These PS recognition receptors (Figure 2) come in two primary flavors – those that can bind directly to PS, and those that indirectly bind PS *via* soluble bridging molecules. Direct-binding PS receptors include members of the TIM family (the prototype TIM-4, and also TIM-1 and TIM-3; Kobayashi N et al., 2007; Miyanishi et al., 2007; Santiago et al., 2007; Ichimura et al., 2008; DeKruyff et al., 2010; Rodriguez-Manzanet et al., 2010;

Wong et al., 2010), BAI1 (brain angiogenesis inhibitor 1; Park D et al., 2007a), and Stabilin-2 (Park SY et al., 2007b).

In addition, some phagocytic receptors, such as integrin $\alpha v\beta 3$; the scavenger receptor CD36; Axl, Sky, Tyro-3 and Mer receptor tyrosine kinases require bridging molecules - e.g. TSP1 (thrombospondin 1), Gas6 (growth arrest-specific 6), protein S, MFG-E8 (milk-fat globule epidermal growth factor 8), $\beta 2$ -glycoprotein I - for the proper recognition of apoptotic cell-associated PS (Moodley et al., 2003., Elliott and Ravichandran, 2010, Gregory and Pound, 2010; Weigert et al., 2009; Kruse et al., 2010; McColl et al., 2009).

3. Engulfment and processing of apoptotic cells

The activation of phagocytic receptors leads to downstream signaling events culminating in the activation of Rho GTPases (e.g. Rac-1, RhoA) (Vandivier et al., 2006). The engulfed corpse goes through a series of phagosome maturation steps, eventually leading to its degradation (Kinchin and Ravichandran, 2008, 2010). This step is loosely denoted as post-engulfment consequences, involves the release of anti-inflammatory cytokines and other modifiers by the phagocyte that are elicited during recognition and processing (Savill and Fadok, 2000; Savill et al., 2002; Henson, 2005). Collectively, these events mediate the selective and immunologically silent versus immunogenic removal of apoptotic cells *in vivo* (Green et al., 2009).

1.1.2. The anti-inflammatory effects of apoptotic cell clearance

Whereas the phagocytosis of a variety of pathogenic targets, especially bacteria and virally-infected cells, normally triggers a pro-inflammatory response in macrophages (including the generation of reactive oxygen-derived intermediates, the release of proteolytic enzymes, and the production of numerous inflammatory cytokines), ingestion of apoptotic cells by macrophages usually induces an anti-inflammatory phenotype.

Apoptotic cells do not simply fail to provide pro-inflammatory signals; rather, they can initiate signaling pathways in macrophages which can actively interfere with the inflammatory program. For example, pre-incubation of macrophages with apoptotic cells strongly suppresses the inflammatory response induced *via* Toll-like receptor 4 (TLR4) by lipopolysaccharide (LPS), a component of the cell wall of Gram-negative bacteria (Voll et al., 1997; Fadok et al., 1998; Cvetanovic and Ucker, 2004). This inhibitory property appears to be

a common attribute acquired post-translationally by all cells undergoing apoptotic cell death, regardless of the cell type or the particular death stimulus (Cvetanovic and Ucker, 2004; Cocco and Ucker, 2001; Patel et al., 2007).

1. Direct anti-inflammatory effect of apoptotic cell recognition

The earliest anti-inflammatory activity of the apoptotic cell is manifest as an immediate-early inhibition of macrophage pro-inflammatory cytokine gene transcription and is exerted directly upon binding to the macrophage, independent of subsequent engulfment and soluble factor involvement (Cvetanovic and Ucker, 2004).

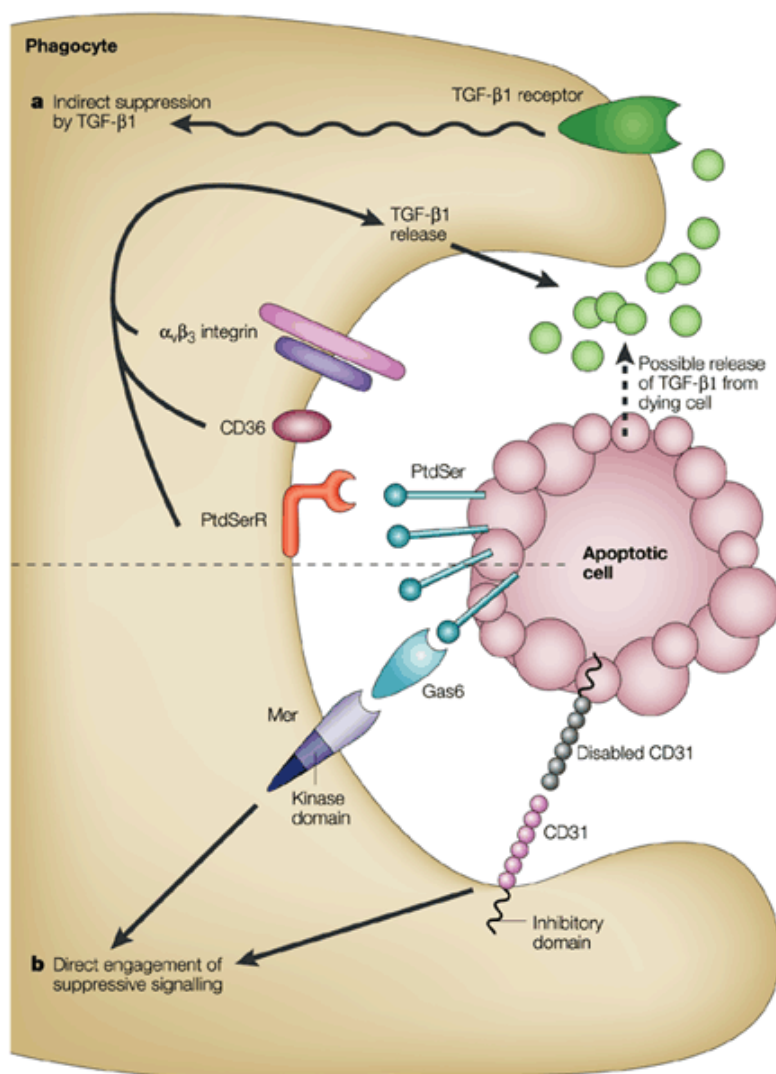
This transcriptional inhibition is evident, for example, on the level of NF- κ B-dependent transcription and occurs without effect on proximal signaling events induced by inflammatory receptors, including innate immune receptors of the TLR family (Cvetanovic and Ucker, 2004).

In case of IL-12, for example, cell–cell contact with apoptotic cells is sufficient to induce the inhibition of the cytokine production by activated macrophages *via* a novel zinc finger nuclear factor, GC-BP, which selectively inhibits IL-12 p35 gene transcription (Kim et al., 2004).

2. Soluble mediators involved in the anti-inflammatory effects of apoptotic cells

Subsequently, soluble mediators are released from macrophages, which act in a paracrine or autocrine fashion to amplify and sustain the anti-inflammatory response. For example, Voll et al. observed an inverse relationship between the secretion of the pro-inflammatory cytokines TNF- α , IL-1 β , and IL-12 and the transient release of IL-10 from LPS-stimulated macrophages after their interaction with apoptotic cells. Fadok et al. reported a similarly inverse relationship between the release of TGF- β , platelet-activating factor, and PGE2 and the secretion of TNF- α , IL-1 β , and several other inflammatory cytokines by macrophages after engulfment of apoptotic targets. Although the pharmacologic blockade of platelet-activating factor and PGE2 signaling had only slight effect (McDonald et al., 1999), the addition of TGF- β -specific neutralizing Ab to cultures of macrophages and apoptotic targets partially restored pro-inflammatory cytokine release (Fadok et al., 1998; McDonald et al., 1999) indicating a central role of this cytokine in mediating anti-inflammatory responses.

Köröskényi et al. demonstrated that adenosine (ADO) is one of the soluble factors produced by macrophages engulfing apoptotic cells, which is involved in the downregulation of pro-inflammatory responses (Köröskényi et al., 2011). Adenosine acting *via* A_{2A}Rs prevents the apoptotic cell-induced NO formation (*via* gene expression regulation of inducible nitric oxide synthase and arginase II) and the consequent NO-dependent neutrophil chemoattractant MIP-2 (macrophage inflammatory protein 2) and KC (CXCL1, chemokine C-X-C motif ligand 1) production in macrophages engulfing apoptotic cells (Köröskényi et al., 2011). Therefore ADO/A_{2A}R signaling protects tissues against pro-inflammatory immune responses during the clearance of apoptotic cells.



Cells undergoing apoptosis release ATP, which is required for macrophage migration toward the apoptotic cells (Elliott et al., 2009). The channel, which opens in a caspase-dependent manner during apoptosis, has been identified as the pannexin channel (Chekeni et al., 2010). Apoptotic cell derived adenine nucleotides released *via* pannexin-1 channels can be converted to adenosine by macrophages (Sándor et al., 2016) to trigger adenosine A_{2A} receptor (A_{2A}R) anti-inflammatory signaling in macrophages (Köröskényi et al., 2011; Yamaguchi et al., 2014).

Figure 3. The direct and indirect anti-inflammatory effects of apoptotic cells

(Savill et al., 2002)

3. Nuclear receptors involved in the anti-inflammatory effects of apoptotic cell clearance.

Apoptotic cell recognition and engulfment promote the transcriptional activity of LXR and PPAR nuclear receptors. Lipids derived from the engulfed apoptotic cells serve as source of endogenous ligands (fatty acids, oxysterols) to activate lipid sensing receptors (LXR α and β , PPAR γ and δ , PPARs; Figure 2). Nuclear receptor activation upon apoptotic cell phagocytosis leads to the upregulation of phagocytic receptors (e.g., MerTK, CD36; Rószler et al., 2011), TG2 (Réb   et al., 2009), and opsonins (e.g., MFG-E8, C1qb, Gas6 and thrombospondin; Mukundan et al., 2009), and phagocyte metabolism (A-Gonzalez and Hidalgo, 2014).

In addition, apoptotic cell engulfment and the consequent LXR activation leads to the synthesis of a dihydro-retinoic acid derivative (Sarang et al., 2011) which – acting *via* RARs - contributes to the upregulation of further phagocytic receptor, metabolic genes (e.g. UCP2, CYP27) as well as enhance the expression of all the lipid sensing receptors (Garabuczi et al., 2015).

1.2. ADENOSINE

Adenosine was first recognized as a physiologic regulator of coronary vascular tone by Drury and Szent-Gyorgyi (1929), however it was not until 1970 that Sattin and Rall showed that adenosine regulates cell function *via* occupancy of specific receptors on the cell surface (Sattin and Rall, 1970). Adenosine is an endogenous purine nucleoside that, following its release from cells or after being formed extracellularly, diffuses to the cell membrane of surrounding cells, where it binds to its receptors (Ralevic and Burnstock, 1998; Fredholm et al., 2001).

1.2.1. Adenosine metabolism

There are several important producer cell types of extracellular adenosine. Neutrophils and endothelial cells release large amounts of adenosine at sites of metabolic distress, inflammation and infection (Cronstein et al., 1983; Gunther and Herring, 1991; Madara et al., 1993; Rounds et al., 1994). Activated macrophages (e.g. LPS-treated) can also serve as a major source of extracellular adenosine *via* ATP release and degradation (Ruiz-Garc  a et al., 2011). Because the inhibitory effects of exogenously added ATP on macrophage TNF- α production can be abrogated by administering exogenous adenosine deaminase (Hask  -

Kuhel-Salzman et al., 2000), it can be proposed that endogenously released ATP is also degraded to adenosine by macrophages.

Adenosine concentrations at its receptors are determined by a variety of processes, which include extracellular and intracellular adenosine generation, adenosine release from cells, cellular reuptake and metabolism. These processes are closely intertwined and strictly regulated. For example, under hypoxic conditions, the increased intracellular dephosphorylation of adenosine 5'-triphosphate (ATP) to adenosine by the metabolic enzyme 5'-nucleotidase is accompanied by a suppression of the activity of the salvage enzyme adenosine kinase, which prevents the rephosphorylation of adenosine (Deussen, 2000). These processes lead to adenosine reaching high concentrations inside the cell and the release of adenosine into the extracellular space through nucleoside transporters (Hyde et al., 2001; Pastor-Anglada et al., 2001). The other major pathway that contributes to high extracellular adenosine concentrations during metabolic stress is release of precursor adenine nucleotides (ATP, ADP and AMP) from the cell (Figure 4). This is followed by extracellular degradation to adenosine by a cascade of ectonucleotidases, which include CD39 (nucleoside triphosphate diphosphohydrolase [NTPDase]) and CD73 (5'-ectonucleotidase) (Kaczmarek et al., 1996; Resta et al., 1998; Zimmermann, 1999; Eltzschig et al., 2004; Thompson et al., 2004; Sperl agh et al., 2006). Adenosine accumulation is limited by its catabolism to inosine by adenosine deaminase. Inosine is finally degraded to the stable end product uric acid (Jennings and Steenbergen, 1985; Hask o-Kuhel-N emeth et al., 2000; Cristalli et al., 2001; Hask o et al., 2004).

Although adenosine is constitutively present in the extracellular space at low concentrations (<1 μM), its concentration can dramatically increase under inflammatory conditions reaching concentrations high enough (Martin et al., 2000; Sottofattori et al., 2001) to exert immunomodulatory and especially immunosuppressive effects (Hask o and Cronstein, 2004).

1.2.2. Adenosine receptors and signaling pathways

There are four adenosine receptors, all of which are G protein-coupled receptors (GPCR) and are abundantly expressed by macrophages (Hask o and Cronstein, 2004). The genes for these receptors have been analyzed in detail and are designated A_1 (A_1R), A_{2A} (A_{2AR}), A_{2B} (A_{2BR}) and A_3 (A_3R) (Figure 4).

The G_i -coupled adenosine A_1 receptors are stimulated by 10^{-10} – 10^{-8} M concentrations of adenosine and mediate decreases in intracellular cyclic AMP (cAMP) levels, G_s -coupled

adenosine A_{2A} and A_{2B} receptors are stimulated by higher (5×10^{-7} M and 1×10^{-5} M, respectively) concentrations of adenosine and mediate increases in cAMP levels, while G_i -coupled adenosine A_3 receptors are stimulated by 10^{-6} M concentrations of adenosine and mediate adenylate cyclase inhibition (Fredholm et al., 2001).

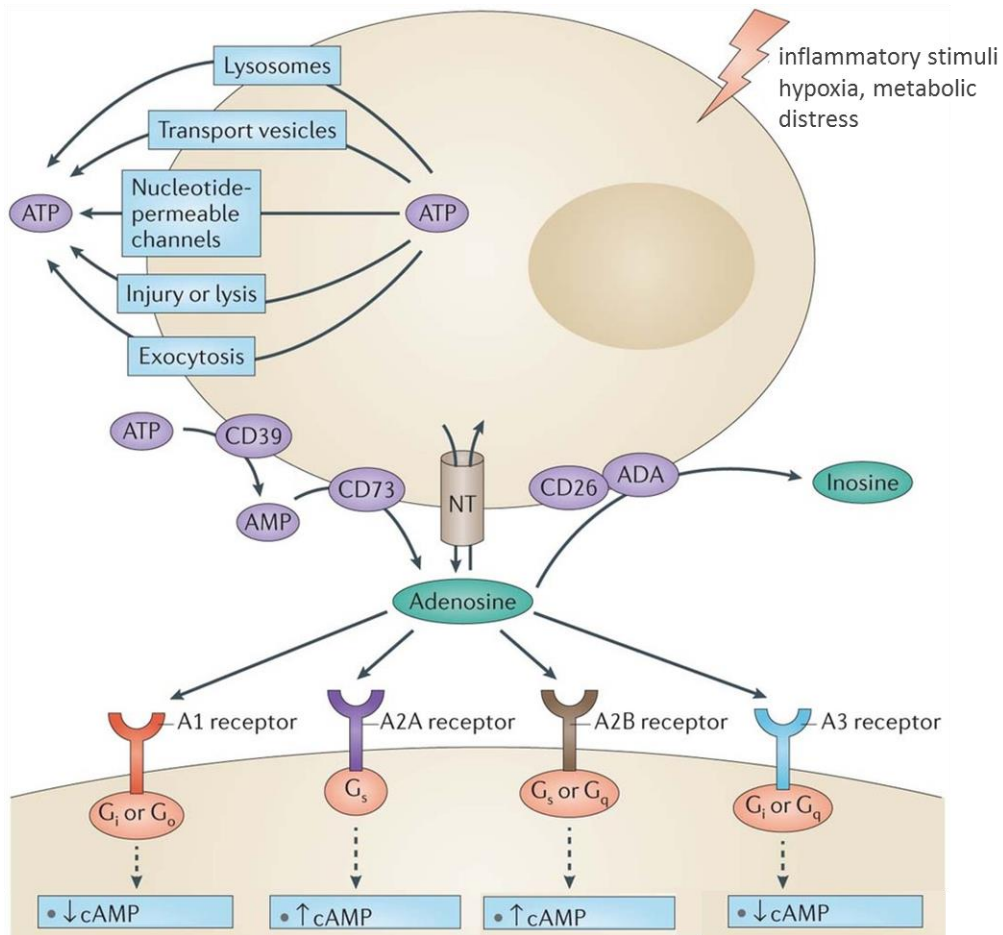


Figure 4. Adenosine and adenosine signaling

(modified from Antonioli et al, 2013)

Lower concentrations of adenosine activate the high affinity A_1 , A_{2A} , and A_3 receptors, and high adenosine concentrations stimulate the low affinity A_{2B} receptors. Thus, since the degree of metabolic distress can determine the concentration of extracellular adenosine, the more pronounced the metabolic distress is, the more likely it is that A_{2B} receptors are activated. Further factors that determine the net effect of adenosine on macrophage function are adenosine receptor expression and coupling efficacy to intracellular signaling pathways, factors that are all very dynamically regulated (Haskó et al., 2007).

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

The general extracellular signaling molecule adenosine acts as a modulator of various biological processes in many cell types, tissues and organs. Its effect is critical in the normal functions of the cardiovascular (CV) system, gastrointestinal tract, central nervous system (CNS) and immune system. In the CNS adenosine is an inhibitory neurotransmitter. Under physiological conditions, it is involved in the development and maturation of neuronal networks, in the process of cognition and memory, as well as in the regulation of sleep and arousal (Ribeiro and Sebastiao, 2010). In the CV adenosine exerts vasodilatory, antithrombotic, blood pressure and heart rate lowering effects (Layland et al, 2014).

In the immune system, adenosine acting *via* different types of its receptors expressed by various immune cells modulates wide spectra of innate immune responses e.g. (Haskó et al., 2008):

- Adenosine receptor ligation on monocytes or macrophages inhibits production of IL-12 upon binding of lipopolysaccharide (LPS) through Toll-like receptor 4 (TLR-4) (Haskó-Kuhel-Chen et al., 2000; Haskó-Kuhel-Németh et al., 2000). The stimulation of adenosine A_{2A} receptor leads to a decrease in TLR-4 induced release of TNF- α , MIP-1 α , and nitric oxide (NO) and augments production of anti-inflammatory cytokine IL-10 (Haskó et al., 1996). Broussas et al. (2002) investigated that adenosine also inhibited the release of tissue factor (TF) from LPS stimulated human monocytes *via* binding to adenosine A₃ receptors. Adenosine increases vascular endothelial growth factor (VEGF) production from macrophages, thus facilitates the process of angiogenesis (Adair, 2005).
- During metabolic stress, without TLR activation, adenosine acts as a chemotactic molecule and promotes chemotaxis of immature human dendritic cells *via* binding to adenosine A₁ and A₃ receptors and increases intracellular calcium concentration and actin reorganization (Panther et al., 2001; Fossetta et al., 2003). However, mature plasmacytoid dendritic cells express adenosine A_{2A} receptors and binding of adenosine to these receptors leads to a decrease in cytokine (IL-6, IL-12 and IFN- α) production in response to CpG oligodeoxynucleotides (ODN) (Kumar and Sharma, 2009).
- In chronic inflammatory conditions like asthma and chronic obstructive pulmonary disorder (COPD), adenosine mediated stimulation of adenosine A_{2B} and A₃ receptors expressed on human mast cells causes degranulation of mast cells and release of histamine, prostaglandins, leukotrienes, serotonin, pro-inflammatory cytokines (IL-4

IL-13, IL-8, IL-1 β), angiogenic factors (IL-8 and VEGF), chemokines and various tissue damaging enzymes, proteases (Feoktistov and Biaggioni, 1995; Ramkumar et al., 1993; Feoktistov et al., 2003; Ryzhov et al., 2004).

- Adenosine A₁ receptor activation induces chemotaxis and increases adherence of neutrophils to endothelial cells. While adenosine A_{2A} receptor activation decreases neutrophil adherence to endothelium, uncouples chemoattractant receptors from their stimulus transduction proteins and inhibits the synthesis of toxic free radicals (Kumar and Sharma, 2009).
- Adenosine *via* binding to adenosine A₃ receptor increases the antitumor activity of NK cells (Harish et al., 2003).

1.3. GENERAL ASPECTS OF TNF BIOLOGY

The principle of an antitumoral response of the immune system *in vivo* has been recognized already about 100 years ago by the physician William B Coley. About 30 years ago, a soluble cytokine termed tumor necrosis factor (TNF) has been identified that is produced upon activation by the immune system, able to exert significant cytotoxicity on many tumor cell lines and to cause tumor necrosis in certain animal model systems. In 1984, the cDNA of TNF was cloned, the structural and functional homology to lymphotoxin (LT) α was realized, and several years later, two membrane receptors, each capable of binding both cytokines, were identified. Subsequently, it was recognized that TNF is the prototypic member of a large cytokine family, the TNF ligand family (Wajant et al., 2003).

1.3.1. Structure and synthesis of TNF- α

Human TNF- α is expressed as a 27-kDa (233 amino acid) protein. It is primarily produced as a type II transmembrane protein arranged in stable homotrimers (Kriegler et al., 1988; Tang et al., 1996; Figure 5). From this membrane-integrated form 27-kDa TNF- α (mTNF- α) the soluble homotrimeric cytokine (sTNF) 17-kDa is released *via* proteolytic cleavage by the metalloprotease TNF alpha converting enzyme (TACE) (Black et al., 1997). The soluble 51-kDa trimeric sTNF tends to dissociate at concentrations below the nanomolar range, thereby losing its bioactivity. The 17-kDa TNF- α protomers are composed of two antiparallel β -pleated sheets with antiparallel β -strands, which form a 'jelly roll' β -structure, typical for the TNF ligand family, but also found in viral capsid proteins. It is believed that mTNF- α and

sTNF- α regulate biological responses at autocrine/paracrine and endocrine levels, respectively (Bazan, 1993; Perez et al., 1990; Grell, 1995).

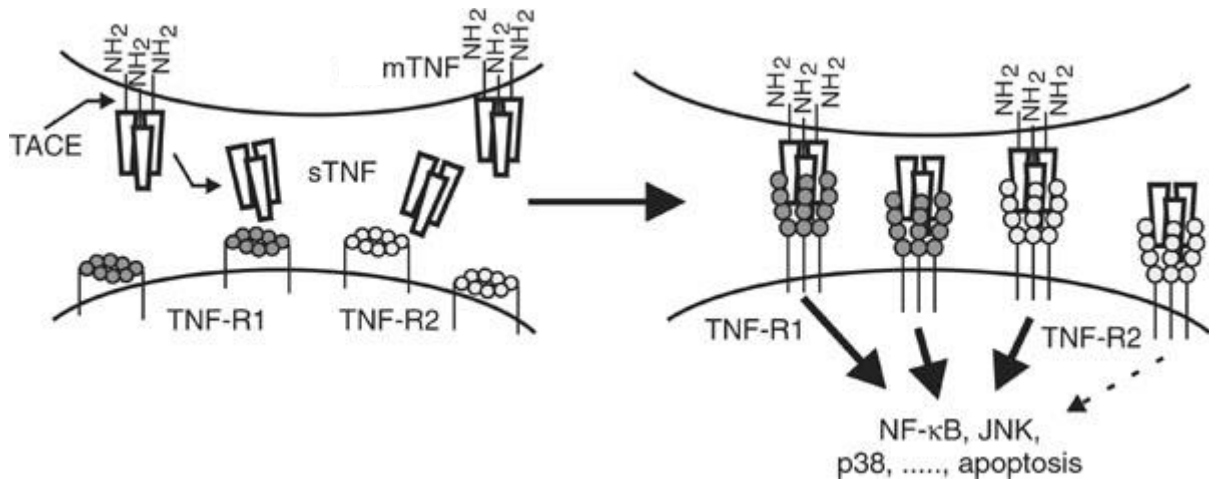


Figure 5. mTNF and sTNF bind to two members of the TNF-receptor superfamily, TNF-R1 and TNF-R2 (Wajant et al., 2003)

Though TNF- α is a pleiotropic cytokine produced by many different types of cells in the body, cells of the monocytic lineage - such as macrophages, astroglia, microglia, Langerhans cells, Kupffer cells, and alveolar macrophages - are the primary synthesizers of it (Pfeffer et al., 1993; Flynn et al., 1995). TNF- α gene expression is regulated at the transcriptional level by several factors, including nuclear factor kappa B (NF- κ B) and nuclear factor activated T cells (NF-AT). TNF- α production is also regulated at the translational level *via* the UA-rich sequence in the 3' untranslated region of human TNF- α mRNA (Spriggs et al., 1992; Vilcek and Lee, 1991).

Large amounts of sTNF- α are released in response to lipopolysaccharide and other bacterial products. In concert with other cytokines, TNF- α is considered to be a key player in the development of septic shock (Männel and Echtenacher, 2000). Whereas high concentrations of TNF- α induce shock-like symptoms, the prolonged exposure to low concentrations of TNF- α can result in a wasting syndrome, that is, cachexia (Beutler et al., 1985).

1.3.2. TNF receptors

The members of the TNF ligand family exert their biological functions *via* interaction with their cognate membrane receptors, comprising the TNF receptor (TNF-R) family (Locksley et al., 2001), whose members include TNF receptor 1 (TNF-R1), also known as CD120a, p55 or p60, TNF receptor 2 (TNF-R2), also known as CD120b, p75 or p80, Fas antigen, CD27, CD30, CD40, 4-1BB and several other receptors (Smith et al., 1994).

TNF- α binds to both TNF-R1 and TNF-R2 with high affinity. There is, however, some species specificity in terms of the receptor subtype and TNF- α binding. It has been shown that human TNF- α binds only to the mouse TNF-R1 (Lewis et al., 1991; Tartaglia and Goeddel, 1992). There are also other unique differences between TNF-R1 and TNF-R2. For example, the cytoplasmic portion of TNF-R1, but not TNF-R2, contains a death domain.

Binding of TNF- α onto TNF-R1 is considered to be an irreversible mechanism, whereas binding of TNF- α onto TNF-R2 has both rapid on and off kinetics. Therefore, it has been suggested that TNF-R2 might act as a “ligand passer” to TNF-R1 in some cells, increasing the local concentration of TNF- α at the cell surface through rapid ligand binding and dissociation (Tartaglia et al., 1993).

TNF-R family members (TNF-R1/2, CD40, HVEM, TRAILR1, RANK, 4-1BB, CD30, Fas, OX40, CD27; Sun and Fink, 2007) contain one to six cysteine-rich repeats in their extracellular domain, typically each with three cysteine bridges and usually no similarities in their intracellular domain. In response to an inflammatory signal, the extracellular domains of both receptors can be proteolytically cleaved by matrix metalloproteinase family of enzymes, yielding soluble receptor fragments (sTNFs) (Wallach et al., 1991). The processing enzyme(s) responsible for TNF-R1 cleavage is still undefined, but TNF-R1 cleavage is obviously an important step in the regulation of cellular TNF responsiveness, as cleavage-resistant TNF-R1 mutations are linked with dominantly inherited autoinflammatory syndromes (TNF-R1-associated periodic syndromes; TRAPS) (McDermott et al., 1999). Interestingly, sTNF-Rs retain the ability to bind TNF- α , and thus function as endogenous inhibitors of TNF- α (Van Zee et al., 1992). Owing the lack of cooperativity in ligand binding, however, the affinities of sTNF-Rs are low compared to their membrane-integrated forms.

TNF-R1 is constitutively expressed in most tissues, whereas expression of TNF-R2 is highly regulated and is typically found in cells of the immune system. In the vast majority of cells,

TNF-R1 appears to be the key mediator of TNF signalling, whereas in the lymphoid system TNF-R2 seems to play a major role. Generally, the importance of TNF-R2 is likely to be underestimated, because this receptor can only be fully activated by mTNF- α , but not sTNF- α (Grell et al., 1995). The cause for this difference is not fully understood yet, but the different stabilities, that is half-lives, of the individual ligand/receptor complexes may contribute to this (Grell et al., 1995; Grell-Wajant et al., 1998).

1.3.3. TNF- α signal transduction and biological role

TNF family members can generate bidirectional signals. While on one side, TNF-TNFR interactions initiate multiple signaling pathways (forward signals) promoting cell survival, death, differentiation, or inflammation in TNFR-expressing cells, TNF-R family members (TNF) also serve as ligands to initiate reverse signaling, regulating cell proliferation, cytokine secretion, oxidative burst, class switch, and T cell maturation in cells expressing molecules belonging to the TNF family (Sun and Fink, 2007; Figure 6).

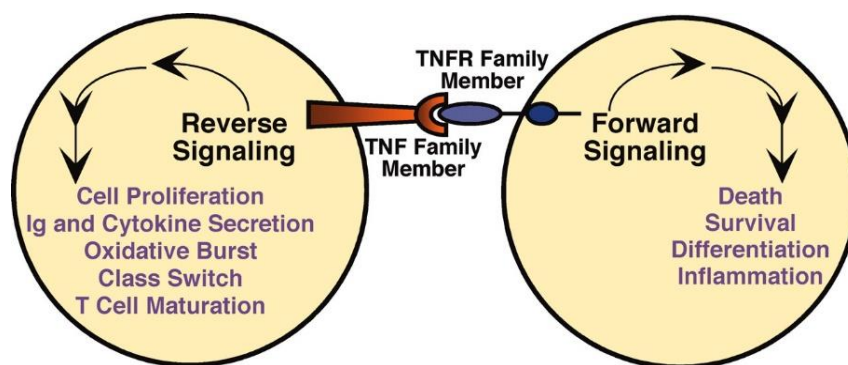


Figure 6. Reverse signaling and forward signaling by members of TNF family

(Sun and Fink, 2007)

TNF- α forward signaling

The pleiotropic biological effects of TNF- α can be attributed to its ability to simultaneously activate multiple signaling pathways in cells. Binding of TNF- α to TNF-R1 on the cell surface triggers trimerization of the receptor and exposes intracellular domain of TNF-R1. This domain recruits a TRADD (TNF-R1-associated death domain protein adaptor protein. TRADD acting as a scaffold protein assembles at least two distinct signaling complexes that initiate opposing signaling pathways (Varfolomeev and Vucic 2008; Figure 7):

- TRADD recruits TRAF2 (TNF receptor associated factor 2) and RIPK1 (Receptor-interacting serine/threonine-protein kinase 1) to form a complex, referred as **complex 1**. This pathway exerts NF- κ B and MAPK-dependent anti-apoptotic and immune-regulatory effects.
- TRADD dissociating from the receptor integrates FADD (FAS-associated death domain protein) and procaspase 8 to form a complex referred to as the **complex 2**. Activated caspase 8 induces the execution of apoptosis.

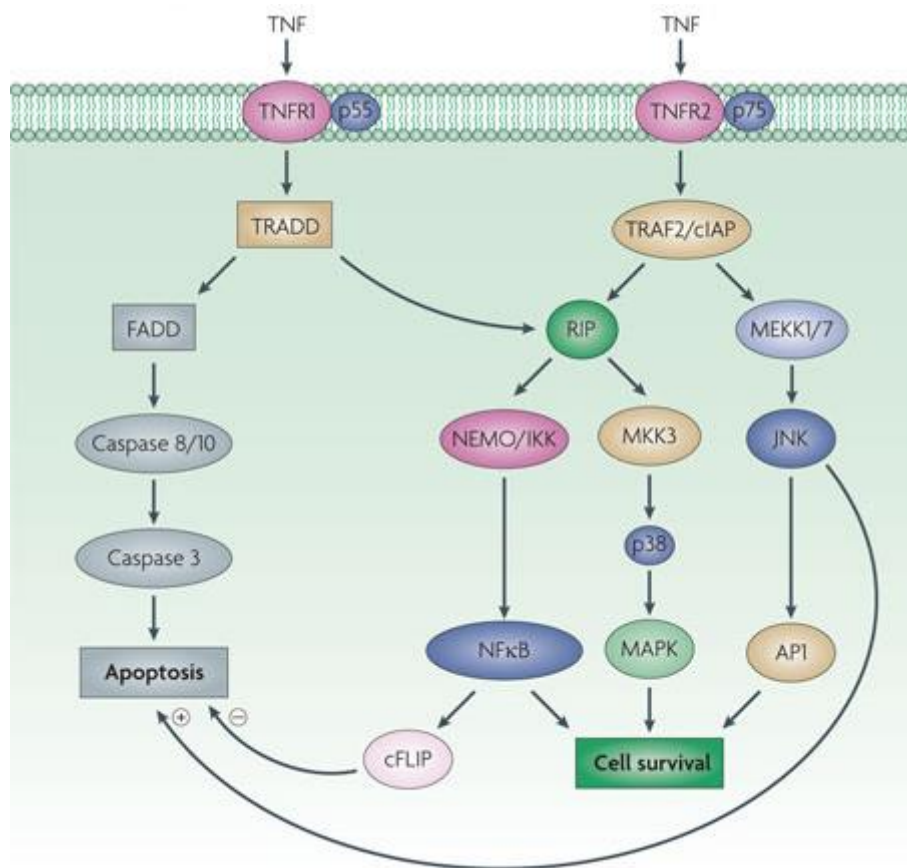


Figure 7. Forward TNF receptor signaling pathways

(Faustman and Davis, 2010)

On the other hand, for TNF-R2 signaling, it is known that the occupancy of TNF-R2 by TNF leads to the recruitment of TRAF1 and TRAF2 as well as cIAP1 and cIAP2 (Rothe et al., 1995). TNF-R2 induces gene expression by a TRAF-2 dependent signaling mechanism and also crosstalk's with TNF-R1. However, it is less clear how these molecules correlate to transduce the diverse TNF signals through TNF-R2 (Liu ZG, 2005).

TNF-R1 activation triggers not only inflammatory responses, but also apoptosis-related signaling events, while TNF-R2 signal, especially in activated T cells, induces cell survival pathways that can result in cell proliferation (Faustman and Davis, 2010; Figure 7).

TNF- α reverse signaling

Transmembrane TNF- α acts as a ligand by binding to TNF- α receptors as well as functioning as a receptor that transmits outside-to-inside (reverse) signals back into the transmembrane TNF- α -bearing cells (TNF- α -producing cells) (Eissner et al., 2004; Zhang H et al., 2008).

mTNF deliver reverse signals initiated by anti-TNF mediated cross-linking, which costimulate T cells to express cytokines and adhesion molecules (Ferran et al., 1994; Higuchi et al., 1997; Harashima et al., 2001). Reverse signaling by mTNF differentially regulates CD4⁺ and CD8⁺ T cell activity against allogeneic endothelial cells by attenuating the proliferative potential of Th2 cells and increasing the cytotoxicity of CD8⁺ T cells (Vudattu et al., 2005).

Surprisingly, reverse signaling initiated by engaging mTNF with soluble TNF-Rs also leads to the desensitization of monocytes and macrophages to LPS by down-regulating the production of soluble TNF, IL-6, IL-1, and IL-10 (Eissner et al., 2000; Kirchner-Holler et al., 2004). Soluble TNF-R shed during inflammation might have the potential to bind mTNF and thereby downregulate inflammation. CKI is likely involved in the signaling pathway because it phosphorylates serine residue 5 of the mTNF cytoplasmic domain upon cell activation *in vitro* (Watts et al., 1999).

In addition, Duda et al. have shown that on the intracellular portion the serine side chain is phosphorylated, and that the 10 kDa peptide generated by the proteolytic processing of the 26 kDa form of TNF carries a functional nucleus localisation and IL-1 inflammatory cytokine production was affected (Pócsik et al., 1995; Domonkos et al., 2001).

Biological functions of TNF- α

Owing to its strong pro-inflammatory and immunostimulatory activities, TNF is, in general, an important mediator of progression of many autoimmune diseases. Important examples are rheumatoid arthritis and inflammatory bowel disease (Crohn's disease), where significant clinical improvement can be achieved when patients are treated with TNF neutralizing agents (Taylor et al., 2000; Blam et al., 2001).

Biological activities of transmembrane TNF- α as a ligand

Transmembrane TNF- α on the cell surface of TNF- α -producing cells binds to TNF receptors on the target cells and exerts various biological functions that will contribute to the modulation of local inflammation in a cell-to-cell contact manner as well as in a cell-type-specific fashion. Expression of transmembrane TNF- α on various cell types would contribute to the physiological as well as pathological responses in health and diseases (Table 1).

Target	Function
Tumour cells (various types)	Cytotoxicity
HIV-infected lymphocyte	Cell death
Intracellular parasite-infected macrophage	Inhibit the growth of intracellular pathogens
<i>Mycobacterium infection</i>	T-cell and macrophage migration, granuloma formation
Monocyte	IL-10 production
B cell	Proliferation, Ig production
T cell	HLA-DR and CD25 expression, GM-CSF production
NK cell	Enhancement of cytotoxic activity
Endothelial cell	Cell death, induction of pro-coagulant agents, adhesion molecules and pro-inflammatory cytokines
Adipose tissue	Inhibition of adipocyte differentiation, insulin resistance
Heart	Concentric cardiac hypertrophy
Lung	Interstitial inflammation
Liver	Hepatitis

Table 1. Biological activities of transmembrane TNF- α as a ligand

(Horiuchi et al., 2010)

Biological activities of transmembrane TNF- α as a receptor

Transmembrane TNF- α -bearing cells show their biological activity when transmembrane TNF- α on their cell surface is bound to its receptor, TNF-R1 or -R2. The biological activity is induced by the transmembrane TNF- α -mediated signal, also called an ‘outside-to-inside signal’ or ‘reverse signal’. The biological activities of transmembrane TNF- α as a receptor have been demonstrated in T cells, monocytes/macrophages and NK cells in humans (Harashima et al., 2001; Vudattu et al., 2005; Eissner et al., 2000; Xin et al., 2006; Yu et al., 2009).

1.3.4. Therapeutic agents targeting TNF- α in inflammatory diseases

Even though TNF- α is important for normal homeostatic mechanisms including host defense, dysregulated production of TNF- α has been found in several inflammatory diseases. In addition, TNF- α -activated macrophages are the principal components of the immunopathology of many autoimmune diseases (Flavell, 2002). Thus, macrophage-derived TNF- α is now implicated in a number of diseases, including rheumatoid arthritis, inflammatory bowel disease, psoriatic arthritis, ankylosing spondylitis, juvenile chronic arthritis, atherosclerosis, and sepsis (Feldmann et al., 1995; Kollias et al., 1999; Beutler and Cerami, 1989; Kleemann et al., 2008; Figure 8).

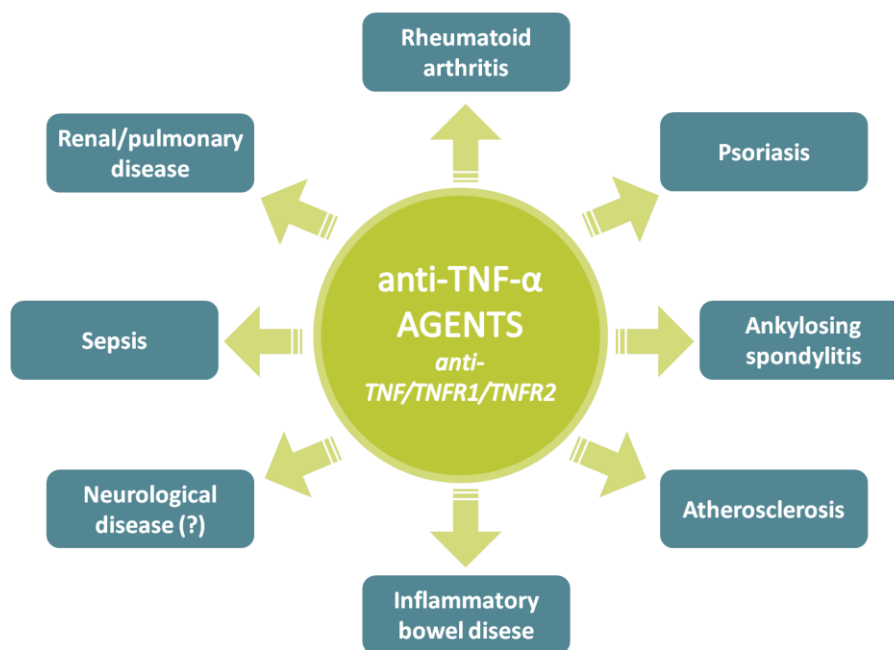


Figure 8. Clinical applications of anti-TNF- α agents

(modified from Parameswaran and Patial, 2010)

Nowadays five drugs (TNF- α blocking agents) are currently licensed for the treating of some diseases. These five drugs are: 1) etanercept is a recombinant human soluble fusion protein of TNF-R2 coupled to the Fc portion of IgG (Weinblatt et al., 1999); 2) infliximab is an anti-TNF human-murine chimeric IgG1 monoclonal antibody (Maini RN et al., 1998); 3) adalimumab is a human anti-human TNF- α antibody that was produced by phage display (den Broeder et al., 2002); (4) certolizumab pegol is a PEGylated TNF- α antibody (Choy et al., 2002); and 5) golimumab is a human anti-TNF- α IgG1 κ monoclonal antibody that can be

administered by the patient (Zhou et al., 2007). These drugs almost equally neutralize the TNF- α , however significant differences can be found as to the disease they are effective. This suggests that in addition to the neutralization of TNF- α , other effects may dictate the therapeutic results.

2. AIMS OF THE STUDIES

In my thesis I decided to investigate the potential involvement of two macrophage cell surface receptors (adenosine A₃ receptor, mTNF- α) in the apoptotic cell-mediated anti-inflammatory effects on macrophages. Our main objectives were:

1. It is known, that adenosine is produced by macrophages in from the adenine nucleotide released by apoptotic cells and exerts anti-inflammatory effects in an adenosine A_{2A}R-dependent manner. In the present study, I investigated the involvement adenosine A₃R in the regulation of inflammatory responses of macrophages engulfing apoptotic cells.
2. Since both apoptotic cell uptake and mTNF- α signaling have been shown to inhibit pro-inflammatory cytokine production of LPS-treated macrophages, I decided to investigate whether apoptotic cells trigger mTNF- α to inhibit LPS-induced pro-inflammatory production of macrophages. Since mTNF- α signaling was not known I decided also to identify its signaling elements.

3. MATERIALS AND METHODS

3.1. REAGENTS

Galardin, Wortmannin and SB-203580 were obtained from Calbiochem, rTGF- β was from AbD Serotec, and FITC-labeled anti-mouse TNF- α and its FITC-labeled isotype control, PE-labeled anti-mouse TNF-R type I/p55 and its PE-labeled isotype control Abs, were from BioLegend. TNF-RII PE-conjugated Ab, anti-mouse TNF- α and anti-mouse TGF- β Abs were from R&D Systems, and DAPI was from Life and Technologies. TCS JNK60 was from Tocris Bioscience. Anti-mouse MKK3/4/7, p-MKK4/7, and p-MKK3 (Ser¹⁸⁹)/MKK6 (Ser²⁰⁷) Abs were from Cell Signaling and Technology. Ficoll-Paque Plus was obtained from Amersham Biosciences. CD14 microbeads, human specific, were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). Human M-CSF was from PeproTech. All other reagents were obtained from Sigma-Aldrich (Budapest, Hungary).

List of synthetic compounds (Inhibitors, Agonist and Antagonist)

Name	Target
Cytochalasin D	Actin polymerization inhibitor
Forskolin	Adenylyl cyclase activator
Galardin	Inhibitor of metalloproteinases
H89	Protein kinase A inhibitor
IB-MECA	A ₃ Receptor-specific-agonist
L-NAME	Non-selective nitric oxide synthase (NOS) inhibitor
MRS1523	A ₃ Receptor-specific-antagonist
Rp-cAMP triethylamine	Membrane-permeable inhibitor of cAMP-dependent PKA I and II
SB-203580	p38 α/β kinases inhibitor
SCH442416	Selective A _{2A} R antagonist
TCS JNK60	Jun kinases inhibitor
Wortmannin	PI3K inhibitor

3.2. ANIMALS

The experiments were done using 3-month-old wild-type, adenosine A₃ receptor deficient mice developed and provided to us by the Merck Co., NJ, USA (Lee et al., 2003) generated on a C57BL/6 background. Some of the studies were carried out on adenosine A_{2A} receptor deficient mice (Ledent et al., 1997) generated on a FVB background.

In second part of the experiments 2-mo-old NMRI male mice were used, whereas in some experiments TNF- α –deficient male mice (Pasparakis et al., 1996) and their C57BL/6 wild-type littermates were used after being killed by ether anesthesia. Mice were maintained in a

specific pathogen-free condition in the Central Animal Facility and all animal experiments were approved by the Animal Care and Use Committee of the University of Debrecen [DEMÁB].

3.3. CELL CULTURE PROCEDURES

Macrophages were obtained by peritoneal lavage with sterile physiological saline. Cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 2 mM glutamine, 1 mM Na-pyruvate, 50 μ M 2-mercaptoethanol, 100 U/ml penicillin and 100 μ g/ml streptomycin at 37°C in 5% CO₂ for 2 days before use. After 3–4 h incubation, the non-adherent cells were washed away. Before the experiments the cells were cultured for 2 days replacing media daily.

For bone marrow-derived macrophage (BMDMs), bone marrow was isolated from femurs with sterile physiological saline and cultured in DMEM supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 10% L929 conditioned media at 37°C in 5% CO₂ for 7 days before use. RAW264.7 cells were obtained from the American Type Culture Collection and were maintained in a DMEM culture medium containing glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μ g/ml), and 10% FBS. Peripheral human blood mononuclear cells were isolated by density gradient centrifugation with Ficoll-Paque Plus from buffy coats obtained from the blood collected from healthy volunteers by the Blood Donation Center, Debrecen. Buffy coats not used up therapeutically were provided to us with the permit (RKEB 3582-2012) from the Regional Research Ethical Committee. CD14⁺ cells were separated by magnetic sorting with MACS, followed by washing with PBS containing 0.5% BSA and 2 mM EDTA. Freshly isolated monocytes were cultured for a period of 5 days in IMDM supplemented with 10% AB serum and 5 ng/ml M-CSF to differentiate them from macrophages.

3.4. THYMOCYTE APOPTOSIS INDUCTION *IN VITRO*

Thymocytes were prepared from 4-week-old wild-type or pannexin null mice (Corriden and Insel, 2012). The isolated thymocytes were cultured for 18 or 24 h (10^{6-7} cells/ml) in RPMI 1640 medium supplemented with penicillin/streptomycin in the absence of serum. This method typically resulted in >80% apoptotic cells (as assessed by propidium iodide/annexin V-FITC staining) (Köröskényi et al., 2011). In some experiments apoptosis was induced by 0.1 μ M dexamethasone or by a combination of 5 ng/ml phorbol dibutyrate and 1 μ g/ml

ionomycin. Apoptotic cells that died spontaneously were used in the immune downregulation assays at a 10:1 (apoptotic cell:macrophage) ratio.

3.5. DETERMINATION OF ADENOSINE A₃ RECEPTOR EXPRESSION ON THE CELL SURFACE

Wild-type and A₃R null peritoneal macrophages were co-incubated with apoptotic thymocytes for 1 h in 1:10 ratio. After replacing the media and washing away the apoptotic cells, macrophages were incubated for additional 1, 3 or 5 h. After the treatments the macrophages were washed (1× PBS), collected, blocked with 50% FBS for 30 min and labeled with the anti-mouse A₃R antibody (Santa Cruz Biotechnology) or goat IgG isotype control (R&D Systems). For detection the cells were stained with FITC-conjugated anti-goat IgG. The stained cells were analyzed on a FACSCalibur (BD Biosciences). The results were analyzed by using the WinMDI 2.9 software.

3.6. DETERMINATION OF ADENOSINE A₃ RECEPTOR mRNA EXPRESSION

Wild-type peritoneal macrophages were co-incubated with various target cell types: apoptotic, living, heat killed (45 min, 55°C) or anti-CD3-pretreated (10 µg/ml, 20 min; R&D Systems) adenosine A₃ null thymocytes for 1 h in the 1:10 ratio. After washing away the apoptotic cells and replacing the media, mRNA was collected 2 h later.

3.7. DETERMINATION OF ADENOSINE A₃ RECEPTOR PROTEIN LEVELS

Peritoneal macrophages were homogenized in an ice-cold lysis buffer containing 0.5% Triton X-100. The protein concentration of samples was diluted to 2 mg/ml, then the samples were boiled with an equal volume of Laemmli buffer. Electrophoresis was performed in a 10% SDS-polyacrylamide gel. The separated proteins were transferred to an Immobilon-P transfer membrane (Millipore, Budapest, Hungary) and were probed with an anti-mouse A₃R antibody (Santa Cruz Biotechnology). To detect A₃R signals, HRP-labeled anti-goat antibodies were used. Protein bands were visualized by the Immobilon Western Chemiluminescent HRP substrate (Millipore, Budapest, Hungary).

3.8. DETERMINATION OF CYTOKINE PRODUCTION

Wild-type and A₃R null peritoneal macrophages were plated onto 24-well plates at a density of 1×10^6 cells/well. To determine cytokine production by macrophages exposed to apoptotic cells, the macrophages (A₃R^{+/+} or A₃R^{-/-}) were exposed to apoptotic cells for 1 h in the

presence or absence of the A₃R-selective agonist IB-MECA (10 μM) or the A₃R-selective inhibitor MRS1523 (10 μM). Then the apoptotic cells were washed away, the compounds were readed and the macrophages were cultured for an additional 5 h.

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

3.9. PHAGOCYTOSIS ASSAY

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

3.10. DETERMINATION OF NO PRODUCTION OF MACROPHAGES ENGULFING APOPTOTIC CELLS

Wild-type or A₃R null macrophages were exposed to apoptotic cells for 1 h. The media were replaced and macrophages were incubated for an additional 1 h. Cell culture supernatants were analyzed for NO production by measuring nitrite, a stable oxidation product of NO, using the Griess-Ilosvay method (Kleinbongard et al., 2002).

3.11. IMMUNOHISTOCHEMISTRY

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

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

3.12. DETERMINATION OF TNFR I AND II EXPRESSION ON THE APOPTOTIC THYMOCYTE CELL SURFACE

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

3.13. IMMUNOFLUORESCENCE STAINING AND CONFOCAL MICROSCOPY

BMDMs were plated in eight-well chamber slides (2.5×10^5 /well) and cultured for 7 days before use. The cells were treated with 100 ng/ml LPS for 1 hour. After the exposure of macrophages to apoptotic cells stained overnight with 10 μ M 5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine (1:5) for 30 min, the cells were washed with HEPES buffer and fixed in methanol/acetone (1:1) for 10 min at -20°C . The macrophages were blocked with the 10% normal donkey serum in HEPES for 2 hours at 4°C , then washed with ice-cold HEPES buffer twice and stained with goat anti-mouse TNF- α primary Ab overnight at 4°C . After three washes, samples were incubated with Alexa Fluor 488-conjugated donkey anti-goat secondary Ab for 4 hours. The cells were then stained with DAPI for 10 min, washed four times, left in the HEPES buffer, and imaged immediately using a Zeiss LSM 510 confocal laser scanning microscope. For visualizing the distribution of mTNF- α , overview images and three-dimensional stacks were acquired at 1.5-mm optical thickness. Red fluorescence of 5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine-stained apoptotic cells and blue fluorescence of nuclei were imaged for the same fields in the multitrack mode to avoid spectral overlap.

3.14. DETERMINATION OF mTNF- α EXPRESSION ON THE CELL SURFACE

The BMDM and RAW264.7 cells were stimulated with 100 ng/ml bacterial LPS for the indicated time points after pre-incubation with galardin (5 μ g/ml) for 4 hours or alone. After the treatment, macrophages were washed (1x PBS), collected, blocked with 50% FBS for 30 min, stained with FITC-labeled anti-mouse TNF- α or FITC-labeled rat IgG1 isotype control

Abs, and fixed in 1% paraformaldehyde. The stained cells were analyzed on a FACSCalibur (BD Biosciences). The results were analyzed by WinMDI 2.9 software.

3.15. ISOLATION OF TOTAL RNA AND QUANTITATIVE RT-qPCR

Total RNA was isolated from control and bacterial LPS-treated BMDMs and RAW264.7 macrophages (3×10^6 cells/sample) by TRI reagent. After various treatments, the macrophages were washed with ice-cold PBS. RNA was extracted with TRI reagent. cDNA was synthesized with a high-capacity cDNA archive kit (Applied Biosystems) according to the manufacturer's instructions. TNF- α , TGF- β 1, and IFN- α mRNA levels were determined with TaqMan PCR using FAM-MGB-labeled probes (Applied Biosystems). Samples were run in triplicates on a Roche (LC480) platform using SDS2.1 software for evaluation (Applied Biosystems). The gene expression was normalized to the GAPDH expression (Table 2).

REVERSE TRANSCRIPTION		RT-qPCR	
Component	Volume/sample	Component	Volume/sample
Nuclease free water	4,2 μ l	Nuclease free water	2,04 μ l
RT puffer (10x)	2 μ l	Taq puffer (10x)	1 μ l
		MgCl ₂ (25 mM)	1,2 μ l
dNTP mix (100 mM)	0,8 μ l	dNTP mix (2,5 mM)	0,5 μ l
Random primers (10x)	2,0 μ l	20x oligo mix	0,2 μ l
MultiScribe RT enzyme	1 μ l	Taq polimerase (5 U/ μ l)	0,0625 μ l
RNA	10 μ l	cDNA	5 μ l
Total	20 μl	Total	10,0025 μl
THERMAL PROFILE			
1. 25 °C/10 min		1. 94 °C/1 min denaturation	
2. 37 °C/120 min		2. 94 °C/12 sec hybridization	
3. 85 °C/5 min		3. 60 °C/30 sec elongation	
4. 4 °C/ ∞		4. 40 cycles repeat	

Table 2. Details of reverse transcription and RT-qPCR measurement.

Calculation:

$$\text{Relative gene expression: } \frac{2^{-(Ct_{\max} - Ct_{\text{gene of interest}})}}{2^{-(Ct_{\max} - Ct_{\text{GAPDH}})}}$$

Ct_{max}: 40
Ct: threshold cycle

3.16. PHOSPHORYLATION CHANGES IN THE MAPK SIGNALING PATHWAY FOLLOWING TRIGGERING mTNF- α

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

3.17. WESTERN BLOT ANALYSIS TO DETECT PHOSPHORYLATION LEVELS OF MKK3/MKK4/MKK6/MKK7

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

3.18. STATISTICAL ANALYSIS

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

4. RESULTS

4.1. ADENOSINE A₃ RECEPTORS NEGATIVELY REGULATE THE ENGULFMENT-DEPENDENT APOPTOTIC CELL SUPPRESSION OF INFLAMMATION

4.1.1. The expression of adenosine A₃ receptors is decreased on the cell surface of macrophages during the engulfment of apoptotic cells

Previous studies have shown that adenosine A₃Rs are expressed by macrophages and contribute to the directed migration of these cells (Kronlage et al., 2010). Since our studies have shown that macrophages engulfing apoptotic cells produce adenosine and express increasing levels of A_{2A}Rs (Köröskényi et al., 2011; Sándor et al., 2016), we decided to test whether the expression levels of the A₃R are altered during the phagocytosis of apoptotic cells. As shown in Fig. 9A, we can confirm the expression of the cell surface adenosine A₃ receptors in mouse peritoneal macrophages, but this expression is significantly decreased following the incubation with apoptotic cells. The inhibition partially involved transcriptional regulation, since not only the cell surface expression of A₃R, but its mRNA and total protein levels also decreased during long term phagocytosis (Fig. 9B). However, the decrease in the cell surface expression could be detected much earlier than that of the mRNA and the protein, indicating that fast posttranslational mechanisms might also be involved in the downregulation of A₃R.

Cytochalasin D does inhibit the engulfment process, but it does not influence the recognition of apoptotic cells (Cvetanovic and Ucker, 2004). The binding of phosphatidylserine on the surface of apoptotic cells plays a key role in their recognition and subsequent uptake by the macrophages, and this recognition can be inhibited by the pre-incubation of apoptotic cells with recombinant annexin V (which binds to phosphatidylserine; Hoffmann et al., 2001). While recombinant annexin V prevented the downregulation of A₃R mRNA and protein expression by apoptotic cells (Fig. 9C), cytochalasin D had no effect on it, suggesting that not the engulfment, but the recognition *per se* triggers the decrease in the A₃R mRNA and protein expression. The inhibition of the A₃R expression during phagocytosis was specific for the engulfment of apoptotic cells, as the uptake of neither necrotic nor antibody-coated cells affected it (Fig. 9D).

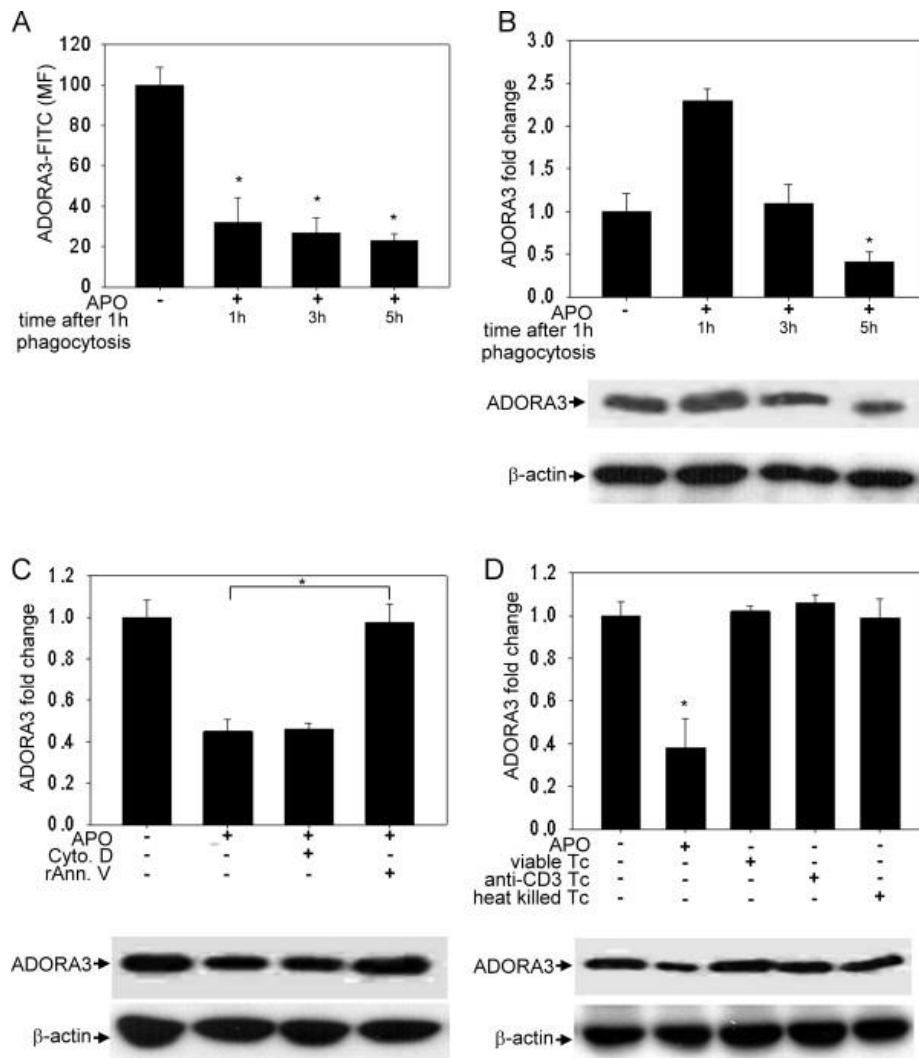


Figure 9. Apoptotic cell recognition inhibits the cell surface expression and the *de novo* adenosine A₃ receptor synthesis in peritoneal macrophages.

Wild-type peritoneal macrophages were exposed for 1 h to A₃R null apoptotic thymocytes and cell surface A₃R (A) and A₃R mRNA and protein levels (B) were determined by flow cytometry, quantitative PCR and Western Blot respectively, at the indicated time periods following washing away of apoptotic cells. (C) Blocking of apoptotic cell recognition abolishes the decrease of A₃ receptor level. Phagocytosis was inhibited by pretreating macrophages with 50 mM cytochalasin D or by masking the phosphatidylserine on the apoptotic cell surface with recombinant annexin V (10 μg/10⁵ apoptotic cell). After 1 h co-incubation apoptotic cells were washed away and macrophages were further cultured for 3 h. Adenosine A₃ receptor mRNA and protein levels were determined by quantitative PCR and Western Blot respectively. (D) Only apoptotic cell recognition decreases the *de novo* adenosine A₃ receptor synthesis in peritoneal macrophages. Macrophages were co-incubated with apoptotic, living, heat killed (45 min, 55°C) or anti-CD3-pretreated (10 μg/ml, 20 min) A₃R null thymocytes for 1 h. Thymocytes were then washed away and macrophages were further incubated for 3 h. The gene expression

and protein levels were measured by quantitative PCR and Western Blot respectively. MFI: mean fluorescence intensity. The results are expressed as mean \pm SD of three or four independent experiments (*P < 0.05).

4.1.2. Loss of adenosine A₃ receptor influences the pro-inflammatory cytokine production by macrophages engulfing apoptotic cells

The evaluation of the cytokine secretion profile of unstimulated macrophages was performed using a highly sensitive cytokine antibody array method, enabling the simultaneous detection of low concentrations of multiple cytokines in one assay (picogram per milliliter range). The map of the 40 cytokines detected on the membranes is diagrammed in Fig. 10A. The cytokines in our experimental systems were first evaluated by the experiments using untreated wild-type and A₃R^{-/-} macrophages *in vitro*. The results reported in Fig. 10B show that the loss of the A₃R did not affect significantly the composition of the cytokines released. In the levels of the cytokines we found individual animal variations, but when the MIP-2 and KC levels were determined in many samples by ELISA, no significant difference could be detected in their basal levels when we compared the wild-type and the A₃R^{-/-} (Fig. 10D). However, as shown in Fig. 10C, when macrophages were exposed to apoptotic cells, we found four cytokines whose production was increased by the wild-type macrophages engulfing apoptotic cells compared to their non-engulfing counterparts, but decreased in A₃R^{-/-} macrophages. These cytokines include the cytokine-induced neutrophil-attracting chemokine (KC), and the macrophage inflammatory protein-2 (MIP-2), which act as chemoattractants for neutrophils and/or other cell types (Iida and Grotendorst, 1990; Wolpe et al., 1989; Wolpe and Cerami, 1989), the pro-inflammatory cytokine IL-1 α (Dinarello, 1994) and the antagonist of the IL-1 receptor (IL-1ra). Among the cytokines released in altered amounts in the supernatant by cultured A₃R^{-/-} macrophages, MIP-2 showed the most dynamic change in response to the apoptotic cell exposure, and MIP-2 and KC levels were detected in the highest amounts (Fig. 10B and C). These observations correlate with those found in the A_{2A}R null macrophages, in which the loss of the A_{2A}R affected the production of the same cytokines in the presence of the apoptotic cells (Köröskényi et al., 2011), but the direction of the alteration was the opposite: the loss of A_{2A}Rs lead to an increase in the production of these cytokines. To confirm further the effect of A₃R on the expression of the above cytokines during the apoptotic cell engulfment, we decided to test the effect of A₃R on the pro-inflammatory cytokine production by exposing wild-type macrophages either to the highly specific A₃R agonist IB-MECA or to the A₃R antagonist MRS1523.

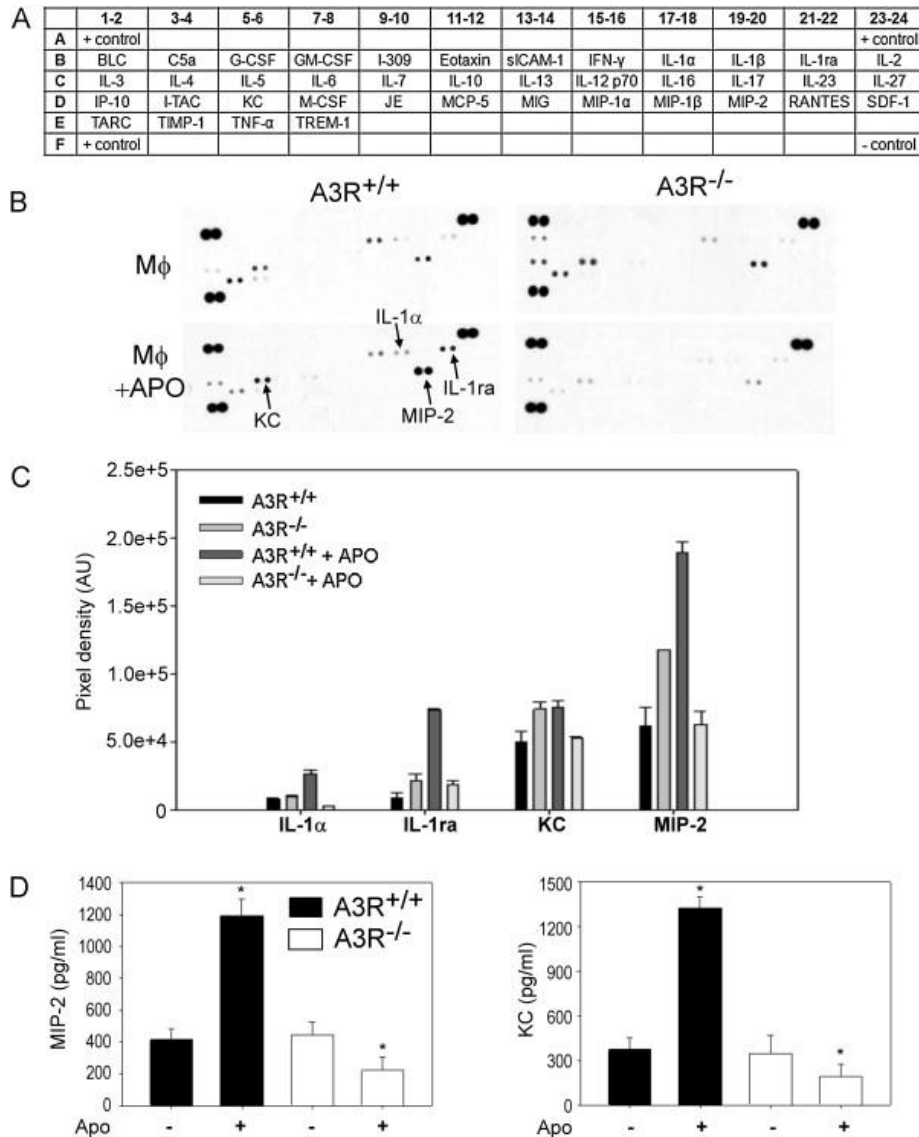


Fig. 10. Adenosine A₃ receptor-deficient macrophages respond to apoptotic cells with a decreased MIP-2 and KC production.

(A) The map of the 40 cytokines detected on the membranes. (B) Cytokine panel of control and apoptotic cell treated wild-type and adenosine A₃ receptor null peritoneal macrophages. Peritoneal macrophages were co-incubated with apoptotic thymocytes for 1 h (M ϕ :APO = 1:10) followed by the removal of apoptotic cells and addition of fresh medium. Supernatants were collected 5 h later and cytokine levels were determined by cytokine array. The arrows highlight KC, MIP-2, IL-1 and IL-1ra neutrophil chemoattractants, the production of which is significantly downregulated by A₃R null macrophages. (C) Cytokines, whose levels were significantly different ($P < 0.05$) in the supernatants analyzed by the cytokine array. (D) Wild-type and A₃R null macrophages were exposed to apoptotic thymocytes for 1 h (M ϕ :APO = 1:10). 5 h after the removal of the apoptotic cells supernatants were collected. The levels of MIP-2 and KC were determined by ELISA. The results are expressed as mean \pm SD of 10 independent experiments (* $P < 0.05$). The results are expressed as mean \pm SD of three independent experiments.

The modification in the levels of cytokines was not due to a difference in the extent of phagocytosis, as a similar rate of engulfment by wild-type and $A_3R^{-/-}$ macrophages was observed by counting the number of apoptotic cells taken up by 500 individual macrophages (Fig. 11).

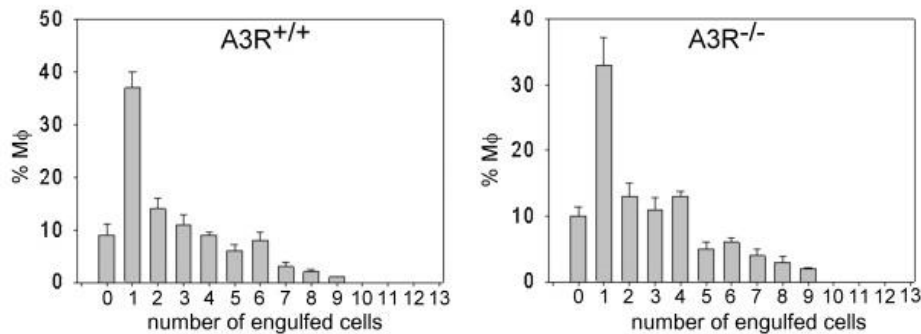


Figure 11. Adenosine A_3 receptor deficiency is not accompanied by altered phagocytotic capacity in macrophages.

The number of engulfed fluorescently labeled apoptotic cells within WT or A_3R null macrophages counted by a confocal microscopy following 1 h of phagocytosis. The results are expressed as mean \pm SD of three independent experiments.

As shown in Fig. 12, using wild-type macrophages we could confirm the decreased expression of the pro-inflammatory cytokines in the presence of the A_3R antagonist, indicating that altered cytokine response of engulfing A_3R null macrophages is not due to an altered differentiation of the cells. In addition, the presence of the A_3R agonist enhanced the expression of these cytokines indicating that A_3R signaling indeed negatively regulates the engulfment-dependent apoptotic cell suppression of inflammation, at least if these cytokines are concerned. The same compounds had no effect on the apoptotic cell-induced pro-inflammatory cytokine production, when they were administered to $A_3R^{-/-}$ macrophages (data not shown) indicating that they indeed act *via* the A_3R s. To confirm the results, MIP-2 and KC protein levels were also assessed by ELISA. In harmony with the cytokine array results, while the production of MIP-2 and KC was enhanced by wild-type macrophages exposed to apoptotic cells as compared to non-engulfing macrophages, the release of both MIP-2 and KC by $A_3R^{-/-}$ macrophages was decreased under the same conditions (Fig. 10D).

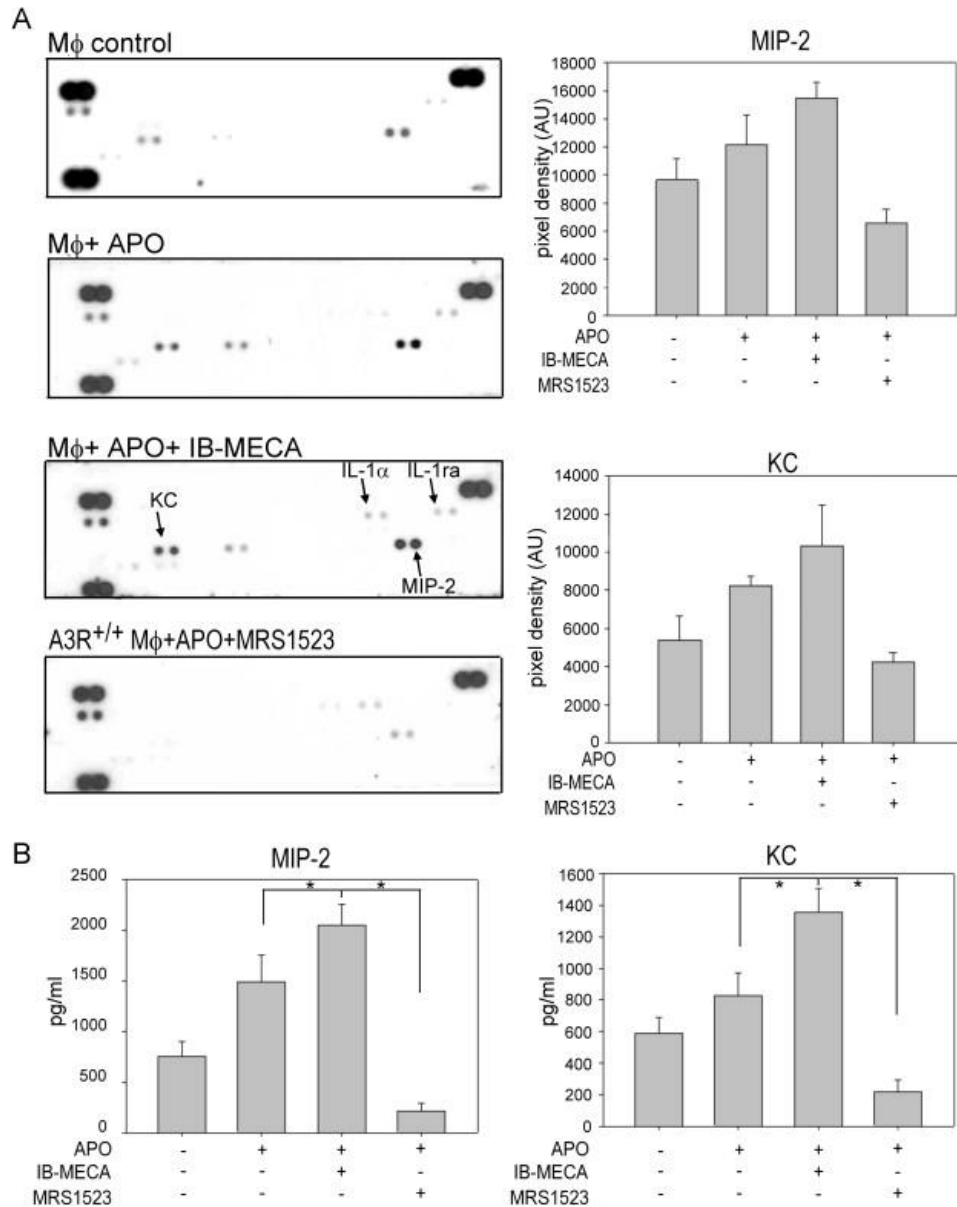


Figure 12. As compared to controls, MIP-2 and KC production is enhanced by A₃R agonist-treated, while decreased by A₃R antagonist-treated wild-type macrophages engulfing apoptotic cells.

(A) The cytokine panel of control, apoptotic cell exposed non-treated, IB-MECA (A₃R-specific agonist)-treated or MRS1523 (A₃R-specific antagonist)-treated wild-type macrophages. (B) MIP-2 and KC production of control, apoptotic cell exposed non-treated, IB-MECA (A₃R-specific agonist)-treated or MRS1523 (A₃R-specific antagonist)-treated wild-type macrophages determined by ELISA. Peritoneal macrophages were preincubated with 10 μM IB-MECA or with 10 μM MRS1523 for 30 min, then they were exposed to apoptotic thymocytes for 1 h (Mφ:APO = 1:10). 5 h after the removal of the apoptotic cells supernatants were collected and cytokine levels were determined by a cytokine array. The arrows highlight KC and MIP-2 overproduced by IB-MECA-treated macrophages. The results are expressed as mean ± SD of three independent experiments (*P < 0.05).

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

Since among the neutrophil chemoattractants, the release of which was simultaneously altered by the loss of both the $A_{2A}R$ and the A_3R , the production of MIP-2 and KC was studied in our previous experiments in detail, we decided to investigate further the regulation of the expression of these two cytokines. Previously we have shown (Köröskényi et al., 2011) that the production of these two cytokines would be triggered by the uptake of apoptotic cells, but the simultaneous activation of the adenylyate cyclase pathway triggered by the $A_{2A}Rs$ attenuated the response. Since A_3Rs are known to inhibit the adenylyate cyclase activity *via* stimulating G_i (Fredholm et al., 2001), we speculated that if adenosine is produced by engulfing macrophages in an amount that can trigger A_3Rs , the loss of A_3R signaling would lead to the enhanced adenylyate cyclase signaling by $A_{2A}Rs$ in A_3R null macrophages. If it is so, the enhanced adenylyate cyclase signaling could explain the more effective inhibition of KC and MIP-2 production by engulfing A_3R null macrophages. Indeed, the addition of SCH442416, a selective $A_{2A}R$ antagonist, H89, a protein kinase A inhibitor, or Rp-cAMPS triethylamine, a specific membrane-permeable inhibitor of activation by cAMP of cAMP dependent protein kinase I and II (van Haastert et al., 1984), all significantly enhanced the production of MIP-2 and KC in apoptotic cell-triggered A_3R null macrophages (Fig. 13A). In contrast, the addition of forskolin, an adenylyate cyclase activator (Seamon et al., 1981) decreased the MIP-2 and KC production in wild-type macrophages. The same concentration of forskolin, however, was ineffective in A_3R null cells indicating that adenylyate cyclase is fully activated in these cells (Fig. 13B). When the adenylyate cyclase pathway inhibitor Rp-cAMPS triethylamine was applied to $A_{2A}R$ or A_3R null macrophages and their wild-type counterparts, a similar MIP-2 and KC production was found in the paired macrophages indicating that in the absence of the adenylyate cyclase signaling, the difference in apoptotic cell triggered-MIP-2 and KC production disappears (Fig. 13C). At the same time a significant strain difference was detected in the apoptotic cell-induced pro-inflammatory cell response. However, as we do not know at the moment how apoptotic cells induce the adenosine-controlled pro-inflammatory response, we cannot identify the molecular determinants for the observed difference between the mouse strains. These data indicate that in wild-type macrophages the adenylyate cyclase pathway is not fully activated, and a balance in the $A_{2A}R$ and A_3R signaling, which regulates the activity of the adenylyate cyclase pathway in an

opposite way, will decide the outcome of MIP-2 and KC production in the presence of apoptotic cells.

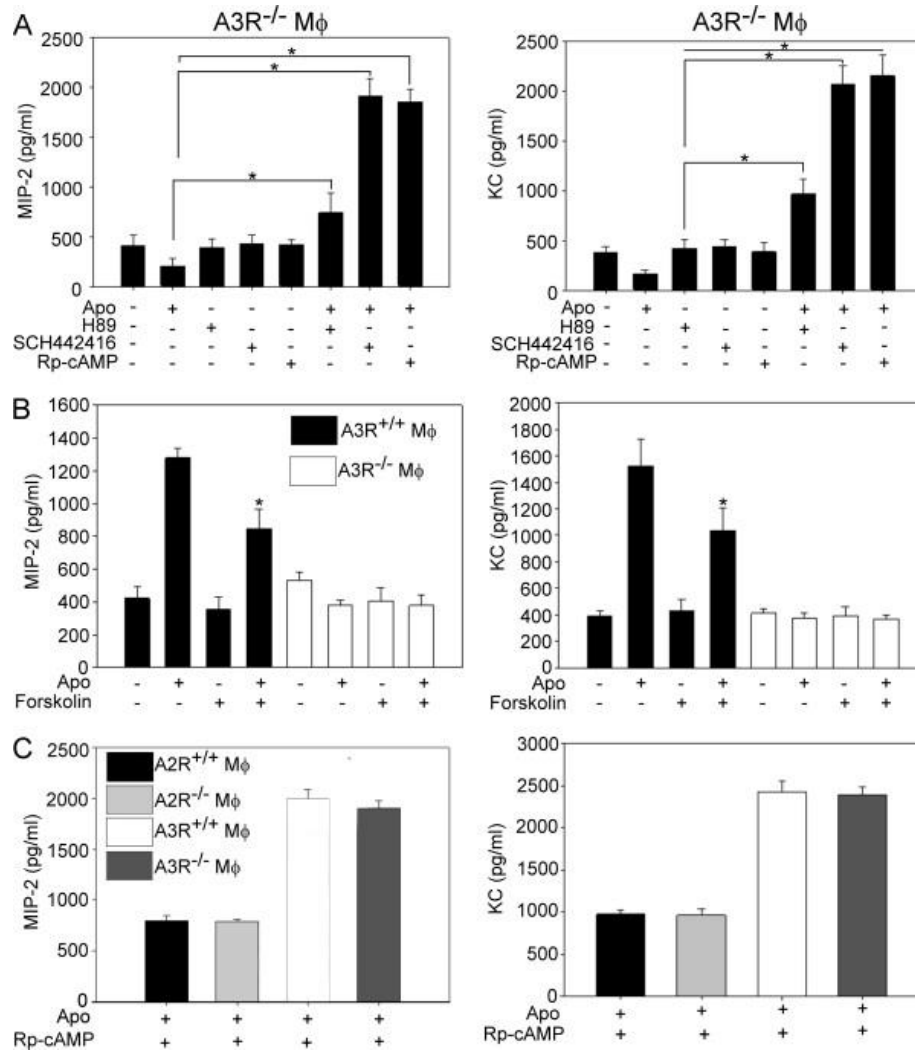


Figure 13. The altered cytokine production of A₃R null engulfing macrophages is related to an enhanced adenylyate cyclase signaling induced by A_{2A}Rs.

(A) A₃R null macrophages were preincubated in the presence of 10 nM SCH442416, an A_{2A} receptor antagonist, 100 μ M Rp-cAMP triethylamine or 10 μ M H89, inhibitors of protein kinase A for 30 min, then exposed to apoptotic thymocytes for 1 h (M ϕ :APO = 1:10). (B) Wild-type and A₃R null macrophages were preincubated in the presence of 10 μ M forskolin, an adenylyatecyclase activator for 30 min, then exposed to apoptotic thymocytes for 1 h (M ϕ :APO = 1:10). (C) A₃R and A_{2A}R null macrophages and their wild-type counterparts were preincubated in the presence of 100 μ M Rp-cAMP triethylamine for 30 min, then exposed to apoptotic thymocytes for 1 h (M ϕ :APO = 1:10). 5 h after the removal of the apoptotic cells supernatants were collected in all the above experiments and the levels of MIP-2 and KC were determined by ELISA. The different basal levels of cytokines might be related to the fact that the mice were of different genetic background. The results are expressed as mean \pm SD of three independent experiments (*P < 0.05).

4.1.4. Altered MIP-2 and KC production by A₃R null macrophages is a consequence of altered NO production

Our previous studies have shown that the upregulation of NO production in macrophages exposed to apoptotic cells contributes to MIP-2 release (Köröskényi et al., 2011). To confirm that the regulation of NO production is involved in the altered MIP-2 and KC response of A₃R null macrophages as well, the macrophages were exposed to the nitric oxide synthase (NOS) inhibitor L-(G)-nitro-L-arginine methyl ester(L-NAME) before the addition of apoptotic cells. As we have previously reported (Köröskényi et al., 2011), the addition of L-NAME attenuated apoptotic cell-induced the MIP-2 protein expression by wild-type macrophages (Fig. 14A), L-NAME had no effect on the MIP-2 production generated by A₃R null macrophages. In accordance with these results, the wild-type macrophages produced detectable NO levels upon the apoptotic cell exposure while the A₃R null macrophages did not (Fig. 14B). In the presence of Rp-cAMP, however, the apoptotic cell exposed to the A₃R^{-/-} macrophages were capable of producing similar amounts of NO as wild-type macrophages indicating that their NO production is suppressed by the adenylate cyclase pathway. Previous studies have shown that the addition of sodium nitropusside, a potent NO donor, alone did not induce the MIP-2 production, but significantly enhanced the apoptotic cell-induced MIP-2 production in wild-type macrophages (Köröskényi et al., 2011). Interestingly, sodium nitropusside had no effect on the MIP-2 production in the A₃R null engulfing macrophages (Fig. 14C). When, however, sodium nitropusside was applied to them together with Rp-cAMP, an enhanced MIP-2 production could be detected by A₃R^{-/-} engulfing macrophages as well. These data indicate that the enhanced adenylate cyclase signaling in A₃R null macrophages must inhibit simultaneously not only the NO production, but also other signaling element(s) which contribute to the apoptotic cell-induced MIP-2 production.

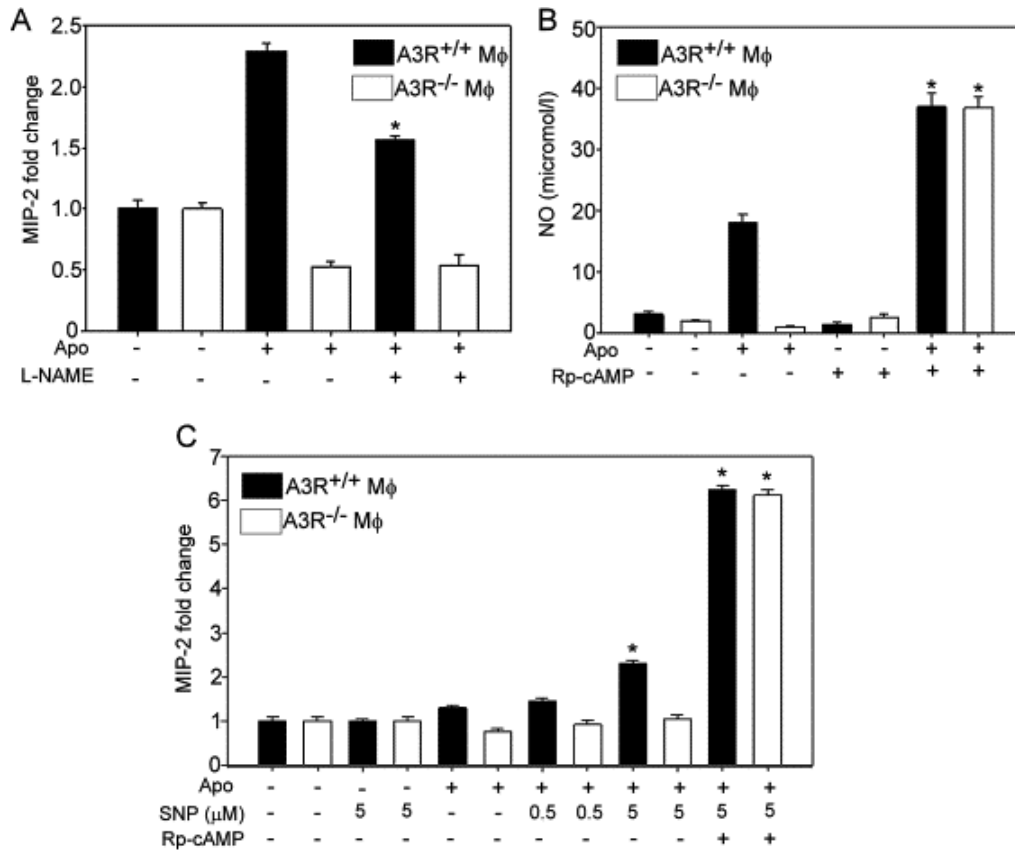


Figure 14. The altered MIP-2 production by A₃R null macrophages engulfing apoptotic cells is related to an altered NO production.

(A) L-NAME, an inhibitor of nitric oxide synthetase, prevents the apoptotic cell-induced MIP-2 production in wild-type engulfing macrophages, but has no effect on it in the A₃R null engulfing macrophages. Macrophages were preincubated in the presence of 5 μM L-NAME for 30 min prior to the exposure to the apoptotic cells for 1 h. MIP-2 mRNA levels were determined 2 h after the removal of the apoptotic cells. (B) The engulfing A₃ null macrophages do not produce NO, unless the adenylate cyclase pathway is inhibited. Macrophages were preincubated with 100 μM Rp-cAMP triethylamine for 30 min, than exposed to the apoptotic thymocytes for 1 h (Mφ:APO = 1:10). NO levels were determined 1 h later. (C) Exogenously produced NO stimulates the apoptotic cell-triggered MIP-2 production in A₃R null macrophages only if the adenylate cyclase pathway is simultaneously inhibited. Wild-type and A₃R null peritoneal macrophages were exposed to the indicated concentrations of the NO donor sodium nitropusside (SNP) in the presence or absence of apoptotic cells. In some cases macrophages were pretreated with 100 μM Rp-cAMP triethylamine for 30 min prior to the exposure to apoptotic cells. Apoptotic cells were washed away after 1 h. MIP-2 mRNA levels were determined 2 h later. The results are expressed as mean ± SD of five independent experiments (*P < 0.05).

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

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

First, we investigated with confocal microscopy whether TNF- α is detectable in non-stimulated BMDMs or RAW264.7 macrophages. As shown in Fig. 15A, TNF- α is detectable in both BMDMs and RAW264.7 macrophages in intracellular vesicles. Although the protein could be readily seen, only a minimal amount of TNF- α mRNA was detected in non-stimulated cells.

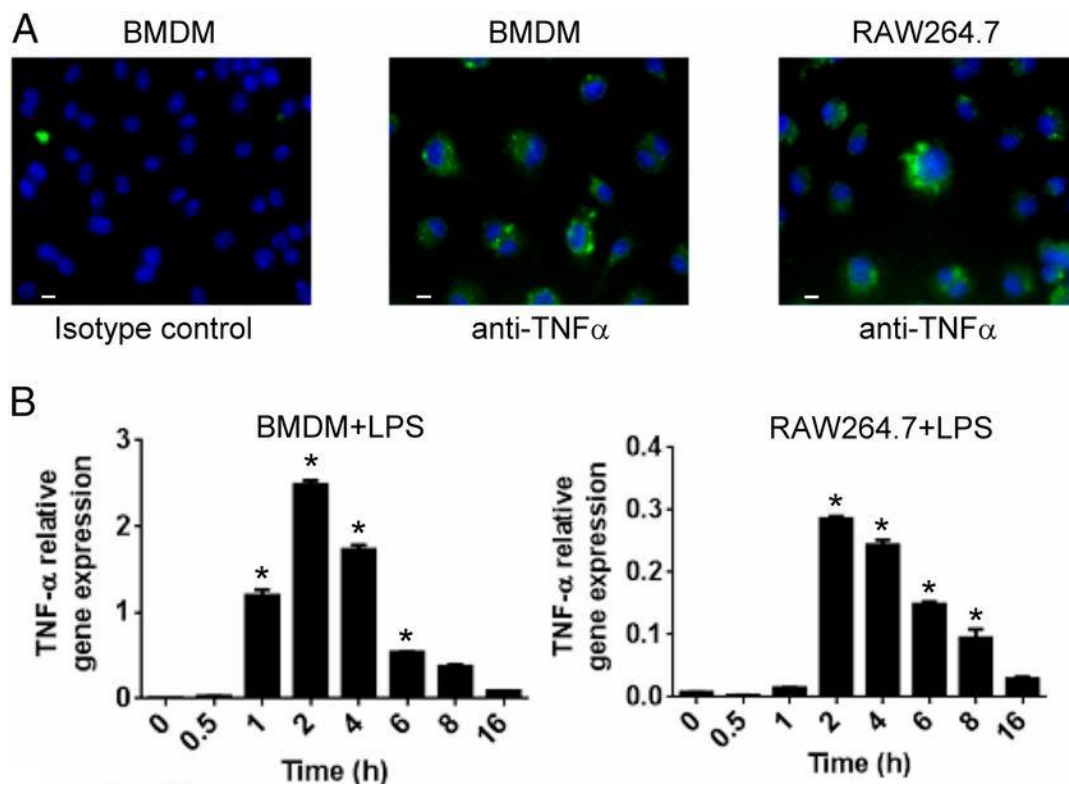


Figure 15. TNF- α is expressed by resting macrophages and is further induced by LPS.

(A) TNF- α is expressed by resting BMDMs and RAW264.7 macrophages detected by a confocal microscopy. Green indicates TNF- α ; blue indicates cell nuclei stained with DAPI. The scale bar, 10 μ m. (B) LPS induces the mRNA levels of TNF- α in both BMDMs and RAW264.7 macrophages. Macrophages were exposed to 100 ng/ml LPS for the indicated times followed by RNA isolation and reverse transcription. The gene expression levels of TNF- α were determined by quantitative RT-PCR using GAPDH as a normalizing gene. The results are mean \pm SD from three independent experiments. The results are mean of three independent experiments. *p < 0.05 versus control.

When, however, macrophages were exposed to LPS (100 ng/ml), a significant increase in TNF- α mRNA levels was detected in both cell types, reaching its maximum following a 2-hour stimulation (Fig. 15B). Then the mRNA levels gradually decreased.

In line with these observations, the cell surface levels of mTNF- α of both BMDMs and RAW264.7 macrophages (Fig. 16A), which were not detectable in non-stimulated cells, started to rise after the LPS stimulation and became more readily detectable when the cell surface cleavage of mTNF- α was inhibited by galardin, a synthetic metalloproteinase inhibitor (Grobelyny et al., 1992).

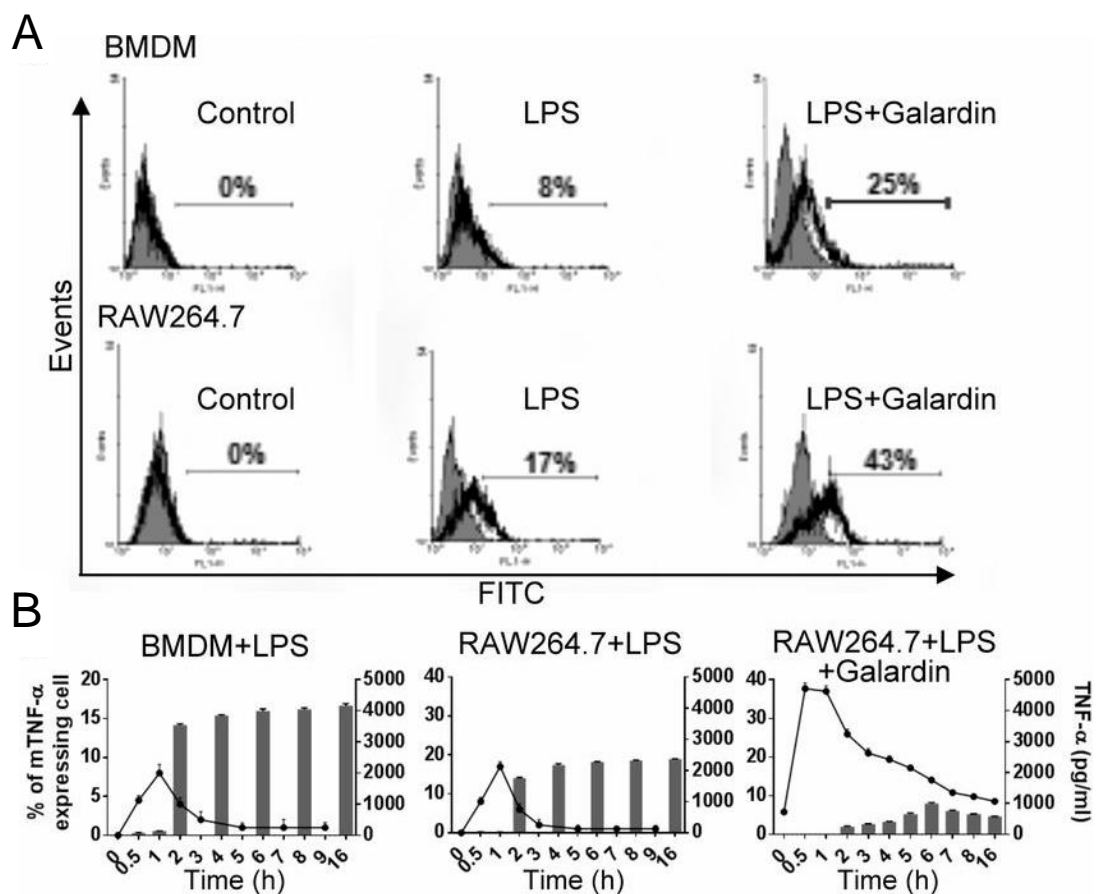


Figure 16. TNF- α is induced by LPS and Galardin.

(A) LPS induces the expression of mTNF- α detected by the FACS analysis in 1 h following the LPS (100 ng/ml) exposure. The addition of galardin, a metalloprotease inhibitor (5 μ g/ml) 4 hours before the LPS exposure, enhances the percentage of mTNF- α -expressing cells. (B) The time course of the percentage of mTNF- α -expressing macrophages (line graph) and the amount of soluble TNF- α in the cell culture medium (bar graph) following the LPS (100 ng/ml) exposure in the presence or absence of galardin detected by the FACS analysis and ELISA, respectively. The results are mean for the mTNF- α -expressing cells and mean \pm SD for the ELISA data from three independent experiments. * p < 0.05 versus control.

The galardin treatment tested in RAW264.7 cells alone also increased the cell surface expression of mTNF- α (Fig. 16B), indicating that 1) the metalloprotease responsible for the cleavage of mTNF- α must be expressed in non-stimulated cells as well, and 2) although we could not detect the presence of mTNF- α without galardin, there must also be some basal level of mTNF- α production in non-stimulated cells. The cell surface expression of mTNF- α of LPS-treated BMDMs and RAW264.7 cells reached its maximum in 1 hour (Fig. 16B), and then it started to gradually decrease. In galardin-treated RAW264.7 cells, the increase in the cell surface mTNF- α expression could be detected as soon as 15 min after the LPS stimulation, indicating that mTNF- α at the beginning must come from the already existing intracellular TNF- α pool. The sharp decrease of LPS-induced mTNF- α expression following 1 hour of stimulation (Fig. 16B) despite the increase in TNF- α mRNA expression (Fig. 15B) indicates that the expression of the metalloprotease, which is responsible for the mTNF- α cleavage, must also be induced by the LPS stimulation in both types of macrophages. The galardin treatment considerably delayed the decrease in the mTNF- α expression of macrophages and also delayed and decreased the appearance of soluble TNF- α in the cell culture medium (Fig. 16B).

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

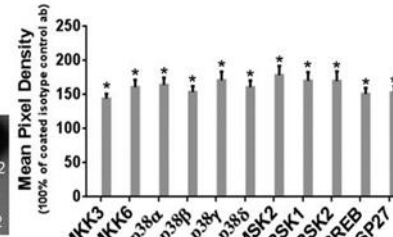
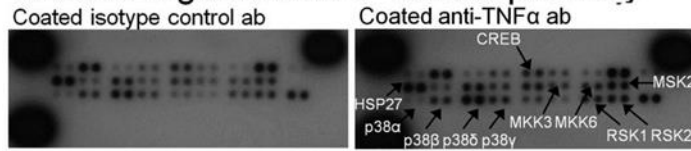
Since our preliminary data indicated that mTNF- α must be present on the surface of non-stimulated macrophages as well, we decided to investigate mTNF- α signaling in BMDMs stimulated with the help of a phospho-MAPK array kit, which enables the parallel determination of relative levels of phosphorylation of MAPKs and those of several related proteins. The data received in the presence of coated anti-mTNF- α Abs were compared with those that resulted in the presence of isotype control Abs. The stimulation of mTNF- α by coated anti-mTNF- α Abs led to the activation of the MKK3/6-regulated MAPK, the JNK, the ERK kinase, and the PI3K pathways (Fig. 17A, 17B). Since preliminary experiments, which we used as a read-out to detect the activation of mTNF- α signaling, demonstrated that soluble anti-TNF- α Abs are not able to inhibit LPS-induced IL-6 and MIP-2 production, we applied coated Abs to trigger mTNF- α signaling (Fig. 18).

A

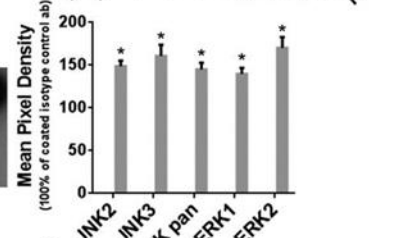
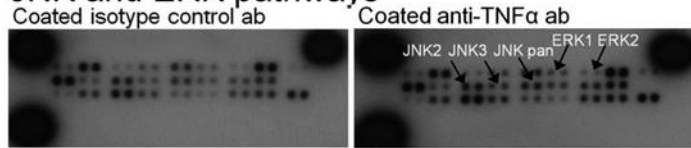
	1-2	3-4	5-6	7-8	9-10	11-12	13-14	15-16	17-18	19-20	21-22
A	+control										+control
B		Akt1	Akt2	Akt3	Akt pan	CREB	ERK1	ERK2	GSK-3 α/β	GSK-3 β	
C		HSP27	JNK1	JNK2	JNK3	JNK pan	MKK3	MKK6	MSK2		
D		p38 α	p38 β	p38 δ	p38 γ	p53	p70 S6 Kinase	RSK1	RSK2	TOR	
E										-control	
F	+control										

B

MKK3/6 regulated MAP kinase pathway



JNK and ERK pathways



PI3 kinase pathway

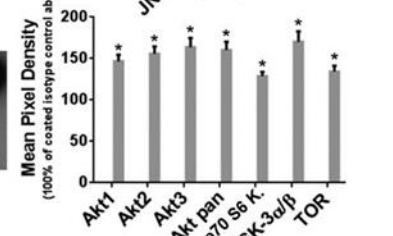
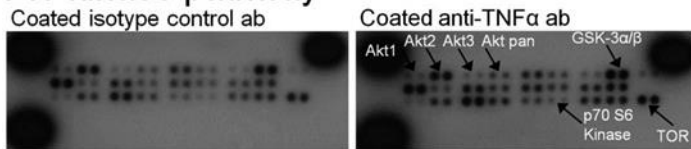


Figure 17. mTNF- α induces the MKK3/6-regulated MAPK, Jun kinase, ERK kinase, and PI3K pathways in mouse BMDMs.

(A) The map of 26 phosphorylated proteins detected on the membranes. (B) The phospho-MAPK panel of coated isotype control or coated anti-mTNF- α Ab-exposed macrophages. BMDMs were exposed to the coated isotype control or anti-mTNF- α Abs for 2 hours. The macrophages were then lysed, and the amount of phosphorylated proteins was detected by a phospho-MAPK array. The arrows highlight those proteins for which phosphorylation was enhanced in BMDMs exposed to the coated anti-mTNF- α Abs as compared with the isotype control. One representative array of a series of three is shown. Elevated phosphorylation is expressed as a percentage of mean pixel density of the respective isotype Ab control (mean \pm SD from three independent experiments). * $p < 0.05$ versus respective control.

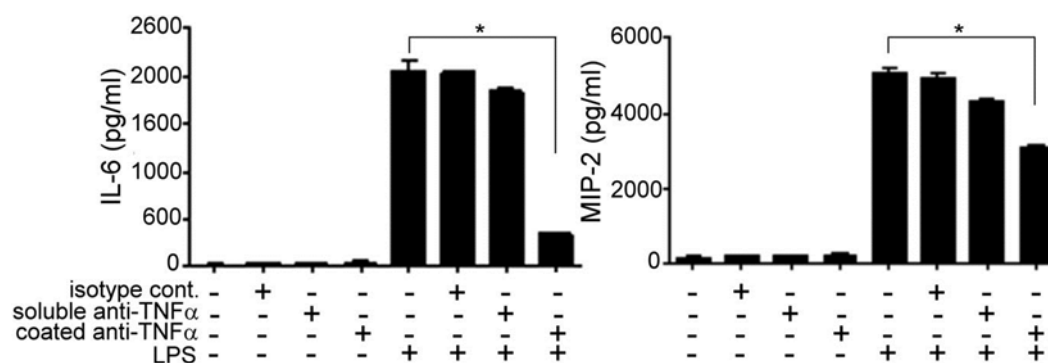


Figure 18.

Soluble anti-TNF- α Abs do not significantly inhibit LPS-induced IL-6 and MIP-2 production detected at 6 h by ELISA, whereas coated anti-mTNF- α Abs inhibit such production. The macrophages were exposed to either soluble anti-TNF- α (2 μ g/ml) or coated anti-mTNF- α (2 μ g/ml) Abs for 2 hours before exposing the macrophages to LPS (100 ng/ml). The cytokine levels were determined 6 hours later. The results are mean \pm SD from three independent experiments. * $p < 0.05$ versus the respective LPS exposed control.

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

Because these alterations in the phosphorylation status of various proteins were detected 2 hours after the macrophages were seeded onto the coated Abs, we could not exclude the possibility that mTNF- α signaling induced the expression of a cytokine that drives the above-mentioned three signaling pathways. Since TNF- α , IFN- α , and TGF- β were previously shown to activate the MKK3/6, Jun kinase, and PI3K pathways (Wysk et al., 1999; Zhang YE, 2009; Li Y et al., 2005; Wu et al., 2005), we decided to test whether the expression of these cytokines is induced by triggering mTNF- α . Although we could not detect the induction of IFN- α or TNF- α at mRNA levels (data not shown), the induction of TGF- β was readily detected by a pan anti-TGF- β Ab at protein levels (Fig. 19A) and that of TGF- β 1 at mRNA levels (Fig. 19B). To determine whether TGF- β is indeed responsible for the mTNF- α triggering-induced MKK3/6, JNK, ERK kinase, and PI3K pathway activation, the experiments described in Fig. 17 were repeated in the presence of neutralizing anti-TGF- β Abs as well. As shown in Fig. 19C, mTNF- α -induced phosphorylation of those proteins, which are known to be activated by the TGF- β signaling pathway (Massagué, 2012) (Fig 19D), was completely or partially prevented by the administration of neutralizing TGF- β Abs. Indeed, these data confirm that the mTNF- α signaling-induced TGF- β is responsible for their phosphorylation.

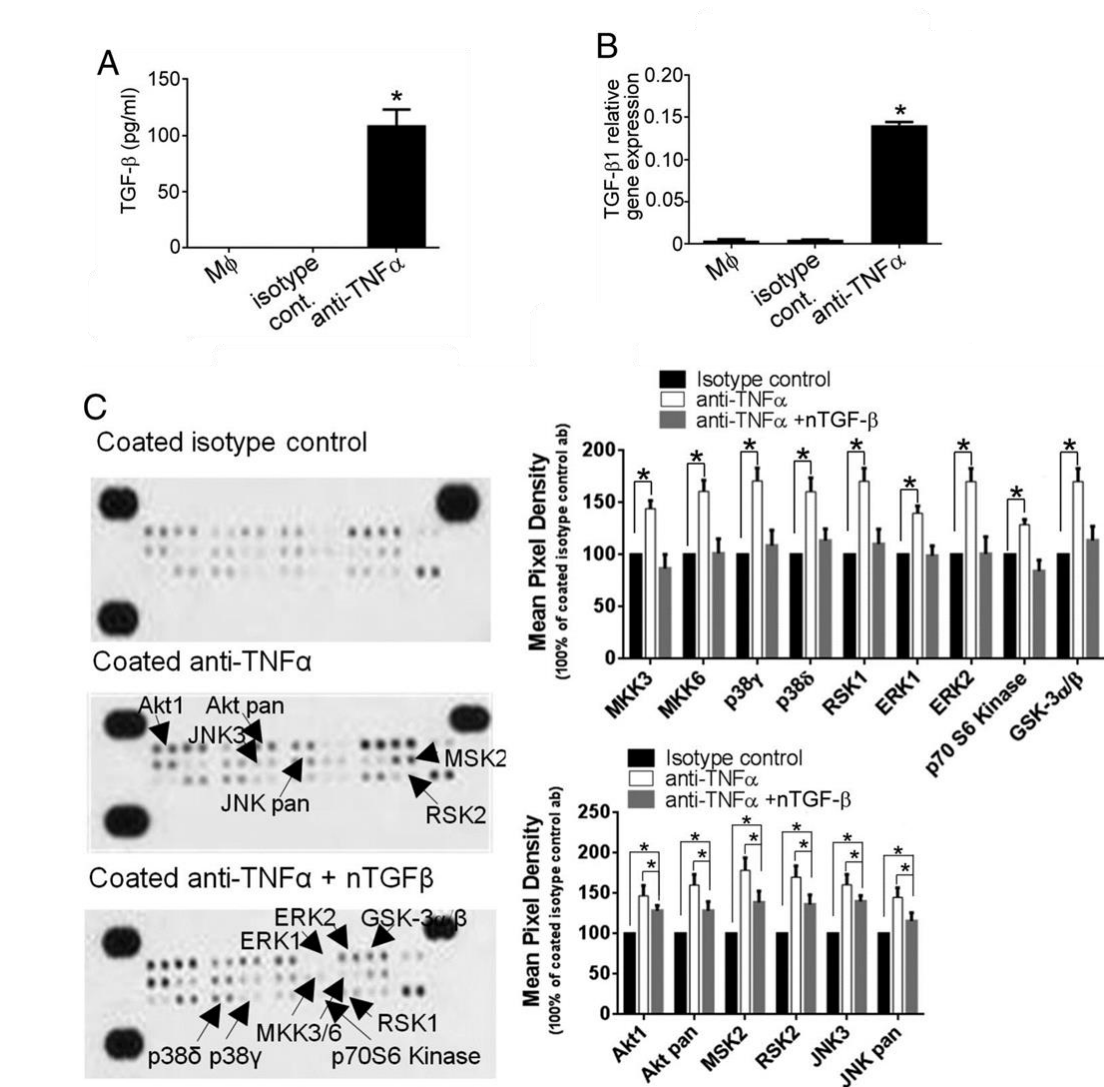


Figure 19. Triggering of mTNF-α induces TGF-β production in mouse BMDMs.

(A) BMDMs were exposed to coated anti-mTNF-α (2 μg/ml), and the TGF-β produced was detected 6 hours later by ELISA using an anti-pan-TGF-β Ab. (B) BMDMs were exposed to coated anti-mTNF-α Abs for 2 hours followed by RNA isolation and reverse transcription. The gene expression levels of TGF-β1 were determined by quantitative RT-PCR using GAPDH as a normalizing gene. The results are mean ± SD from three independent experiments. *p < 0.05 versus isotype Ab-exposed control.

(C) The phospho-MAPK panel of coated isotype control, coated anti-mTNF-α Ab-, or coated anti-mTNF-α and neutralizing anti-TGF-β Ab-exposed macrophages. BMDMs were exposed to coated isotype control (2 μg/ml),

anti-mTNF- α (2 μ g/ml), or anti-mTNF- α and neutralizing anti-TGF- β (5 μ g/ml) Abs for 2 hours. The macrophages were then lysed, and the number of phosphorylated proteins was detected by a phospho-MAPK array. The arrows highlight those proteins for which the anti-mTNF- α -enhanced phosphorylation was fully or partially prevented by neutralizing anti-TGF- β Abs. One representative array series of three is shown. The enhancement in phosphorylation is expressed as a percentage of mean pixel density of the respective isotype Ab control (mean \pm SD from three independent experiments). **(D)** The phosphorylated proteins identified by the usage of neutralizing TGF- β Abs are all known participants of the TGF- β signaling pathway. * $p < 0.05$ versus respective control.

4.2.4. mTNF- α signaling induces the MKK4 signaling pathway

The same blots were then analyzed further to determine the kinases that belong directly to mTNF- α signaling. As shown in Fig. 20, those kinases were selected to belong to the mTNF- α signaling pathway, the activation of which was not or was only partially affected by the administration of neutralizing TGF- β Abs. The elements of the PI3K (TOR, Akt 1, 2, and 3), the p38 α/β , MAPK, and JNK pathways were found to be activated directly by mTNF- α . The MAPK pathways are evolutionally conserved, and it was shown that the p38 α/β pathways can be activated by either MKK3/6 or MKK4/MKK7 (Li Y et al., 2005; Wu et al., 2005). Since MKK3 and MKK6 were not activated by the mTNF- α signaling pathway (Fig. 20), whereas MKK4 and MKK7 can also activate Jun kinases (Davis, 2000), we decided to determine their phosphorylation levels following mTNF- α triggering.

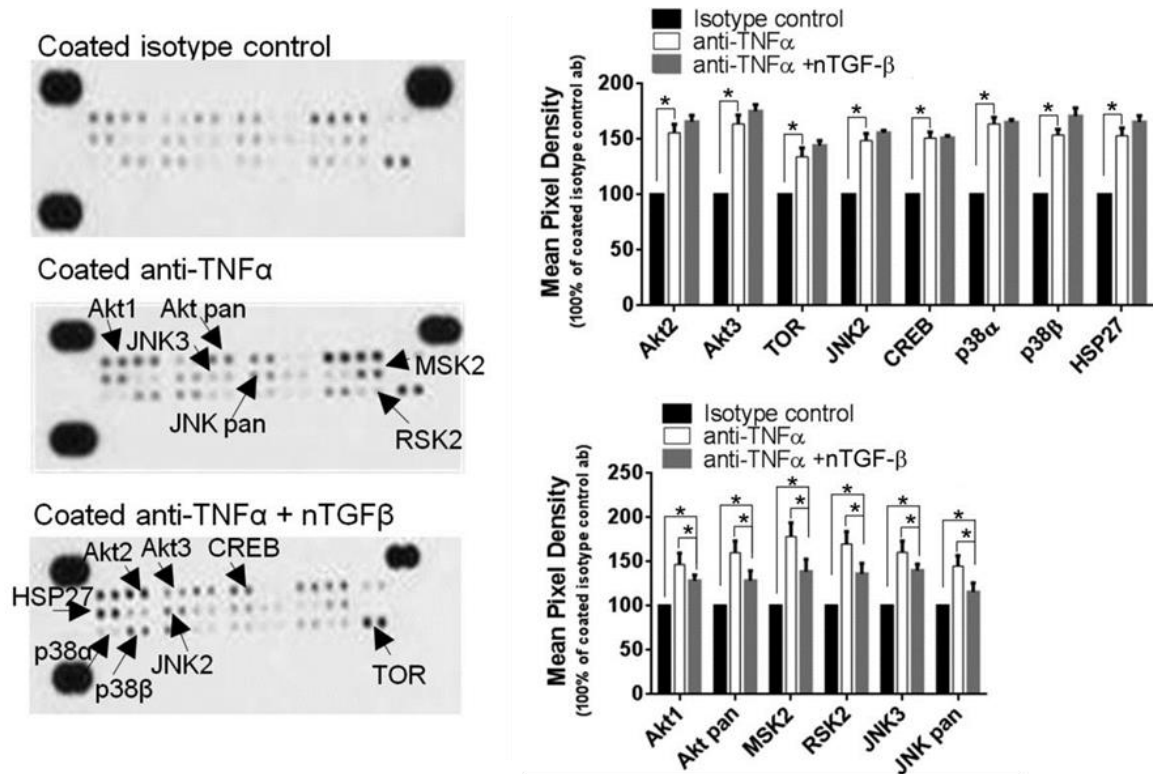


Figure 20.

Phospho-MAPK panel of coated isotype control-, coated anti-mTNF- α Ab-, or coated anti-mTNF- α and neutralizing anti-TGF- β -exposed BMDMs. BMDMs were exposed to coated isotype control (2 μ g/ml), anti-mTNF- α (2 μ g/ml), or anti-mTNF- α and neutralizing anti-TGF- β (5 μ g/ml) Abs for 2 h. Macrophages were then lysed, and the amount of phosphorylated proteins was detected by a phospho-MAPK array. Arrows highlight those proteins for which the anti-mTNF- α -enhanced phosphorylation was not altered or was only partially prevented by neutralizing anti-TGF- β Abs. One representative array series of three is shown. Enhancement in phosphorylation is expressed as a percentage of mean pixel density of the respective isotype Ab control (mean \pm SD from three independent experiments). *p < 0.05 versus respective control.

To avoid interference with the TGF- β signaling, the experiments were performed in the presence of neutralizing TGF- β Abs. As shown in Fig. 21A, the phosphorylation of MKK4 transiently increased, whereas that of MKK7 was not detectable during mTNF- α signaling. These data indicate that MKK4 mediates the effect of mTNF- α . In the absence of neutralizing TGF- β Abs, the induced phosphorylation levels of MKK4 remained high even at 2 hours (Fig. 21B), in line with the fact that TGF- β also triggers the activation of MKK4 (Dong et al., 2002). The phosphorylation of MKK3/6 induced by TGF- β appeared with a delay as compared to that of MKK4 without alterations in MKK3 levels. The protein levels of MKK7, alternatively, gradually decreased, indicating that TGF- β downregulates MKK7 signaling (Fig. 21B).

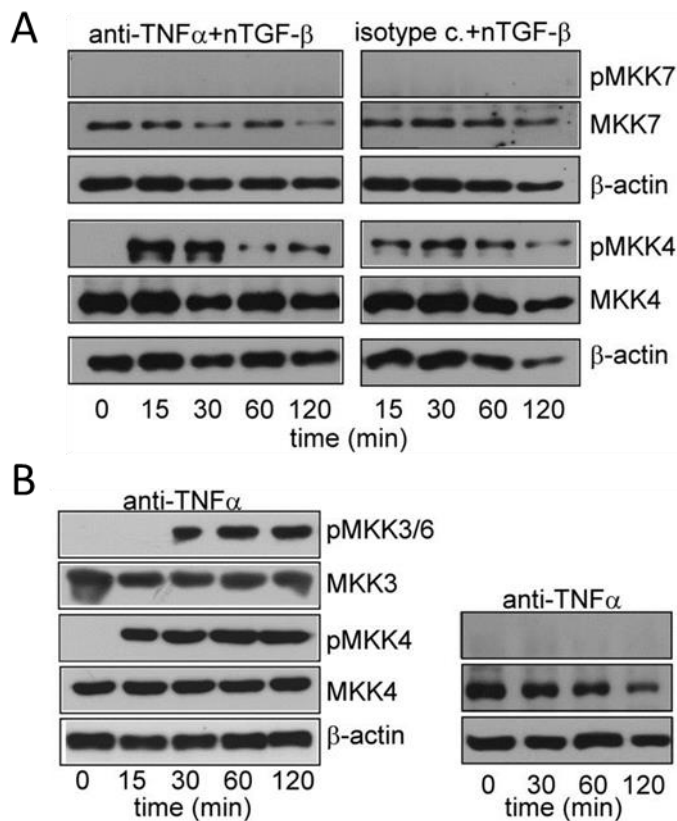


Figure 21.

(A) The Western blot analysis of the time-dependent phosphorylation levels of MKK4 and MKK7 in BMDMs exposed to coated anti-mTNF- α Abs in the presence of neutralizing anti-TGF- β Abs (5 μ g/ml). (B) The Western blot analysis of the time-dependent phosphorylation levels of MKK4, MKK7, and MKK3/MKK6 in BMDMs exposed to coated anti-mTNF- α Abs. β -Actin was used as a loading control.

Next, we decided to investigate which of the mTNF- α activated signaling pathways contribute to the induction of TGF- β . The phosphorylation levels of both mitogen and stress-activated kinase 2 and ribosomal S6 kinase 2 known to be activated by p38 MAPKs were induced by mTNF- α signaling (Fig. 20). These kinases can phosphorylate CREB at Ser¹³³ (detected by the phosphokine array, Fig. 20) and activate CREB-dependent gene expression (Wiggin et al., 2002; Xing et al., 1996).

Because the promoter of both TGF- β 2 (Kelly et al., 1995) and - β 3 (Liu G et al., 2006; Roberts et al., 1991) contains cAMP response element, we decided to investigate whether selective inhibition of p38 MAPKs by SB203580 (10 μ M) could interfere with the mTNF- α -induced TGF- β production. As seen in Fig. 22A, the inhibition of p38 MAPKs attenuated the mTNF- α -induced TGF- β production. The TGF- β 1 promoter, alternatively, was reported to be regulated principally by AP-1 sites (Roberts et al., 1991). In line with this observation, inhibition of Jun kinases by TCS JNK 60 also attenuated mTNF- α -induced TGF- β production (Fig. 22A) and completely prevented the mTNF- α -induced TGF- β 1 mRNA formation (Fig. 22B). The inhibition of p38 α/β kinases by SB203580, alternatively, had no effect on the

TGF- β 1 mRNA production. However, when the p38 kinase α/β inhibitor was added together with the Jun kinase inhibitor, the mTNF- α -induced TGF- β production was completely prevented.

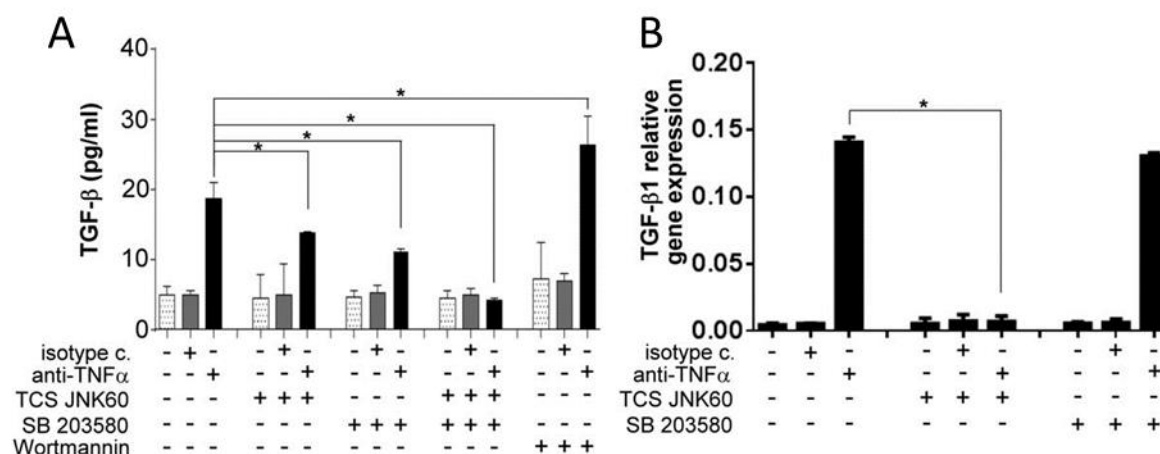
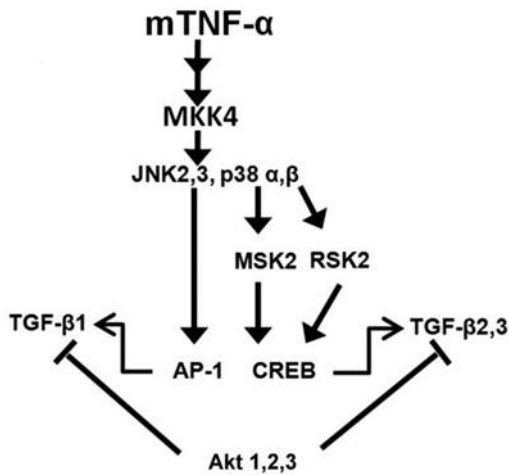


Figure 22.

(A) mTNF- α -induced TGF- β production is fully prevented by the simultaneous inhibition of the Jun kinase and p38 MAPK pathways, whereas it is further induced when PI3K is inhibited. BMDMs were exposed to coated isotype control (2 μ g/ml) or coated anti-mTNF- α (2 μ g/ml) Abs for 2 hours in the presence or the absence of the indicated compounds SB203580 (10 μ M), a p38 MAPK inhibitor, TCS JNK 60 (10 mM), a Jun kinase inhibitor, and wortmannin (1 mM), a PI3K inhibitor. TGF- β levels in the cell culture medium were determined by ELISA using an anti-pan-TGF- β Ab. The results are mean \pm SD from three independent experiments. * p < 0.05 versus control. (B) mTNF- α -induced TGF- β 1 mRNA production is fully prevented by the inhibition of Jun kinases, whereas it is not affected by the inhibition of p38 MAPKs. BMDMs were exposed to coated isotype control (2 μ g/ml) or anti-mTNF- α (2 μ g/ml) Abs for 2 hours in the presence or the absence of the indicated compounds followed by RNA isolation and reverse transcription. The gene expression levels of TGF- β 1 were determined by quantitative RT-PCR using GAPDH as a normalizing gene. The results are mean \pm SD from three independent experiments. * p < 0.05 versus anti-mTNF- α -treated control.

These data indicate that the Jun kinase pathway regulates primarily TGF- β 1, whereas the p38 kinase α/β pathway regulates the production of other TGF- β s (Fig. 22B). In striking contrast, inhibition of PI3K (which is known to activate the Akt pathway) by wortmannin (a PI3K inhibitor) resulted in the increased TGF- β formation (Fig. 22A). This observation indicates that this pathway might initiate a negative autoregulatory loop in the regulation of the TGF- β production (Fig. 23).



The mechanism of Akt activation was not investigated in our studies, but in other studies it was found that the MKK4 pathway can down-regulate the levels of PIP3 (phosphatidylinositol-3,4,5-trisphosphate) 3-phosphatase, leading to the activation of Akt (Xia et al., 2007).

Figure 23. Proposed signaling pathways induced by mTNF- α in macrophages leading to TGF- β production.

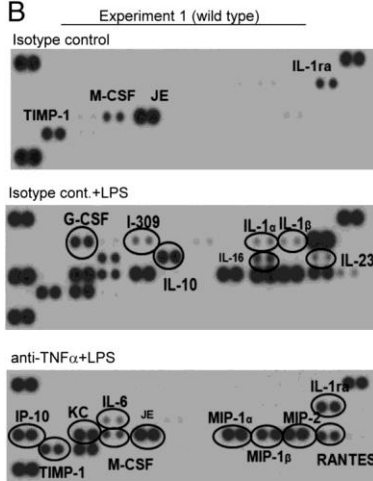
4.2.5. mTNF- α signaling inhibits the production of a subset of LPS-induced pro-inflammatory cytokines

Because our preliminary experiments (Fig. 18) indicated that mTNF- α signaling inhibits LPS-induced IL-6 and MIP-2 formation to a different degree, we decided to examine the effect of mTNF- α signaling on the LPS-induced pro-inflammatory cytokine formation by simultaneously detecting the production of pro-inflammatory cytokines using a cytokine array (Fig. 24A). BMDMs generated from NMRI animals generally produced TIMP-1, M-CSF-1, and JE pro-inflammatory cytokines at basal levels (Fig. 24B, 24C). Exposure to LPS did not affect the production of these cytokines. Although we always exposed the cells to the same amount of LPS, as shown in Fig. 24B and 24C, their response to LPS varied from isolation to isolation. A 2-hour exposure of BMDMs to coated anti-mTNF- α always attenuated the LPS-induced pro-inflammatory cytokine formation, but the effect was more dramatic when the cells showed a lower LPS response (Fig. 24C). However, not each cytokine demonstrated the same sensitivity to mTNF- α inhibition.

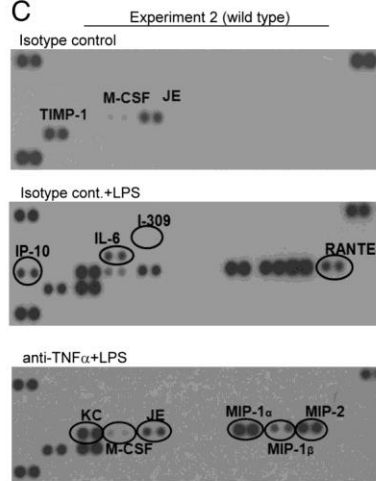
A

	1-2	3-4	5-6	7-8	9-10	11-12	13-14	15-16	17-18	19-20	21-22	23-24
A	+control											+control
B	BLC	C5/C5a	G-CSF	GM-CSF	I-309	Eotaxin	sICAM-1	IFN- γ	IL-1 α	IL-1 β	IL-1ra	IL-2
C	IL-3	IL-4	IL-5	IL-6	IL-7	IL-10	IL-13	IL-12p70	IL-16	IL-17	IL-23	IL-27
D	IP-10	I-TAC	KC	M-CSF	JE	MCP-5	MIG	MIP-1 α	MIP-1 β	MIP-2	RANTES	SDF-1
E	TARC	TIMP-1	TNF- α	TREM-1								
F	+control											-control

B



C



D

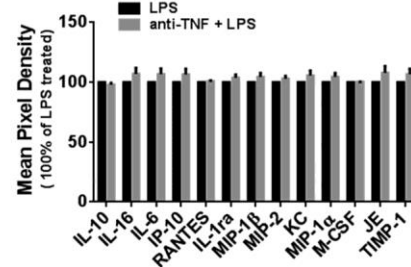
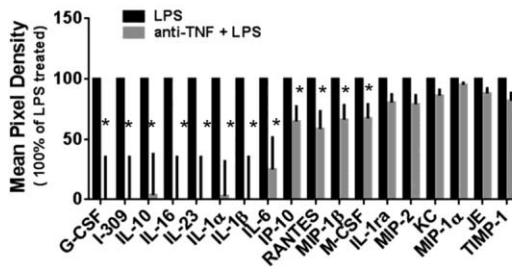
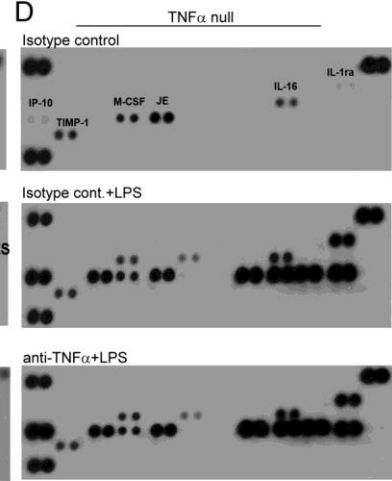


Figure 24. Triggering of mTNF- α selectively inhibits the LPS-induced pro-inflammatory cytokine formation in mouse BMDMs.

(A) Map of the 40 cytokines detected on the membranes. (B and C) Cytokine panel of isotype control-, isotype control and LPS-, or anti-mTNF- α Ab and LPS-exposed wild-type macrophages from two independent experiments. Macrophages were exposed to coated isotype control or coated anti-mTNF- α Abs for 2 hours and then to LPS (100 ng/ml) for additional 6 hours. Supernatants were collected and cytokine levels were determined by cytokine array. The circles highlight those cytokines on the isotype control Ab/LPS-treated samples for which the expression was fully inhibited by mTNF- α signaling, whereas for cytokines on the anti-mTNF- α Ab/LPS-treated samples, the LPS-induced expression was only attenuated by mTNF- α signaling. Alterations in cytokine levels are expressed as a percentage of mean pixel density of the respective LPS-treated isotype Ab control (mean \pm SD from four independent experiments). (D) One representative cytokine panel of isotype control-, isotype control and LPS-, or anti-mTNF- α Ab and LPS-exposed TNF- α -null macrophages. Experiments were performed as above. Alterations in cytokine levels are expressed as a percentage of mean pixel density of the respective LPS-treated isotype Ab control (mean \pm SD from three independent experiments). * $p < 0.05$ versus isotype Ab/LPS-treated control.

The inhibition of LPS-induced G-CSF, I-309, IL-10, IL-23, IL-1 α , IL-1 β , and IL-16 production was nearly complete, IL-6, IP-10, MIP-1 β , IL-1ra, M-CSF, and RANTES production was significantly inhibited (calculated from the pixel densities of cytokine arrays generated from four independent experiments), but MIP-1 α , MIP-2, TIMP-1, JE, or KC productions were also reduced. To prove that coated anti-mTNF- α Abs indeed act *via* mTNF- α , we also exposed TNF- α -null macrophages to LPS and checked whether anti-mTNF- α Abs affect their cytokine production. As shown in Fig. 24D, coated anti-mTNF- α Abs did not significantly alter the LPS-induced pro-inflammatory cytokine production of macrophages isolated from TNF- α -null mice (Pasparakis et al., 1996).

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

Next we decided to examine whether TGF- β is involved in the suppression of the LPS-induced pro-inflammatory cytokine formation by mTNF- α by applying neutralizing anti-TGF- β Abs. As shown in Fig. 25A, neutralizing anti-TGF- β Abs did not affect the LPS induced pro-inflammatory cytokine production in the presence of isotype control Abs with the exception of IL-23, IL-1 α , and IL-1 β .

However, they strongly interfered with the mTNF- α -mediated inhibition of the LPS-induced pro-inflammatory cytokine formation with the exception of those three cytokines, which were affected by the neutralizing anti-TGF- β Abs in the isotype control Ab-treated samples as well. A summary of the inhibitory effect of mTNF- α signaling on the LPS-induced IL-6 and MIP-2 production detected by ELISA and the effect of neutralizing TGF- β Abs from 10 independent experiments is shown in Fig. 25B. Additionally, pre-incubation with recombinant TGF- β for 2 hours also attenuated the LPS-induced pro-inflammatory cytokine formation in BMDMs in a cytokine-specific manner (Fig. 26).

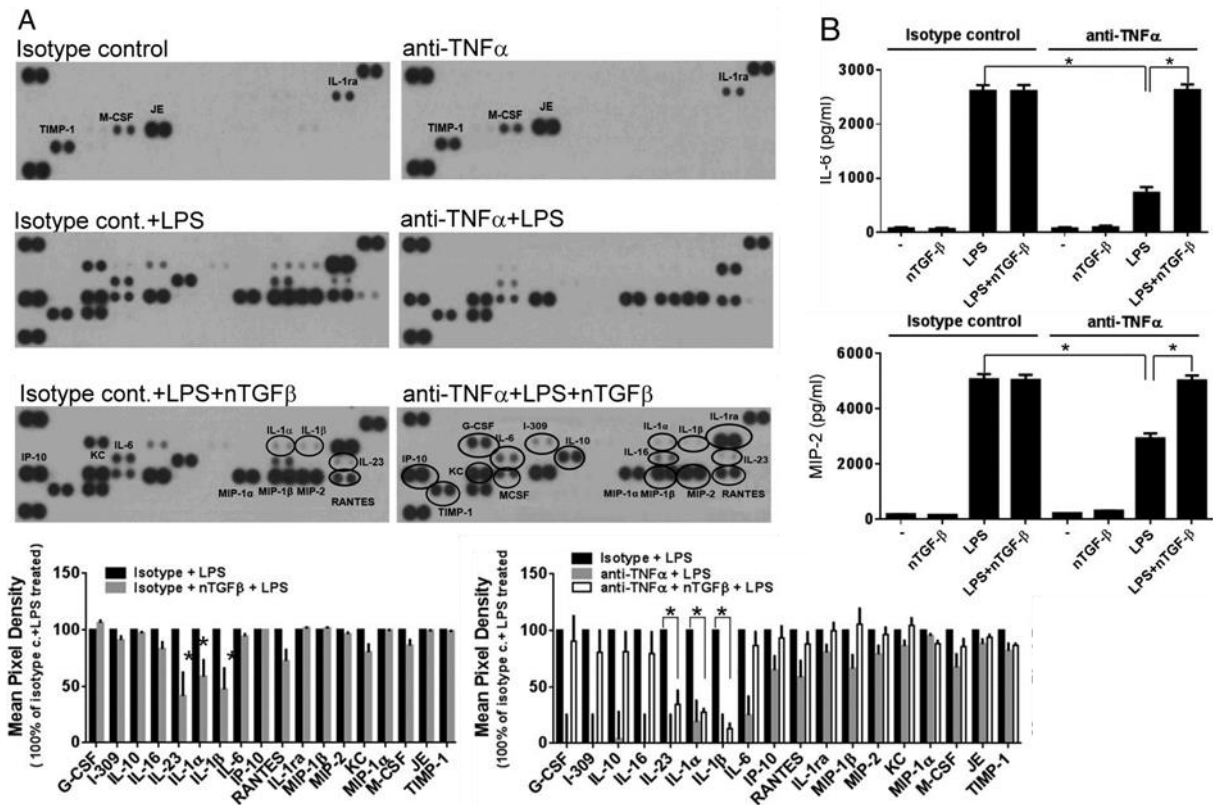


Figure 25. mTNF- α signaling interferes with the LPS-induced pro-inflammatory cytokine production via upregulating TGF- β .

(A) One representative cytokine panel series of isotype control or anti-mTNF- α Ab-exposed macrophages triggered by LPS in the presence and absence of neutralizing anti-TGF- β Abs. Macrophages were exposed to coated isotype control (2 μ g/ml) or coated anti-mTNF- α Abs (2 μ g/ml) for 2 hours and then to LPS (100 ng/ml) for an additional 6 hours in the presence and absence of neutralizing anti-TGF- β Abs (5 μ g/ml). Supernatants were collected, and cytokine levels were determined by cytokine array. Those cytokines are highlighted for which the mTNF- α -induced downregulation was prevented by neutralizing TGF- β . Alterations in cytokine levels are expressed as a percentage of mean pixel density of isotype control Ab/LPS-treated macrophages (mean \pm SD from three independent experiments). * p < 0.05 versus respective control. (B) Effect of neutralizing of TGF- β on the mTNF- α -mediated inhibition of LPS-induced IL-6 and MIP-2 production detected at 6 h by ELISA. Macrophages were exposed to either coated isotype control or anti-mTNF- α Abs (2 μ g/ml) for 2 hours before exposing macrophages to LPS (100 ng/ml) in the presence and absence of neutralizing anti-TGF- β Abs (5 μ g/ml). The results are mean \pm SD from 10 independent experiments. * p < 0.05 versus respective LPS-exposed control.

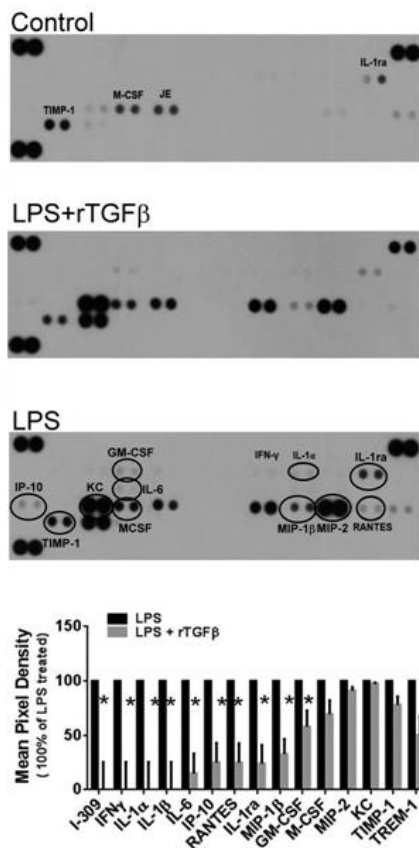


Figure 26.

One representative cytokine panel series of non-treated or LPS-exposed macrophages in the presence and absence of rTGF-β. Macrophages were exposed or not to 1 μg/ml rTGF-β for 2 hours before the addition of LPS (100 ng/ml) for an additional 6 hours. Supernatants were collected, and cytokine levels were determined by cytokine array. Those cytokines are highlighted for which the LPS-induced expression is downregulated by rTGF-β. Alterations in cytokine levels are expressed as a percentage of mean pixel density of LPS-treated macrophages (mean ± SD from three independent experiments). *p < 0.05 versus LPS-treated control.

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

Whereas the phagocytosis of a variety of pathogenic targets, especially bacteria and virally infected cells, normally triggers a pro-inflammatory response in macrophages, the ingestion of apoptotic cells by macrophages usually induces an anti-inflammatory phenotype. Apoptotic cells do not simply fail to provide pro-inflammatory signals; rather, they actively interfere with the inflammatory program. For example, the pre-incubation of macrophages with apoptotic cells strongly suppresses the inflammatory response induced by LPS, and the mechanism involves the release of TGF-β (Fadok et al., 1998). Since most mammalian cells express TNFRs, and thymocytes express both TNFR I and II (Grell-Becke et al., 1998), we decided to research, by using the macrophages of TNF-α-null mice (Pasparakis et al., 1996), whether mTNF-α could contribute to the immune silencing effects of apoptotic cells. As seen in Fig. 27A, the loss of TNF-α did not affect the basal pro-inflammatory cytokine formation of macrophages and the downregulation of them when they were engulfing apoptotic cells.

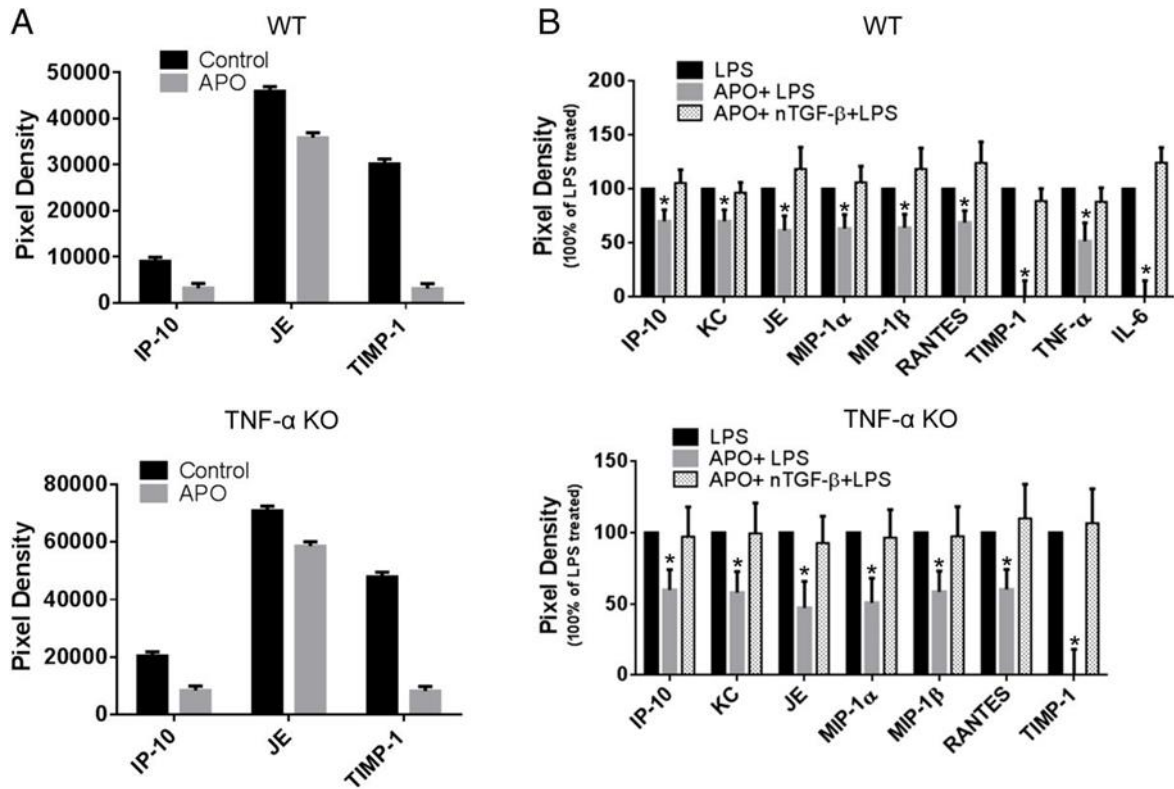


Figure 27. Apoptotic thymocytes do not use the mTNF- α signaling pathway to downregulate the LPS-induced pro-inflammatory cytokine formation of macrophages.

(A) Representative cytokine panel of control- and apoptotic cell-exposed wild-type or TNF- α -null macrophages. (B) Representative cytokine panel of LPS-, LPS and apoptotic cell-, or LPS, apoptotic cell, and neutralizing TGF- β Ab-exposed wild-type or TNF- α -null macrophages. Macrophages either exposed or not to apoptotic thymocytes (10:1 apoptotic cell/macrophage ratio) for 2 hours were washed and were or were not exposed to LPS (100 ng/ml) in the presence or absence of neutralizing TGF- β Abs (5 μ g/ml). Supernatants were collected 24 hours later, and cytokine levels were determined by cytokine array. The pixel density results are mean \pm SD from three independent experiments. Alterations in cytokine levels are expressed as a percentage of mean pixel density of LPS-treated macrophages (mean \pm SD from three independent experiments). * p < 0.05 versus respective LPS-treated control.

The exposure of apoptotic cells to macrophages resulted in the downregulation of the LPS-induced response of a number of pro-inflammatory cytokines in a TGF- β -dependent manner, but the loss of mTNF- α tested on TNF- α -null macrophages did not affect their capability of responding to apoptotic cells (Fig. 27B).

Additionally, we did not observe a correlation between the location of TNF- α -containing vesicles of LPS-treated macrophages and the position of phagocytic portals for apoptotic cells (Fig. 28A). These data indicate that mTNF- α signaling does not play a role in the apoptotic

cell-induced anti-inflammatory response. Thus, we checked whether apoptotic thymocytes still express TNFRs.

As shown in Fig. 28B, thymocytes express both TNFR I and II, but as they enter apoptosis following the serum withdrawal, they downregulate the expression of both TNFRs. The down-regulation was not apoptotic signal specific, as thymocytes exposed to dexamethasone or phorbol dibutyrate/ionomycin also responded with full downregulation of their TNFRs within 5 hours. Interestingly, apoptotic neutrophils known to encounter LPS-activated macrophages in an inflammatory situation also downregulate their TNFRs during apoptosis (Hart et al., 2000). The data indicate that apoptotic cells do not trigger an mTNF- α response for immune silencing (Weinblatt et al., 1999).

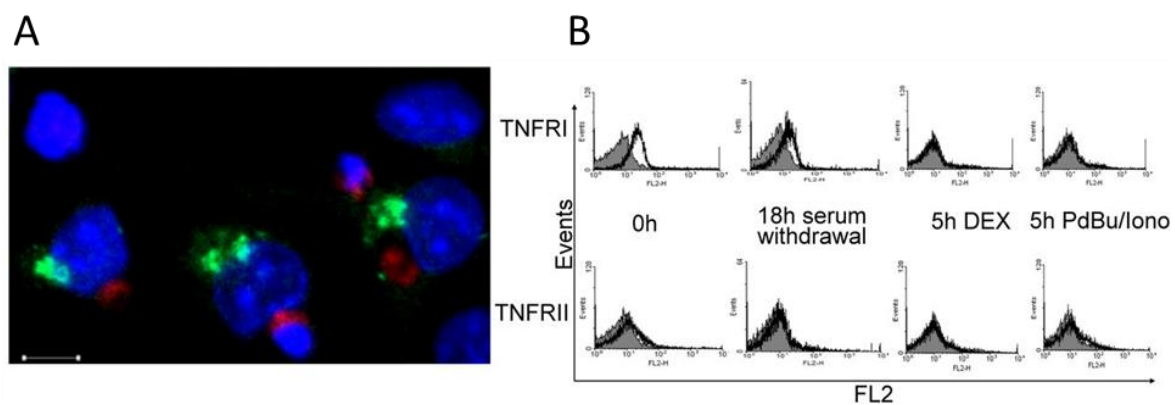


Figure 28.

(A) Distribution of TNF- α (green) protein in LPS-treated (100 ng/ml for 2 hours) wild-type macrophages engulfing apoptotic cells (red) detected by a confocal microscopy. The nuclei appear in the blue color following with DAPI staining. The scale bar, 10 μ m. (B) Apoptotic thymocytes downregulate their cell surface TNFR I and II. Thymocytes either dying following the serum withdrawal for 18 hours or induced to die by 0.1 μ M dexamethasone or by 5 ng/ml phorbol dibutyrate/1 μ g/ml ionomycin for 5 hours were analyzed for their TNFR expression by FACS analysis.

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

TNF- α -targeting molecules are widely used in the therapy of various inflammatory diseases. Although all compounds neutralize TNF- α with the same efficiency and are similarly effective in the treatment of rheumatoid arthritis (Maini R et al., 1999; Zhou et al., 2007; Shull et al., 1992), golimumab and infliximab are effective (Löwenberg and D'Haens, 2015;

Sandborn and Hanauer, 2002; Bartolucci et al., 2002; Yee and Pochapin, 2001), whereas etanercept failed in the trials of Crohn's disease (Sandborn et al., 2001), Wegener's granulomatosis (Tracey et al., 2008), and sarcoidosis (Ramos-Casals et al., 2008). Thus, we decided to study whether these compounds can trigger the TGF- β production in human macrophages. Human macrophages generated from buffy coats in three separate experiments were exposed to either etanercept, infliximab, or golimumab all in 50 μ g/ml concentration for 4 hours, and then the TGF- β levels were determined in the cell culture fluid. In the case of nontreated macrophages, 39 ± 5 pg/ml TGF- β could be detected. Exposure to etanercept (TGF- β , 40 ± 4 pg/ml) did not affect the basal levels of TGF- β , whereas in the cultures of infliximab (78 ± 15 pg/ml) and in those of golimumab (96 ± 12 pg/ml) TGF- β could be detected, the levels of which were significantly higher ($p < 0.05$) than basal levels.

5. DISCUSSION

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

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

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

In the present study, on one hand we investigated whether the A₃R could be involved in the regulation of the anti-inflammatory response induced in macrophages by apoptotic cells. We found that in contrast to A_{2A}Rs, engulfing macrophages downregulate A₃Rs during phagocytosis. While upregulation of A_{2A}Rs involves activation of the nuclear lipid sensing receptors following phagocytosis (Köröskényi et al., 2011), expression of A₃Rs seems to be downregulated *via* cell surface macrophage receptors recognizing phosphatidylserine on the surface of apoptotic cells.

Similar to A_{2A}Rs, loss of adenosine A₃Rs did not affect the rate of phagocytosis. However, when exposed to apoptotic cells, A₃R^{-/-} macrophages notably produced decreased amounts of

MIP-2 and KC acting as chemoattractants for various cell types, especially for neutrophils. We could confirm these data by using a specific A₃R antagonist indicating that lack of the actual A₃R signaling rather than altered macrophage differentiation in the absence of A₃R explains the phenomenon. Interestingly, the production of a similar set of cytokines, namely the neutrophil chemoattractants, was affected by the loss of A₃Rs as that of the loss of A_{2A}Rs, but in an opposite direction indicating that A₃R receptor signaling in wild-type macrophages might inhibit the A_{2A}R signaling.

The data presented indicate that in A₃R null macrophages the loss of A₃Rs, which would normally negatively regulate the adenylate cyclase activity during engulfment (Fredholm et al., 2001), results in an enhanced adenylate cyclase signaling by A_{2A}Rs leading to full suppression of the apoptotic cell-induced NO production and the consequent neutrophil chemoattractant formation. As a result, neither addition of the forskolin, which could further activate adenylate cyclase, nor inhibition of NO synthesis, since no NO production was detected, had an effect on the amount of MIP-2 produced by A₃R null macrophages engulfing apoptotic cells.

Previous studies have shown that ingestion of apoptotic cells is accompanied also by a release of PGE₂ (Fadok et al., 1998) which acting *via* EP₂₋₄ receptors (Hubbard et al., 2001) could also activate adenylate cyclase. However, when we inhibited adenylate cyclase signaling by Rp-cAMPS triethylamine, we found no difference in the apoptotic cell-induced neutrophil chemoattractant formation between A₃R or A_{2A}R null macrophages and their wild-type counterparts indicating that the main receptor that triggers adenylate cyclase activity and inhibits neutrophil chemoattractant formation in the absence of A₃R signaling must be the A_{2A}R. This observation is further supported by the finding that inhibition of A_{2A}R by signaling in A₃R null engulfing cells results in similar amount of pro-inflammatory cytokine formation as the inhibition of the adenylate cyclase pathway by Rp-cAMPS. Our data also indicate that the main receptor that inhibits the adenylate cyclase pathway is the A₃R.

Previously we have shown that exogenously added NO alone is not sufficient to trigger neutrophil chemoattractant formation in macrophages, but enhances it, when it is administered to engulfing wild-type macrophages. This observation indicated that apoptotic cells induce neutrophil chemoattractant formation not only *via* inducing NO formation, but also simultaneously triggering additional signaling pathway(s). Since in A₃R null engulfing macrophages, in which the adenylate signaling pathway is strongly activated, exogenous NO

could enhance neutrophil chemoattractant formation only when it was added together with Rp-cAMPS triethylamine, our data indicate that the enhanced adenylate cyclase signaling must interfere with the additional pathway(s) as well.

Our data demonstrate that in wild-type macrophages engulfing apoptotic cells initially both A_{2A}Rs and A₃Rs are activated by adenosine, and a balance between the activities of the two receptors decides the strength of the adenylate cyclase signaling and the consequent degree of suppression of apoptotic cell-induced chemoattractant formation. But because A_{2A}Rs are upregulated, while A₃Rs disappear from the cell surface of engulfing macrophages, adenosine on long term will mediate suppression of inflammation when macrophages engulf apoptotic cells.

Besides studying the involvement of adenosine A₃ receptor in the apoptotic cell-mediated immune silencing, we also investigated the potential involvement of mTNF- α signaling in the inhibition of LPS-triggered inflammatory responses of macrophages by apoptotic cells. We found that TNF- α is expressed, although at low levels, in unstimulated macrophages, and some of it appears also on the cell surface as mTNF- α . Exposure to LPS increased both the synthesis of TNF- α and its cell surface expression, but this increase was only transiently due to the activation of the metalloprotease, which is responsible for its cleavage. Already the basal cell surface levels of mTNF- α allowed an efficient mTNF- α signaling, and we found that the signaling pathway led to the production of TGF- β . The induction of TGF- β production, however, is not an universal response of cells to mTNF- α signaling, because monocytes were shown to produce TNF- α and not TGF- β when their mTNF- α was triggered by the TNF-Rs of activated T cells (Rossol et al., 2007).

We have also identified the signaling pathway that regulates mTNF- α -induced TGF- β production in macrophages. This pathway involves the MKK4-initiated MAPK pathway, which leads the activation of both p38 MAPKs and JNKs. Whereas the Jun kinase pathway regulates the production of TGF- β 1, the p38 MAPKs are responsible for the production of the rest of TGF- β s. In contrast, the Akt pathway also activated by mTNF- α seems to act as a negative autoregulatory loop in the control of mTNF- α -induced TGF- β production. Although previous studies have suggested that mTNF- α triggers the ERK kinase pathway (Eissner et al., 2000, Kirchner-Boldt et al., 2004), our data indicate that the activation of these kinases is only secondary and a consequence of TGF- β signaling. We have also demonstrated that mTNF- α signaling alone induces only a transient MKK4 and p38 MAPK activation when TGF- β is

neutralized; however, the appearance of TGF- β sustains MKK4 and the consequent p38 MAPK signaling.

We have confirmed that mTNF- α signaling indeed inhibits the LPS-induced pro-inflammatory cytokine formation, but we found that different subsets of cytokines show different susceptibility for this inhibition. We have also demonstrated that mTNF- α signaling interferes with the LPS signaling *via* the autocrine production of TGF- β . Because previous studies have shown that different elements of the LPS induced signaling pathway are involved in the induction of individual cytokines (Björkbacka et al., 2004), our data indicate that TGF- β signaling might selectively interfere with the various LPS-induced signaling pathways. Our data do confirm previous findings, in which it was demonstrated that in the presence of TGF- β the balance between the LPS-induced ERK and p38 MAPK activities is dysregulated owing to the simultaneous TGF- β -stimulated ERK activation. As a result, the NF- κ B-driven transcription was shown to be attenuated, whereas the AP-1-driven transcription was shown to be enhanced (Xiao et al., 2002).

We have also tested whether three TNF- α -targeting molecules introduced already in the therapy of various inflammatory diseases could also trigger the mTNF- α -driven TGF- β production in human macrophages. We found that etanercept is not able to trigger such a mTNF- α -driven TGF- β production, whereas infliximab and golimumab are able to do so, indicating that infliximab and golimumab trigger mTNF- α signaling. In support of our observation, infliximab in patients was reported to induce the transient phosphorylation of p38 MAPKs and Hsp70, the elements of the mTNF- α signaling pathway (Waetzig et al., 2003). Previous studies have suggested that the therapeutic efficacy of TNF- α -targeting molecules might be related not only to their capability of neutralizing TNF- α , but it might also be affected by their biological nature or whether they could also trigger mTNF- α signaling (Nesbitt et al., 2007; Kirchner-Holler et al., 2004). Indeed, although all three compounds neutralize TNF- α with the same efficiency and are similarly effective in the treatment of rheumatoid arthritis (Maini R et al., 1999; Zhou et al., 2007; Shull et al., 1992), etanercept, which in our assay, failed to upregulate TGF- β , and it also failed in the trials of Crohn's disease (Sandborn et al., 2001), Wegener's granulomatosis (Tracey et al., 2008), and sarcoidosis (Ramos-Casals et al., 2008). Because in other biological assays etanercept responded similarly to infliximab and golimumab (Nesbitt et al., 2007), our data indicate that its failure in the treatment of these above-mentioned diseases might be related to the lack of TGF- β induction. Interestingly, Crohn's disease, Wegener's granulomatosis, and sarcoidosis

are all characterized by altered TGF- β signaling (Fiocchi, 2001; Muraközy et al., 2001; Piotrowski et al., 2015). Although in Crohn's disease TGF- β signaling was reported to be impaired due to the elevated levels of TNF- α and IFN- γ , which interfere with the TGF- β signaling (Fiocchi, 2001), the administration of a diet rich in TGF- β 2 was shown to be beneficial in its long-term maintenance (Hartman et al., 2008). Thus, simultaneous neutralization of the pro-inflammatory and anti-TGF- β signaling effects of TNF- α and promotion of TGF- β signaling by elevating its levels might all contribute to the therapeutic effects of those anti-TNF- α targeting molecules, which also trigger the TGF- β production.

Every day billions of cells die by apoptosis as part of the normal tissue homeostasis. Anti-inflammatory effects of apoptotic cells result in a silent removal of dying cells as well as strongly contribute to the resolution of inflammation (Griffith and Ferguson, 2011). We have studied whether apoptotic cells known to interfere with the LPS signaling of macrophages also by upregulating TGF- β (Fadok et al., 1998) do use mTNF- α signaling to act in this manner. Our data indicate that apoptotic cells cannot trigger the mTNF- α signaling pathway in macrophages, because they downregulate their TNF-Rs. As a result, none of the TNF- α -targeting molecules is expected to interfere with the anti-inflammatory effects of apoptotic cells.

Collectively, our data indicate that the induction of TGF- β , a strong anti-inflammatory cytokine (Li MO et al., 2006), might be an essential contributing factor in determining the therapeutic efficacy of TNF- α -targeting molecules in chronic inflammatory diseases, such as Crohn's disease. Because in Crohn's disease patients, who do not respond to infliximab therapy, phosphorylation of both ATF-2 and Hsp70 was found to be impaired (Hartman et al., 2008), it is very likely that in these patients the mTNF- α -signaling pathway is suppressed. For such patients as an additional therapy to the TNF- α -targeting molecules, triggering of receptors used by apoptotic cells to induce TGF- β production, such as stabilin-2 (Park SY et al., 2008), could be considered.

As a new result we demonstrated that adenosine A₃ receptor in contrast to A_{2A} receptor enhances the production of MIP-2 and KC chemoattractants in the uptake of apoptotic cells, but the expression disappears during phagocytosis. The mTNF- α is not involved in the apoptotic cell-mediated anti-inflammatory effect, since the apoptotic cells downregulate their TNF receptors. Triggering of mTNF- α signaling pathway in macrophages leads to production of TGF- β . Certain molecules used in TNF- α therapy are able

to activate the mTNF- α signaling pathway which can be effective in the treatment of several chronic inflammatory diseases.

Taken together, the present studies revealed the involvement of adenosine/A₃R and mTNF- α signaling in the regulation of the inflammatory responses of macrophages. Our results not only improve our knowledge about the macrophage-associated immune functions, but they also could serve potential therapeutic targets for 'personalized medicine' in the treatment of various autoimmune and chronic inflammatory diseases.

6. SUMMARY

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

It is well known that the apoptotic cell uptake induces a strong anti-inflammatory response in macrophages. Previously it has been thought that TGF- β released upon apoptotic cell uptake is the most important, moreover the only one anti-inflammatory cytokine mediating this inhibitory effect. However, results coming from the last few years contradict this obligate function of TGF- β . Our group has found that apoptotic cell-exposed macrophages produce also adenosine, which inhibits the apoptotic cell-induced pro-inflammatory response in a cytokine dependent manner by triggering macrophage adenosine A_{2A} receptors (A_{2A}Rs). In the present study we showed that the expression of adenosine A₃ receptors (A₃R) is decreased on the cell surface of macrophages during engulfment of apoptotic cells. The pro-inflammatory behavior of this receptor is confirmed by the fact that A₃R deficiency is accompanied by decreased production of neutrophil chemoattractant MIP-2 and KC during engulfment of apoptotic cells. The decreased MIP-2 and KC production of A₃R null macrophages is a consequence of an attenuated apoptotic cell-induced NO production, which is inversely regulated by the adenosine A_{2/3} receptor/adenylate cyclase/protein kinase A signaling pathway.

Apoptotic cells do not simply fail to provide pro-inflammatory signals; rather, they actively interfere with the inflammatory program. For example, pre-incubation of macrophages with apoptotic cells strongly suppresses the inflammatory response induced *via* Toll-like receptor 4 by lipopolysaccharide (LPS), a component of the cell wall of Gram-negative bacteria. mTNF- α was shown to act both as a ligand by binding to TNF- α receptors, as well as a receptor that transmits outside-to-inside (reverse) signals back into the mTNF- α bearing cells. Since the reverse mTNF- α signaling, similar to apoptotic cells, was shown to suppress LPS-induced pro-inflammatory cytokine formation, we tested the potential involvement of mTNF- α in the apoptotic cell-induced immunosuppression. We found that even non-activated macrophages

express basal levels of mTNF- α and triggering mTNF- α induces an MKK4-dependent signaling pathway, which leads to TGF- β production. The production of TGF- β 1 is regulated *via* Jun kinases, while that of other TGF- β s *via* p38 MAP kinases. Exposure to LPS further induced the expression of mTNF- α , and triggering of mTNF- α strongly suppressed the LPS-induced pro-inflammatory response in a TGF- β -dependent manner. Apoptotic cells, however, do not use this signaling pathway to achieve immune silencing, because they downregulate their TNF receptors.

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

Taken together, our results highlight the involvement of adenosine A₃ receptor and mTNF- α in the regulation of inflammatory responses exerted by macrophages. Agents, targeting these molecules – A₃R antagonists or mTNF- α inducers – represent potential therapeutic tools for the treatment of various inflammatory diseases.

7. ÖSSZEFOGLALÁS

A makrofágok szervezet védelmét biztosító három fő feladata a következő: a kórokozók és a károsodott, vagy elhalt sejtek bekebelezése és eliminálása; antigén prezentáció és immunmoduláció különböző citokinek és növekedési faktorok felszabadulásán keresztül. Az, hogy milyen mediátorok szabadulnak fel, nagyban függ a makrofágok által felismert sejt típusától, és ez meghatározza a környező szövet és immunrendszer válaszait is.

Ismert, hogy az apoptotikus sejtfevétel erős gyulladáscsökkentő választ vált ki makrofágokban. Korábban azt gondolták, hogy TGF- β az apoptotikus sejtfevétel során a legfontosabb, sőt az egyetlen gyulladáscsökkentő citokin, mely közvetíti ezt a gátló hatást. Az elmúlt évek eredményei azonban cáfolják a TGF- β kizárólagos szerepét. Munkacsoportunk megállapította, hogy az apoptotikus sejteknek kitett makrofágok adenzint is termelnek, amely gátolja az apoptotikus sejt által indukált gyulladási választ citokin függő módon a makrofág adenzin A_{2A} receptorok aktiválásán keresztül. Jelen vizsgálatunkban kimutattuk, hogy apoptotikus sejtfevétel során az adenzin A₃ receptorok expressziója csökken a makrofágok sejt felszínén. E receptor gyulladáshoz viselkedését megerősíti az a tény is, hogy hiánya neutrofil kemoattraktáns MIP-2 és KC csökkent termeléséhez vezet apoptotikus sejtek bekebelezése során. Az A_{3R} hiányos makrofágok csökkent MIP-2 és KC termelése egy gyengített apoptotikus sejt által indukált NO termelés következménye, amely fordítottan szabályozódik adenzin A_{2/3} receptor/adenilát cikláz/protein kináz A jelátviteli útvonal által.

Ez nem egyszerűen azért történik, mert az apoptotikus sejtek nem nyújtanak gyulladási szignálokat, sokkal inkább aktívan beleszólnak a gyulladási programba. Erre jó példa, hogy a makrofágok apoptotikus sejtekkel történő előinkubálása képes lenyomni az LPS (lipopoliszacharid; a Gram-negatív baktériumok sejt falának egy komponense) által Toll-like receptor 4-en keresztül indukált gyulladási választ. Az mTNF- α -ról korábban kimutatták, hogy ligandként kötődik a TNF- α receptorokhoz, valamint receptorként is működik, amely reverz jelátvitelre képes az mTNF- α -expresszáló sejtekben. Mivel a fordított mTNF- α jelátvitel, hasonlóan az apoptotikus sejtekhez, képes gátolni az LPS-indukált gyulladási citokin kifejeződést, ezért teszteltük a mTNF- α lehetséges szerepét az apoptotikus sejt-indukált immunszuppresszióban. Azt találtuk, hogy a még nem aktivált makrofágok alapszintű mTNF- α -t termelnek. Az mTNF- α fokozása egy MKK4-függő jelátviteli útvonalat indukál, amely TGF- β termeléséhez vezet. A TGF- β 1 termelése Jun kinázokon keresztül, míg más

TGF- β -k termelése p38 MAP kinázokon keresztül szabályzott. LPS hatására tovább indukálódott az mTNF- α expressziója, valamint az mTNF- α fokozása erősen gátolta az LPS-indukált gyulladási választ TGF- β -függő módon. Az apoptotikus sejtek azonban az immunrendszer csendesítéséhez nem ezt a jelátviteli útvonalat használják, mert down-regulálják a TNF receptoraikat.

A legfontosabb következtetéseink, hogy az apoptotikus sejtek fagocitózisa során a makrofágokban beinduló gyulladás gátló folyamatokban a TGF- β mellett az adenzinnak és receptorainak van szerepe, míg az mTNF- α -nak nincs. Ezzel szemben az LPS-sel indukált makrofág aktiváció lefékezésében az mTNF- α által indukált TGF- β részvétele figyelhető meg.

Mindent összevetve eredményeink rámutatnak az adenzin A₃ receptor és mTNF- α szerepére makrofágok által kiváltott gyulladási válaszok szabályozásában. Az ezeket a molekulákat megcélzó szerek - A₃R antagonisták vagy mTNF- α induktorok - lehetséges terápiás eszközök lehetnek különböző gyulladási betegségek kezelésében.

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9. LIST of PUBLICATIONS



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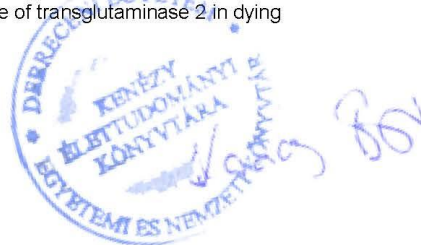
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List of publications related to the dissertation

1. **Pallai, A.**, Kiss, B., Vereb, G., Armaka, M., Kollias, G., Szekanez, Z., Szondy, Z.:
Transmembrane TNF-[alfa] Reverse Signaling Inhibits Lipopolysaccharide-Induced
Proinflammatory Cytokine Formation in Macrophages by Inducing TGF-[béta]: therapeutic
Implications.
J. Immunol. 196 (3), 1146-1157, 2016.
DOI: <http://dx.doi.org/10.4049/jimmunol.1501573>
IF: 4.985 (2015)
2. Duró, E., **Pallai, A.**, Köröskényi, K., Sarang, Z., Szondy, Z.: Adenosine A3 receptors negatively
regulate the engulfment-dependent apoptotic cell suppression of inflammation.
Immunol. Lett. 162 (2PtB), 292-301, 2014.
DOI: <http://dx.doi.org/10.1016/j.imlet.2014.06.014>
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List of other publications

3. Sándor, K., **Pallai, A.**, Duró, E., Legendre, P., Couillin, I., Sággy, T., Szondy, Z.: 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. [Epub ahead of print], 2016.
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4. Yen, J. H., Lin, L. C., Chen, M. C., Sarang, Z., Leong, P. Y., Chang, I. C., Hsu, J. D., Chen, J. H., Hsieh, Y. F., **Pallai, A.**, Kőröskényi, K., Szondy, Z., Tsay, G. J.: The metastatic tumor antigen 1-transglutaminase-2 pathway is involved in self-limitation of monosodium urate crystal-induced inflammation by upregulating TGF- β 1.
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Immunol. Lett. 138 (1), 71-78, 2011.
DOI: <http://dx.doi.org/10.1016/j.imlet.2011.03.004>
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Total IF of journals (all publications): 22,986

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

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10. KEYWORDS

Apoptotic cell clearance

Adenosine A₃ receptor

Nitric oxide (NO)

Transmembrane/membrane-bound tumor necrosis factor alpha (mTNF- α)

Reverse signaling

Transforming growth factor beta (TGF- β)

11. TÁRGYSZAVAK

Apoptótikus sejt eltakarítás

Adenozin A₃ receptor

Nitrogén-monoxid

Membránkötött-tumor nekrosis faktor alfa

Fordított jelátvitel

Transzformáló növekedési faktor béta

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