

THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
(PhD)

The role of adenosine A3 receptors and retinoids in the regulation of efferocytosis

by

GERGELY JOÓS MD, DMD

SUPERVISOR: PROFESSOR ZSUZSA SZONDY MD, PhD, DSc



UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF DENTAL SCIENCES

DEBRECEN, 2017

TABLE OF CONTENTS

List of abbreviations	4
1. Introduction	6
1.1.Efferocytosis: The clearance process of apoptotic cells.....	6
1.2.The role of efferocytosis in a healthy organism.....	6
1.3.“Effectors” of efferocytosis.....	7
1.3.1. Macrophages.....	7
1.4.Steps of efferocytosis.....	9
1.4.1. Recruitment of macrophages to the dying cells (“find-me” signals).....	10
1.4.1.1. The role of autocrine purinergic signaling in the chemotactic movement of phagocytes.....	12
1.4.2. Recognition of apoptotic cells: “Eat me” signals on the dead cells and uptake receptors on the phagocyte surface.....	14
1.4.2.1. Regulation of the expression of the phagocytotic receptors.....	15
1.4.3. Digestion of apoptotic corpses (downstream signaling from the receptors)....	18
1.4.4. “Post-engulfment” consequences (release of anti-inflammatory cytokines by engulfing phagocytes).....	19
1.5.Defective clearance of dead cells.....	20
1.5.1. Diseases related to disturbed efferocytosis.....	21
1.5.2. Therapeutic possibilities: Enhancing efferocytosis when clearance of apoptotic cells is impaired.....	22
1.5.2.1. Affecting recognition and binding of apoptotic cells to the phagocytes.....	22
1.5.2.2. Targeting lipid-sensing nuclear receptors with the aim of increasing the expression of phagocytic receptors or their bridging molecules.....	23
1.5.2.3. Affecting the Rac-1/RhoA balance.....	24
1.5.2.4. Altering the membrane lipid composition of macrophages.....	24
2. Aims of the studies	25
3. Materials and methods	26
3.1. Reagents.....	26
3.2. Experimental animals.....	26
3.3. Peritoneal macrophage isolation and culturing before chemotactic migration assay....	26
3.4. Bone marrow derived macrophage (BMDM) cell culture and treatment.....	27
3.5. Thymocyte apoptosis induction <i>in vivo</i> and related measurements.....	27
3.6. Thymocyte apoptosis induction <i>in vitro</i>	28
3.7. Chemotaxis assays.....	28
3.8. Phagocytosis of injected apoptotic thymocytes by peritoneal macrophages.....	29
3.9. Immunohistochemistry.....	29
3.10. <i>In vitro</i> apoptotic cell phagocytosis.....	30
3.11. Measuring the <i>in vivo</i> production of RAR-activating derivatives.....	30
3.12. Analysis of mRNA expression.....	31
3.13. Western blot analysis.....	31
3.14. Immunofluorescence staining and confocal microscopy.....	31
3.15. LXR receptor silencing.....	32
3.16. Retinoid measurement by HPLC-MS-MS.....	32
3.17. Statistical analyses.....	33
4. Results	33
4.1. Involvement of adenosine A3 receptors in the clearance of apoptotic thymocytes.....	33
4.1.1. Proper <i>in vitro</i> chemotactic migration of macrophages towards apoptotic cells is affected by adenosine receptor signaling.....	33

4.1.1.1. Find-me signals released by apoptotic thymocytes induce chemotaxis and increase the velocity of migration of macrophages in a purinergic signaling-dependent manner.....	33
4.1.1.2. Loss of A3R signaling in macrophages results in a loss of chemotactic navigation.....	35
4.1.1.3. Administration of exogenous adenosine does not affect migration of wild type macrophages toward the apoptotic cells, but promotes that of A3R null macrophages.....	38
4.1.1.4. Adenosine receptor signaling alone is also capable of maintaining chemotactic migration of macrophages toward the apoptotic thymocytes.....	38
4.1.1.5. A2AR signaling does not significantly contribute to the chemotactic orientation of macrophages migrating toward apoptotic thymocytes.....	38
4.1.1.6. Simultaneous A2BR and A3R signaling are required to mediate the adenosine-promoted chemotactic navigation of macrophages toward the apoptotic thymocytes.....	39
4.1.2. Loss of A3Rs delays the <i>in vivo</i> clearance of injected apoptotic thymocytes in the peritoneum.....	39
4.1.3. Surprisingly, loss of A3Rs does not affect the <i>in vivo</i> clearance of apoptotic thymocytes in dexamethasone-treated mouse thymus.....	41
4.2. Macrophages engulfing apoptotic cells produce nonclassical retinoids to enhance their phagocytic capacity.....	43
4.2.1. Upregulation of TG2 alone in wild-type macrophages is not sufficient to promote phagocytosis of apoptotic cells.....	43
4.2.2. Ligation of LXRs induces retinoid production in peritoneal macrophages.....	46
4.2.3. Ligation of LXR enhances phagocytosis of apoptotic cells in a retinoid-dependent manner.....	49
4.2.4. LXR stimulation has a long-term effect on expression of multiple phagocytic receptors. Induction of the expression of various phagocytic receptors is time-dependent after LXR ligation.....	50
4.2.5. A potentially dihydro-retinoic acid derivative is produced by macrophages engulfing apoptotic cells.....	51
5. Discussion.....	54
6. Summary.....	63
Keywords/Kulcsszavak.....	64
7. Összefoglalás.....	66
8. Acknowledgements.....	68
9. References.....	70
10. Publications.....	88

LIST OF ABBREVIATIONS

9cRA: 9-cis retinoic acid
A1R: adenosine 1 receptor
A2AR: adenosine 2A receptor
A2BR: adenosine 2B receptor
A3R: adenosine 3 receptor
ABCA1: ATP-binding cassette transporter
ACAMP: Apoptotic Cell Associated Molecular Pattern
ADP: adenosine diphosphate
AMP: adenosine monophosphate
ApoE: Apolipoprotein E
ATP: adenosine triphosphate
ATRA: all-trans retinoic acid
BAI1: brain-specific angiogenesis inhibitor 1
BMDM: bone marrow derived macrophage
C1q: complement component 1q
C5a: complement component C5a
cAMP: cyclic AMP
COPD: chronic obstructive pulmonary disease
CRABP: Cellular retinoic acid-binding protein
CRBP: Cellular retinol-binding protein
CRT: calreticulin
DEAB: diethylaminobenzaldehyde
Dex: dexamethasone acetate
DMEM: Dulbecco's Modified Eagle's Medium
DMSO: dimethyl sulfoxide
FBS: fetal bovine serum
FKN: fractalkine
fMLP: *N*-Formylmethionyl-leucyl-phenylalanine
GAPDH: to glyceraldehyde-3-phosphate dehydrogenase
Gas6: growth-arrest-specific 6
ICAM-3: intracellular adhesion molecule-3
IFN γ : interferon gamma
IL-1 β : interleukin 1 β
IL-10: interleukin 10
Jak-STAT: Janus kinase- Signal Transducer and Activator of Transcription
LDL: low density lipoprotein
LOX1: oxidized low-density lipoprotein receptor 1
LPC: lysophosphatidylcholine
LTF: lactoferrin
LXR: liver X receptor
Mertk: Mer tyrosine kinase
MFG-E8: milk fat globule epidermal growth-factor
NECA: 5'-*N*-ethylcarboxamidoadenosine
NF- κ B: nuclear factor kappa-light-chain-enhancer of activated B cells
NO: nitric oxide
PAF: platelet-activating factor
PGE2: prostaglandin E2

PS: phosphatidylserine
Pol II: RNA polymerase II
PPAR: peroxisome proliferator-activated receptor
RA: retinoic acid
RAR: retinoic acid receptor
RARE: RA-response element
RALDH: retinaldehyde dehydrogenase
RGD: arginine– glycine–aspartic acid motif
RDH: retinol dehydrogenase
ROS: reactive oxygen species
RXR: retinoid X receptor
S1P: sphingosine-1-phosphate
SLE: systemic lupus erythematosus
SOCS 1/3: suppressors of cytokine signaling 1/3 proteins
SREBP: sterol response element binding protein
TAF: TBP-associated factor
TBP: TATA-binding protein
TG2: tissue transglutaminase
TGF- β : transforming growth factor- β
TIM4: T cell immunoglobulin and mucin-domain-containing molecule 4
TNF α : tumor necrosis factor α
TSP1: trombospondin 1
TSP1-bs: trombospondin 1-binding site
UCP2: uncoupling protein 2
UTP: uridine triphosphate

1. INTRODUCTION

1.1. Efferocytosis: The clearance process of apoptotic cells

Most cell types have a limited life span, which ends physiologically through the process of apoptosis or programmed cell death. Upon apoptotic cell death, another fundamental biological process starts, whereby dead cells are cleared by living cells in a rapid and efficient manner. This clearance process is a special form of phagocytosis and due to its importance it's been recently called by a separate name, efferocytosis. (Henson et al., 2001). The term is derived from the latin verb “efferre” meaning: “to take to the grave” or “to bury”. Phagocytosis is in a broad sense the internalization and procession of larger, solid particles (such as bacteria, nutrients or debris) within a membrane-bound vesicle called the phagosome, while efferocytosis is specifically designated to the uptake of apoptotic cells. Compare to other forms of phagocytosis, efferocytosis has distinctive molecular regulatory features and unique signaling consequences.

1.2. The role of efferocytosis in a healthy organism

Efferocytosis is highly conserved process throughout evolution. In healthy individuals billions of cells die and get cleared every day (roughly one million cells in every second) without initiating inflammation and immune response (Hart et al., 2008; Ravichandran 2010). Apoptotic cell death, in contrast to necrosis (which is coupled to cell disintegration), does not provoke pro-inflammatory responses and plays a fundamental role in almost all physiological processes. The immunologically silent character of the apoptotic cell death is in fact closely related to the uptake of the dying cells: professional phagocytes (macrophages, dendritic cells, neutrophil granulocytes) and nonprofessional phagocytes (neighboring cells, such as epithelial cells or fibroblasts) remove the dead apoptotic bodies rapidly and efficiently. This way the potentially harmful intracellular cell contents are not released to the surrounding tissues and the materials of the cells can be reutilized (Ravichandran 2010). Moreover, beyond preventing the release of potentially cytotoxic or immunogenic cellular contents, the uptake of apoptotic cells triggers production of different compounds, which subsequently shape the outcome of the process. Such molecules are for example the transforming growth factor- β (TGF- β) (Fadok et al., 1998), retinoic acids (RAs) (Garabuczi et al., 2013) or adenosine (Köröskényi et al., 2011), which act both on the phagocytes and on the surrounding cells to regulate inflammation (Köröskényi et al., 2011), tissue regeneration (Biancheri et al., 2014) or apoptosis and differentiation (Sarang

et al., 2013) under various conditions. Maintaining the homeostasis in most tissues is also closely related to efferocytosis. Efficient execution of the process is determinant, where the cellular turnover is extremely high (bone marrow or thymus) or where many excess cells need to be deleted (tissue differentiation or morphogenesis). The resolution phase of inflammation or the normal response to tissue injury (wound healing) is also accompanied with high rate of apoptotic cell removal (deCathelineau 2003). Interestingly, efferocytosis occurs throughout the body, but the specific molecular pathways that orchestrate the process can vary by tissue (Elliott et al., 2010).

1.3.“Effectors” of efferocytosis

As it was already mentioned above, different cell types are capable of engulfing dead cells. Although the process can be carried out by most of the cell types, including epithelial cells, fibroblasts and other non-professional phagocytes, there is a division of cells, the so called professional phagocytes, which are capable of engulfing dead cells more effectively than others. Such “professionals” are the macrophages, dendritic cells and neutrophil granulocytes (Ravichandran, 2010). Many studies have demonstrated that the effector molecules (receptors, signal transducers, etc.) of efferocytosis appear to be widely distributed, unique and evolutionarily very highly conserved, but it is not completely clear, whether the mechanism of uptake vary between different cell types (Monks et al., 2008). Evidence for differences has been presented, but could largely reflect rates of uptake rather than qualitative differences (Parnaik et al., 2000). Other studies, however, suggest that uptake mechanisms, receptors, and signaling are conserved, not only between different cell types but also evolutionally (Monks et al., 2008). Despite of the fact that apoptotic cell removal is evident in the absence of macrophages -for example before macrophages are not present during embryonic development or in organisms that don't have macrophages-, there is a prevailing perception in the literature that apoptotic cell removal *in vivo* is largely mediated by macrophages (Monks et al., 2008).

1.3.1. Macrophages

Macrophages belong to the mononuclear phagocyte system, that was previously called by the famous german pathologist Karl Albert Ludwig Aschoff, reticuloendothelial system (Aschoff, 1924). The system consists of cell types with common developmental origin and similar functions, but with different appearance and name because of their various locations: microglia in the central nervous system, dermal macrophages in the skin, splenic marginal zone and metallophilic macrophages, type A synovial lining cells in synovial joints and tendon sheath,

Kupffer cells in the liver, alveolar macrophages in lungs, osteoclasts in bones and histiocytes in connective tissue (Nich et al., 2013). Monocytes and macrophages are critical effectors and regulators of inflammation and the innate immune response, the immediate, pre-programmed arm of the immune system. They originate from the multipotent hematopoietic stem cells that also give rise to other types of lymphoid and myeloid cells. The proliferating myeloid progenitors of macrophages leave the bone marrow, their birth-cradle, as monocytes and after circulating in the blood they subsequently populate tissues as macrophages in the steady state and during inflammation (van Furth et al., 1968).

Circulating monocytes represent immune effector cells, equipped with chemokine receptors and pathogen recognition receptors that mediate migration from blood to tissues during infection or tissue injury. Beyond producing inflammatory cytokines, they take up cells, toxic molecules and debris by phagocytosis. They are capable of differentiating into inflammatory dendritic cells or macrophages during inflammation, and possibly, less efficiently, in the steady state. The eventual differentiation to inflammatory dendritic cells and macrophages is likely determined by the recruitment to a specific tissue site due to local inflammatory conditions and activation of the pattern recognition receptors (Serbina et al., 2008).

Resident tissue macrophages are highly specialized cells uniquely adapted to their location, and are believed to be involved in steady-state tissue homeostasis via the clearance of apoptotic cells, and the production of growth factors. They are also equipped with a broad range of pathogen recognition receptors that make them efficient at phagocytosis and at induction of inflammatory cytokine production (Gordon, 2002).

External signals in different tissues and circumstances can significantly alter macrophages (called macrophage activation) by changing the expression of wide range of cell-surface markers and modulating their functions including efferocytosis (Martinez et al., 2014). In general terms there are two ends of the macrophage activation and the related functional phenotypes: The „classically activated” or M1 macrophages demonstrate diminished efferocytosis, increased phagocytosis of foreign organisms and enhanced production of pro-inflammatory cytokines, reactive oxygen species (ROS), and nitric oxide (NO). Such macrophages are present early in the course of infection or injury and are termed by some authors „efferocytotic-low” macrophages (Korns et al., 2011). On the opposite end of the spectrum, various „alternatively activated” or M2 macrophages are, that exhibit increased expression and/or activity of the nuclear receptors peroxisome proliferator-activated receptor (PPAR) γ and δ , increased levels of certain receptors (e.g., the macrophage mannose receptor) and anti-inflammatory cytokines, whereas production of pro-inflammatory cytokines and ROS

are downregulated. The „alternative activation” states have been associated with enhanced efferocytosis of apoptotic cells (and likely also necrotic cells and cellular debris) supporting a role in the resolution phase of inflammation. The different programming state of the activated macrophage subgroups results from stimulation with different external stimuli (cytokines, interleukins, growth factors, bacterial derivatives, etc) found in their local environment (Korns et al., 2011; Martinez et al., 2014).

As I have already mentioned in the upper section of my thesis, there is a prevailing perception in the literature that apoptotic cell removal *in vivo* is largely mediated by macrophages (Monks et al., 2008). Additionally, most of the experimental data related to efferocytosis research is derived from studies, in which murine or human peripheral and/or bone marrow derived macrophages are used. In the further sections of the introduction chapter (and also in the Results part) efferocytosis is going to refer on to macrophage related efferocytosis or the given cell type will be specifically indicated.

1.4. Steps of efferocytosis

The quick and efficient removal of unwanted cells is important for “making space” for living cells, and for maintaining the function of tissue, and in turn, a healthy organism (Savill et al., 2002). Under physiological conditions efferocytosis is so effective that even in tissues with high cellular turnover (bone marrow, spleen or thymus), very few apoptotic cells can be detected. Many authors claim that a dynamic balance between the numbers of apoptotic cells, the numbers of phagocytes, and their capacity for uptake is set (Gardai et al., 2006; Ravichandran 2010). This suggests, that the capacity of cell clearance has to be tightly regulated. To clarify the regulation or the “fine-tuning” of the efferocytotic capacity, the specific steps by which apoptotic cells are recognized and removed have to be defined.

Efferocytosis can be broadly broken down into four major steps (Lauber et al., 2004; Fig. 1.): First the phagocytes are **recruited** to the sites of death via specific “find-me” signals (Gregory 2009). Dead cells are **recognized** via the interaction of “eat-me” signals exposed on the apoptotic cell surface and efferocytosis related receptors on the phagocyte cell surface. After this, the internalization, procession and **digestion** of the ingested cargo is the third step (Grimsley et al., 2003). The so called “**post-engulfment consequences**” are the final actions of the uptake process that covers the induction of anti-inflammatory cytokine release and active inhibition of pro-inflammatory cytokine production in the engulfing phagocyte (Savill et al., 2002).

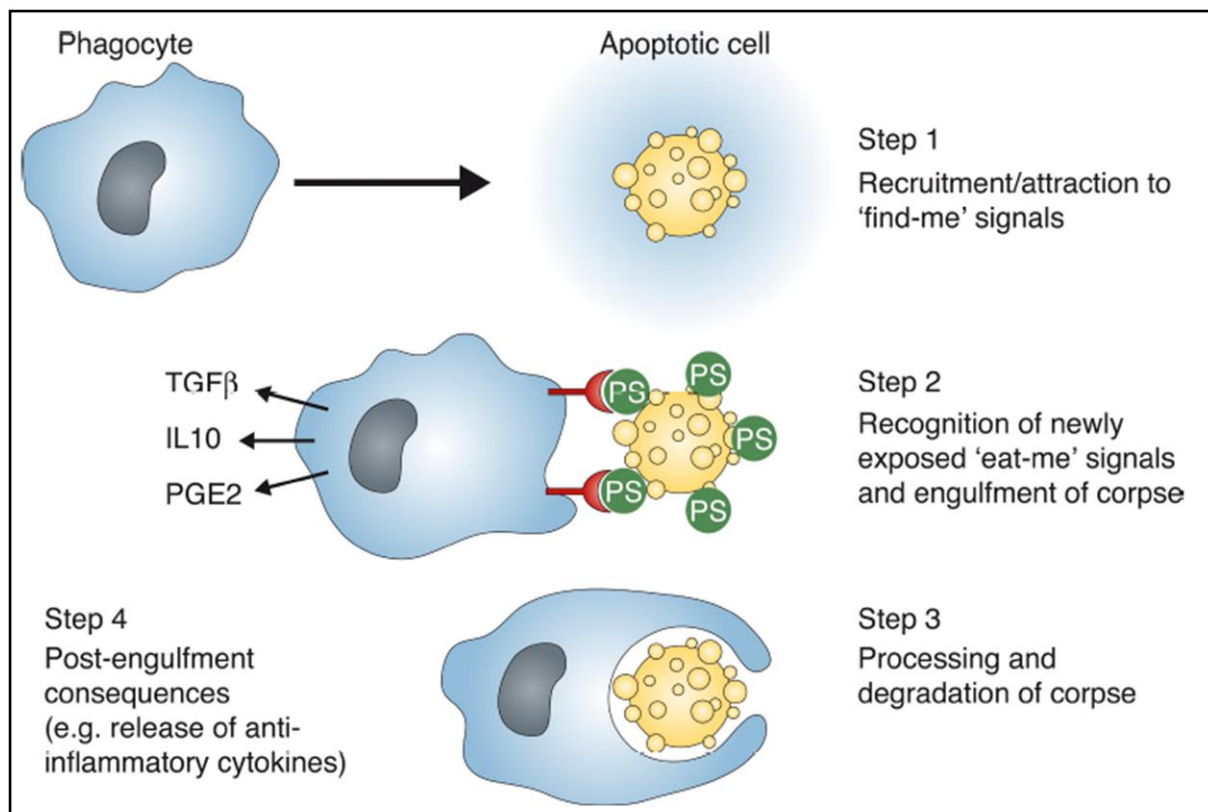


Figure 1. Steps of the apoptotic cell clearance (Ravichandran KS, 2010)

1.4.1. Recruitment of macrophages to the dying cells (“find-me” signals)

Migration is a prominent component of tissue repair and immune surveillance, in which leukocytes from the circulation migrate into or within the surrounding tissue to destroy invading microorganisms, infected cells and to clear debris (Ridley et al., 2003). Dying apoptotic cells release soluble factors which attract the motile phagocytes for prompt efferocytosis. This way the capacity of efferocytosis can be increased by recruiting the main effector cells to the site of action.

Up to now several so called “find-me” signals were reported to be released by apoptotic cells: The first discovered lipid “find-me” signal was the lysophosphatidylcholine (LPC). LPC is released from apoptotic cells due to caspase-3 mediated activation of the calcium-independent phospholipase A2 (Lauber et al., 2003). The G-protein-coupled receptor G2A is responsible for binding of LPC, which promotes the chemotaxis of monocytic cells (Peter et al., 2008).

Sphingosine-1-phosphate (S1P), a sphingolipid metabolite produced by the ubiquitously expressed sphingosine kinase 1 enzyme, is another chemotactic factor secreted by apoptotic cells. Low nanomolar concentrations of S1P were shown to elicit chemotactic movement of

macrophages. Moreover, S1P markedly induces macrophage cytoskeletal rearrangements (Gude et al., 2008).

The chemokine and adhesion molecule CX3CL1 also known as fractalkine (FKN), together with its cognate receptor CX3CR1 plays an active role in the chemotaxis of macrophages towards apoptotic cells (Truman et al., 2008). The soluble fractalkine has chemoattractive activity for monocytes, natural killer cells, and T cells and is generated by two sheddases, the disintegrin-like metalloproteinases ADAM 10 and ADAM 17. The membrane-bound fractalkine supports integrin-independent leukocyte adhesion (Jones et al., 2010).

Adenosine triphosphate (ATP) has been identified as “find-me” signal released by apoptotic cells during the early stages of apoptosis (Elliott et al., 2009). Plasma membrane channel pannexin 1 opening and effector caspase activation has been reported to mediate the regulated release of ATP (Chekeni et al., 2010). As a “find-me” signal, ATP has been proposed to create an ATP gradient that attracts phagocytes via the P2Y2 receptor (Elliott et al., 2009). However, outside of cells, ATP is rapidly (in seconds) degraded by a family of ectonucleotidases (Yegutkin, 2008), which presumably limits the reach of ATP as a potential chemoattractant (Elliott et al., 2009, Isfort et al., 2011).

Whether these “find-me” signals are always formed, or whether they are released in a cell type specific manner is not known yet. However, the types of signals might determine the signaling that drives migration. Thus for neutrophils it has been shown that phosphatidylinositol 3-kinase plays a role in chemotaxis mediated by chemokines, whereas migration toward the bacteria derived *N*-Formylmethionyl-leucyl-phenylalanine (fMLP) or toward the complement component C5a is strongly dependent on the signaling of the mitogen-activated protein kinase p38 (Heit et al., 2002; Heit et al., 2008). In contrast, experiments performed on macrophages indicated that their chemotaxis to C5a is phosphatidylinositol 3-kinase- and mitogen-activated protein kinase p38-independent indicating that the signaling that drives the migration is not only signal, but also cell type-specific (Isfort et al., 2011).

It should be noted that phagocytosis of apoptotic cells is generally recognized as a non-immunogenic, non-inflammatory process with minimal, if any, neutrophil recruitment (Savill et al., 2002). However, LPC, S1P and nucleotides are all known to stimulate neutrophil chemotaxis as well (Chen et al., 2006; Florey et al., 2009). A possible explanation for the absence of neutrophils in the recruitment phase of efferocytosis is that apoptotic cells might secrete “stay away” signals as well (Hochreiter-Hufford et al., 2013). Such a signal could be the release of lactoferrin (LTF) by apoptotic cells, which appears to specifically inhibit neutrophil migration (Bournazou et al., 2009).

1.4.1.1. The role of autocrine purinergic signaling in the chemotactic movement of phagocytes

Cellular movement of neutrophils and macrophages is achieved by coordinately generating membrane protrusions (lamellopodia) at the front of the cell and contractions at the rear (Ridley et al., 2003; Friedl et al., 2008), whereas chemotactic navigation requires that the leading edge is directed toward the source of chemoattractant (Kamimura et al., 2008). Cell migration can be conceptualized as a cyclic process, by which the initial response of a cell to a migration-promoting agent, is to polarize and extend protrusions in the leading edge toward the direction of migration (Ridley et al., 2003).

Previous studies in neutrophils and macrophages have shown that in addition to the chemotactic signal, an autocrine purinergic signaling on the leading edge is needed to amplify and translate migration-promoting signals into directional motility. Thus the purine nucleotides might not only function in the course of efferocytosis as potential “find-me” signals released by apoptotic cells (as it was observed in the case of ATP), but they also represent ligands released by the moving phagocytes themselves that bind to the cell surface purinergic receptors and amplify the chemotactic signal. (Fig. 2.)

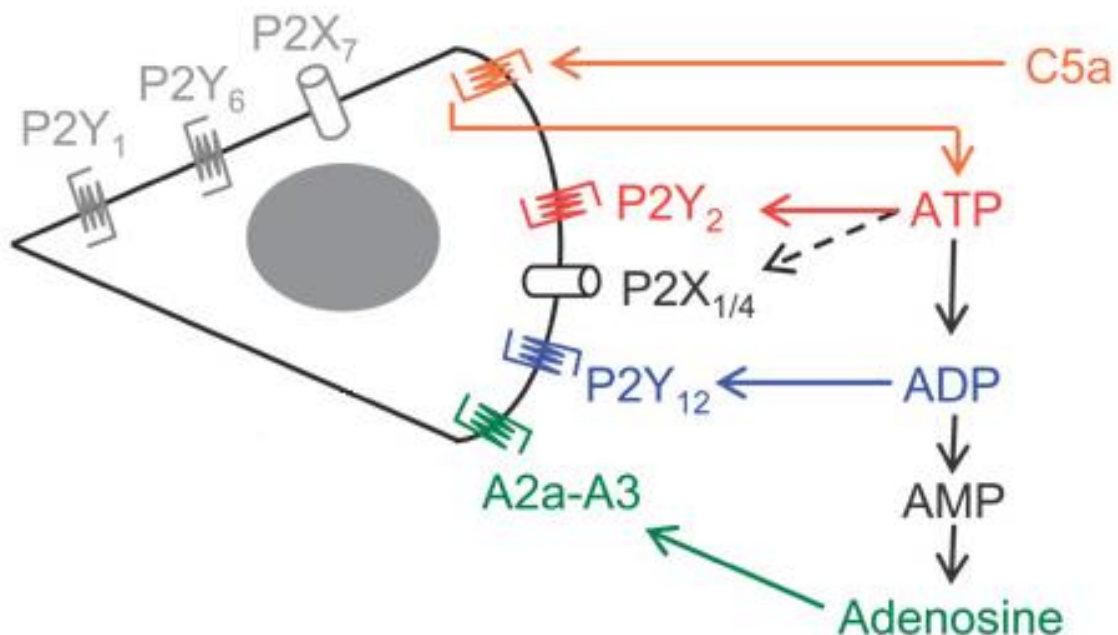


Figure 2. Autocrine purinergic receptor signaling in macrophages during chemotactic movement toward C5a (based on Kronlage et al., 2010)

Upon sensing the chemotactic signal, phagocytes release ATP at the leading edge of the cell, which is then degraded to adenosine diphosphate (ADP), adenosine monophosphate (AMP) and

adenosine. In neutrophils ATP acting on the P2Y2 nucleotide receptors promotes directional movements, while adenosine acting on the adenosine 3 receptors (A3R) promotes the velocity of migration toward a chemotactic signal (Chen et al., 2006). Interference with either P2Y2 or A3R signaling strongly inhibits the chemotactic movement of neutrophils, and loss of A3Rs impacted neutrophil immune function *in vivo* (Butler et al., 2012).

In macrophages it was shown that activation of one of the three purinergic receptors (P2Y2 by ATP, P2Y12 by ADP or the adenosine receptors together by adenosine) alone is sufficient, but is also needed to initiate migration toward a chemotactic signal (Kronlage et al., 2010). Surprisingly, in these studies apyrase (a plant derived ATP/ADPase enzyme) was found to initiate an alternative degradation pathway for ATP, resulting in no ADP and adenosine formation. Thus, in the presence of apyrase, an undefined degradation product is formed, that none of the purinergic receptors activate and consequently the chemotaxis is severely affected (Kronlage et al., 2010) explaining why injection of apyrase could delay migration of macrophages toward apoptotic cells also *in vivo* (Elliott et al., 2009). Although some authors claim, that ATP cannot act as a chemotactic signal for macrophages (Kronlage et al., 2010) as it was suggested previously (Elliott et al., 2009), apoptotic cell-derived ATP and its degraded products ADP and adenosine might promote the autocrine purinergic signaling that amplify and translate chemotactic “find-me” signals into directional motility.

There are four adenosine receptors, all of which are G protein-coupled receptors. The genes for these receptors have been analyzed in detail. The receptors are designated adenosine 1 receptor (A1R), adenosine 2A receptor (A2AR), adenosine 2B receptor (A2BR) and adenosine 3 receptor (A3R). A1R receptors are stimulated by 10^{-10} - 10^{-8} M concentrations of adenosine and mediate decreases in intracellular cyclic AMP (cAMP) levels, A2ARs and A2BRs are stimulated by higher (5×10^{-7} M and 1×10^{-5} M, respectively) concentrations of adenosine and mediate increases in cAMP levels, while adenosine A3Rs are stimulated by 10^{-6} M concentrations of adenosine and mediate adenylate cyclase inhibition (Fredholm et al., 2011). However, the response of adenosine receptors is also determined by their cell surface expression, thus, when one compares ligand potencies to modulate cAMP levels at comparative receptor densities, it is observed that adenosine is nearly equipotent at A1R, A2AR, and A3R but is some 50 times less potent at A2BRs (Fredholm et al., 2001). Peritoneal macrophages have been reported to express adenosine A2AR, A2BR and A3R receptors (Kronlage et al., 2010).

1.4.2. Recognition of apoptotic cells: “Eat me” signals on the dead cells and uptake receptors on the phagocyte surface

After phagocytes are recruited to the close proximity of dying cells, dead cells have to be distinguished from living cells (recognition phase). This phase of efferocytosis consists of a complex molecular interaction between the surface of the dead cells and the phagocytes. Due to the importance of this cell surface interaction it has been also termed “the third synapse” (Fig.3.).

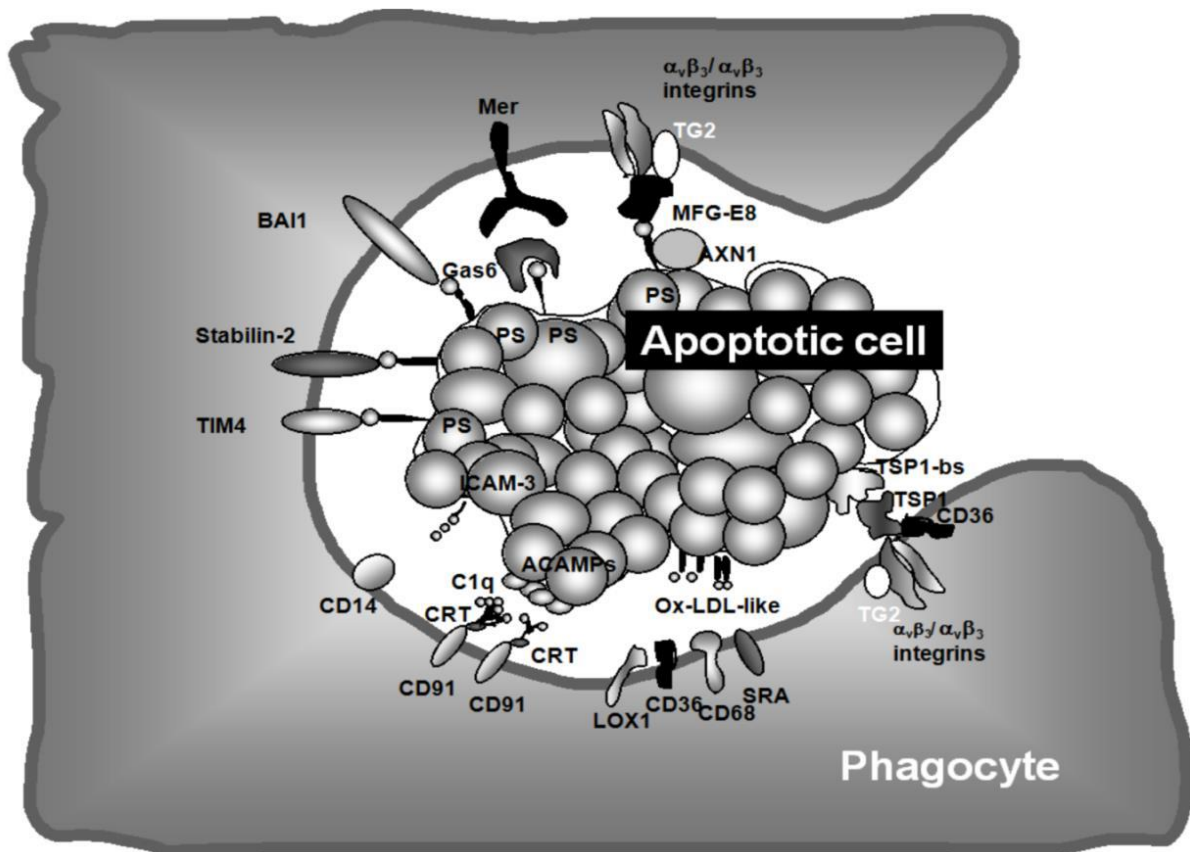


Figure 3. The „third synapse”: Molecules connecting apoptotic cells to the phagocyte surface. Apoptotic cell phosphatidylserine (PS) can be recognized directly via receptors displayed on phagocyte, such as T cell immunoglobulin and mucin-domain-containing molecule 4 (TIM4), brain-specific angiogenesis inhibitor 1 (BAI1) or stabilin-2, and also via the bridging molecules milk fat globule epidermal growth-factor (MFG-E8), growth-arrest-specific 6 (Gas6) which bind to Mer tyrosine kinase (Mertk) and $\alpha_v\beta_3/\alpha_v\beta_5$ integrins, respectively. The tissue transglutaminase (TG2) serves as a co-receptor for integrins. The membrane glycoprotein CD14 interacts with the intracellular adhesion molecule-3 (ICAM-3). The complement component 1q (C1q) regulates the recognition of Apoptotic Cell Associated Molecular Patterns (ACAMPs) via calreticulin (CRT) and CD91. Several scavenger receptors (oxidized low-density lipoprotein receptor 1 (LOX1), CD36, CD68, and SRA) recognize oxidized-LDL-like sites on apoptotic cell surface. $\alpha_v\beta_3/\alpha_v\beta_5$ integrins associate to CD36 and interact with thrombospondin 1 (TSP1), which is recognized by thrombospondin 1-binding site on apoptotic cell surface (TSP1-bs)(Szondy et al., 2011).

As Fig. 3. highlights there are several molecular markers on an apoptotic cell that are recognized by the phagocytic receptors. Since there is not only one single, characteristic cell surface change on the apoptotic cells, macrophages seem to recognize the sum of all the cell surface alterations by utilizing number of phagocytic receptors acting simultaneously (Stuart et al., 2005, Ravichandran et al., 2007). The best characterized mark that distinguishes cellular life from death is the redistribution of phosphatidylserine (PS) on the surface of the apoptotic cell (Fadok et al., 2003). Many of the macrophage receptors recognize PS or its oxidized form binding to it directly or indirectly via bridging molecules, such as milk fat globule EGF-factor 8 (MFG-E8) (Hanayama et al., 2002), thrombospondin-1 (TSP1) (Savill et al., 1992) or complement component 1q (C1q) (Botto et al., 1998). Macrophages are capable of both expressing the cell surface receptors and releasing the bridging molecules for the recognition and engulfment of apoptotic cells. Some of these receptors, such as CD14 (Devitt et al., 2004) or T cell immunoglobulin and mucin-domain-containing molecule 4 (Tim4) (Park et al., 2009), mediate tethering, while other receptors, such as CD36 (Greenberg et al., 2006), integrin $\beta 3$ (Savill et al., 1992), integrin $\beta 5$ (Albert et al., 2000), Mer tyrosine kinase (Mertk) (Cohen et al., 2002), stabilin-2 (Park et al., 2008) and ATP-binding cassette transporter (ABCA1) (Hamon et al., 2006) seem to activate two evolutionally conserved parallel signaling pathways promoting cytoskeletal reorganization via the activation of the low molecular weight GTPase Rac1, which is obligatorily required for the uptake (Kinchin et al., 2005). Previous work in our laboratory has shown that transglutaminase 2 (TG2) acting as a coreceptor for integrin $\beta 3$ is required for proper phagocytosis of apoptotic cells (Tóth et al., 2009). In the absence of TG2 systemic lupus erythematosus (SLE) like autoimmunity develops in mice (Szondy et al., 2003), similarly to other mice characterized by a deficiency in the clearance of apoptotic cells (Tanaka et al., 2007).

1.4.2.1. Regulation of the expression of the phagocytotic receptors

Macrophages are exposed to varying number of apoptotic cells *in vivo*, thus they must have a sensing mechanism that prepares them, when it is needed, for an increased apoptotic cell uptake by the upregulation of different effector molecules, including surface “eat-me” receptors. The capacity of apoptotic cell clearance can be set this way on molecular level.

Increasing evidence suggests that the receptors that sense the amount of engulfed apoptotic cells, and increase the phagocytic capacity of macrophages in response, are the lipid sensing receptor liver X receptor (LXR) (A-Gonzalez et al., 2009), peroxisome proliferator-activated receptors (PPAR) γ (Roszer et al., 2011) and δ (Mukundan et al., 2009) (Fig. 4.). Triggering of these receptors leads to enhanced expression of phagocytic receptors and bridging molecules

such as Mertk, C1qb, CD36 and ABCA1 (A-Gonzalez et al., 2009, Roszer et al., 2011, Mukundan et al., 2009).

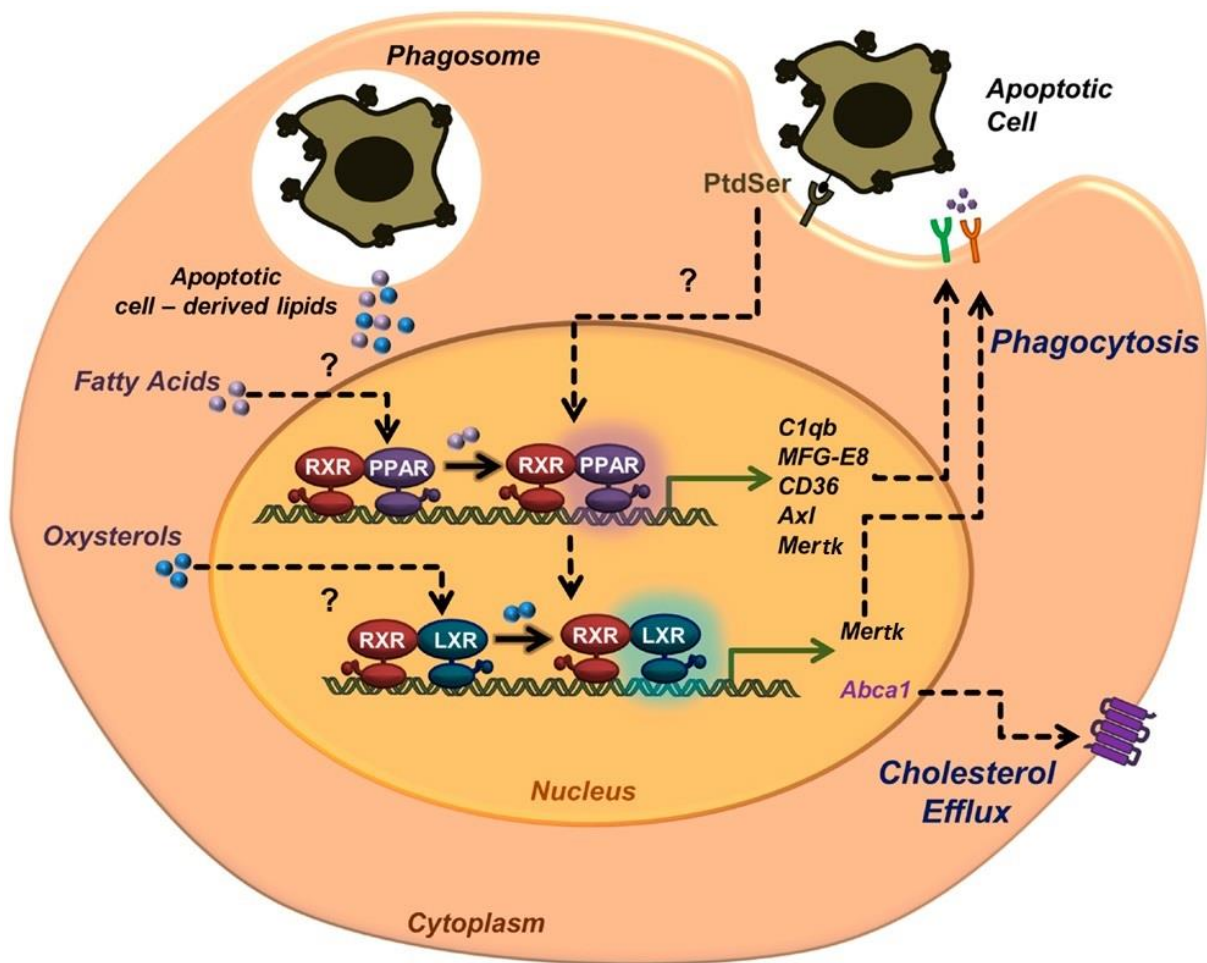


Figure 4. Activation of nuclear receptors in phagocytes during apoptotic cell clearance. Apoptotic cell recognition and engulfment promote the transcriptional activity of nuclear receptors LXRs and PPARs. Recognition of phosphatidylserine in the outer leaflet membrane of the apoptotic cell leads to transcriptional activation of ABCA1 and cholesterol efflux. Nuclear receptor activation upon apoptotic cell phagocytosis also leads to upregulation of phagocytic receptors (e.g., Mertk, CD36, and Axl) and opsonins (e.g., MFG-E8 and C1qb). Lipids derived from the engulfed apoptotic cells may also serve as source of endogenous ligands to activate PPARs (fatty acids) and LXRs (oxysterols) (based on A-Gonzales and Hidalgo, 2014).

Lipid sensing receptors belong to the nuclear retinoid X receptor (RXR) heterodimer family and can be triggered independently by both the lipid content of the engulfed cells on the LXR or PPAR side and by a vitamin A (retinol) derivative on the retinoid X receptor (RXR) side (rexinoids or permissive RXR heterodimers) (Mangelsdorf et al., 1995, Tzameli et al., 2003). It is believed that the RXR ligand in tissues is 9-cis retinoic acid (9cRA) which is thought to be produced by a non-enzymatic reaction from all-trans retinoic acid (ATRA) (Heyman et al., 1992). Interestingly, however, so far 9cRA could never be detected *in vivo* (Rühl, 2006). ATRA

is an oxidative derivative of retinol, and is generated from it by two subsequent dehydrogenase steps (Kam et al., 2012). In addition to the lipid sensing receptors, ATRA and 9cRA, can activate the retinoic acid receptors (RARs) as well (Fig. 5). RAR/RXR receptors, unlike the lipid sensing receptors, cannot be activated via the RXR side alone (non-permissive RXR heterodimers) (Germain et al., 2006, Tzameli et al., 2003).

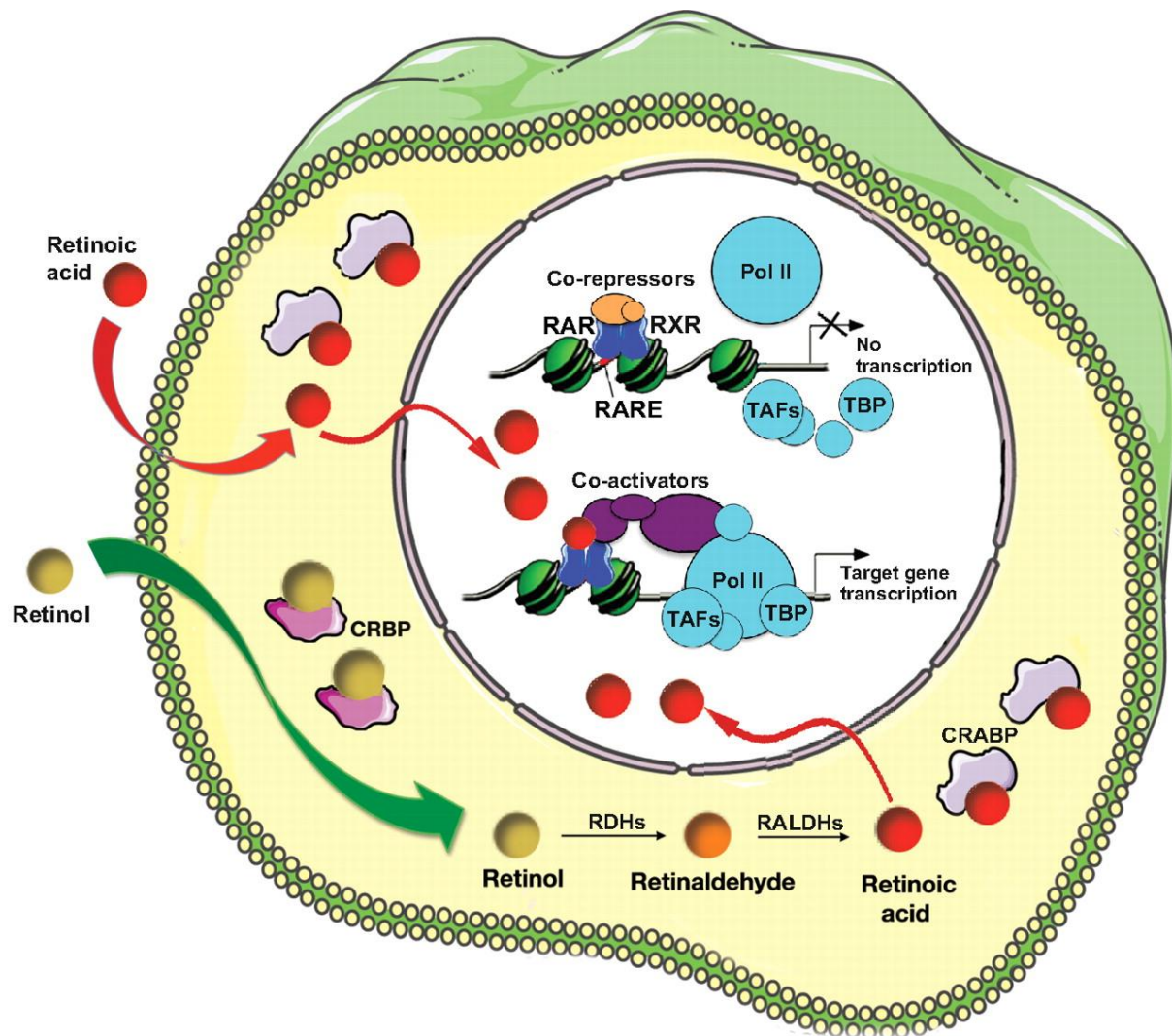


Figure 5. Summary of the retinoic acid (RA) signalling pathway. RA, synthesized intracellularly from circulating retinol or diffusing from an adjacent cell (curved red arrow), eventually reaches the nucleus. Cellular retinoic acid-binding proteins (CRABPs) may be involved in this transfer. Cellular retinol-binding proteins (CRBPs) may help present retinol to retinol dehydrogenases (RDHs). Dimers of RA receptors (RARs) and retinoid X receptors (RXRs), termed RAR/RXR, are able to bind to RA-response elements (RAREs) in their target genes in the absence of ligand, interacting with protein complexes (co-repressors) that stabilise the chromatin nucleosomal structure and prevent access to the promoter. Upon RA binding, a conformational change in the helicoidal structure of the RAR ligand-binding domain changes its protein-protein interaction properties, releasing the co-repressors and recruiting co-activator complexes that destabilise the nucleosomes and/or facilitate assembly of the transcription pre-initiation complex, which contains RNA polymerase II (Pol II), TATA-binding protein (TBP) and TBP-associated factors (TAFs) (Rhinn et al., 2012).

Following ligation, lipid sensing receptors regulate the transcription of various genes including those that belong to the lipid homeostasis (Calkin et al., 2012). Thus LXRs can directly promote transcription of sterol response element binding protein 1c (SREBP-1c) through two LXR response elements in the mouse SREBP-1c promoter (Yoshikawa et al., 2001) and also that of RAR α (Rébé et al., 2009) and ABCA1 (Costet et al., 2000). In addition, Mertk was also found to be a direct LXR target gene (A-Gonzalez et al., 2009). LXR binding sites exist also in the promoter of LXR, thus following ligation LXRs promote their own transcription via an autoregulatory loop mechanism (Li et al., 2002).

TG2 expression in macrophages has also been shown to be induced by ATRA and interferon gamma (IFN γ) (Moore et al., 1984; Bayardo et al., 2012). Our research group found previously that enhanced retinoid production can be observed in the thymus after the *in vivo* apoptosis induction of thymocytes. Interestingly, the cells that express retinaldehyde dehydrogenases (RALDHs), that is responsible for RA production, are the engulfing macrophages. Indeed, apoptotic cell uptake *in vitro* also triggered the expression of RALDHs in macrophages indicating that the uptake of apoptotic cells is coupled to retinoid synthesis (Garabuczi et al., 2013). Since the three lipid-sensing nuclear receptors (LXR, PPAR γ and PPAR δ) have been implicated in the macrophage response to the engulfed apoptotic cells (A-Gonzalez et al., 2009; Roszer et al., 2011; Mukundan et al., 2009) Éva Garabuczi from our laboratory tested whether triggering of these receptors affect the expression of RALDH1. She found that agonists of all these three receptors promoted the mRNA expression of RALDH1 in macrophages, but the most effective was the LXR agonist GW3965 (Garabuczi et al., 2013).

1.4.3. Digestion of apoptotic corpses (downstream signaling from the receptors)

After ligation of the efferocytotic receptors by the apoptotic “eat-me” signals, two distinct signaling pathways are activated that subsequently mediate the initiation of the cytoskeletal rearrangements necessary for the internalization of the corpse. The first signaling pathway is the CrkII–Dock180–ELMO that is involved also in cell migration, neurite growth, and myoblast fusion (Nagata et al., 2010). Several phagocytic receptors have been shown to use this pathway for clearance of apoptotic cargo. These include integrin α v β 3/ α v β 5 receptors, Mertk and BAI1 (Hochreiter-Hufford et al., 2013). The second pathway is related to the interaction of the adaptor protein GULP with either CD91 or ABCA1 or stabilin-2 (Su et al., 2002). Both signaling pathways converge into the activation of the small GTPase Rac1. Upon activation Rac1 mediates actin reorganization and the subsequent “phagocytic cup” formation (Nagata et al., 2010). On the other hand RhoA activation, which is an other small GTPase, inhibits the same

process. The relative balance between Rac1 and RhoA determines macrophage efferocytotic ability (Nakaya et al., 2006).

The further events downstream from the internalization of the apoptotic corpse are collectively termed phagosome maturation. The phagosome, which is a membrane bound compartment containing the phagocytosed target, becomes increasingly acidic and ultimately fuses with lysosomes gaining digestive enzymes required for degradation (Hochreiter-Hufford et al., 2013). The regulation of phagosome maturation is an actively studied, evolving field of the efferocytosis research (it may influence the phagocytic capacity of a cell to internalize additional targets), however the more detailed description of it is beyond the scope of my thesis. Apoptotic cells are digested in the phagosomes into their building blocks including nucleotides, lipids (fats/sterols) and peptides (amino acids). Several mechanisms have been identified that help a phagocyte to maintain homeostasis and cellular metabolism after consuming apoptotic cell constituents: Such a mechanism is the upregulation of the mitochondrial uncoupling protein 2 (UCP 2), which has been shown to positively regulate the engulfment capacity of phagocytes by uncoupling oxidative phosphorylation from ATP synthesis and reducing mitochondrial membrane potential (Park et al., 2011). Effective degradation of target cell DNA depends on the lysosomal enzyme DNase II, which's normal function seems to be critical in keeping the anti-inflammatory character of the efferocytosis. It has been observed that the deletion of DNase II in a mouse model led to the development of polyarthritis and an increase of inflammatory cytokines like tumor necrosis factor α (TNF α) in the joint tissues (Kawane et al., 2006). Excessive cholesterol load has to be also strictly regulated in an efferocytosing cell. As it was already described above lipids derived from the engulfed apoptotic cells may also serve as source of endogenous ligands to activate PPARs (fatty acids) and LXRs (oxysterols) that in turn regulate the expression of other phagocytosis related molecules (A-Gonzalez et al., 2014). Nevertheless, it has been also shown that phagocytes increase their basal cholesterol efflux mechanism via ABCA1 (Gerbod-Giannone et al., 2006).

1.4.4. “Post-engulfment” consequences (release of anti-inflammatory cytokines by engulfing phagocytes)

The hallmark feature of apoptotic cell clearance, on the contrary to necrotic cell clearance, is the non-inflammatory, tolerogenic nature of the process. The immunologically silent character was first observed by Voll and his colleagues in the late 90's. They found that the LPS-induced pro-inflammatory TNF- α and interleukin 1 β (IL-1 β) expression was reduced in monocytes after co-culturing them with apoptotic lymphocytes, while the anti-inflammatory interleukin 10 (IL-

10) was upregulated (Voll et al., 1997). Since then, it has been generally accepted that in contrast to the uptake of pathogens or necrotic cells, which is accompanied with subsequent inflammatory responses, uptake of apoptotic cells by phagocytes has non-inflammatory character both *in vitro* and *in vivo*. Moreover, apoptotic cell uptake is not only immunologically silent process, but it has immunosuppressive effects (Fadok et al., 1998): First, apoptotic cell binding to the macrophages, independent of subsequent engulfment, directly inhibits the production and release of pro-inflammatory cytokines. This effect is a consequence of multiple signaling steps like blocking the NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells) pathway after ligation of MERTK (Sen et al., 2007) or induction of suppressors of cytokine signaling 1/3 proteins (SOCS1 and SOCS 3) and suppression of the Jak-STAT (Janus kinase- Signal Transducer and Activator of Transcription) signaling (Tassiulas et al., 2007). Second, efferocytosis is going along with active secretion of soluble anti-inflammatory molecules like IL-10, TGF- β , platelet-activating factor (PAF), prostaglandin E2 (PGE2), and S1P (Voll et al., 1997, Fadok et al., 1998). Inhibition of pro-inflammatory cytokine release and secretion of anti-inflammatory compounds are both thought to be related to interaction of phagocytes with PS containing cell membranes (Fadok et al., 2001).

Interestingly, adenosine and adenosine receptor related signaling have been shown to be involved not only in the regulation of chemotactic migration of phagocytes, but also in the regulation of the immunosuppressive effects of apoptotic cell uptake. It is widely accepted that certain naturally occurring purines can exert powerful effects on the immune system. Adenosine is the best characterized nucleoside from these, as it affects almost all aspects of an immune response (Haskó et al., 2007). Recent work in our laboratory has also shown that adenosine is produced by the macrophage 5' nucleotidase during the interaction of engulfing macrophages and apoptotic cells (Sandor et al., 2016). Krisztina Köröskényi from our laboratory found previously, that A2AR contributes to the immunosuppressive nature of efferocytosis by mediating the inhibition of the macrophage inflammatory protein-2 (MIP-2) (Köröskényi et al., 2011). On the other hand, A3Rs have been found to have opposite effects (Dúró et al., 2014).

1.5. Defective clearance of dead cells

In healthy tissues, the regulated balance between the numbers of apoptotic cells, the numbers of phagocytes and their capacity for uptake keeps the process of efferocytosis so rapid and effective that even in tissues with high cellular turnover, very few apoptotic cells can be detected. By disturbance of the proper clearance, apoptotic cells undergo secondary necrosis and start to release pro-inflammatory cell contents that damages their environment. In addition,

detection and removal of apoptotic cells induces generally an anti-inflammatory response, the absence of which might also contribute to the pathological consequences of improper apoptotic cell clearance.

Indeed, increasing evidence suggest that improper clearance of apoptotic cells, being the result of either genetic anomalies and/or a persistent disease state, contributes to the establishment and progression of a number of human diseases via effects on the maintenance of tissue homeostasis, tissue repair, and inflammation (Elliot et al., 2010). On the other hand, proper clearance of dead cells is important by the initiation of tissue repair processes following injury (Wan et al., 2013) and it is a key event in the resolution phase of inflammation (Savill et al., 2002).

1.5.1. Diseases related to disturbed efferocytosis

Autoimmune disorders, in which both animal models and human research indicate a strong relationship between improper clearance and the development of the disease, represent the best characterized example of disturbed efferocytosis related diseases. The regulated nature of apoptotic cell death normally prevents the leakage of the immunogenic intracellular contents. However, when dead cells are not cleared, they subsequently undergo secondary necrosis and start to release intracellular antigens and DNA, which provoke in the long-term an auto-inflammatory response (Muñoz et al., 2010). Thus, in most of the knock out mice in which efferocytosis is impaired, systemic lupus erythematosus (SLE) like autoimmunity develops (Hanayama et al., 2004; Scott et al., 2001; Botto et al., 1998; Szondy et al., 2003; Lewis et al., 2006). Human SLE is also accompanied by improper efferocytosis (Muñoz et al., 2010), and can develop also as a result of a genetic deficiency of the phagocytosis process (Lewis et al., 2006).

While in SLE improper clearance of apoptotic cells affects all the tissues, in several chronic inflammatory respiratory diseases, such as chronic obstructive pulmonary disease (COPD), cystic fibrosis, and asthma, increased numbers of apoptotic cells are seen only in the sputum and lung tissue (Henson et al., 2008). Though so far no evidence was provided for a definite linkage between genetic anomalies affecting efferocytosis and lung disease, inefficient apoptotic clearance in the lung was detected in all these respiratory diseases (McCubrey et al., 2013).

Macrophages play a key role in the development of atherosclerosis, and impaired clearance of apoptotic macrophages characterizes the late plaques, in which uncleared apoptotic cells undergo secondary necrosis leading to the formation of an unstable necrotic core and the

maintenance of inflammation (Seimon et al., 2009). Impaired efferocytosis, however, might also contribute to the development of the disease, as knock out mice deficient in efferocytosis are prone to develop atherosclerosis on LDL (low density lipoprotein) or ApoE (apolipoprotein E) null genetic back-grounds as well (Thorp et al., 2008; Bhatia et al., 2007; Moura et al., 2008). An excess of apoptotic cells was detected in a numerous neurodegenerative diseases as well, such as Parkinson's, Alzheimer's, and Huntington's disease (Mattson et al., 2000). Though the elevated levels of apoptotic cells might also be the result of an increased neuronal cell death, in these diseases loss of signaling by fractalkine (again: an apoptotic cell "find-me" signal) resulted in an increase in the number of dying cells and worsening of the disease (Cardona et al., 2006).

Interestingly, type 2 diabetes and obesity were also shown to be associated with impaired phagocytosis of apoptotic b-cells in the pancreas in autoimmune diabetes-prone rats (O'brien et al., 2002) and in ob/ob and db/db mice (Li et al., 2009).

Chronic periodontitis, one of the leading cause of tooth loss worldwide, might be also associated with elevated apoptotic cell numbers in the periodontium as a result of the potential defects of their clearance process (Joós G., 2014).

1.5.2. Therapeutic possibilities: Enhancing efferocytosis when clearance of apoptotic cells is impaired

It is of great importance to clarify and understand the background of the diseases, which are potentially associated with the defective clearance. By the manipulation of the engulfment machinery, novel therapeutic perspectives could be reached (Ravichandran, 2010). Since improper efferocytosis might contribute to both the initiation and the maintenance of human diseases, enhancing phagocytosis might provide a therapeutic possibility to influence the progression of them.

1.5.2.1. Affecting recognition and binding of apoptotic cells to the phagocytes

If lack of sufficient MFG-E8 production leading to improper efferocytosis participates in the pathomechanism of a disease, providing MFG-E8 in recombinant protein form to the site of acute inflammation might enhance the efficiency of efferocytosis. Indeed, a decreased MFG-E8 expression was found in inflamed colons during the acute phase of murine experimental colitis, and intrarectal treatment with recombinant MFG-E8 ameliorated colitis by reducing inflammation and improving disease parameters (Otani et al., 2012).

MFG-E8 contains a PS binding domain, as well as an arginine–glycine–aspartic acid (RGD) motif, which enables its binding to integrins. Opsonization of the apoptotic cells and binding to integrins on the surface of phagocytic cells, mediates the engulfment of the dead cell. Based on this observation, an RGD–annexin A5 was designed, and it was shown that introduction of RGD transformed the annexin A5, a molecule that binds to PS of apoptotic cells, from an inhibitor into a stimulator of efferocytosis (Schutters et al., 2013).

While MFG-E8 acts as a bridging molecule for integrins, Gas6, and protein S are bridging molecules for Mertk. Thus in cases, where Mertk plays a driving role in efferocytosis, such as cardiac repair after myocardial infarction (Wan et al., 2013), provision of Gas6 or protein S could similarly accelerate phagocytosis of apoptotic cells and tissue repair. Glucocorticoids enhance phagocytosis by making efferocytosis Mertk dependent (McColl et al., 2009), thus combining glucocorticoids and Gas6 or protein S might have a synergistic effect.

Other bridging molecules, such as collectins, were also reported to promote efferocytosis. Macrolide antibiotics, which have wideranging anti-inflammatory effects, were found to enhance efferocytosis by enhancing the expression of collectins (Hodge et al., 2008). The therapeutic potential of these drugs has already been recognized, as they are successfully used in the treatment of COPD, cystic fibrosis, or asthma (Yamaryo et al., 2003).

1.5.2.2. Targeting lipid-sensing nuclear receptors with the aim of increasing the expression of phagocytic receptors or their bridging molecules

Since nuclear receptor signaling is strongly associated with enhanced efferocytosis and suppression of inflammation, glucocorticoids, PPAR γ , PPAR δ , and LXR agonists or retinoids are logical therapeutic targets in diseases in which efferocytosis is impaired. Glucocorticoids, the most widely used anti-inflammatory drugs, were shown to enhance phagocytosis of apoptotic cells by increasing the expression of the phospholipid binding protein annexin A1 and its receptor ALXR (Elliot et al., 2010), as well as that of Mertk (Zahuczky et al., 2011).

LXR agonists were shown to be effective in the treatment of mouse models of atherosclerosis and inflammation by lowering the serum cholesterol (Joseph et al., 2002), and inhibiting the expression of inflammatory mediators (Joseph et al., 2003). In addition, ligation of LXR was shown to prevent the development of SLE like autoimmunity in lpr mice (A-Gonzalez et al., 2009) and decrease the disease severity in Alzheimer disease (Sodhi et al., 2013).

In addition to LXR agonists, PPAR γ , PPAR δ agonists were also shown to be effective in disturbed efferocytosis related experimental animal models (Fernandez-Boyanapalli et al., 2010; Feinstein et al., 2002).

1.5.2.3. Affecting the Rac-1/RhoA balance

Since previous studies have shown that Rac activation is required, while RhoA activation is inhibitory for effective clearance of apoptotic cells (Nakaya et al., 2006), compounds that alter the Rac-1/RhoA balance, by either increasing the level of active Rac-1 or decreasing the levels and/or activity of RhoA/Rho kinase, would be potential candidates for use in therapy. Among the anti-inflammatory drugs glucocorticoids were shown to alter the Rac-1/RhoA balance in macrophages (Giles et al., 2001). Another molecule that was shown to affect the Rac-1/RhoA balance is lipoxin A4, which enhances phagocytosis via a protein kinase A-dependent manner (Godson et al., 2000).

Statins are 3-hydroxy-3-methylglutaryl coenzyme A-reductase inhibitors with potent anti-inflammatory effects, largely due to their ability to inhibit the prenylation of Rho GTPases, including Rac-1 and RhoA. Since proper membrane localization of these proteins determines their function, statins inhibit the effectiveness of G protein signaling. Lovastatin was shown to enhance efferocytosis *in vitro* both in naïve murine lung and in alveolar macrophages taken from COPD patients (Morimoto et al., 2006).

During inflammation oxidant-mediated activation of RhoA and inhibition of efferocytosis might be reversed by antioxidant treatment. Thus, in an LPS-induced lung injury model, antioxidants enhanced efferocytosis and reduced inflammation by inhibiting RhoA activation (Moon et al., 2010).

1.5.2.4. Altering the membrane lipid composition of macrophages

Finally, altering the membrane lipid composition of macrophages e.g. with fish oil diet might also serve to facilitate efferocytosis under certain conditions (Li et al., 2009). ω -3 fatty acids provided by fish oil are known substrates for the biosynthesis of pro-resolving mediators, such as resolvins, protectins, and maresin which, similar to glucocorticoids or opsonization of apoptotic cells by iC3b (Schif-Zuck et al., 2011; Verbovetski et al., 2002), act as enhancers of efferocytosis.

2. AIMS OF THE STUDIES

- I. Previous studies have shown that in addition to the chemotactic signals, purinergic autocrine signaling is also required for macrophages to amplify and translate chemotactic signals into directional motility (Kronlage et al., 2010). However, the contribution of individual adenosine receptors to the chemotactic movement of macrophages has not been characterized yet. Our first aim was:
 - to build up an *in vitro* experimental set up, in which migration of macrophages toward apoptotic cells can be studied
 - to study the involvement of A3 receptors in the chemotactic migration of macrophages directed by apoptotic thymocyte-derived “find-me” signals in *in vitro* and *in vivo* experimental setups

- II. Previous work in our laboratory has shown that TG2 acts as an integrin $\beta 3$ coreceptor during efferocytosis (Tóth et al., 2009). TG2 is a retinoid regulated gene (Moore et al., 1984), while LXR stimulation can upregulate retinoid synthesis in macrophages (Garabuczi et al., 2013). In the second part of my work my goal was to investigate:
 - how triggering of LXR receptors in macrophages lead to enhanced retinoid production
 - whether LXR-induced retinoid production is involved in the LXR induced enhanced efferocytosis
 - how retinoids enhance efferocytosis
 - what is the exact retinoid, which's production might be accompanied with efferocytosis after apoptosis induction in the thymus

3. MATERIALS AND METHODS

3.1. Reagents

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

3.2. Experimental animals

The experiments were carried out with four-week-old or 2-4-month-old C57BL/6 and FVB (wild type/WT) mice. To study the effect of loss of A2AR, A3R, TG2 or CD14 on efferocytosis, macrophages were isolated from A2AR^{-/-} (Ledent et al., 1997), A3R^{-/-} (Lee et al., 2003), TG2^{-/-} (Szondy et al., 2003) and CD14^{-/-} (Devitt et al., 2004) mice, respectively. In some experiments RARE-hsp68-lacZ reporter transgenic mice (Rossant et al., 1991) were used to indicate *in vivo* retinoic acid production. Mice were bred and maintained under specific pathogen-free conditions in the Life Science Building Animal Facility of the University of Debrecen. Before isolating thymocytes or macrophages, mice were euthanized with ether anesthesia. All animal experiments were approved by the Animal Care and Use Committee of University of Debrecen (DEMÁB).

3.3. Peritoneal macrophage isolation and culturing before chemotactic migration assay

Macrophages (under nonelicited conditions) were obtained by peritoneal lavage with sterile physiological saline from 2-4 month-old mice. 2×10^5 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 1 mM Na-pyruvate, 100 U/ml penicillin and 100 µg/ml streptomycin at 37 °C in 5% CO₂ on 20-mm glass bottom dishes (In Vitro Scientific, Sunnyvale, California, USA). After 4 h incubation, the non-adherent cells were washed away. The cells were then cultured for 1 day replacing the medium with a final medium containing apyrase and/or the indicated adenosine receptor agonist or antagonist right before the start of the *in vitro* migration experiment. Since all

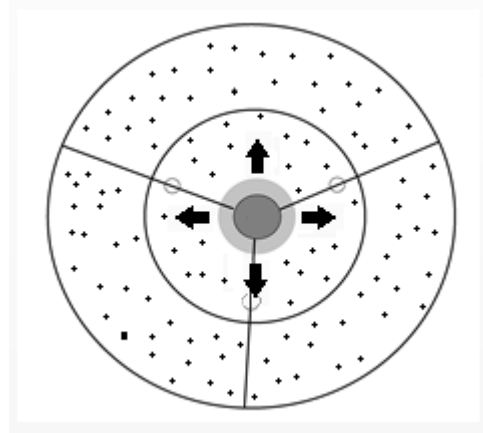


Figure 6. Schematic view of the dish configuration. Dots represent seeded cells, arrows show the direction of the chemoattractant gradient

the adenosine receptor agonists and antagonists were dissolved in DMSO, DMSO was present in all the cultures at a final concentration of 0.5 v/v%. To investigate migration of resident peritoneal macrophages toward apoptotic thymocytes *in vitro*, thymocytes were induced to die by administering 1 μ M dexamethasone for 18 h resulting in >70 % Annexin V positive cells. Chemotactic migration was initiated by inserting in the middle of the dish a sterilized Whatman GF/C paper filter ring surrounding 6×10^6 apoptotic thymocytes. Through the Whatman GF/C paper filter the apoptotic thymocyte-derived find-me signals smaller than 0.7 μ m, could be released into the culture medium, but the apoptotic cells remained together to generate chemotactic gradient. To maintain the chemotactic gradient, the paper filter ring was covered by a 12mm diameter VWR Microscope Cover Glass. Chemotaxis of macrophages was followed for 5h in the area located around the Whatman GF/C ring under the VWR Microscope Cover Glass. The optical field of the microscope allowed observation of the migration of 25-30 macrophages on each dish.

3.4. Bone marrow derived macrophage (BMDM) cell culture and treatment

Bone marrow progenitors were obtained from the femur of 2-4-month-old mice lavage with sterile physiological saline. Cells were allowed to differentiate for 6 days in DMEM medium supplemented with 10% FBS, 10% conditioned medium derived from L929 cells, 2 mM glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin at 37°C in 5% CO₂. Non-adherent cells were washed away every second day. BMDMs were treated with 1 μ M GW3965 (Glaxo Smith Kline, Budapest, Hungary), a synthetic LXR agonist, 1 μ M LG268 (kind gift of John Schwabe, University of Leicester), a synthetic RXR agonist, 1 μ M AM580 (Tocris Bioscience), a synthetic pan RAR agonist, 500 nM AGN193109, a pan RAR antagonist, 300 nM ATRA, a natural pan RAR agonist or 300 nM 9cRA, a natural RXR and RAR agonist for the indicated time periods. In some experiments 25 μ M DEAB (diethylaminobenzaldehyde) was used to block aldehyde dehydrogenase enzyme activities.

3.5. Thymocyte apoptosis induction *in vivo* and related measurements

Four-weeks-old C57BL/6 mice were injected i.p. with 22 μ g/bwg dexamethasone (Dex) dissolved in DMSO (or with DMSO-saline for the control experiments). At the indicated times, mice were sacrificed, thymuses were gently isolated. Thymus lobes were excised and cell suspension was prepared. After counting total cell numbers, thymocytes were stained with Alexa Fluor 647 conjugated Annexin V (Thermo Fisher Scientific, Waltham, MA, USA)

to determine the percentage of apoptotic cells. Cells were analyzed on Becton Dickinson FACSCalibur (BD Biosciences) by Flowing 2.5 software. Alternatively, after excision the thymuses were fixed and prepared for immunohistological staining or gene expressions were analyzed, respectively.

3.6. Thymocyte apoptosis induction *in vitro*

Thymocytes were prepared from 4 week-old wild-type or adenosine A3R null mice. Thymus lobes were minced in RPMI 1640 media supplemented with 10% FBS, 2 mM glutamine, and 100 IU penicillin/100 µg streptomycin/ml. Thymocytes were washed three times and diluted to a final concentration of 10^7 cells/ml in RPMI 1640 medium supplemented with penicillin/streptomycin in the presence of 1 µM Dex before incubation at 37°C in a humidified incubator under an atmosphere of 5% CO₂/95% air. At the end of the culture the percentage of apoptotic thymocytes was determined by propidium iodide/annexin V–FITC staining. Cells were analyzed on FACSCalibur (BD Biosciences) by Flowing 2.5 software. For the peritoneal *in vivo* phagocytosis experiments wild type thymocytes were stained with CellTracker Deep Red Dye (Thermo Fisher Scientific, Waltham, MA, USA) during cell death induction for 6h with 2 µM Dex resulting in >60% apoptotic cells (as assessed by propidium iodide/annexin V–FITC staining). At the end of culture, cells were washed three times in physiological saline, and the indicated number of apoptotic cells was taken up and injected into the peritoneum in 500 µl physiological saline.

3.7. Chemotaxis assays

A SANYO MCO18-AC (Wood Dale, IL, US) CO₂ incubator was used with a back-side instrument port. The chamber was modified to host four microscopes. Olympus (Tokyo, Japan) upright microscopes were modified for inverted usage, so the revolver turrets were installed to replace the original illumination. Four identical DigiMicro 2.0 megapixel USB 2.0 digital microscope cameras were modified to host Carl Zeiss (Jena, Germany) plan achromatic objective ($\times 10$: 0.25 NA), that were used to enable a broad field of view to be imaged. The original illumination of the digital microscopes was removed and diodes emitting light at 940 nm (LED: 5- mm diameter; 1.2 V and 50 mA, driven at 5 V using a serial 82 Ohm resistor) were used to illuminate cells for the sake of minimizing heat and phototoxicity. During the course of time-lapse image acquisition, images of the field of view were captured every minute for a time span of 5 hours using the eTox Long Term Scan system³⁶ and eTox LTS software. The resulting image sequences were processed in ImageJ software

(<http://rsbweb.nih.gov/ij/docs/install/windows.html>) using custom-developed macros. Processing including transformation to 8-bit grayscale, stack deflickering, fast fourier transformation and background subtraction resulted in a binary image sequence where the cells have been separated from the background. The binarized sequence was then used to define the entire path of each cell in the field of view. The cell paths were determined using temporal color coding (ImageJ>Image>Hyperstacks>Temporal Color Code) with fire LUT. The temporal color coded LUT was then skeletonized (Image J>Plugins>Skeletonize>Skeletonize 2D/3D) and the resulting cell tracks were arranged to a point of origin. The tracks were arranged so, that the vector of the main direction of movement (gradient source) is parallel to axis-y and the point of origin is marked by x,y=0. The entire path of each cell was measured using Image J Particle Analysis (Image J>Analyse>Analyse Particles) along with the y component of movement, which is the largest y value the cell reached along its way. To characterize chemotactic migration, we determined the chemotaxis index defined as the displacement along the y axis divided by the accumulated distance.

3.8. Phagocytosis of injected apoptotic thymocytes by peritoneal macrophages

3-month-old male wild-type or A3AR-deficient mice were injected intraperitoneally with various numbers of CellTracker Deep Red Dye-labeled apoptotic thymocytes suspended in 500 µl sterile physiological saline. 30 min later macrophages were collected by peritoneal lavage with sterile physiological saline. Cells were fixed in 200 µl of 2% paraformaldehyde and stored at 4°C in the dark for 10 min. Cells were collected by centrifugation at 100 RCF for 10 min, transferred into a 50 µl blocking buffer (50% BSA in PBS) for 10 min, then stained with anti-F4/80 antibody conjugated to FITC (Thermo Fisher Scientific, Waltham, MA, USA). Stained cells were analyzed on a FACSCalibur (BD Biosciences) by Flowing 2.5 software.

3.9. Immunohistochemistry

Thymuses were fixed in Sainte-Marie fixative for 24 h. After fixation, samples were dehydrated in graded series of alcohol, xylol and embedded into paraffin. Serial histological sections (4-6 µm) were made from the samples. They were exposed to 6% H₂O₂ for 7 min to inhibit endogenous peroxidase activity. After incubation with 1% bovine serum albumin for 30 min at room temperature, primary antibody against F4/80 (Abcam, Cambridge, UK (ab6640) was applied in 1:200 overnight at 4 °C. Anti-rat antibody was used in 1:400 (30 min at room temperature) as secondary antibody. After washing, samples were incubated by Vectastain Elite ABC (avidin-biotin complex) Kit in 1:100 (Vector Laboratories Ltd., Peterborough, UK) to

label the secondary antibody. Control samples were stained using the same procedure but the primary antibody was replaced with PBS (phosphate-buffered saline). The ABC was visualized with di-amino-benzidine method (Vector Laboratories Ltd., Peterborough, UK) for conventional light microscopy. No specific signal was recorded from control sections. Samples were counterstained with dimethylmethylene-blue in order to visualize the cortex-medulla border in the samples and mounted with DPX mounting media. 24-bit RGB bitmap images of immunostained thymus sections were analyzed using National Institute of Health 'ImageJ' open-source software bundle. Bitmaps were loaded separately for every timepoint, then six identical copies created about each images for further analysis. The medullary (M), corticomedullary (CM) and subcapsular (SC) areas were selected using 'Versatile Wand Tool' plugin. Area selection was based on the greyscale, HUE values and pixel interconnectivity. Brown staining indicating the presence of the macrophages was segmented from the selected areas. Image segmentation was carried out using greyscale and HUE values of non-interconnecting pixels in M, CM and SC areas. Segmented images representing the M, CM and SC areas and their stained pixels were transformed to 8-bit greyscale images. Binary images were created by thresholding the greyscale images. Area sizes were measured using 'Analyze Particles' plugin on each binary image. Percentages of immunopositive pixels were determined for M, CM and SC areas.

3.10. *In vitro* apoptotic cell phagocytosis

BMDMs were stained for 24 hrs with 5 μ M CellTracker™ Orange (CMTMR, Invitrogen). To generate apoptotic thymocytes, thymus was collected from 4-week-old C57BL/6 mice, thymocytes were isolated and cultured for 24 h (10^7 cells/ml) in RPMI 1640 medium supplemented with 2 mM glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin and 10 μ M carboxyfluorescein diacetate succinimidyl ester (CFDA-SE, Invitrogen) in the absence of serum. CFDA stained apoptotic thymocytes were added to the BMDMs in 10:1 (apoptotic cells:macrophage) ratio for 30 minutes. After coculture, apoptotic cells were washed away and macrophages were detached by trypsinization. Cells were analyzed on Becton Dickinson FACSCalibur.

3.11. Measuring the *in vivo* production of RAR-activating derivatives

To measure the *in vivo* production of RAR-activating derivatives RARE-hsp68-lacZ reporter transgenic mice were injected with 20 mg/kg GW3965 or vehicle. Twenty-four hours later, peritoneal cells were collected by lavage. Cells were allowed to adhere for 3 h; then the

nonadherent cells were washed away. Adherent cells were fixed with 0.25 mM glutaraldehyde for 5 min on ice. Fixed cells were incubated at 37°C for 24 h in X-gal staining solution (35 mM potassium ferrocyanide, 35 mM potassium ferricyanide, 2 mM MgCl₂, 0.02% Nonidet P-40, 1 mg/ml X-gal). Images were taken using an AMG EVOS inverted microscope at original magnification x20.

3.12. Analysis of mRNA expression

Total RNA was isolated from BMDMs, thymic samples and from peritoneal cells of RARE-hsp68-lacZ reporter transgenic mice by TRI reagent according to the manufacturer's guidelines. Total RNA was reverse transcribed into cDNA using High Capacity cDNA Reverse Transcription Kit (Life Technologies, Budapest, Hungary) according to the manufacturer's instruction. qRT-PCR was carried out in triplicate using pre-designed FAM-labeled MGB assays (Life Technologies, Budapest, Hungary) on a Roche LightCycler LC 480 real-time PCR instrument. To detect lacZ mRNA the following primers and FAM-TAMRA-labeled TaqMan probes (designed and ordered from Eurogentec, Seraing, Belgium) were used: forward, 5'-TGC-CGT-CTG-AAT-TTG-ACC-TGA-G-3'; reverse, 5'-CCG-CCA-CATATC-CTG-ATC-TTC-C-3'; probe, FAM-ACT-CCA-ACG-CAG-CAC-CAT-CAC-CGCTAMRA. Relative mRNA levels were calculated using comparative CT method and were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA.

3.13. Western blot analysis

BMDMs were homogenized in ice-cold lysis buffer containing 0.5% Triton X-100. Protein concentration of each samples were diluted to 2 mg/ml, then the samples were boiled with an equal volume of Laemmli buffer. Electrophoresis was performed in 10% SDS-polyacrylamide gel. Separated proteins were transferred to an Immobilon-P transfer membrane (Millipore, Budapest, Hungary) and were probed with rabbit polyclonal anti-TG2 (SantaCruz, Heidelberg, Germany), anti-mouse LXR α/β (ABGENT), GAPDH or mouse anti- β -actin (Sigma-Aldrich, Budapest) antibodies. Protein bands were visualized by Immobilon Western Chemiluminescent HRP substrate (Millipore, Budapest, Hungary).

3.14. Immunofluorescence staining and confocal microscopy

Bone marrow progenitors from WT mice were plated in 8-well chamber slides (5 × 10⁵/well) and allowed to mature for 6 days. Phagocytosis assay was carried out as described previously. After coculturing macrophages with apoptotic cells for 30 min, cells were washed and fixed in

4% paraformaldehyde. For integrin $\beta 3$ and CD14 staining, macrophages were blocked with 50% FBS for 30 min at 37°C, then washed with ice-cold HEPES buffer and stained with PE-conjugated anti- mouse $\beta 3$ integrin (BD Biosciences) or FITC-conjugated anti-CD14 (BD Biosciences) Ab for 15 min on ice. After washing cells were fixed in 4% paraformaldehyde. Images were taken with a Zeiss LSM 510 or Olympus FV1000 confocal laser scanning microscope.

3.15. LXR receptor silencing

Five-days-matured BMDMs were transfected with TransIT-siQUEST® Transfection Reagent (Mirus Bio LLC) according to user's instruction. Briefly, 1.5×10^6 BMDMs/well in 6-well plate were cultured in 1.25 ml DMEM medium supplemented with 10% FBS, 10% conditioned medium derived from L929 cells, 2 mM glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin. Transfection cocktail containing 250 μ l OptiMem, 6 μ l TransITsiQUEST reagent and 37.5 μ l (1 μ M) scrambled or LXR α and β specific siRNA was added drop-wise to the cells. Medium was replaced after 3 hrs of incubation to complete DMEM.

3.16. Retinoid measurement by HPLC-MS-MS

Four-weeks-old C57B6 mice were injected ip. with either 0.3 mg Dex dissolved in DMSO alone or with DEAB (0.24mg/g body weight) or vehicle. 24 hrs later thymi were removed in dark and snap-frozen in liquid nitrogen and stored on -70 °C. Concentrations of retinoic acids were determined in mouse thymuses by our HPLC-MS-MS method (Rühl, 2006). In summary, 100 mg of the thymic samples (if samples were under 100 mg, water was added up to the used standard weight: 100 mg) was diluted with a threefold volume of isopropanol, the tissues were minced by scissors, vortexed for 10 seconds, put in a ultra sonic bath for 5 minutes, shaken for 6 minutes and centrifuged at 13000 rpm in a Heraeus BIOFUGE Fresco at +4°C. After centrifugation, the supernatants were dried in an Eppendorf concentrator 5301 (Eppendorf, Germany) at 30°C. The dried extracts were resuspended with 60 μ l of methanol, vortexed, shaken, diluted with 40 μ l of 60 mM aqueous ammonium acetate solution and transferred into the autosampler and subsequently analysed using HPLC MS-MS equipment. In addition, we focussed on detecting novel non-identified dihydro-retinoic acid derivatives like the ones we described previously (Aydemir et al., 2013) and we switched our MS-MS to single ion recording (SIR) modus and focussed on 303 m/z signals in relative intensity. Relative retinoid concentrations were given as “area under the curve” (AUC) and normalized to thymic weight.

3.17. Statistical analyses

All the data are representative of at least three independent experiments carried out on three different days. Values are expressed as mean $\pm$ SD. P values were calculated by using two-tailed Student's t-test for two samples of unequal variance. Statistical significance is indicated by an asterisk ($P < 0.05$). Statistical calculations were run on GraphPad Prism 6 software.

4. RESULTS

4.1. Involvement of adenosine A3 receptors in the clearance of apoptotic thymocytes

4.1.1. Proper *in vitro* chemotactic migration of macrophages toward apoptotic cells is affected by adenosine receptor signaling

4.1.1.1. Find-me signals released by apoptotic thymocytes induce chemotaxis and increase the velocity of migration of macrophages in a purinergic signaling-dependent manner

Resident peritoneal macrophages isolated from wild-type mice migrated robustly toward the apoptotic cells (Fig. 7A) with an average velocity of 0.3 ± 0.19 $\mu\text{m}/\text{min}$. As seen in Fig. 7A, while macrophages in RPMI migrated randomly with a chemotactic index of 0.12 ± 0.07 , exposure to apoptotic cells resulted in a migration characterized with a chemotactic index of 0.55 ± 0.2 , and also in a 25% increase in the velocity. Exposure of macrophages to apyrase lead to a loss of chemotactic navigation in agreement with the previously published data on the chemotaxis of macrophages (Kronlage et al., 2010). However, unlike previously published in the case of complement C5-driven migration (Kronlage et al., 2010), we detected also a significant decrease in the velocity. These data underlie the importance of the previously reported purinergic signaling in amplifying and translating chemotactic signals into directional macrophage motility also in the context of apoptotic cell-derived find-me signals.

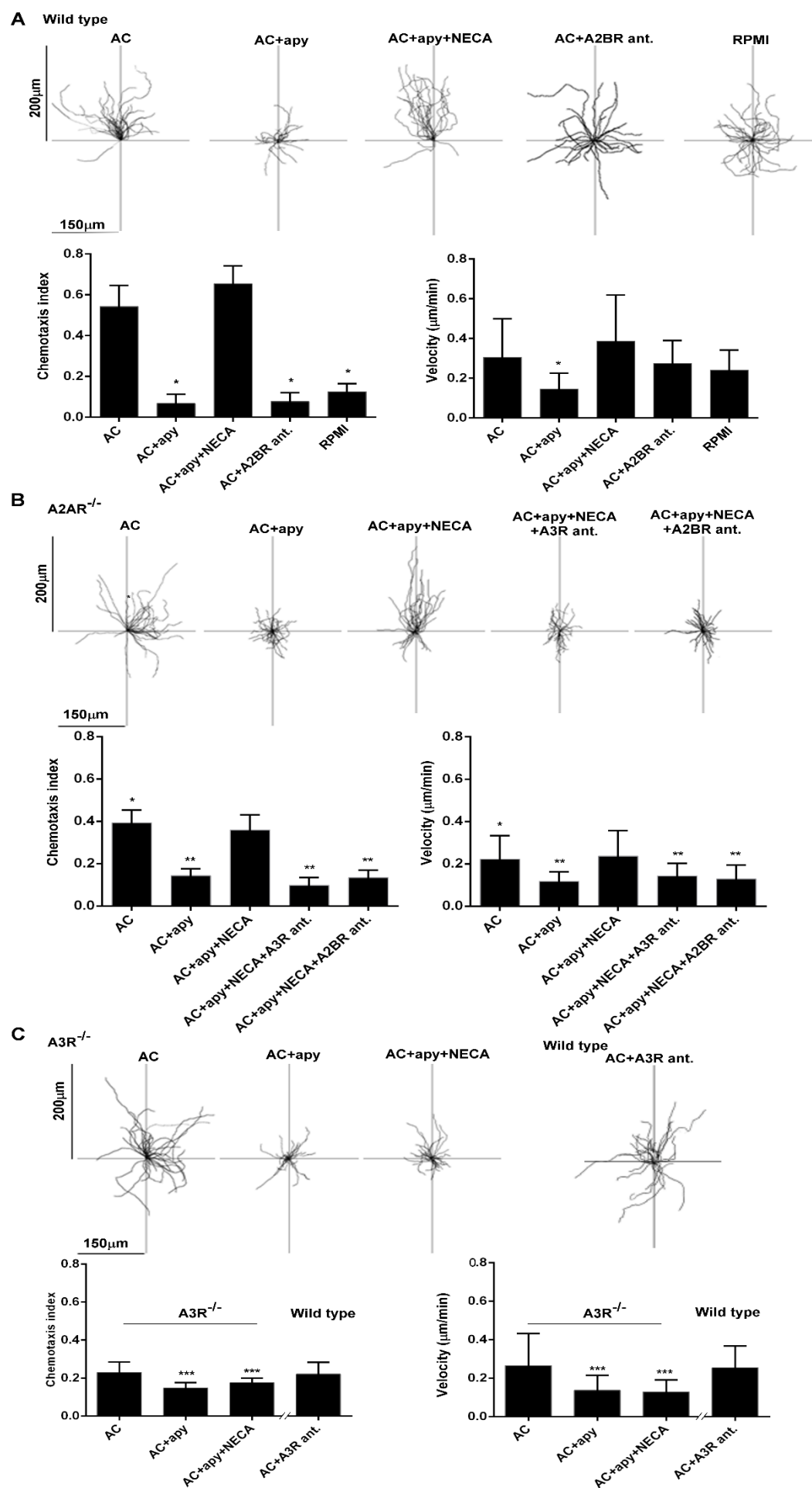


Figure 7. Autocrine adenosine receptor signaling is required for macrophage chemotaxis driven by apoptotic thymocyte-derived find-me signals. (A) Migration plots, mean chemotaxis indexes and mean velocities of wild type (C57BL/6) macrophages in RPMI alone, or exposed to apoptotic cells (AC) alone, with apyrase (40U/ml), with apyrase and NECA (10 μ M) or with the A2BR antagonist MRS1754 (1 μ M). (B) Migration plots, mean chemotaxis indexes and mean velocities of A2AR null macrophages exposed to apoptotic cells alone, with apyrase (40U/ml), with apyrase and NECA (10 μ M), with apyrase, NECA (10 μ M) and the A2BR antagonist MRS1754 (1 μ M) or with apyrase, NECA (10 μ M) and the A3R antagonist MRS3777 (1 μ M). (C) Migration plots, mean chemotaxis indexes and mean velocities of A3R null macrophages exposed to apoptotic cells alone, with apyrase (40U/ml) or with apyrase and NECA (10 μ M) and that of wild type macrophages exposed apoptotic cells with the A3R antagonist MRS3777 (1 μ M). Since all the adenosine receptor agonists and antagonists were dissolved in DMSO, DMSO was added to all the cultures at a final concentration of 0.5 v/v%. Migration plots were obtained by tracking 25 cells for 5 h in RPMI alone or after being exposed to apoptotic thymocytes alone or together with the indicated compounds. The start point of each track was normalized to $x=0$ and $y=0$, and positive y axis values represent movement in the direction of the apoptotic thymocyte-derived find-me signals. Mean chemotactic indexes and velocities represent mean $\pm$ S.D. results from three independent experiments ($n=75$ cells). *Significantly different from wild type macrophages exposed to apoptotic cells alone. **Significantly different from A2AR null macrophages exposed to apoptotic cells alone. ***Significantly different from A3R null macrophages exposed to apoptotic cells alone ($p<0.05$).

4.1.1.2. Loss of A3R signaling in macrophages results in a loss of chemotactic navigation toward apoptotic thymocytes

We decided to further characterize the phenomenon by investigating the contribution of individual adenosine receptors to the chemotactic migration of macrophages. We did so by video imaging the migration of various adenosine receptor null or adenosine receptor antagonist-treated macrophages toward apoptotic thymocytes. Thus, we tested the migration of peritoneal macrophages isolated from both A2AR (Ledent et al., 1997) and A3R (Lee et al., 2003) null mice. Since the adenosine receptor null mice were generated on different genetic background, in our preliminary experiments we compared the characteristics of the migration of wild type FVB and C57Bl/6 macrophages, but found no difference (Fig. 7 and 8). While the loss of individual adenosine receptor signaling did not affect the migration of macrophages in RPMI alone (Fig. 8A), as shown in Fig. 7B, as compared to wild type mice, loss of A2ARs resulted in about 30% loss in the velocity of migration toward the apoptotic cells. In addition, A2AR null cells lost partially, while A3R null macrophages (Fig. 7C) lost completely their ability of chemotactic navigation. The inability of A3R null macrophages to migrate toward the apoptotic cells was not related to a change in their differentiation, because administration of MRS3777, an A3R antagonist had similar effects (Fig. 7C). To test the potential involvement of A2BRs, we also followed the migration of wild type macrophages in the presence of the

A2BR antagonist MRS 1754. As seen in Fig. 7A, inhibition of A2BRs also lead to a complete loss of chemotactic orientation, while the velocity of migration was not affected. These data indicate that when macrophages migrate toward apoptotic thymocytes, sufficient adenosine is formed to trigger A2BRs, which have the lowest affinity for adenosine, and also that the A2BRs and the A3Rs play the determining role in promoting chemotactic orientation. In addition, although in the context of complement C5-driven chemotaxis, it was suggested that ATP and ADP receptors alone can promote chemotactic migration in the full absence of adenosine receptor signaling (Kronlage et al., 2010), our data demonstrate that when only one single adenosine receptor signaling (either A2BR or A3R) is affected, the chemotactic orientation becomes impaired.

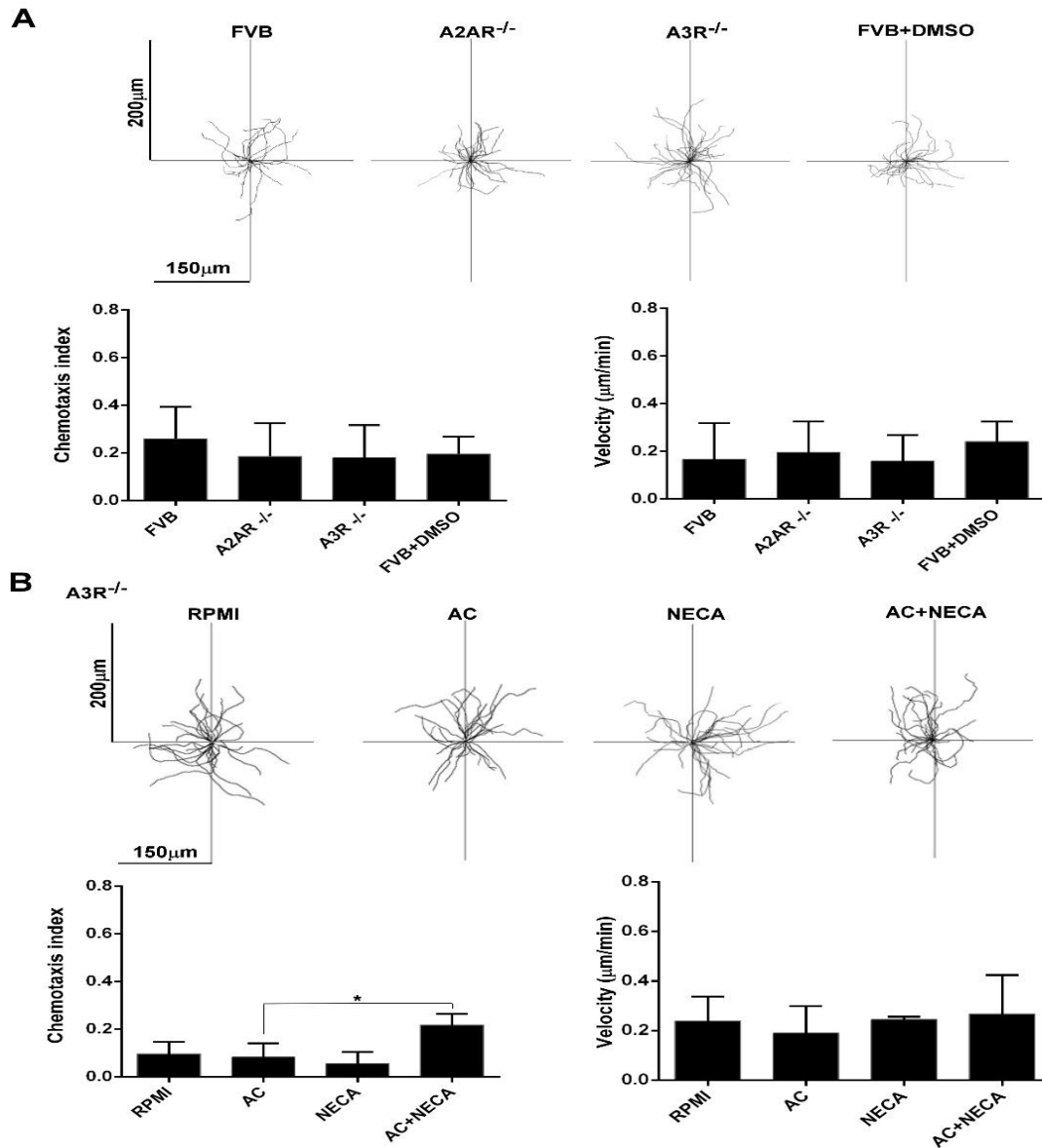


Figure 8. Loss of adenosine receptors or exposure to DMSO or to NECA does not significantly affect the spontaneous migration of macrophages, but exposure to NECA enhances the chemotactic index of A3R null macrophages migrating toward apoptotic cells. (A) Migration plots, mean chemotaxis indexes and mean velocities of wild type (FVB), A2AR^{-/-} and A3R^{-/-} macrophages in RPMI alone, or of wild type (FVB) macrophages exposed to 0.5 v/v% DMSO. (B) Migration plots, mean chemotaxis indexes and mean velocities of A3R^{-/-} macrophages alone, exposed to apoptotic cells, exposed to NECA (10 mM) or to apoptotic cells and NECA (10 mM) all in the presence of 0.5 v/v% DMSO. Migration plots were obtained by tracking 25 cells for 5 h on each dish. The start point of each track was normalized to x=0 and y=0, and positive y axis values represent movement in the direction of the apoptotic thymocyte-derived find-me signals. Mean chemotactic indexes and velocities represent mean±S.D. results from three independent experiments (n=75 cells). *Significantly different (p<0.05).

4.1.1.3. Administration of exogenous adenosine does not affect migration of wild type macrophages toward the apoptotic cells, but promotes that of A3R null macrophages

Next we decided to investigate whether higher adenosine concentrations could overcome the chemotactic migration loss related to A3R deficiency. For this purpose both wild type and A3R null macrophages were exposed to 5'-N-ethylcarboxamidoadenosine (NECA), a non-metabolisable analogue of adenosine. NECA was administered at 10 mM concentration to activate all the adenosine receptors. While addition of NECA did not affect the migration of wild type macrophages in RPMI alone or toward the apoptotic thymocytes (data not shown), NECA slightly increased the chemotactic index of A3R null macrophages migrating toward the apoptotic cells (Fig. 8B). These data indicate that in the presence of ATP and ADP signaling higher adenosine concentrations can partially overcome the migration defect related to loss of A3R signaling.

4.1.1.4. Adenosine receptor signaling alone is also capable of maintaining chemotactic migration of macrophages toward the apoptotic thymocytes

To test whether triggering of adenosine receptors alone (in the absence of ATP and ADP signaling) is indeed sufficient to maintain chemotactic migration as it was suggested (Kronlage et al., 2010), wild type macrophages were exposed to apyrase, to initiate removal of ATP and ADP, and to prevent the formation of adenosine (Kronlage et al., 2010), together with NECA, the non metabolisable analogue of adenosine. NECA was administered at 10 μ M concentration to activate all the adenosine receptors. As shown in Figure 7A, in the presence of apyrase NECA alone was capable of maintaining the proper migration of wild type macrophages toward the apoptotic cells confirming that intact adenosine signaling alone is sufficient to amplify the chemotactic signaling into chemotactic migration. Since we added NECA mixed into the whole medium, our data also demonstrate that for this effect no concentration gradient of adenosine is required. Since apyrase degrades ATP, but in the presence of NECA we could observe chemotactic navigation, our data confirm that ATP is not an obligatory 'find-me' signal for the orientation of macrophages toward apoptotic thymocytes as it was suggested (Elliot et al., 2009).

4.1.1.5 A2AR signaling does not significantly contribute to the chemotactic orientation of macrophages migrating toward apoptotic thymocytes

Next, to investigate the involvement of individual adenosine receptors in the chemotactic movement of macrophages toward apoptotic cells, A2AR null cells were exposed to apyrase

(Fig. 7B). Similar to wild type cells, removal of purine nucleotides and nucleosides resulted in lower migration velocity ($0.1 \pm 0.04 \mu\text{m}/\text{min}$), which was not significantly different from the velocity observed in the case of wild type cells, and in loss of chemotactic orientation of A2AR null macrophages as well. However, when adenosine signaling was reconstituted by administering NECA, both the chemotactic index and the velocity of migration did increase, though the later could not reach the degree of migration velocity observed of wild type cells observed under similar conditions.

4.1.1.6 Simultaneous A2BR and A3R signaling are required to mediate the adenosine-promoted chemotactic navigation of macrophages toward the apoptotic thymocytes

The results received with A2AR null macrophages indicated that in the absence of A2ARs, A3Rs and A2BRs can still maintain the adenosine-promoted chemotactic migration toward the apoptotic cells. To test, which of these receptors is responsible for the effect, we exposed A2AR null macrophages either to an A2BR (MRS1754) or to an A3R (MRS3777) antagonist, and found that in the presence of these antagonists NECA could not restore the migration. These data demonstrate that both A2BRs and A3Rs contribute to the NECA-promoted migration of macrophages in the absence of A2AR signaling. Next we tested the migration of A3R null macrophages toward the apoptotic cells in the presence of apyrase. As shown in Fig. 7C, apyrase treatment again resulted in a similar decrease in both the velocity and the orientation of migration that we observed in the case of both wild type and A2AR null cells. However, unlike in the case of A2AR null cells, administration NECA to A3R null macrophages could not restore the apyrase-blocked migration demonstrating that A2R signaling alone cannot maintain the velocity and the navigation of macrophage chemotaxis toward apoptotic cells. These data all together indicate that in the absence of adenine nucleotides adenosine signaling alone, but only a simultaneous adenosine A2R and A3R signaling is able to promote chemotactic migration of macrophages toward the apoptotic cells. In addition, surprisingly, among the A2Rs the A2BRs seem to be dominant.

4.1.2. Loss of A3Rs delays the *in vivo* clearance of injected apoptotic thymocytes in the peritoneum.

Since we found that A3R null macrophages lost their capability of chemotactic orientation toward apoptotic thymocytes *in vitro*, we decided to test whether loss of A3Rs also affect the *in vivo* chemotactic migration and the consequent phagocytic clearance of injected apoptotic

thymocytes in the peritoneum. For this purpose increasing numbers of CellTracker Deep Red Dye-labeled apoptotic thymocytes were injected into the peritoneal cavity of wild type or A3R null mice. Previous studies from our laboratory have shown that loss of A3Rs does not affect the engulfing capacity of macrophages *in vitro* exposed to various apoptotic cell densities (Duró et al., 2014). In the peritoneum most of the resident macrophages have capability for clearing apoptotic cells, but we hypothesized that at a limiting apoptotic cell number, when macrophages have to find their prey on a longer run, navigation deficiency would affect clearance. Indeed, tested with 2, 8 and 30 million injected apoptotic cells within a 30 min time frame, as compared to the wild type macrophages a lower percentage of A3R null macrophages were able to engulf apoptotic cells indicating that less A3R null macrophages found at least one apoptotic cell (Fig. 9A). The difference was more pronounced at the lowest injected apoptotic cell number (2×10^6), where two times more wild types macrophages were engulfing than A3R null cells. When the injected apoptotic cells number was increased to 30×10^6 , the percentage of wild type engulfing macrophages also increased (from $21 \pm 4\%$ to $93.5 \pm 4.5\%$), and the difference in the percentage between the two types of engulfing macrophages decreased to 10%. But even in the latter case, those A3R null macrophages, which engulfed, took up a smaller number of apoptotic cells as an average reflected in their lower mean fluorescence intensities (Fig. 9B).

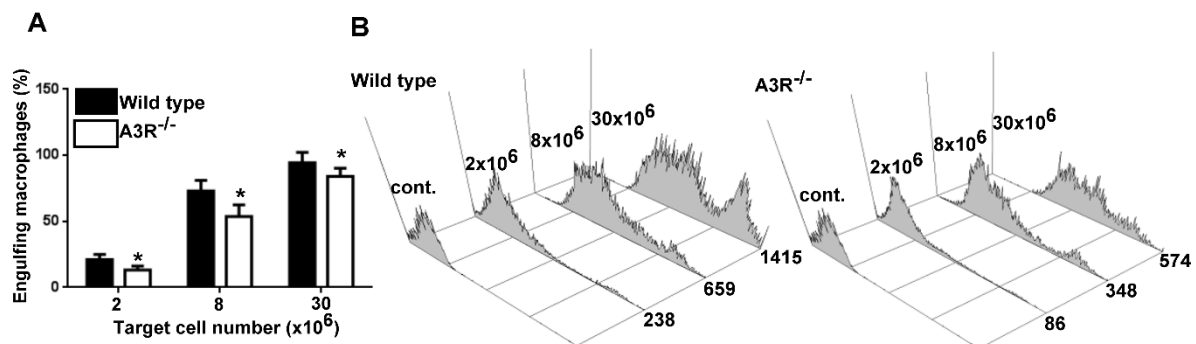


Figure 9. Delayed *in vivo* clearance of intraperitoneally injected apoptotic thymocytes by A3R null macrophages. 3-month-old male wild-type or A3AR-deficient mice were injected intraperitoneally with the indicated numbers of CellTracker Deep Red Dye-labeled apoptotic thymocytes. 30 min later macrophages were collected, stained with FITC-labelled anti-F4/80 antibody and analyzed. (A) Percentage of wild type and A3R null macrophages engulfing apoptotic cells. Data represent mean $\pm$ S.D of three independent experiments. (B) Mean fluorescence intensities of F4/80+ macrophages, which are related to the amount of labelled apoptotic cells engulfed, are shown as a function of injected apoptotic cells. Data represent one of three independent experiments. *Significantly different from the wild type macrophages ($p < 0.05$).

4.1.3. Surprisingly, loss of A3Rs does not affect the *in vivo* clearance of apoptotic thymocytes in dexamethasone-treated mouse thymus

Next we decided to test whether the loss of A3Rs also affects the *in vivo* chemotactic migration and the consequent phagocytic clearance in the mouse thymus exposed to dexamethasone. Previous studies from our laboratory have shown that loss of A3Rs does not affect the engulfing capacity of macrophages tested at various apoptotic cell densities and even at saturating apoptotic cell concentrations that occurs in the thymus, when it is exposed to high concentration of dexamethasone (Duró et al., 2014). To make sure then that the potential difference in the amount of apoptotic cells following *in vivo* apoptosis induction in the thymus *in vivo* is indeed related to an altered chemotactic migration and not to an altered apoptosis rate, first we tested, whether loss of A3Rs affects the dexamethasone sensitivity of mouse thymocytes. However, as seen in Fig. 10A, tested by Annexin V/propidium iodide staining, we found no difference in the dexamethasone sensitivity of wild type and A3R null thymocytes. Thus we followed the time dependent loss in the thymic cell number (Fig. 10B) and the accumulation of apoptotic Annexin V+ thymocytes (Fig. 10C) following dexamethasone injection in the thymus of both wild type and A3R null mice. To our surprise, however, despite of the fact that A3R null macrophages do not orient properly, no difference in the *in vivo* clearance was found. Thus we decided to detect the *in vivo* migration of macrophages following dexamethasone injection on histological sections of the mouse thymuses.

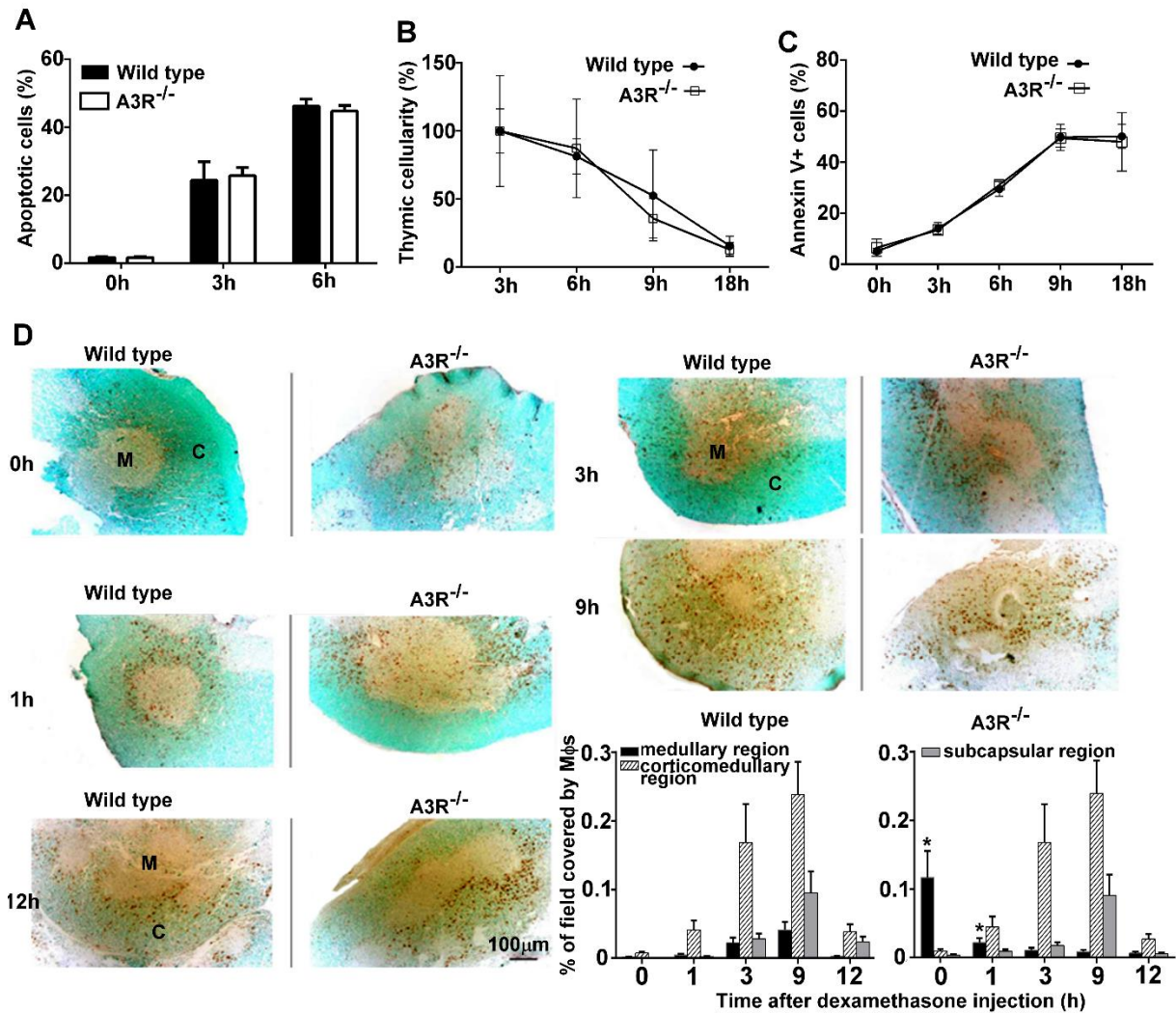


Figure 10. Loss of A3Rs does not affect the in vivo clearance of apoptotic thymocytes in the dexamethasone-treated thymus. (A) No difference in the vitro apoptosis rate of wild type and A3R null apoptotic thymocytes exposed to 1 μ M dexamethasone acetate. Percentage of apoptotic cells were determined by propidium iodide/annexin V-FITC staining at the indicated time points. (B) No difference in the time-dependent loss of thymic cell number in wild type and A3R null mice exposed to 22 μ g/bwg dexamethasone acetate. Number of thymocytes was determined at the indicated time points. (C) No difference in the accumulation of annexin V positive apoptotic thymocytes in wild type and A3R null mice exposed to 22 μ g/bwg dexamethasone acetate. Percentage of annexin V positive thymocytes was determined at the indicated time points following intraperitoneal injection of dexamethasone acetate. Data represent mean $\pm$ S.D. of three independent experiments. (D) Histological sections of thymuses from wild type and A3R null mice removed at the indicated time points following dexamethasone acetate (22 μ g/bwg) injection. Dark immunoprecipitation indicates the distribution pattern of macrophages. Sections were counterstained with dimethylmethylene-blue. The light blue territory indicates the medulla (M), while the dark blue indicates the cortex (C) of the thymus. For the quantifications cortex was subdivided into a corticomedullary and a subcapsular region by a ratio of two to one. All the figures show representative territories of the samples. Scale 100 μ m. Data represent mean $\pm$ S.D. of three independent experiments. *Significantly different from the wild type macrophages ($p < 0.05$).

As seen in Fig. 10D, while in non-treated wild type thymuses most of the macrophages were localized in the cortical zone, in A3R null thymuses many macrophages were detected in the medullary zone as well. Already at 1 h following dexamethasone treatment we started to see immigrating macrophages entering the cortex from the corticomedullary region, but, detected at 3 h, the migration of A3R null macrophages toward the subcapsular region was slightly delayed as compared to the wild type macrophages. However, from 6 h following dexamethasone treatment no difference between the two strains could be detected, and by 12 h the immigration of macrophages into thymus in both strains seemed to have completed, since all the macrophages could be detected in the remained cortical zone of the thymus, while the medullary zone became clear.

4.2. Macrophages engulfing apoptotic cells produce nonclassical retinoids to enhance their phagocytic capacity

4.2.1. Upregulation of TG2 alone in wild-type macrophages is not sufficient to promote phagocytosis of apoptotic cells

Previous studies have shown that both ATRA (Moore et al., 1984) and interferon γ (Bayardo et al., 2012) are capable of inducing TG2 expression in macrophages. In our experiments we decided to test whether induction of TG2 expression is sufficient to enhance phagocytosis of apoptotic cells by macrophages. Though both compounds increased the expression of TG2 in macrophages (Fig. 11A), only ATRA was capable of significantly inducing the engulfment of apoptotic cells (Fig. 11B and C). However, ATRA could significantly induce phagocytosis of TG2 null macrophages as well indicating that besides TG2, ATRA must also affect the expression of other phagocytosis-related genes. (Fig. 11D).

Indeed, when the expression of various other phagocytic receptors were tested by quantitative PCR analysis after 24 h of retinoid treatment, we found that, in addition to TG2, the expression of six other phagocytosis-related molecules, namely, Mertk, Stabilin-2, Tim4, ABCA1, CD14, and C1q, were also significantly enhanced by retinoids (Fig. 11E). From the additional phagocytosis-related molecules tested, retinoids did not have significant effect on the expression of integrin β 1, MFG-E8, thrombospondin, CD91, or CD36, whereas the expression levels of scavenger receptor A, integrin α_v , β_3 , and β_5 were decreased (Fig. 11E and 11F).

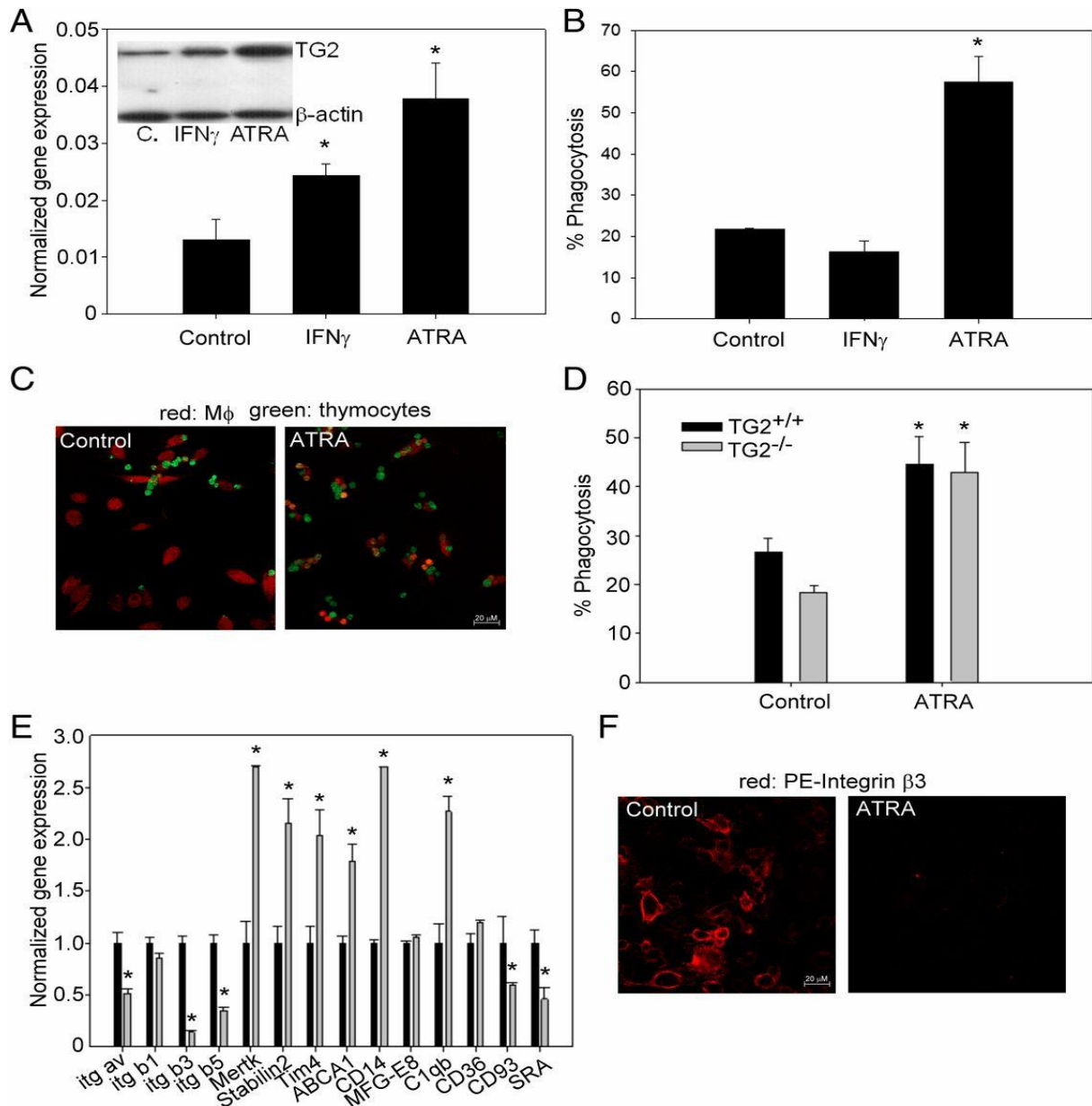


Figure 11. ATRA increases apoptotic cell phagocytosis in BMDMs. (A) BMDMs were treated with 20 μ g/ml IFN- γ or 300 nM ATRA for 24 h followed by RNA isolation and reverse transcription. TG2 expression was determined by qRT-PCR, normalized to GAPDH, and expressed as mean value $6 \pm SD$. Inset shows TG2 protein measured by Western blot. (B) BMDMs were treated with 20 μ g/ml IFN-g or 300 nM ATRA for 24 h in the presence of 5 μ M CMTMR. The next day, cells were coincubated with CFDA-stained apoptotic thymocytes for 30 min followed by washing and trypsinization. Phagocytosis was measured by flow cytometry or (C) demonstrated by confocal microscopy. (D) TG2 $^{+/+}$ or TG2 $^{-/-}$ BMDMs were treated with 300 nM ATRA as described earlier, and phagocytosis was measured by flow cytometry. (E) BMDMs were treated with 300 nM ATRA for 24 h followed by RNA isolation and reverse transcription. Gene expression level of phagocytosis-associated genes was determined by qRT-PCR and expressed as fold inductions $6 \pm SD$ relative to nontreated cells after normalization of samples with GAPDH expression. Results are representative of at least three independent experiments (* $p < 0.05$, significantly different from respective control). (F) Control and 300 nM ATRA-treated BMDMs were stained with PE-labeled anti-integrin β 3 Abs. Cellsurface integrin β 3 level was determined by confocal microscopy. Representative images are shown.

Besides ATRA, a natural pan RAR agonist, which, however, can be converted to 9cRA in cells (Heyman et al., 1992), all the retinoids tested (9cRA, a pan RAR and RXR agonist; Am580, a synthetic pan RAR agonist; and LG268, a synthetic RXR agonist) could induce both the expression of TG2 (Fig. 12A) and the percentage phagocytosis (Fig. 12B). Among these retinoids, ATRA and 9cRA were found to be the most effective, indicating that, although both RXR and RAR receptors can contribute to TG2 induction and the enhancement of phagocytosis, the effect is more pronounced when both receptors are stimulated.

The retinoids affected the expression of the other ATRA sensitive phagocytic receptors as well (Fig. 12C). Although, however, all the ATRA-sensitive phagocytic receptors could be induced also by 9cRA or LG268, with LG268 being less effective, expression of Mertk and ABCA1 were not Am580-sensitive, indicating that, unlike the expression of others, theirs was not regulated via RAR/RXRs. These data implied that retinoids might facilitate phagocytosis via triggering both RAR/RXR and other RXR heterodimers in macrophages.

Similar to the loss of TG2, the loss of CD14 alone had no significant effect on the ATRA responsiveness of phagocytosis tested in CD14 null macrophages (Fig. 12D). This indicates that very likely upregulation of all the ATRA-sensitive phagocytic receptors together results in enhanced engulfment also in the absence of CD14.

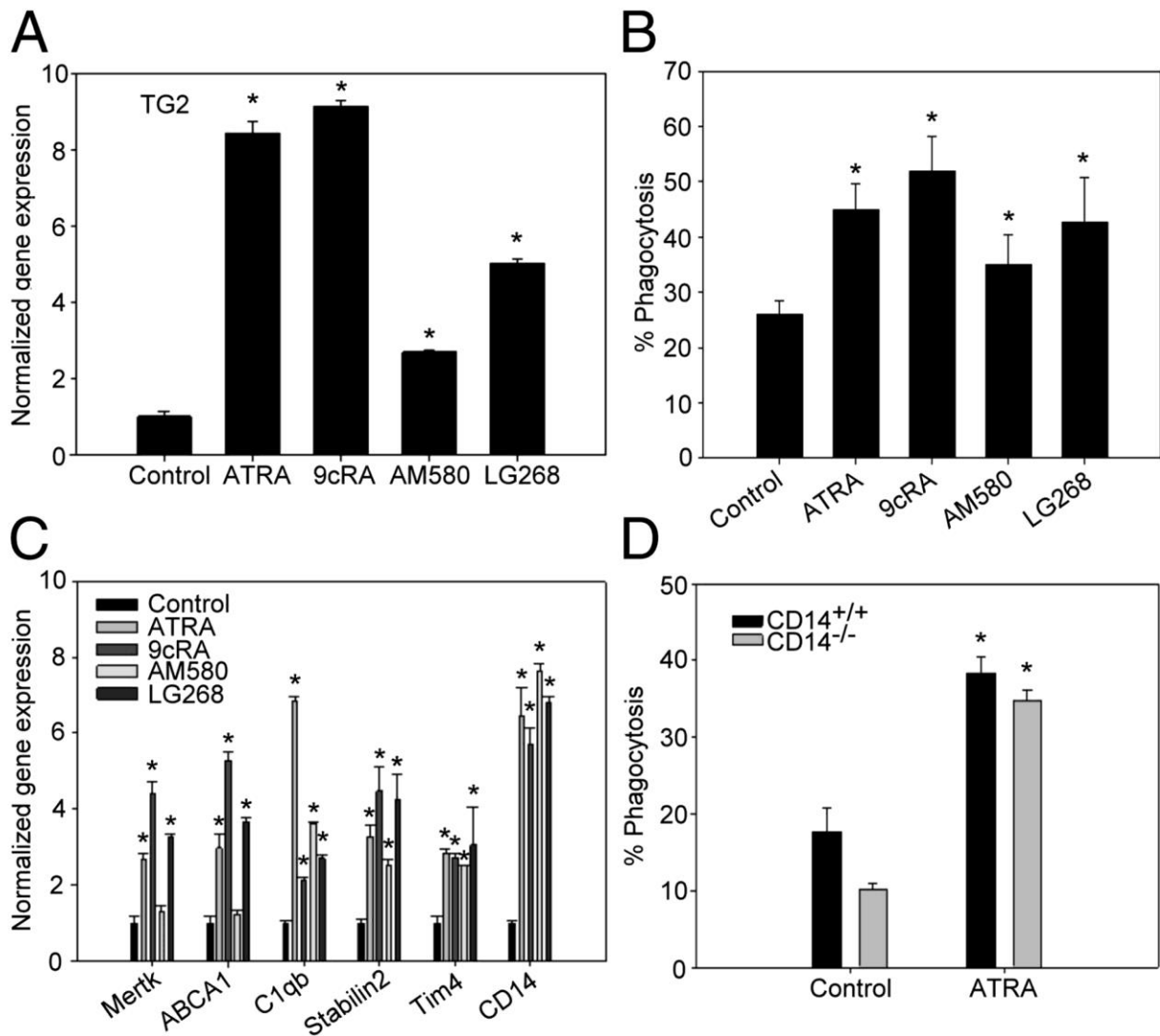


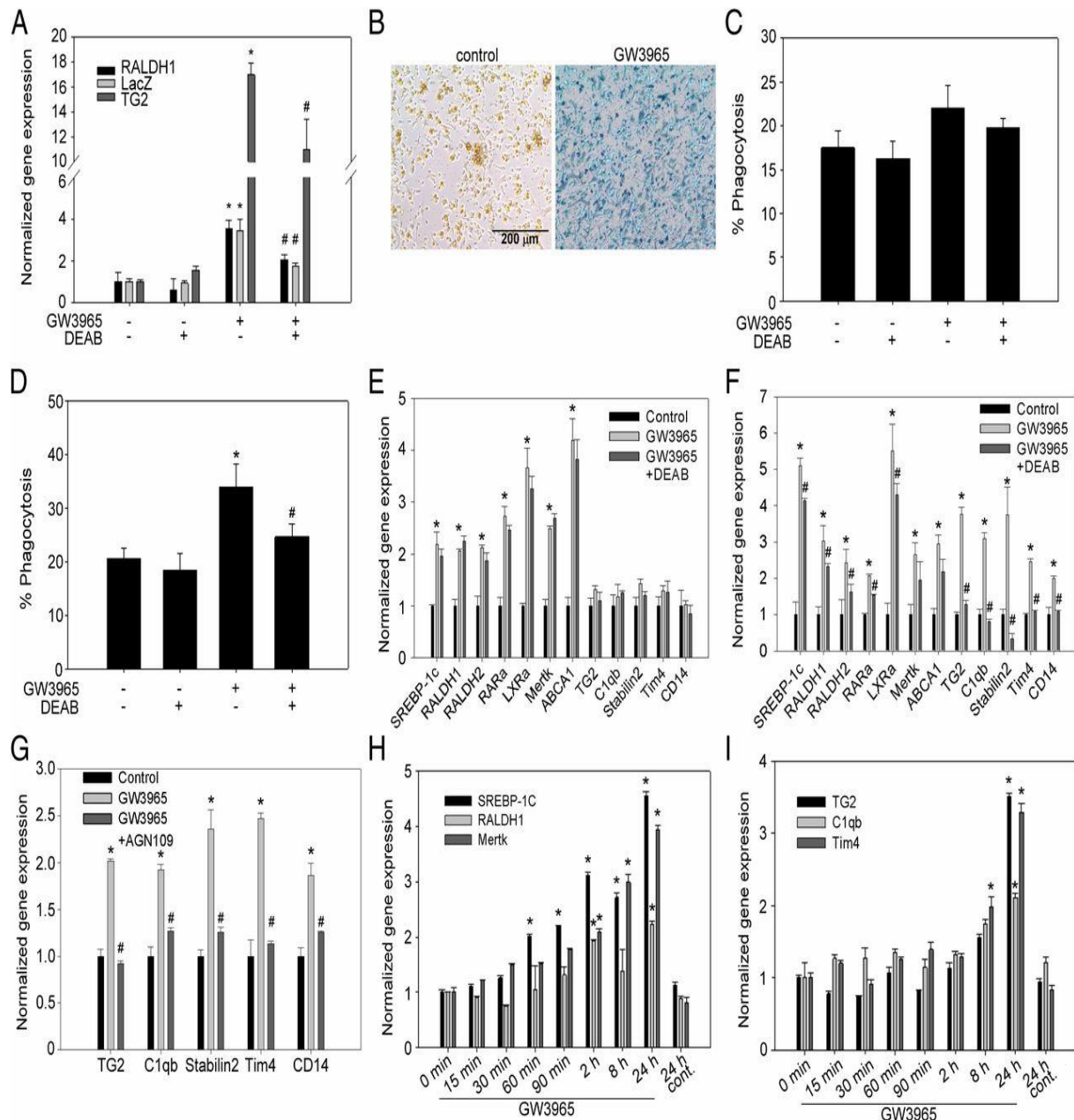
Figure 12. Natural and synthetic RAR and RXR agonists induce the expression of phagocytosis-related genes and increase apoptotic cell phagocytosis. (A) BMDMs were treated for 24 h with 300 nM ATRA, 300 nM 9cRA, 1 μ M AM580 (an RAR agonist), 1 μ M LG268 (an RXR agonist), or vehicle. TG2 expression was determined by qRT-PCR as described in Fig.6. (B) BMDMs were treated the same way as described earlier and 24 h later, phagocytosis was measured as described in Fig.6. (C) BMDMs were treated for 24 h with 300 nM ATRA, 300 nM 9cRA, 1 μ M AM580, 1 μ M LG268, or vehicle. Expression of phagocytosis-related genes was measured by qRT-PCR. (D) Wild type or CD14^{-/-} BMDMs were treated with 300 nM ATRA or vehicle for 24 h, and phagocytosis was measured by flow cytometry. Results are representative of at least three independent experiments (* p < 0.05, significantly different from respective control).

4.2.2. Ligation of LXRs induces retinoid production in peritoneal macrophages

Previous studies in our laboratory have shown that the *in vivo* apoptosis induction of thymocytes is coupled to enhanced retinoid production in the thymus, and the cells that expressed retinaldehyde dehydrogenases (RALDHs) responsible for retinoic acid production were the engulfing macrophages (Garabuczi et al., 2013). Apoptotic cell uptake *in vitro* also

triggered the expression of RALDHs in macrophages, indicating that the uptake of apoptotic cells is coupled to retinoid synthesis. Because the three lipid-sensing nuclear receptors (LXR, PPAR γ , and PPAR δ) have been implicated in the macrophage response to engulfed apoptotic cells (A-Gonzalez et al., 2009; Roszer et al., 2011; Mukundan et al., 2009), we have also tested whether triggering of these receptors affects the expression of RALDH1. As our group have reported previously, agonists of all these three receptors were found to promote the mRNA expression of RALDH1 in macrophages (data published in Garabuczi et al., 2013).

Because the LXR agonist, GW3965, was the most effective in inducing RALDHs (Garabuczi et al., 2013), we decided to check *in vivo*, using the RARE lacZ mice (Rossant et al., 1991), whether triggering LXR indeed leads to enhanced retinoid production. For this purpose, mice were injected i.p. with 20 mg/kg GW3965, and both the RALDH1 and the β -galactosidase mRNA expression were determined in the freshly isolated peritoneal macrophages 24 h later. In these mice, lacZ expression demonstrates the *in vivo* retinoid-dependent transcriptional activity. As shown in Fig. 12A, LXR ligation *in vivo* induced the expression of both RALDH1 and lacZ, indicating that LXR signaling is coupled to both enhanced RALDH synthesis and enhanced retinoid production in macrophages. In addition, we could detect the increased expression of TG2 as well. We could also demonstrate the induced expression of β -galactosidase protein by detecting its enzymatic activity, which results in blue staining of macrophages (Fig. 13B). Although *in vivo* we could not apply DEAB, an inhibitor of RALDHs, at optimum concentration because it in higher concentrations killed the mice, induction of both lacZ and TG2 could be attenuated by pretreating mice with DEAB administered at the highest tolerable concentration, proving further that the induction is the result of *in vivo* synthesis of retinoic acid (Fig. 13A).



4.2.3. Ligation of LXR enhances phagocytosis of apoptotic cells in a retinoid-dependent manner

Because previous studies indicated that LXR signaling enhances phagocytosis of apoptotic cells (A-Gonzalez et al., 2009), we decided to investigate whether LXR-induced retinoid synthesis contributes to the enhancement of phagocytosis by macrophages. Administration of GW3965 for 2 h had no effect on the *in vitro* phagocytosis of macrophages, and addition of DEAB had no effect on it (Fig. 13C). However, exposure to GW3965 for 24 h significantly enhanced the engulfment capacity of peritoneal macrophages, and inhibition of retinoid synthesis by DEAB nearly completely prevented this effect (Fig. 13D).

Although 2 h of LXR stimulation had no effect on the phagocytosis of apoptotic cells, it already affected the expression of those two phagocytic genes (Mertk and ABCA1), the induction of which could not be induced by the pan RAR agonist Am580 (Fig. 12C), and that of LXR α , which together with Mertk and ABCA1 is a known LXR target gene (A-Gonzalez et al., 2009; Rébé et al., 2009; Costet et al., 2000) (Fig. 13E). RALDHs, which were also induced, are not known LXR target genes, but they are known to be induced by SREBP-1c (Huq et al., 2006), which contains two LXR response elements in its promoter (Yoshikawa et al., 2001). Thus, we checked the expression of SREBP-1c as well, and detected its induction by GW3965 within 2h. However, the genes (TG2, C1q, stabilin-2, Tim4, and CD14) found to be induced by the pan RAR agonist AM580 (Fig. 11A and 11C) were not induced by GW3965 at 2 h (Fig. 13E). In addition, at this time point, the expression of none of the LXR-induced genes was affected by simultaneous administration of DEAB, an inhibitor of all aldehyde dehydrogenases including RALDHs (Fig. 13E).

If, however, we determined the expression of the same genes 24 h later (Fig. 13F), we found that all the phagocytic receptors, which were induced by ATRA or 9cRA (Fig. 12C), were induced by LXR stimulation as well. From these phagocytosis-related genes, the LXR-induced expression of those receptors (TG2, C1q, stabilin-2, Tim4, and CD14), which were inducible by the pan RAR agonist Am580, was fully prevented by coadministration of DEAB. In addition, administration of a pan RAR antagonist (AGN193109) also prevented the induction of these five genes by the LXR agonist (Fig. 13G). Taken together, these data indicate that these five phagocytosis-related molecules are true RAR-regulated genes in the LXR pathway, and their expression is fully dependent on the newly synthesized retinoid.

To test whether the RAR expression is also affected by LXR stimulation, we detected RAR expression after exposure to GW3965. From the retinoid receptors, we detected only the

increase in the expression of RAR α (Fig. 13E and 13F), in line with a previous publication, which has shown that RAR α is an LXR target gene (R     et al., 2009).

4.2.4. LXR stimulation has a long-term effect on expression of multiple phagocytic receptors. Induction of the expression of various phagocytic receptors is time-dependent after LXR ligation.

The fact that the expression of some phagocytic genes was RAR dependent, whereas that of the others were not, indicated that the expression of the various phagocytic receptors is regulated in a different way after LXR stimulation. Thus, we checked the time curve for the expression of both the phagocytic receptors and the genes responsible for retinoid synthesis. As shown in Fig. 9H, SREBP-c1 and Mertk appeared early after LXR stimulation, in line with the fact that these are all known LXR target genes (A-Gonzalez et al.,2009, Yoshikawa et al., 2001; Costet et al., 2000). The increase in the expression of RALDHs followed that of the direct LXR target genes, whereas it preceded the upregulation of the RAR-dependent phagocytic genes TG2, C1q, and Tim4 (Fig. 13I). DEAB tested at 24 h (Fig. 13F) inhibited not only the expression of the RAR-dependent genes, but partially affected the induction of the LXR target genes as well, indicating that the newly synthesized retinoid contributes also to their long-term induction. It seems very likely that this occurred via targeting the RXR ligand binding site of the LXR/RXR heterodimer by the retinoid forming in this way an autoregulatory positive feedback loop in the LXR signaling pathway.

To prove that the responses we see upon the addition of GW3965 are indeed the consequence of LXR stimulation, we checked the expression of several genes after LXR α/β silencing as well. As shown in Fig. 14A, silencing of LXRs efficiently reduced the protein levels of LXR α and LXR β . Silencing of LXRs prevented the induction of SREBP-1c, Mertk, Tim4, and TG2 by GW3965 (Fig. 14B).

In addition, silencing of LXRs also prevented the increase in phagocytosis of apoptotic cells induced by GW3965 (Fig. 14C), indicating that GW3965 acts indeed via the LXRs in macrophages.

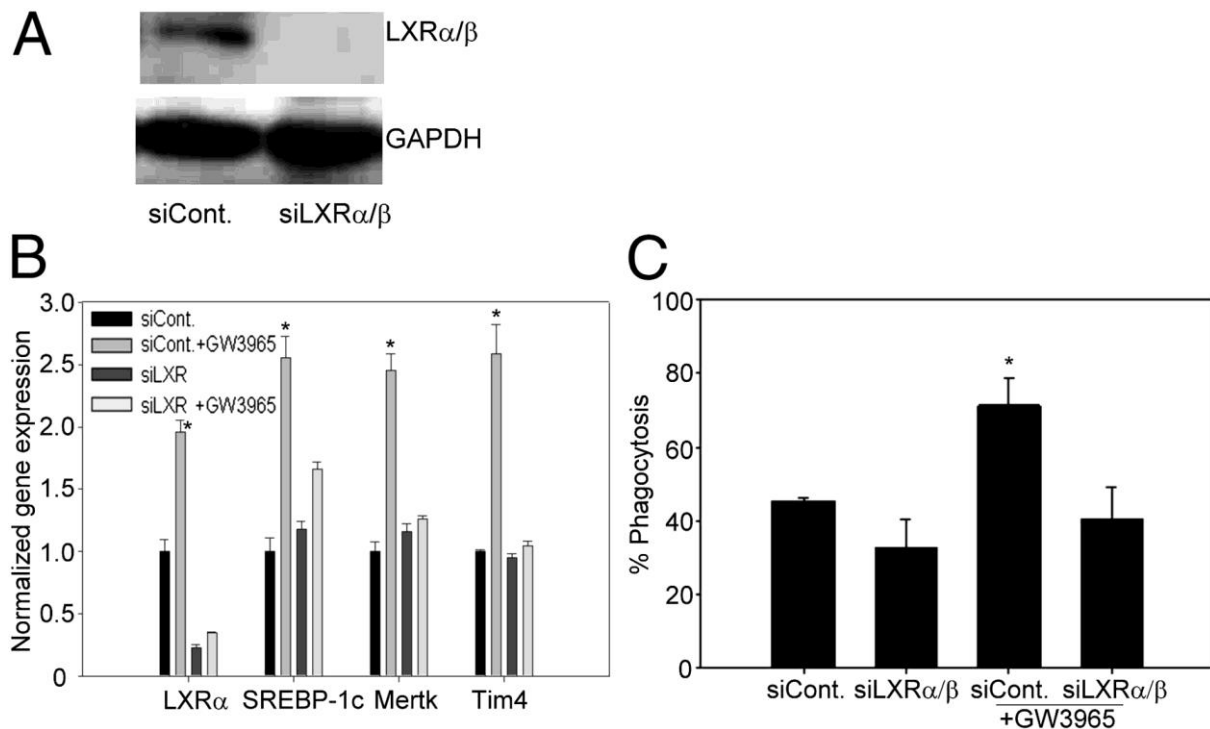


Figure 14. GW3965 mediates its effects on phagocytosis via stimulating LXRs. (A) BMDMs were transfected with 25 nM siLXRα/β or scrambled siRNA. Twenty-four hours later, macrophages were tested for LXRα/β expression by Western blot analysis. (B) BMDMs transfected with 25 nM siLXRα/β or scrambled siRNA were treated with 1 μM GW3965 or vehicle for 24 h. Gene expression was measured by qRT-PCR and expressed as fold inductions ± SD relative to scrambled siRNA-transfected, vehicle-treated cells after normalization of samples with GAPDH expression. (C) siLXRα/β or scrambled siRNA transfected BMDMs were treated with 1 μM GW3965 or vehicle for 24 h in the presence of 5 μM CMTMR. Phagocytosis of apoptotic thymocytes was determined by flow cytometry. Results are representative of three independent experiments (**p* < 0.05, significantly different from respective control).

4.2.5. A potentially dihydro-retinoic acid derivative is produced by macrophages engulfing apoptotic cells

Next, we decided to analyze the RAR-ligating compound produced by macrophages. Because it is difficult to isolate sufficient macrophages to detect their retinoid production, we decided to analyze whole thymus tissue. In the thymus, we have shown previously the increased RALDH expression of macrophages and that of LacZ in the thymus of RARE LacZ mice after *in vivo* apoptosis induction (Garabuczi et al., 2013). LacZ expression in RARE LacZ mice indicates the production of endogenously formed RAR-activating ligands, and because its dexamethasone-induced induction was prevented by DEAB, our data suggested that these bioactive compounds were produced via an RALDH-dependent step *in vivo*. Dexamethasone injected at 0.3 mg/mice induces a high rate of thymocyte apoptosis followed by effective phagocytosis with a 60% loss of total thymic weight within 24 h (Szondy et al., 1997). In line

with the enhanced phagocytosis, this treatment significantly induced the thymic expression of LXR, RALDH, TG2, Tim4, CD14, C1q, and Mertk (Fig. 15A).

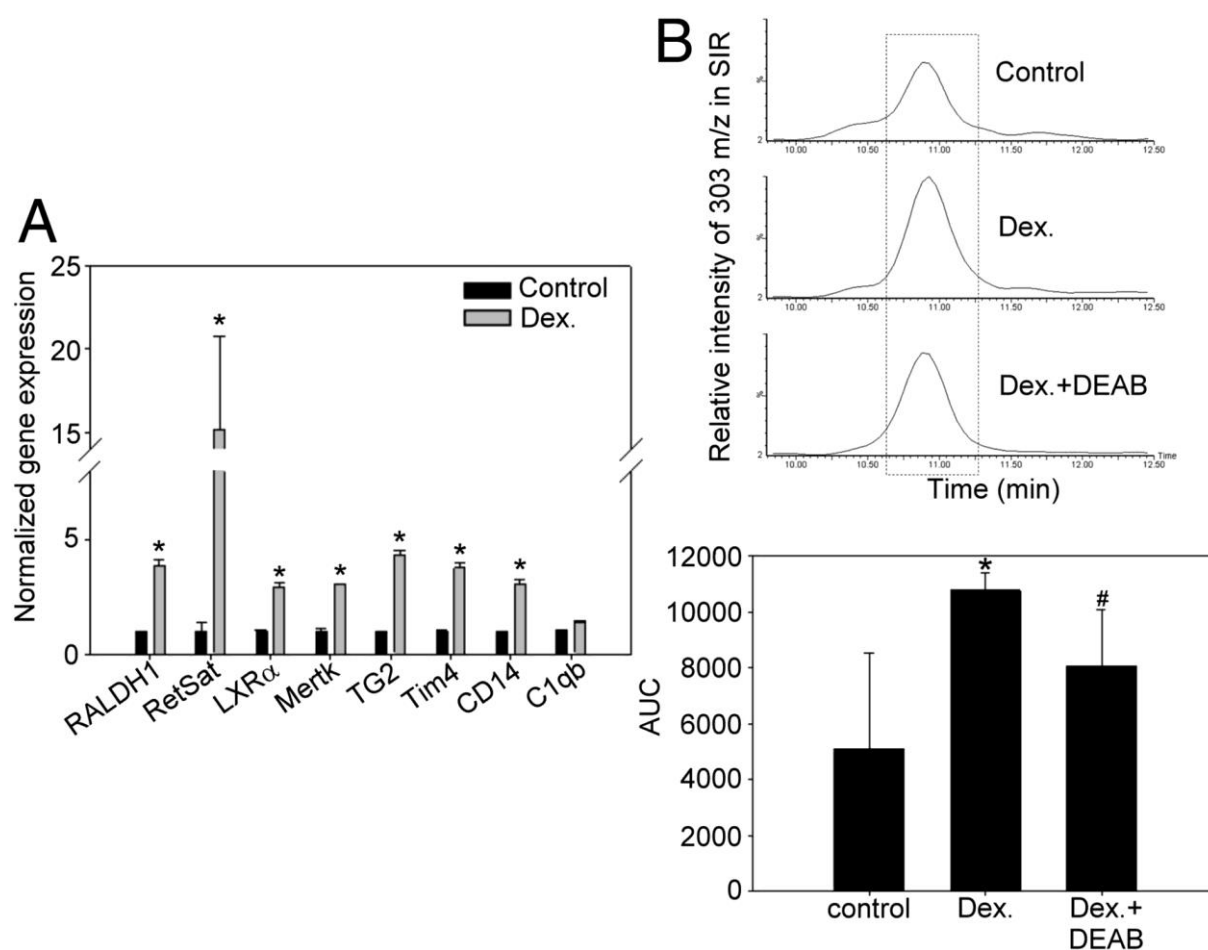


Figure 15. Novel, endogenous retinoid production in the mouse thymus after apoptosis induction with dexamethasone. (A) Four-week-old mice were injected i.p. with 0.3 mg dexamethasone-acetate or vehicle. Twenty-four hours later, thymi were collected and gene expression was determined by qRT-PCR. (B) Four-week-old mice were injected i.p. with 0.3 mg Dex dissolved in DMSO alone or with DEAB (0.25 mg/g body weight) or vehicle. Twenty-four hours later, thymi were collected and retinoid concentration was measured by LC-MS-MS analysis. By using single ion recording at 303 m/z, we found a novel peak, which is regulated in response to Dex and Dex+ DEAB. Data were normalized in relation to sample weight to ensure comparable values and represent mean $\pm$ SD of three independent experiments. (* $p < 0.05$, significantly different from control. # $p < 0.05$, significantly different from the dexamethasone-treated macrophages). AUC, area under the curve.

To detect retinoic acid production, we treated 4-wk-old mice for 24 h with dexamethasone, and the thymi collected were analyzed for retinoic acid before and after the treatment. In line with our previous publication (Kiss et al., 2008), neither the cis-isomers 13-cis, 9-cis RA, nor ATRA were detectable or were around the detection limit of our LC MS/MS technique ($\sim 10^{-9}$ M),

indicating that they were present in much lower concentrations than that responsible for potential RAR activation. In addition, there was no indication that their levels were altered by administration of dexamethasone.

Thus, we decided to search for further dexamethasone-regulated peaks and found various unknown peaks, from which only one was reduced by simultaneous DEAB treatment. This compound has a potential molecular mass of 302 Da indicated by a MS-signal of 302+1 Da using atmospheric pressure chemical ionization at positive ionization mode. A representative chromatogram is shown in Fig. 15B. These data indicate that an unknown retinoid of 302 Da is present and regulated by dexamethasone in the mouse thymi. Based on the MS settings specific for this peak, it seems to be a dihydro-retinoic acid derivative. The location of this dehydrogenation at the various double bonds of the retinoic-acid conjugated system and potential presence of retinoid isomers, however, cannot be decided at present because of the lack of available standard compounds.

Previous studies have reported that there exists an alternative retinoid metabolism in several tissues, in which retinol is converted to dihydro-retinol by the retinol saturase enzyme (Moise et al., 2004). Dihydro-retinol can be converted further via RALDHs to dihydro-retinoic acids (Moise et al., 2005), which can also act as low-affinity RAR ligands (Moise et al., 2009). We therefore investigated whether retinol saturase is expressed in the thymus and whether its expression is enhanced after *in vivo* apoptosis induction. As shown in Fig. 15A, retinol saturase was expressed by the mouse thymus, and its expression was significantly increased after dexamethasone injection.

5. DISCUSSION

Cell-turnover is coupled physiologically in most of the tissues to programmed cell death. When the clearance of dead cells' particles is disturbed, disintegrating cells release their contents, that subsequently induce tissue damage, as well as inflammation on long-term. Inflammation is defined as part of the non-specific immune response that occurs in reaction to harmful stimuli -such as pathogens or damaged cells- and serves as a protective attempt by the organism to remove and demarcate these stimuli and to initiate the healing process (Ferrero-Miliani et al., 2007). It is widely accepted that improper phagocytosis of apoptotic cells (efferocytosis) provokes inflammation and can contribute to the establishment and progression of a number of human chronic inflammatory diseases. During the past decade, our knowledge about the mechanisms involved in the clearance of apoptotic cells increased significantly. Understanding the underlying molecular mechanisms of efferocytosis provides potential pharmacological strategies and targets through which the efficiency of apoptotic cell clearance could be influenced. The further precise determination and clarification of the regulation behind efferocytosis might provide additional perspectives to the understanding of the pathogenesis and also to the treatment of a several diseases that are in connection with the defective clearance.

Chronic inflammation of periodontal tissues, called periodontitis is a morbidity with such characteristics. It is a classical multifactorial disease, that definitely can not proceed in the absence of the pathogen bacterial flora, that could be however hardly achieved in the mouth. The oral cavity harbours one of the most diverse microbiom in our body. It is widely accepted that periodontal disease is basically a chronic infection, but defective regulation of apoptosis and subsequent efferocytosis seems to profoundly influence the progression of the disease. In this aspect it is important to note that the regulation of apoptosis and efferocytosis are tissue and cell type specific, moreover chemotactic signals induce cell type specific signaling pathways that must have great relevance in a histologically so complex structure like the periodontium (Joós G., 2014).

Generally efferocytosis can be broken down into four main steps. It is initiated by chemotactic migration of phagocytes, mainly macrophages, towards the apoptotic cells that is followed by the engulfment of the dead corpses with the participation of numerous cell surface receptors and adaptor molecules.

Increasing evidences suggest that purinergic signaling on the site of the migrating phagocytes is necessary for proper directed movement (Chen et al., 2006; Kronlage et al., 2010) and that in the course of the engulfment process vitamin A derivatives- retinoids are synthesized (Garabuczi et al., 2013). In the present studies, on one hand we investigated the contribution of individual adenosine receptors, especially the A3 receptor, to the chemotactic movement of macrophages. On the other hand we analysed how the regulation of the engulfment process is influenced by LXR stimulation and the subsequent retinoid production.

Although ATP and its degradation products have been previously claimed to serve as potential “find me” signals in the recruitment phase of efferocytosis, it turned out in the context of neutrophil chemotactic migration that they might function differentially (Chen et al., 2006). In this model the authors claimed that the role of the purinerg signaling is more like to amplify the external signals and to transform the gradient sensing into directed movement. Purinerg receptors seem to be recruited to the leading edge of the migrating neutrophils and a dominantly P2Y2 and A3 receptor mediated feedback signaling promote the effective, oriented cell motility (Chen et al., 2006). Later similar regulation behind the macrophage migration has been confirmed: ATP, ADP or adenosine signaling induce lamellopodial extensions of preexisting lamellopodia in the presence of the chemotactic signal (Kronlage et al., 2010). However, the contribution of individual adenosine receptors to the chemotactic migration of macrophages has not been elucidated.

We decided to investigate the involvement of individual adenosine receptor signaling in the chemotactic migration of macrophages in the context of apoptotic thymocyte-derived “find-me” signals. Therefore we set different *in vitro* and *in vivo* experimental approaches. Our results confirm that adenosine receptors have significant impact on the proper execution of macrophage migration during efferocytosis. We could also demonstrate, that the individual adenosine receptors contribute differently to the chemotactic migration of macrophages. Our data demonstrate that by intact ATP and ADP signaling loss of both A3R and the A2BR signaling leads to disturbed chemotactic navigation while the velocity of migration just slightly decreases. Our findings confirm the conclusions of previous studies (Kronlage et al., 2010), that adenosine receptor signaling alone can maintain the chemotactic navigation of macrophages: When we added apyrase to the media of the migrating cells, that led to the absence of ATP, ADP or adenosine, proper directed migration could be restored with the co-administration of

the synthetic adenosine analogue NECA. Interestingly, only simultaneous A2R and A3R signaling was able to maintain proper migration. A2BRs, which have the lowest affinity for adenosine, seem to play more determinant role in the regulation of efferocytotic migration than A2ARs.

Both A2Rs can elevate cAMP levels, however our results suggest in this context a cAMP-independent regulation for macrophage orientation. Though the mechanism of signaling was not investigated yet in our study, we cannot exclude the possibility that cAMP might contribute to the enhancement of the velocity of migration, for example by promoting the Epac-Rap1 pathway, as it was suggested for dendritic cells (Ring et al., 2015). Previous studies have shown that most of the signaling in macrophages that initiates chemotactic migration involves either p38 α MAP kinase- or PI3 kinase-regulated pathways (Cuenda et al., 2007, Jones, 2000). From the apoptotic cell-derived find me signals lysophosphatidylcholine and sphingosine-1-phosphate were shown to induce PI3K-dependent migration (Yang et al., 2005, Rosen et al., 2009), while fractalkine was reported to induce a syk-dependent (Gevrey et al., 2005) PI3 kinase and MAP kinase activation (Kansra et al., 2001). In this context it is worth to note, that while A2ARs do not affect these signaling pathways, the A3Rs were shown to activate both the PI3 kinase and the p38 α MAP kinase (Hammarberg et al., 2004), while the A2BRs all the MAP kinase pathways (Aherne et al., 2011). However, what signal regulates the movement of macrophages towards apoptotic thymocytes is not known, since none of the above signals seem to participate (Elliot et al., 2009), and though ATP was suggested to be the determining “find me” signal (Elliot et al., 2009), according to our present data and to Kronlage’s group (Kronlage et al., 2010) ATP does not act so. Interestingly, in the context of apoptotic thymocyte-derived signaling the velocity of migration was strongly affected by apyrase treatment, while no change in velocity was reported, if apyrase was added to C5a-exposed macrophages (Kronlage et al., 2010). Unfortunately, for technical reasons, we could not repeat their experiments. However, if their results could be confirmed, these data would indicate that the contribution of purinergic signaling to chemotactic navigation might depend on the presence of different chemotactic signals, which regulate chemotaxis via different signaling pathways.

An earlier study has already addressed the question, whether adenosine could act as an apoptotic cell-derived chemotactic signal in the context of macrophage chemotactic navigation towards apoptotic thymocytes (Elliot et al., 2009). They found that adenosine cannot act so. In agreement with their finding, our data indicate that adenosine can enhance chemotaxis without forming a chemotactic gradient supporting the view that it promotes the action of the apoptotic

cell-derived ‘find-me’ signals, rather than acts as a chemoattractant itself. In addition, previous studies have shown that though apoptotic thymocytes can convert the released ATP to AMP (Yamaguchi et al., 2014), adenosine is not formed (Köröskényi et al., 2011) due to the lack of 5’ nucleotidase expression by these cells. Indeed, recent work in our laboratory has elucidated that the apoptotic cell-derived adenine nucleotides are converted to adenosine by the macrophage 5’ nucleotidase (Sándor et al., 2016), indicating that endogenous adenosine will be formed directly on the surface of macrophages, where it can immediately trigger the macrophage adenosine receptors.

Adenosine seems to influence the macrophage migration also in tumor tissue environment. Tumors can attract myeloid cells via generating adenosine (Montalbán Del Barrio et al., 2016, Koszałka et al., 2015). Moreover, in an adenosine free tumor environment in the presence of α,β -methyleneadenosine 5’-diphosphate (which can trigger ADP receptors) stimulation of individual adenosine receptors promoted macrophage migration. In line with our results, in those tests the A3R agonist was found to be the most effective, but stimulation of the A2BRs was not investigated (Koszałka et al., 2016). In our tests addition of NECA could not further enhance migration of wild type macrophages towards apoptotic cells, but acted so in the case of A3R null cells.

We investigated the effect of the loss of A3Rs in two different *in vivo* models. Previous *in vitro* studies from our group have already proved that A3Rs does not determine the rate of apoptotic cell engulfment (Duró et al., 2014), so our hypothesis was that potential *in vivo* consequences could be related to disturbed chemotactic migration of phagocytes. We detected impaired *in vivo* clearance of apoptotic thymocytes, when we injected them into the peritoneum of A3R null mice that indicates, loss of navigation delays the *in vivo* apoptotic clearance process, even if the phagocytic capacity itself is not affected. Surprisingly, we didn’t explored similar consequences, when we studied the *in vivo* apoptotic cell clearance in the dexamethasone-treated thymus. One possible explanation is that A3Rs are not involved in the chemotactic navigation of thymic macrophages. Additionally, in the thymic cortex the dexamethasone sensitive double positive thymocytes form clusters with the cortical macrophages, indicating a strong interaction between macrophages and immature thymocytes during their differentiation, even before they die (Rezzani et al., 2008). Thus the removal of the firstly formed apoptotic cells might not require chemotactic migration of macrophages, moreover, dexamethasone can also enhance their phagocytic capacity (McColl et al., 2009, Garabuczi et al., 2015). However,

improper migration of macrophages has been reported to affect the *in vivo* clearance of apoptotic cells in the thymus as well (Elliot et al., 2009). In accordance with previous findings (Odaka et al., 2002), we also observed that additional F4/80⁺ macrophages immigrate into the thymic cortex following glucocorticoid treatment. The process was reported to be induced by oxidized membrane vesicles and blebs from apoptotic cells containing biologically active oxidized phospholipids that induce monocyte-endothelial interactions (Huber et al., 2002). The entry was detected within 1h following dexamethasone injection, and while wild type macrophages moved right away towards the cortex, in A3R null mice an increased number of macrophages was detected in the thymic medulla as well. Despite of the lack of oriented migration, however, a significant number of A3R null macrophages could also reach the cortex. It is very likely in the thymic cortex macrophages can engulf apoptotic cells even without the need of chemotactic navigation, since the access to apoptotic thymocytes following dexamethasone treatment is unlimited (Szondy et al., 1997). In addition, increased adenosine concentration is present in the thymus, when the high amount of dying cells release ATP (Resta et al., 1997), which might partially overcome the defect in chemotactic navigation resulted from the loss of A3Rs. The lack of importance of A3Rs at later phases of the *in vivo* clearance is also supported by our previous finding that once macrophages find the apoptotic cells and start to engulf them, they significantly downregulate their cell surface A3Rs (Duró et al., 2014). Interestingly, in mice treated with apyrase a delayed apoptotic cell clearance was reported in the dexamethasone-treated thymus (Elliot et al., 2009). However, our data indicate that apyrase not only leads to a loss in chemotactic orientation, but also to a decreased velocity of macrophage migration, moreover the apyrase treatment might affect the engulfment of apoptotic cells, which was not investigated so far.

Thus all together our data demonstrate that adenosine receptors play a determinant role in the chemotactic orientation of macrophages towards apoptotic thymocytes. However, despite the *in vitro* and *in vivo* evidences, loss of A3R does not result in a thymic phenotype because either thymic macrophages do not use A3Rs in the chemotactic navigation, or the loss of chemotactic navigation alone plays less significant role in the *in vivo* apoptotic cell clearance in the thymus.

In the other part of our work, we were focusing on the signaling events behind the engulfment process. Although phagocytosis of various targets requires generally the function of one definite receptor on the macrophages, uptake of apoptotic cells is different. Apoptotic cell clearance involves several phagocytic receptors and adapter molecules, which function in a coordinate

fashion to promote engulfment (Stuart et al., 2005, Ravichandran et al., 2007). Previous studies in our laboratory have shown that TG2, by interacting with both MFG-E8 and integrin $\beta 3$ as a coreceptor (Tóth et al., 2009), belongs to the cell-surface proteins required for engulfment. The loss of TG2 decreases the *in vivo* phagocytic capacity of macrophages and leads to SLE-like autoimmunity (Szondy et al., 2003), similarly to the single loss of other crucial phagocytic receptors (Hanayama et al., 2002, Savill et al., 1992, Botto et al., 1998, Devitt et al., 2004, Park et al., 2009, Greenberg et al., 2006, Albert et al., 2000, Cohen et al., 2002, Park et al., 2008, Hamon et al., 2006).

Other studies have revealed that lipid-sensing nuclear receptors (LXR, PPARs) from the family of RXR heterodimers respond to the lipid content of the engulfed apoptotic cells and influence the expression of various phagocytic related genes. In deed, phagocytic capacity of macrophages can be increased via activation of these receptors in situations where high numbers of apoptotic cells have to be cleared *in vivo* (A-Gonzalez et al., 2009, Roszer et al., 2011, Mukundan et al., 2009). Loss of either LXR or PPAR δ receptors results in impaired phagocytosis of apoptotic cells, moreover their loss also results in development of SLE-like autoimmunity (A-Gonzalez et al., 2009, Mukundan et al., 2009).

Ligation of LXR/RXR heterodimers leads also to endogenous retinoid production in macrophages (Aydemir et al., 2013). Parallel to this finding, one of my colleague has observed that after apoptotic cell engulfment, genes responsible for endogenous retinoid synthesis are induced (Garabuczi et al., 2013). Our group has reported that engulfment-coupled retinoid production of macrophages might play a role in the upregulation of TG2 in the apoptotic thymocytes of the thymus (Garabuczi et al., 2013), as well as in the modulation of the thymic selection processes (Sarang et al., 2013). It was also found that macrophage-produced retinoids contribute to regulatory T cell formation in the airway (Soroosh et al., 2013).

We could confirm in our current study the previous observations that ligation of LXR enhances the expression of Mertk and ABCA1 (A-Gonzalez et al., 2009), two LXR-regulated phagocytic genes, and that LXR stimulation leads to endogenous retinoid production via induction of genes coupled to endogenous retinoid synthesis (Aydemir et al., 2013). The retinoid produced partly enhanced the expression of a group of phagocytosis-related genes including TG2 via activating RARs, but also contributed to the enhanced expression of LXRs, Mertk, and ABCA1 detected at 24 h after LXR ligation, very likely by acting via the RXR ligand binding site of the LXR heterodimer.

Interestingly we found that an increase in the TG2 expression alone is not sufficient to enhance the phagocytic capacity of macrophages. This is in line with our previous observation, that showed, that recombinant TG2 significantly enhances the phagocytic capacity of TG2 null macrophages, but not that of wild-type cells (Tóth et al., 2009). Exogenous administration of retinoids enhanced efferocytosis effectively, also in those macrophages that lacked one of the phagocytosis related receptors (TG2 or CD14). In deed, ATRA, one of the retinoids tested, induced the expression of a number of phagocytic receptors and bridging molecules, indicating that simultaneous increase in the expression of all these phagocytosis-related molecules might contribute to the enhanced phagocytosis. Analysis using various retinoid receptor agonists revealed that some of the retinoid-induced phagocytosis related molecules, such as TG2, Stabilin-2, Tim4, CD14, and C1q, were regulated by the ligation of the RARs, whereas Mertk and ABCA1 were induced by activation of other RXR heterodimers. In line with these observations, those retinoids were the most effective in enhancing phagocytosis of apoptotic cells, which were capable of transactivating both RARs and RXRs.

We tested whether the LXR induced enhancement of phagocytosis requires retinoid production. Inhibition of retinoid production by inhibiting RALDHs prevented the LXR-induced enhancement in the phagocytosis of apoptotic cells, indicating that retinoids are mediate the effect of LXR. Our present report is the first to our knowledge, to indicate that retinoids also affect the phagocytic capacity of macrophages. Based on our data, we propose a model, shown in Fig. 16, for the LXR-induced enhancement of phagocytosis of apoptotic cells by macrophages.

We wanted to exactly identify the retinoid, which is produced during efferocytosis by macrophages. However, in line with our previous publication (Kiss et al., 2008), none of the known classical RAs was detectable in the thymus after apoptosis induction. We detected the “apopto-phagocytosis-dependent” appearance of a novel endogenous derivative, the appearance of which was RALDH dependent and which might be a dihydroretinoic derivative or an apo-159-lycopenoic acid derivative (Aydemir et al., 2013), indicated by its molecular mass of 302 Da.

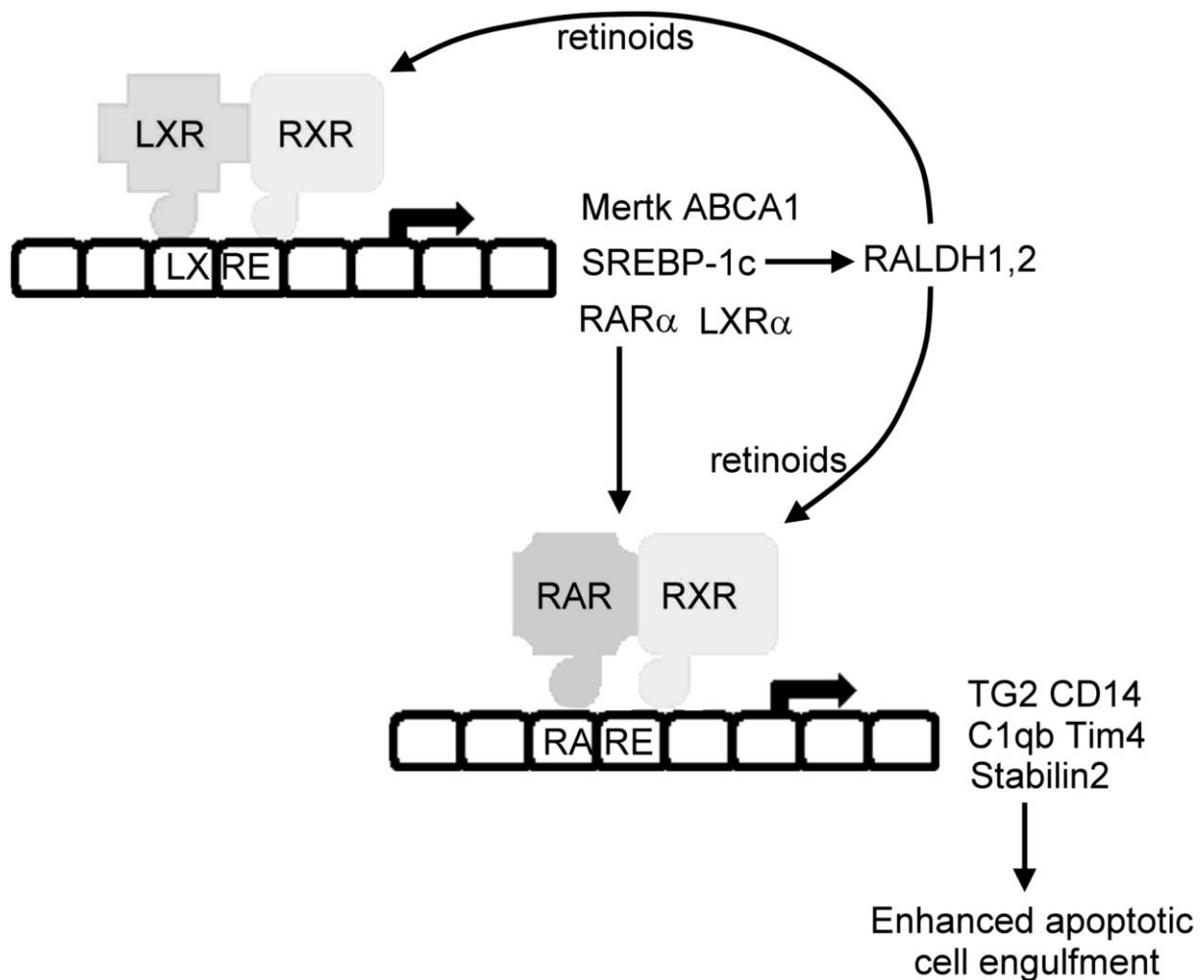


Figure 16. Proposed model for the LXR-induced enhancement of phagocytosis of apoptotic cells by macrophages. LXR triggering leads to the upregulation of LXR target genes such as *Mertk*, *ABCA1*, *SREBPc1*, and *RARα*. LXRs also promote their own transcription via an autoregulatory loop mechanism leading to further expression of their target genes. *SREBP-c1* upregulates the expression of *RALDH1* resulting in a consequent retinoid production. The retinoid produced acts on *RARs* to upregulate the phagocytic genes *TG2*, *Tim4*, *stabilin-2*, *CD14*, and *C1q*, leading to enhanced phagocytosis. In addition, it might facilitate both *LXR/RXR*- and *RAR/RXR*-driven transcription via binding also to the *RXR* ligand binding site.

Besides the classical retinoic acid pathways, a novel retinoid pathway has been recently described, which is initiated by retinol saturase enzyme. This pathway also involves *RALDHs* and produces 13,14-dihydro-retinoic acids, which act also as low-affinity *RAR* ligands (Moise et al., 2004, Moise et al., 2005, Moise et al., 2009). We found that retinol saturase is expressed in the thymus, and its expression is induced after *in vivo* apoptosis induction, indicating that this alternative pathway might be involved in the formation of the novel retinoid. However, its exact chemical nature awaits definition. Unfortunately, the expected concentrations of the

retinoids are in the range of nanograms per gram of tissue, and purification may result in only picogram amounts of compounds when using milligram amounts of thymic tissue. These amounts are too small to determine a chemical structure even with the most sensitive available nuclear magnetic resonance technologies. Thus, with a range of chemist collaborators, we initiated targeted organic synthesis by considering all the possible chemical alternatives to clearly identify the novel derivative and its biological activity. However, based on the observation that the expression of the phagocytic receptors seems to be regulated by both RAR- and RXR-dependent pathways indicates that both RAR and RXR ligands or a ligand with both RAR and RXR activating potential is formed in the LXR signaling pathway. We do not presently understand why two parallel retinol-dependent pathways can generate retinoid receptor ligands in the tissues, but the fact that the retinol saturase knockout mice have phenotype indicates tissue-specific roles for this alternative pathway (Moise et al., 2010). Current work in our laboratory has been recently focused on the retinol saturase knockout mice. On the contrary to our previous expectations, the phagocytosis related genes seem to be similarly regulated in the absence of the gene as in the wild type mice. However, RetSat knock out mice at old age develop mild autoimmunity and they don't produce neuropeptide Y, that is a chemotaxis regulation related molecule in macrophages (Sarang, unpublished data). We suppose that the retinol saturase pathway might also influence the migration of macrophages in the course of efferocytosis.

Increasing evidence suggests that impaired phagocytosis of apoptotic cells caused by the loss of various phagocytic receptors leads to the development of SLE in humans and in mice (Hanayama et al., 2002, Botto et al., 1998, Devitt et al., 2004, Cohen et al., 2002, A-Gonzalez et al., 2009, Mukundan et al., 2009, Szondy et al., 2003). It has also been suggested that SLE is characterized by impaired phagocytosis, even if the phagocytic receptors are genetically not affected, indicating that impaired primary or secondary engulfment deficiency plays a determinant role in the pathogenesis of this disease (Licht et al., 2004, Gaipal et al., 2007). Thus, molecules that can enhance phagocytosis of apoptotic cells might be used in the treatment of SLE. Indeed, glucocorticoids, which are widely used in the treatment of SLE, are capable of increasing the phagocytic capacity of macrophages (McColl et al., 2009). In addition, LXR ligands were shown to decrease the symptoms of SLE in *lpr* mice (A-Gonzalez et al., 2009). Based on our data, we propose that retinoids could also be considered as compounds in targeting phagocytosis of apoptotic cells. Lupus nephritis is a leading symptom and a cause of death in patients with SLE (Walsh et al., 1995). *In vivo* ATRA treatment on the development of lupus

nephritis has already been tested in both mouse models (Kinoshita et al., 2003, Pérez de Lema et al., 2004) and humans (Kinoshita et al., 2010) with promising results. Long-term ATRA treatment in SLE-prone mice resulted in longer survival, significant reduction of proteinuria, renal pathological findings, and glomerular IgG deposits. In humans, it also reduced proteinuria. Retinoids have been long accepted to act as immunomodulators. It has been suggested that they delay the development of autoimmune diseases also via modulating the Th1/Th17 versus Th2 balance and by promoting the formation of regulatory T cells responsible for self-tolerance (Carratù et al., 2012). Based on our data, we propose that the potential beneficial effect of retinoids in preventing the development of SLE might also be related to the fact that they enhance the phagocytosis of apoptotic cells.

Altogether, my experiments identified two molecular targets to affect clearance of apoptotic cells: retinoids and adenosine receptors.

6. SUMMARY

In healthy individuals, billions of cells die by apoptosis every day. Removal of the dead cells by phagocytosis (a process called efferocytosis) must be efficient to prevent secondary necrosis and the consequent release of pro-inflammatory cell contents that damages the tissue environment and provokes chronic inflammation or even autoimmunity. That is why by studying these processes, potential pharmacological targets can be identified, influence of which might be used in the treatment of chronic inflammatory diseases.

The first step in the clearance of apoptotic cells is chemotactic migration of macrophages towards the apoptotic cells guided by “find-me” signals released by the dying cells. Upon sensing the chemotactic signals, macrophages release ATP. ATP is extracellularly degraded to ADP, AMP and adenosine to trigger purinergic receptors concentrated at the leading edge of the cell. Previous studies have shown that in addition to the chemotactic signals, this purinergic autocrine signaling is also required to amplify and translate chemotactic signals into directional motility (Kronlage et al., 2010). In the first part of our studies the involvement of adenosine A3 receptors (A3R) was analyzed in the chemotactic migration of macrophages directed by apoptotic thymocyte-derived “find-me” signals. We demonstrate *in vitro* and *in vivo*, that the purinergic autocrine signaling is required for maintaining both the velocity and the directionality of macrophage migration towards the apoptotic thymocytes. Adenosine receptor signaling alone seems to be sufficient to sustain proper chemotactic migration, but only simultaneous A2R and A3R signaling can act so. Though loss of A3Rs does not affect the phagocytotic capacity of macrophages, intraperitoneally-injected apoptotic thymocytes were cleared with a delayed kinetics by A3R null macrophages due to the impaired chemotactic navigation. Our data demonstrate the involvement of macrophage A3Rs in the proper chemotactic navigation and consequent *in vivo* clearance of apoptotic cells in the peritoneum. Interestingly, loss of A3Rs did not affect the *in vivo* clearance of apoptotic thymocytes in the dexamethasone-treated thymus.

In the second part of my thesis, our studies related to the signaling pathways regulating the efferocytotic engulfment machinery are presented. We have confirmed that upon activation of the lipid-sensing liver X receptor (LXR), that is physiologically ligated by the lipid content of the apoptotic cells, the expression of several efferocytosis related molecules’ and that of retinaldehyde dehydrogenases’ genes are induced. Induction of endogenous retinoid synthesis leads to the production of a nonclassical retinoid. Based on our retinoid analysis, this compound

might be a dihydro-retinoic acid derivative. The novel retinoid then contributes to the upregulation of further phagocytic receptors including TG2 by ligating retinoic acid receptors. Inhibition of retinoid synthesis prevents the enhanced phagocytic uptake induced by LXR ligation. Our data indicate that stimulation of LXR enhances the engulfment of apoptotic cells via regulating directly and indirectly the expression of a range of phagocytosis-related molecules, and its signaling pathway involves the synthesis of a nonclassical retinoid. We propose that retinoids could be used for enhancing the phagocytic capacity of macrophages in diseases where impaired phagocytosis of apoptotic cells plays a role in the pathogenesis of the disease.

KEYWORDS: efferocytosis, macrophage, phagocytosis, adenosine, cell migration

7. ÖSSZEFOGLALÁS

Egészséges egyénekben nap, mint nap sejtek milliárdjai halnak el apoptózis révén. Ha az elhalt sejtek fagocitózis útján történő eltakarítása (amit egyre szélesebb körben efferocitózisnak neveznek) nem kellően hatékony, az apoptotikus sejtek szekunder nekrozisa következik be, ami következményesen a pro-inflammatorikus sejttartalom extracelluláris térbe áramlásához, a környező szövetek károsodásához és krónikus gyulladásához vagy akár autoimmunitással járó betegségek kialakulásához is vezethet. Az efferocitózis tanulmányozása olyan gyógyszerterápiákhoz vezethet, melyek új kezelési stratégiák részévé válhatnak olyan betegségek kapcsán, ahol az elhalt sejtek eltakarításának zavara fontos pathogenetikai tényező.

Az apoptotikus sejtek eltakarításának első lépése során az elhalt sejtekből kiáramló ún. „find-me” szignálokat érzékelő makrofágok kemotaktikus migráció révén az apoptotikus sejtek közelébe vándorolnak. A vándorlás során polarizálódó makrofágok vezető oldalukon ATP-t bocsátanak ki, ami extracellulárisan ADP-re, AMP-re, majd adenzinra bomlik, majd a sejtfelszíni purinerg receptorokhoz kötődnek. Korábbi tanulmányok igazolták, hogy a kemotaktikus szignálokon kívül ezen autokrin purinerg jelátvitelre is szükség van a kemotaktikus jelek felerősítéséhez ill. irányított mozgássá alakításához (Kronlage et al., 2010). Kísérleteink első részében az adenzin A3 receptorok (A3R) szerepét vizsgáltuk a makrofágok apoptotikus timociták irányába történő kemotaktikus vándorlásának szabályozásában. *In vitro* és *in vivo* kísérleti módszerek eredményeivel támasztottuk alá, hogy az autokrin purinerg jelátvitel szükséges a makrofágok apoptotikus timociták irányába történő vándorlása során a mozgás sebességének és direkcionálisának fenntartásához. Az adenzin receptorokhoz kötődő jelátvitel eredményeink alapján önmagában képes hatékony sejtmigrációt biztosítani, de csak abban az esetben, ha szimultán A3R és A2R aktiváció is bekövetkezik. Bár az A3R elvesztése önmagában nem befolyásolja a makrofágok fagocitózis kapacitását, az intraperitoneálisan befecskendezett apoptotikus timociták az A3R hiányos makrofágok zavart kemotaktikus navigációs képessége miatt lassabban kerültek felvételre. Eredményeink igazolják a makrofág A3R jelentőségét a kemotaktikus mozgás direkcionálisának szabályozásában és ezzel összefüggésben az apoptotikus timociták *in vivo* intraperitoneális eltakarításának zavartalanságában. Érdekes módon az A3R elvesztése nem befolyásolta a dexamethasone kezelést követően timuszból zajló efferocitózist.

A dolgozat második felében az elhalt sejtek bekebelezésének hátterében zajló jelátviteli folyamatok vizsgálatát célzó kísérleteink mutatnak be. Megerősítettük, hogy az elhalt sejtek lipidjei által aktivált lipid érzékelő liver X receptor (LXR) stimulációja számos efferocitózis kapcsolt molekula génje mellett az endogén retinoidprodukciónak felelős enzimek génjeit is indukálja. Az endogén retinoid szintézis egy nem-klasszikus, ismeretlen retinoid termeléséhez vezet a makrofágokban. Az ismeretlen retinoid a tömegspektrometriás analízis alapján egy dihidro-retinsav származék lehet, melynek megjelenése további fagocitózis kapcsolt receptorok indukciójához, pl.: TG2, vezet a retinsav receptorok aktivációján keresztül. A retinoid szintézis gátlása az LXR stimuláció hatására bekövetkező fagocitózis fokozódás elmaradását eredményezi. Eredményeink arra utalnak, hogy az LXR aktivációt követően megfigyelhető fagocitózis fokozódás több direkt LXR és indirekt módon endogén retinoid produkcióhoz köthetően szabályozott gén megváltozott kifejeződésével áll kapcsolatban. Mindezek alapján azon kórfolyamatokban, melyekben az apoptotikus sejtek eltakarításának zavara megfigyelhető felmerül a retinoidok potenciális terápiás alkalmazása az elhalt sejtek fagocitózisának fokozása céljából.

KULCSSZAVAK: efferocitózis, makrofág, fagocitózis, adenzin, sejtmigráció

8. ACKNOWLEDGEMENTS

This thesis was nine years of my life. Back then in 2008 I was full with curiosity and enthusiasm, that led me to the department of biochemistry and molecular biology, to Professor Szondy's lab. It was the end of the summer after the difficult exams of the 3rd year on the general medical faculty and I was a little bit hesitating to start a new challenge right before the 4th year. However, as professor Szondy gave me the chance to work in her laboratory on studies of the apoptotic cell uptake, I knew it is an overwhelming opportunity. Our first and early successes on the Scientific Students' Associations Conference encouraged me to continue the way I launched.

I don't want to list up all the important milestones, because it would be definitely too long and for sure not comprehensive, so I'd rather like to thank everybody who was part of this journey.

I'd like to give special thanks to those, who supported and inspired me all these years. First of all to my supervisor, professor Zsuzsa Szondy for helping me continuously, for being patient with me and for teaching me so much!

Thanks to professor László Fésüs for his support during my Phd studies.

Thanks to professor Peter Krammer and his colleagues for accepting me as a member of their team and for giving me the opportunity to work with them in Heidelberg.

Thanks to my dear colleagues and collaborators Dr. Zsolt Sarang, Dr. Tamás Papp, Judit Jákím, Dr. Gábor Szemán-Nagy, Dr. Beáta Kiss, Tünde Terdik, Dr. Ralph Rühl, Dr. Szabolcs Felszeghy, Tibor Sággy and Dr. Attila Pap whose contribution to my work was indispensable.

Thanks to all the members of our group in the Szondy lab: Dr. Krisztina Köröskényi, Dr. Éva Garabuczi, Dr. Katalin Sándor, Edina Duró, Dr. Anna Pallai, Dr. Katalin Tóth, Zsófia Budai, Zsolt Hartman, Edit Kómóczi and also the other colleagues and friends from the institute (Fésüs-lab, Nagy-lab, Petrovski-lab, Szatmári-lab).

Thanks to the leaders of the institute and the doctoral school, who made it possible to complete my work.

Thanks to Zsuzsa Oláh and Dr. Krisztina Köröskényi for helping me so much in the administrative issues of my PhD studies.

Thanks to my dear biology teachers Dr. Edit Futó and Dr. György Tóth for their kindness and genuineness.

Last but not least I'd like to thank everything to my beloved family: my Mother, Father, my sister and her family, my dear friends, and to my girlfriend, who all gave me neverending support, encouragement and love on this long, beautiful, but many times very difficult way.

The studies were supported by Hungarian grants from the National Research Fund (OTKA K83865, T104228, NK105046 and 104228), and the TÁMOP 4.2.2.A-11/1/KONV-2012-0023 “VÉD-ELEM” and GINOP-2.3.2-15-2016-00006 projects which are cofinanced by the European Social Fund and the European Regional Development Fund.

9. REFERENCES

- Aherne CM, Kewley EM, Eltzschig HK. 2011.
The resurgence of A2B adenosine receptor signaling.
Biochim Biophys Acta. 1808(5):1329-39
- A-Gonzalez N, Bensinger SJ, Hong C, Beceiro S, Bradley MN, Zelcer N, Deniz J, Ramirez C, Díaz M, Gallardo G, de Galarreta CR, Salazar J, Lopez F, Edwards P, Parks J, Andujar M, Tontonoz P, Castrillo A. 2009.
Apoptotic cells promote their own clearance and immune tolerance through activation of the nuclear receptor LXR.
Immunity. 21;31(2):245-58.
- A-Gonzalez N, Hidalgo A. 2014.
Nuclear receptors and clearance of apoptotic cells: stimulating the macrophage's appetite.
Front. Immunol. 5:211
- Albert ML, Kim JI, Birge RB. 2000.
alpha5beta1 integrin recruits the CrkII-Dock180-rac1 complex for phagocytosis of apoptotic cells.
Nat. Cell Biol. 2(12):899-905.
- Albert ML, Pearce SF, Francisco LM, Sauter B, Roy P, Silverstein RL, Bhardwaj N. 1998.
Immature dendritic cells phagocytose apoptotic cells via alpha5beta1 and CD36, and cross-present antigens to cytotoxic T lymphocytes.
J Exp Med. 188(7):1359-68.
- Aschoff L. 1924.
Das reticulo-endotheliale system.
Ergeb. Inn. Med. Kinderheilkd. 26, 1–118
- Aydemir G, Kasiri Y, Birta E, Béke G, Garcia AL, Bartók EM, Rühl R. 2013.
Lycopene-derived bioactive retinoic acid receptors/retinoid-X receptors-activating metabolites may be relevant for lycopene's anti-cancer potential.
Mol. Nutr. Food Res. 57(5):739-47.
- Bayardo M, Punzi F, Bondar C, Chopita N, Chirido F. 2012.
Transglutaminase 2 expression is enhanced synergistically by interferon- γ and tumour necrosis factor- α in human small intestine.
Clin. Exp. Immunol. 168(1):95-104.
- Bhatia VK, Yun S, Leung V, Grimsditch DC, Benson GM, Botto MB, Boyle JJ, Haskard DO. 2007.
Complement C1q reduces early atherosclerosis in low-density lipoprotein receptor-deficient mice.
Am. J. Pathol. 170(1):416-26.

- Biancheri P, Giuffrida P, Docena GH, MacDonald TT, Corazza GR, Di Sabatino A. 2014.
The role of transforming growth factor (TGF)- β in modulating the immune response and fibrogenesis in the gut.
Cytokine Growth Factor Rev. 25:45-55
- Botto M, Dell'Agnola C, Bygrave AE, Thompson EM, Cook HT, Petry F, Loos M, Pandolfi PP, Walport MJ. 1998.
Homozygous C1q deficiency causes glomerulonephritis associated with multiple apoptotic bodies.
Nat. Genet. 19(1):56-9.
- Bournazou I, Pound JD, Duffin R, Bournazos S, Melville LA, Brown SB, Rossi AG, Gregory CD. 2009 .
Apoptotic human cells inhibit migration of granulocytes via release of lactoferrin.
J. Clin. Invest. 119(1):20-32.
- Butler M, Sanmugalingam D, Burton VJ, Wilson T, Pearson R, Watson RP. 2012
Impairment of adenosine A3 receptor activity disrupts neutrophil migratory capacity and impacts innate immune function in vivo.
Eur. J. Immunol.; 42:3358-3368.
- Calkin AC, Tontonoz P. 2012.
Transcriptional integration of metabolism by the nuclear sterol-activated receptors LXR and FXR.
Nat. Rev. Mol. Cell Biol. 14;13(4):213-24.
- Cardona AE, Pioro EP, Sasse ME, Kostenko V, Cardona SM, Dijkstra IM, Huang D, Kidd G, Dombrowski S, Dutta R, Lee JC, Cook DN, Jung S, Lira SA, Littman DR, Ransohoff RM. 2006.
Control of microglial neurotoxicity by the fractalkine receptor.
Nat. Neurosci. 9(7):917-24.
- Carratù MR, Marasco C, Mangialardi G, Vacca A. 2012.
Retinoids: novel immunomodulators and tumour-suppressive agents?
Br. J. Pharmacol. 167(3):483-92.
- Chekeni FB, Elliott MR, Sandilos JK, Walk SF, Kinchen JM, Lazarowski ER, Armstrong AJ, Penuela S, Laird DW, Salvesen GS. 2010.
Pannexin 1 channels mediate “find-me” signal release and membrane permeability during apoptosis.
Nature. 467: 863–867.
- Chen Y, Corriden R, Inoue Y, Yip L, Hashiguchi N, Zinkernagel A. 2006.
ATP release guides neutrophil chemotaxis via P2Y2 and A3 receptors.
Science 314: 1792-1795.
- Cohen PL, Caricchio R, Abraham V, Camenisch TD, Jennette JC, Roubey RA, Earp HS, Matsushima G, Reap EA. 2002.
Delayed apoptotic cell clearance and lupus-like autoimmunity in mice lacking the c-mer membrane tyrosine kinase.

J. Exp. Med. 196(1):135-40.

Costet P, Luo Y, Wang N, Tall AR. 2000.
Sterol-dependent transactivation of the ABC1 promoter by
the liver X receptor/retinoid X receptor.
J. Biol. Chem. 275(36):28240-5.

deCathelineau AM, Henson PM. 2003.
The final step in programmed cell death: phagocytes carry apoptotic cells to the grave.
Essays Biochem. 39:105-17.

Cuenda A, Rousseau S. 2007.
p38 MAP-kinases pathway regulation, function and role in human diseases.
Biochim Biophys Acta. 1773(8):1358-75.

Devitt A, Parker KG, Ogden CA, Oldreive C, Clay MF, Melville LA, Bellamy CO, Lacy-
Hulbert A, Gangloff SC, Goyert SM, Gregory CD. 2004.
Persistence of apoptotic cells without autoimmune disease or inflammation in CD14^{-/-} mice.
J. Cell Biol. 167(6):1161-70.

Duró E, Pallai A, Köröskényi K, Sarang Z, Szondy Z. 2014
Adenosine A3 receptors negatively regulate the engulfment-dependent apoptotic cell
suppression of inflammation.
Immunol. Lett. 162(2 Pt B): 292-301.

Elliott MR, Cheken FB, Trampont PC, Lazarowski ER, Kadl A, Walk SF, Park D, Woodson
RI, Ostankovich M, Sharma P, Lysiak JJ, Harden TK, Leitinger N, Ravichandran KS. 2009.
Nucleotides released by apoptotic cells act as a find-me signal to promote phagocytic clearance.
Nature. 461(7261):282-6.

Elliott MR, Ravichandran KS. 2010.
Clearance of apoptotic cells: implications in health and disease.
J. Cell Biol. 189(7):1059-70.

Fadok VA, Bratton DL, Henson PM. 2001.
Phagocyte receptors for apoptotic cells: recognition, uptake, and consequences.
J. Clin. Invest. 108(7):957-62.

Fadok VA, Bratton DL, Konowal A, Freed PW, Westcott JY, Henson PM. 1998.
Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine
production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF.
J. Clin. Invest. 101: 890-898.

Fadok VA, Henson PM. 2003.
Apoptosis: giving phosphatidylserine recognition an assist--with a twist.
Curr. Biol. 13(16):R655-7.

- Feinstein DL, Galea E, Gavriluk V, Brosnan CF, Whitacre CC, Dumitrescu-Ozimek L, Landreth GE, Pershadsingh HA, Weinberg G, Heneka MT. 2002. Peroxisome proliferator-activated receptor-gamma agonists prevent experimental autoimmune encephalomyelitis. *Ann. Neurol.* 51(6):694-702.
- Fernandez-Boyanapalli R, Frasch SC, Riches DW, Vandivier RW, Henson PM, Bratton DL. 2010. PPAR γ activation normalizes resolution of acute sterile inflammation in murine chronic granulomatous disease. *Blood* 116(22):4512-22.
- Ferrero-Miliani L, Nielsen OH, Andersen PS, Girardin SE. 2007. Chronic inflammation: importance of NOD2 and NALP3 in interleukin-1 β generation. *Clin. Exp. Immunol.* 147, 227-35
- Florey O, Haskard DO. 2009. Sphingosine 1-phosphate enhances Fc gamma receptor-mediated neutrophil activation and recruitment under flow conditions. *J. Immunol.* 183(4):2330-6.
- Fredholm BB, Ijzerman AP, Jacobson KA, Linden J, Müller CE. 2011. International Union of Basic and Clinical Pharmacology. LXXXI. Nomenclature and classification of adenosine receptors--an update. *Pharmacol Rev.* 63: 1-34.
- Fredholm BB, Irenius E, Kull B, Schulte G. 2001. Comparison of the potency of adenosine as an agonist at human adenosine receptors expressed in Chinese hamster ovary cells. *Biochem. Pharmacol.* 61: 443–448.
- Friedl P, Weigelin B. 2008. Interstitial leukocyte migration and immune function. *Nat. Immunol.* 9: 960-969.
- Gaipl US, Munoz LE, Grossmayer G, Lauber K, Franz S, Sarter K, Voll RE, Winkler T, Kuhn A, Kalden J, Kern P, Herrmann M. 2007. Clearance deficiency and systemic lupus erythematosus (SLE). *J. Autoimmun.* 28(2-3):114-21.
- Garabuczi É, Kiss B, Felszeghy S, Tsay GJ, Fésüs L, Szondy Z. 2013. Retinoids produced by macrophages engulfing apoptotic cells contribute to the appearance of transglutaminase 2 in apoptotic thymocytes. *Amino Acids.* 44(1):235-44.
- Garabuczi É, Sarang Z, Szondy Z. 2015. Glucocorticoids enhance prolonged clearance of apoptotic cells by upregulating liver X receptor, peroxisome proliferator-activated receptor- δ and UCP2. *Biochim. Biophys. Acta.* 1853(3):573-82.

- Gardai SJ, Bratton DL, Ogden CA, Henson PM. 2006.
Recognition ligands on apoptotic cells: a perspective.
J. Leukoc. Biol.; 79:896–903.
- Gerbod-Giannone MC, Li Y, Holleboom A, Han S, Hsu LC, Tabas I, Tall AR. 2006.
TNFalpha induces ABCA1 through NF-kappaB in macrophages and in phagocytes ingesting apoptotic cells.
Proc Natl Acad Sci U S A. 103(9):3112-7.
- Germain P, Chambon P, Eichele G, Evans RM, Lazar MA, Leid M, De Lera AR, Lotan R, Mangelsdorf DJ, Gronemeyer H. 2006.
International Union of Pharmacology. LXIII. Retinoid X receptors.
Pharmacol. Rev. 58(4):760-72.
- Gevrey JC, Isaac BM, Cox D. 2005.
Syk is required for monocyte/macrophage chemotaxis to CX3CL1 (Fractalkine).
J. Immunol. 175(6):3737-45.
- Giles KM, Ross K, Rossi AG, Hotchin NA, Haslett C, Dransfield I. 2001.
Glucocorticoid augmentation of macrophage capacity for phagocytosis of apoptotic cells is associated with reduced p130Cas expression, loss of paxillin/pyk2 phosphorylation, and high levels of active Rac.
J. Immunol. 167(2):976-86.
- Gregory C. 2009.
Cell biology: Sent by the scent of death.
Nature. 461:181–182.
- Greenberg ME, Sun M, Zhang R, Febbraio M, Silverstein R, Hazen SL. 2006.
Oxidized phosphatidylserine-CD36 interactions play an essential role in macrophage-dependent phagocytosis of apoptotic cells.
J. Exp. Med. 203(12):2613-25.
- Grimsley C, Ravichandran KS. 2003
Cues for apoptotic cell engulfment: eat-me, don't eat-me and come-get-me signals.
Trends Cell Biol. 13:648–656.
- Godson C, Mitchell S, Harvey K, Petasis NA, Hogg N, Brady HR. 2000.
Cutting edge: lipoxins rapidly stimulate nonphlogistic phagocytosis of apoptotic neutrophils by monocyte-derived macrophages.
J. Immunol. 164(4):1663-7.
- Gordon S. 2002.
Pattern recognition receptors: doubling up for the innate immune response.
Cell. 111(7):927-30.
- Gordon S, Taylor PR. 2005
Monocyte and macrophage heterogeneity.
Nat Rev Immunol: 953-964.

Gude DR, Alvarez SE, Paugh SW, Mitra P, Yu J, Griffiths R, Barbour SE, Milstien S, Spiegel S. 2008.

Apoptosis induces expression of sphingosine kinase 1 to release sphingosine-1-phosphate as a “come-and-get-me” signal.

FASEB J. 22:2629–2638.

Hait NC, Oskeritzian CA, Paugh SW, Milstien S, Spiegel S. 2006

Sphingosine kinases, sphingosine 1-phosphate, apoptosis and diseases.

Biochim. Biophys. Acta. 1758: 2016–2026.

Hammarberg C, Fredholm BB, Schulte G. 2004.

Adenosine A3 receptor-mediated regulation of p38 and extracellular regulated kinase ERK1/2 via phosphatidylinositol-3'-kinase.

Biochem. Pharmacol. 67(1):129-34.

Hamon Y, Trompier D, Ma Z, Venegas V, Pophillat M, Mignotte V, Zhou Z, Chimini G. 2006.

Cooperation between engulfment receptors: the case of ABCA1 and MEGF10.

PLoS One. 27;1:e120.

Hanayama R, Tanaka M, Miwa K, Shinohara A, Iwamatsu A, Nagata S. 2002.

Identification of a factor that links apoptotic cells to phagocytes.

Nature. 9;417(6885):182-7.

Hanayama R, Tanaka M, Miyasaka K, Aozasa K, Koike M, Uchiyama Y, Nagata S. 2004.

Autoimmune disease and impaired uptake of apoptotic cells in MFG-E8-deficient mice.

Science. 304(5674):1147-50.

Hart SP, Dransfield I, Rossi AG. 2008.

Phagocytosis of apoptotic cells.

Methods 44:280–5.

Haskó G, Pacher P, Deitch EA, Vizi ES. 2007.

Shaping of monocyte and macrophage function by adenosine receptors.

Pharmacol. Ther. 113, 264-75

Heit B, Tavener S, Raharjo E, Kubes P. 2002.

An intracellular hierarchy determines direction of migration in opposing chemotactic gradients.

J. Cell Biol. 159: 91-102.

Heit B, Robbins M, Downey CM, Guan Z, Colarusso P, Miller BJ, *et al.* 2008.

PTEN functions to 'prioritize' chemotactic cues and prevent 'distraction' in migrating neutrophils.

Nat. Immunol. 9: 743-752.

Henson PM, Bratton DL, Fadok VA. 2001.

Apoptotic cell removal.

Curr Biol. 11:R795–R805.

- Henson PM, Tudor RM. 2008
Apoptosis in the lung: induction, clearance and detection.
Am. J. Physiol. Lung Cell Mol. Physiol. 294(4):L601-11.
- Heyman RA, Mangelsdorf DJ, Dyck JA, Stein RB, Eichele G, Evans RM, Thaller C. 1992.
9-cis retinoic acid is a high affinity ligand for the retinoid X receptor.
Cell. 68(2):397-406.
- Hochreiter-Hufford A, Ravichandran KS. 2013.
Clearing the dead: apoptotic cell sensing, recognition, engulfment, and digestion.
Cold Spring Harb. Perspect Biol. 5(1):a008748.
- Hodge S, Hodge G, Jersmann H, Matthews G, Ahern J, Holmes M, Reynolds PN. 2008.
Azithromycin improves macrophage phagocytic function and expression of mannose receptor
in chronic obstructive pulmonary disease.
Am. J. Respir. Crit. Care Med. 178(2):139-48.
- Huber J, Vales A, Mitulovic G, Blumer M, Schmid R, Witztum JL, Binder BR, Leitinger N.
2002.
Oxidized membrane vesicles and blebs from apoptotic cells contain biologically
active oxidized phospholipids that induce monocyte-endothelial interactions.
Arterioscler. Thromb. Vasc. Biol. 22(1):101-7.
- Huq MD, Tsai NP, Gupta P, Wei LN. 2006.
Regulation of retinal dehydrogenases and retinoic acid synthesis by cholesterol metabolites.
EMBO J. 25(13):3203-13.
- Isfort K, Ebert F, Bornhorst J, Sargin S, Kardakaris R, Pasparakis M *et al.* 2011.
Real-time imaging reveals that P2Y₂ and P2Y₁₂ receptor agonists are not chemoattractants
and macrophage chemotaxis to complement C5a is phosphatidylinositol 3-kinase (PI3K) and
p38 mitogen-activated protein kinase (MAPK)-independent.
J. Biol. Chem. 286: 44776-44787.
- Joós G. 2014.
The role of the apoptotic cell death in the pathogenesis of the periodontitis.
<http://hdl.handle.net/2437/230302>
- Jones GE. 2000.
Cellular signaling in macrophage migration and chemotaxis.
J. Leukoc. Biol. 68(5):593-602.
- Jones B A., Beamer M , Ahmed S. Fractalkine/CX3CL1: A Potential New Target for
Inflammatory Diseases *Mol. Interv.* 2010; 10: 263–270.
- Joseph SB, Castrillo A, Laffitte BA, Mangelsdorf DJ, Tontonoz P. 2003
Reciprocal regulation of inflammation and lipid metabolism by liver X receptors.
Nat. Med. 9(2):213-9.

Joseph SB, McKilligin E, Pei L, Watson MA, Collins AR, Laffitte BA, Chen M, Noh G, Goodman J, Hagger GN, Tran J, Tippin TK, Wang X, Lusic AJ, Hsueh WA, Law RE, Collins JL, Willson TM, Tontonoz P. 2002.

Synthetic LXR ligand inhibits the development of atherosclerosis in mice.

Proc. Natl. Acad. Sci. U S A. 99(11):7604-9.

Kam RK, Deng Y, Chen Y, Zhao H. 2012.

Retinoic acid synthesis and functions in early embryonic development.

Cell Biosci. Mar 22;2(1):11.

Kamimura Y, Xiong Y, Iglesias PA, Hoeller O, Bolourani P, Devreotes PN. 2008

PIP3-independent activation of TorC2 and PKB at the cell's leading edge mediates chemotaxis. *Curr. Biol.* 18:1034-1043.

Kansra V, Groves C, Gutierrez-Ramos JC, Polakiewicz RD. 2001.

Phosphatidylinositol 3-kinase-dependent extracellular calcium influx is essential for CX(3)CR1-mediated activation of the mitogen-activated protein kinase cascade.

J. Biol. Chem. 276(34):31831-8.

Kawane K, Ohtani M, Miwa K, Kizawa T, Kanbara Y, Yoshioka Y, Yoshikawa H, Nagata S. 2006.

Chronic polyarthritis caused by mammalian DNA that escapes from degradation in macrophages.

Nature. 443(7114):998-1002.

Kinchen JM, Cabello J, Klingele D, Wong K, Feichtinger R, Schnabel H, Schnabel R, Hengartner MO. 2005.

Two pathways converge at CED10 to mediate actin rearrangement and corpse removal in *C. elegans*.

Nature 434(7029):93-9.

Kinoshita K, Kishimoto K, Shimazu H, Nozaki Y, Sugiyama M, Ikoma S, Funauchi M. 2010. Successful treatment with retinoids in patients with lupus nephritis.

Am. J. Kidney Dis. 55(2):344-7.

Kinoshita K, Yoo BS, Nozaki Y, Sugiyama M, Ikoma S, Ohno M, Funauchi M, Kanamaru A. 2003.

Retinoic acid reduces autoimmune renal injury and increases survival in NZB/W F1 mice.

J. Immunol. 170(11):5793-8.

Kiss I, Oskolás H, Tóth R, Bouillet P, Tóth K, Fülöp A, Scholtz B, Ledent C, Fésüs L, Szondy Z. 2006.

Adenosine A2A receptor-mediated cell death of mouse thymocytes involves adenylate cyclase and Bim and is negatively regulated by Nur77.

Eur. J. Immunol. 36(6):1559-71.

Kiss I, Rühl R, Szegezdi E, Fritzsche B, Tóth B, Pongrácz J, Perlmann T, Fésüs L, Szondy Z. 2008.

Retinoid receptor-activating ligands are produced within the mouse thymus during postnatal development.

Eur. J. Immunol. 38(1):147-55.

Korns D, Frasch SC, Fernandez-Boyanapalli R, Henson PM, Bratton DL. 2011.

Modulation of macrophage efferocytosis in inflammation.

Front. Immunol. 2:57.

Köröskényi K, Duró E, Pallai A, Sarang Z, Kloor D, Ucker DS, Beceiro S, Castrillo A, Chawla A, Ledent CA, Fésüs L, Szondy Z. 2011.

Involvement of adenosine A2A receptors in engulfment-dependent apoptotic cell suppression of inflammation.

J. Immunol. 186: 7144–7155.

Koszalka P, Gołńska M, Stanisławowski M, Urban A, Stasiłój G, Majewski M, Wierzbicki P, Składanowski AC, Bigda J. 2015.

CD73 on B16F10 melanoma cells in CD73-deficient mice promotes tumor growth, angiogenesis, neovascularization, macrophage infiltration and metastasis.

Int. J. Biochem. Cell Biol. 69:1-10.

Koszalka P, Gołńska M, Urban A, Stasiłój G, Stanisławowski M, Majewski M, Składanowski AC, Bigda J. 2016.

Specific Activation of A3, A2A and A1 Adenosine Receptors in CD73-Knockout Mice Affects B16F10 Melanoma Growth, Neovascularization, Angiogenesis and Macrophage Infiltration.

PLoS One. 11(3):e0151420.

Kronlage M, Song J, Sorokin L, Isfort K, Schwerdtle T, Leipziger J, Robaye B, Conley PB, Kim HC, Sargin S, Schön P, Schwab A, Hanley PJ. 2010.

Autocrine purinergic receptor signaling is essential for macrophage chemotaxis.

Sci. Signal. 3(132):ra55.

Lauber K, Blumenthal SG, Waibel M, Wesselborg S. 2004.

Clearance of apoptotic cells: getting rid of the corpses.

Mol. Cell. 14:277–287.

Lauber K, Bohn E, Kröber SM, Xiao Y-J, Blumenthal SG, Lindemann RK. 2003.

Apoptotic cells induce migration of phagocytes via caspase-3-mediated release of a lipid attraction signal.

Cell. 113: 717–730.

Ledent C, Vaugeois JM, Schiffmann SN, Pedrazzini T, El Yacoubi M, Vanderhaeghen JJ, Costentin J, Heath JK, Vassart G, Parmentier M. 1997.

Aggressiveness, hypoalgesia and high blood pressure in mice lacking the adenosine A2a receptor.

Nature. 388(6643):674-8.

- Lee HT, Ota-Setlik A, Xu H, D'Agati VD, Jacobson MA, Emala CW. 2003.
A3 adenosine receptor knockout mice are protected against ischemia- and myoglobinuria-induced renal failure.
Am. J. Physiol. Renal Physiol. 284(2):F267-73.
- Lewis MJ, Botto M. 2006.
Complement deficiencies in humans and animals: links to autoimmunity.
Autoimmunity. 39(5):367-78.
- Licht R, Dieker JW, Jacobs CW, Tax WJ, Berden JH. 2004.
Decreased phagocytosis of apoptotic cells in diseased SLE mice.
J. Autoimmun. 22(2):139-45.
- Li Y, Bolten C, Bhat BG, Woodring-Dietz J, Li S, Prayaga SK, Xia C, Lala DS. 2002.
Induction of human liver X receptor alpha gene expression via an autoregulatory loop mechanism.
Mol. Endocrinol. 16(3):506-14.
- Li S, Sun Y, Liang CP, Thorp EB, Han S, Jehle AW, Saraswathi V, Pridgen B, Kanter JE, Li R, Welch CL, Hasty AH, Bornfeldt KE, Breslow JL, Tabas I, Tall AR. 2009.
Defective phagocytosis of apoptotic cells by macrophages in atherosclerotic lesions of ob/ob mice and reversal by a fish oil diet.
Circ. Res. 105(11):1072-82.
- Mangelsdorf DJ, Evans RM. 1995.
The RXR heterodimers and orphan receptors.
Cell. 83(6):841-50.
- Martinez FO, Gordon S. 2014
The M1 and M2 paradigm of macrophage activation: time for reassessment.
F1000Prime Rep. 6:13.
- Mattson MP. 2000
Apoptosis in neurodegenerative disorders.
Nat. Rev. Mol. Cell Biol. 1(2):120-9.
- McColl A, Bournazos S, Franz S, Perretti M, Morgan BP, Haslett C, Dransfield I. 2009.
Glucocorticoids induce protein S-dependent phagocytosis of apoptotic neutrophils by human macrophages.
J. Immunol. 183(3):2167-75.
- McCubrey AL, Curtis JL. 2013.
Efferocytosis and lung disease.
Chest. 143(6):1750-7.
- Moise AR, Alvarez S, Domínguez M, Alvarez R, Golczak M, Lobo GP, von Lintig J, de Lera AR, Palczewski K. 2009.
Activation of retinoic acid receptors by dihydroretinoids.
Mol. Pharmacol. 76(6):1228-37.

- Moise AR, Kuksa V, Blaner WS, Baehr W, Palczewski K. 2005.
Metabolism and transactivation activity of 13,14-dihydroretinoic acid.
J. Biol. Chem. 280(30):27815-25.
- Moise AR, Kuksa V, Imanishi Y, Palczewski K. 2004.
Identification of all-trans-retinol:all-trans-13,14-dihydroretinol saturase.
J. Biol. Chem. 279(48):50230-42.
- Moise AR, Lobo GP, Erokwu B, Wilson DL, Peck D, Alvarez S, Domínguez M, Alvarez R, Flask CA, de Lera AR, von Lintig J, Palczewski K. 2010.
Increased adiposity in the retinol saturase-knockout mouse.
FASEB J. 24(4):1261-70.
- Monks J, Smith-Steinhart C, Kruk ER, Fadok VA, Henson PM. 2008.
Epithelial cells remove apoptotic epithelial cells during post-lactation involution of the mouse mammary gland.
Biol Reprod. 78(4):586-94.
- Montalbán Del Barrio I, Penski C, Schlausa L, Stein RG, Diessner J, Wöckel A, Dietl J, Lutz MB, Mittelbronn M, Wischhusen J, Häusler SF. 2016.
Adenosine-
generating ovarian cancer cells attract myeloid cells which differentiate into adenosine-
generating tumorassociated macrophages - a self-amplifying, CD39- and CD73-
dependent mechanism for tumor immune escape.
J. Immunother. Cancer. 4:49
- Moon C, Lee YJ, Park HJ, Chong YH, Kang JL. 2010.
N-acetylcysteine inhibits RhoA and promotes apoptotic cell clearance during intense lung inflammation.
Am. J. Respir. Crit. Care Med. 181(4):374-87.
- Moore WT Jr, Murtaugh MP, Davies PJ. 1984.
Retinoic acid-induced expression of tissue transglutaminase in mouse peritoneal macrophages.
J. Biol. Chem. 259(20):12794-802.
- Morimoto K, Janssen WJ, Fessler MB, McPhillips KA, Borges VM, Bowler RP, Xiao YQ, Kench JA, Henson PM, Vandivier RW. 2006.
Lovastatin enhances clearance of apoptotic cells (efferocytosis) with implications for chronic obstructive pulmonary disease.
J. Immunol. 176(12):7657-65.
- Mosser DM, Edwards JP. 2008.
Exploring the full spectrum of macrophage activation.
Nat. Rev. Immunol. 958-969.
- Moura R, Tjwa M, Vandervoort P, Van Kerckhoven S, Holvoet P, Hoylaerts MF. 2008
Thrombospondin-1 deficiency accelerates atherosclerotic plaque maturation in ApoE^{-/-} mice.
Circ. Res. 103(10):1181-9.

Mukundan L, Odegaard JI, Morel CR, Heredia JE, Mwangi JW, Ricardo-Gonzalez RR, Goh YP, Eagle AR, Dunn SE, Awakuni JU, Nguyen KD, Steinman L, Michie SA, Chawla A. 2009.

PPAR-delta senses and orchestrates clearance of apoptotic cells to promote tolerance.
Nat. Med. 15(11):1266-72.

Muñoz LE, Lauber K, Schiller M, Manfredi AA, Herrmann M. 2010.
The role of defective clearance of apoptotic cells in systemic autoimmunity.
Nat. Rev. Rheumatol. 6(5):280-9.

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

Nakaya M, Tanaka M, Okabe Y, Hanayama R, Nagata S. 2006.
Opposite effects of rho family GTPases on engulfment of apoptotic cells by macrophages.
J. Biol. Chem. 281(13):8836-42.

Nich C, Takakubo Y, Pajarinen J, Ainola M, Salem A, Sillat T, Rao AJ, Raska M, Tamaki Y, Takagi M, Kontinen YT, Goodman SB, Gallo J. 2013.
Macrophages-Key cells in the response to wear debris from joint replacements.
J. Biomed. Mater. Res. A. 101(10):3033-45.

O'Brien BA, Fieldus WE, Field CJ, Finegood DT. 2002.
Clearance of apoptotic beta-cells is reduced in neonatal autoimmune diabetes-prone rats.
Cell Death Differ. 9(4):457-64.

Odaka C, Mizuochi T. 2002.
Macrophages are involved in DNA degradation of apoptotic cells in murine thymus after administration of hydrocortisone.
Cell Death Differ. 9(2):104-12.

Otani A, Ishihara S, Aziz MM, Oshima N, Mishima Y, Moriyama I, Yuki T, Amano Y, Ansary MM, Kinoshita Y. 2012
Int. J. Mol. Med. 29(3):349-56.

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

Park D, Han CZ, Elliott MR, Kinchen JM, Trampont PC, Das S, Collins S, Lysiak JJ, Hoehn KL, Ravichandran KS. 2011.
Continued clearance of apoptotic cells critically depends on the phagocyte Ucp2 protein.
Nature. 477(7363):220-4.

Park D, Hochreiter-Hufford A, Ravichandran KS. 2009
The phosphatidylserine receptor TIM-4 does not mediate direct signaling.
Curr. Biol. 19(4):346-51.

Park SY, Jung MY, Kim HJ, Lee SJ, Kim SY, Lee BH, Kwon TH, Park RW, Kim IS. 2008. Rapid cell corpse clearance by stabilin-2, a membrane phosphatidylserine receptor. *Cell Death Differ.* 15(1):192-201.

Pérez de Lema G, Lucio-Cazaña FJ, Molina A, Luckow B, Schmid H, de Wit C, Moreno-Manzano V, Banas B, Mampaso F, Schlöndorff D. 2004. Retinoic acid treatment protects MRL/lpr lupus mice from the development of glomerular disease. *Kidney Int.* 66(3):1018-28.

Peter C, Waibel M, Radu CG, Yang LV, Witte ON, Schulze-Osthoff K, Wesselborg S, Lauber K. Migration to apoptotic “find-me” signals is mediated via the phagocyte receptor G2A. *J. Biol. Chem.* 2008; 283: 5296–5305.

Ravichandran KS. Find-me and eat-me signals in apoptotic cell clearance: progress and conundrums. *J Exp Med.* 2010; 207(9):1807-17.

Ravichandran KS, Lorenz U. 2007. Engulfment of apoptotic cells: signals for a good meal. *Nat. Rev. Immunol.* 7(12):964-74.

Rezzani R, Bonomini F, Rodella LF. 2008. Histochemical and molecular overview of the thymus as site for T-cells development. *Prog. Histochem. Cytochem.* 43(2):73-120.

Rébé C, Raveneau M, Chevriaux A, Lakomy D, Sberna AL, Costa A, Bessède G, Athias A, Steinmetz E, Lobaccaro JM, Alves G, Menicacci A, Vachenc S, Solary E, Gamber P, Masson D. 2009. Induction of transglutaminase 2 by a liver X receptor/retinoic acid receptor alpha pathway increases the clearance of apoptotic cells by human macrophages. *Circ. Res.* 105(4):393-401.

Resta R, Hooker SW, Laurent AB, Jamshedur Rahman SM, Franklin M, Knudsen TB, Nadon NL, Thompson LF. 1997. Insights into thymic purine metabolism and adenosine deaminase deficiency revealed by transgenic mice overexpressingecto-5'-nucleotidase (CD73). *J. Clin. Invest.* 99(4):676-83.

Rhinn M, Dollé P. 2012. Retinoic acid signalling during development. *Development;* 139(5):843-58.

Ridley AJ, Schwartz MA, Burridge K, Firtel RA, Ginsberg MH, Borisy G *et al.* 2003. Cell migration: integrating signals from front to back. *Science;* 302:1704-0709.

Ring S, Pushkarevskaya A, Schild H, Probst HC, Jendrosseck V, Wirsdörfer F, Ledent C, Robson SC, Enk AH, Mahnke K. 2015.

Regulatory T cell-derived adenosine induces dendritic cell migration through the Epac-Rap1 pathway.

J. Immunol. 194(8):3735-44.

Rosen H, Gonzalez-Cabrera PJ, Sanna MG, Brown S. 2009.

Sphingosine 1-phosphate receptor signaling.

Annu. Rev. Biochem. 78:743-68.

Rossant J, Zirngibl R, Cado D, Shago M, Giguère V. 1991.

Expression of a retinoic acid response element

hsplacZ transgene defines specific domains of transcriptional activity during mouse embryogenesis.

Genes Dev. 5(8):1333-44.

Roszer T, Menéndez-Gutiérrez MP, Lefterova MI, Alameda D, Núñez V, Lazar MA, Fischer T, Ricote M. 2011.

Autoimmune kidney

disease and impaired engulfment of apoptotic cells in mice with macrophage peroxisome proliferator-activated receptor gamma or retinoid X receptor alpha deficiency.

J. Immunol. 186(1):621-31.

Rühl R. 2006

Method to determine 4-oxo

retinoic acids, retinoic acids and retinol in serum and cell extracts by liquid chromatography/diode-array detection atmospheric pressure chemical ionisation tandem mass spectrometry.

Rapid Commun Mass Spectrom. 20(16):2497-504.

Sándor K, Daniel B, Kiss B, Kovács F, Szondy Z. 2016.

Transcriptional control of transglutaminase 2 expression in mouse apoptotic thymocytes.

Biochim Biophys Acta. 1859(8):964-74.

Sándor K, Pallai A, Duró E, Legendre P, Couillin I, Sághy T, Szondy Z. 2016

Adenosine produced from adenine nucleotides through

an interaction between apoptotic cells and engulfing macrophages contributes to the appearance of transglutaminase 2 in dying thymocytes.

Amino Acids. [Epub ahead of print]

Sarang Z, Garabuczi É, Joós G, Kiss B, Tóth K, Rühl R, Szondy Z. 2013.

Macrophages engulfing apoptotic thymocytes produce retinoids to promote selection, differentiation, removal and replacement of double positive thymocytes.

Immunobiology 218:1354-1360

Savill J, Dransfield I, Gregory C, Haslett C. 2002.

A blast from the past: clearance of apoptotic cells regulates immune responses.

Nat. Rev. Immunol. 2: 965-975.

Savill J, Fadok V, Henson P, Haslett C. 1993.

Phagocyte recognition of cells undergoing apoptosis.

Immunol Today 14:131-136.

Savill J, Hogg N, Ren Y, Haslett C. 1992.
Thrombospondin cooperates with CD36 and
the vitronectin receptor in macrophage recognition of neutrophils undergoing apoptosis.
J. Clin. Invest. 90(4):1513-22.

Schif-Zuck S, Gross N, Assi S, Rostoker R, Serhan CN, Ariel A. 2011.
Saturated-efferocytosis generates pro-resolving CD11b low macrophages: modulation by
resolvins and glucocorticoids.
Eur. J. Immunol. 41(2):366-79.

Schutters K, Kusters DH, Chatrou ML, Montero-Melendez T, Donners M, Deckers NM,
Krysko DV, Vandenabeele P, Perretti M, Schurgers LJ, Reutelingsperger CP. 2013.
Cell surface-expressed phosphatidylserine as therapeutic target to enhance phagocytosis of
apoptotic cells.
Cell Death Differ. 20(1):49-56.

Scott RS¹, McMahon EJ, Pop SM, Reap EA, Caricchio R, Cohen PL, Earp HS, Matsushima
GK. 2001.
Phagocytosis and clearance of apoptotic cells is mediated by MER.
Nature. 411(6834):207-11.

Seimon T, Tabas I. 2009.
Mechanisms and consequences of macrophage apoptosis in atherosclerosis.
J. Lipid Res. 50 Suppl:S382-7.

Sen P, Wallet MA, Yi Z, Huang Y, Henderson M, Mathews CE, Earp HS, Matsushima G,
Baldwin AS Jr, Tisch RM. 2007
Apoptotic cells induce Mer tyrosine kinase-dependent blockade of NF-kappaB activation in
dendritic cells.
Blood. 109, 653-60

Serbina NV, Jia T, Hohl TM, Pamer EG. 2008.
Monocyte-mediated defense against microbial pathogens.
Annu. Rev. Immunol. 26:421-52.

Soroosh P, Doherty TA, Duan W, Mehta AK, Choi H, Adams YF, Mikulski Z, Khorram N,
Rosenthal P, Broide DH, Croft M. 2013.
Lung resident tissue macrophages generate Foxp3⁺ regulatory T cells and promote airway tol-
erance.
J. Exp. Med. 210(4):775-88.

Stuart LM, Ezekowitz RA. 2005.
Phagocytosis: elegant complexity.
Immunity. 22(5):539-50.

Su HP, Nakada-Tsukui K, Tosello-Tramont AC, Li Y, Bu G, Henson PM, Ravichandran KS.
2002.

Interaction of CED-6/GULP, an adapter protein involved in engulfment of apoptotic cells with CED-1 and CD91/low density lipoprotein receptor-related protein (LRP).
J. Biol. Chem. 277: 11772–11779.

Sodhi RK, Singh N. 2013.
Liver X receptors: emerging therapeutic targets for Alzheimer's disease.
Pharmacol. Res. 72:45-51

Szondy Z, Garabuczi E, Joós G, Tsay GJ, Sarang Z. 2014.
Impaired clearance of apoptotic cells in chronic inflammatory diseases: therapeutic implications.
Front. Immunol. 1;5:354.

Szondy Z, Korponay-Szabó I, Király R, Fésüs L. 2011.
Transglutaminase 2 dysfunctions in the development of autoimmune disorders: celiac disease and TG2-/- mouse.
Adv Enzymol Relat Areas Mol Biol. 78:295-345.

Szondy Z, Molnar P, Nemes Z, Boyiadzis M, Kedei N, Tóth R, Fésüs L. 1997
Differential expression of tissue transglutaminase during
in vivo apoptosis of thymocytes induced via distinct signalling pathways.
FEBS Lett. 404(2-3):307-13.

Szondy Z, Sarang Z, Molnar P, Nemeth T, Piacentini M, Mastroberardino PG, Falasca L, Aeschlimann D, Kovacs J, Kiss I, Szegezdi E, Lakos G, Rajnavolgyi E, Birckbichler PJ, Melino G, Fesus L. 2003.
Transglutaminase 2-/- mice reveal a phagocytosis-associated crosstalk between macrophages and apoptotic cells.
Proc. Natl. Acad. Sci. U S A. 100(13):7812-7.

Tanaka M, Miyake Y. 2007.
Apoptotic cell clearance and autoimmune disorder.
Curr. Med. Chem. 14(27):2892-7.

Tassiulas I, Park-Min KH, Hu Y, Kellerman L, Mevorach D, Ivashkiv LB. 2007
Apoptotic cells inhibit LPS-induced cytokine and chemokine production and IFN responses in macrophages.
Hum. Immunol. 68, 156-64

Thorp E, Cui D, Schrijvers DM, Kuriakose G, Tabas I. 2008.
Mertk receptor mutation reduces efferocytosis efficiency and promotes apoptotic cell accumulation and plaque necrosis in atherosclerotic lesions of apoe-/- mice.
Arterioscler. Thromb. Vasc. Biol. 28(8):1421-8.

Tóth, B., E. Garabuczi, Z. Sarang, G. Vereb, G. Vámosi, D. Aeschlimann, B. Blaskó, B. Be'csi, F. Erdődi, A. Lacy-Hulbert, et al. 2009.
Transglutaminase 2 is needed for the formation of an efficient phagocyte portal in macrophages engulfing apoptotic cells.
J. Immunol. 182: 2084–2092.

- Truman LA, Ford CA, Pasikowska M, Pound JD, Wilkinson SJ, Dumitriu IE *et al.* 2008. CX3CL1fractalkine is released from apoptotic lymphocytes to stimulate macrophage chemotaxis. *Blood*. 112: 5026–5036.
- Tzamelis I, Chua SS, Cheskis B, Moore DD. 2003. Complex effects of rexinoids on ligand dependent activation or inhibition of the xenobiotic receptor, CAR. *Nuclear Receptor*. 1:2.
- van Furth R, Cohn ZA. 1968. The origin and kinetics of mononuclear phagocytes. *J. Exp. Med.* 128(3):415-35.
- Verbovetski I, Bychkov H, Trahtemberg U, Shapira I, Hareuveni M, Ben-Tal O, Kutikov I, Gill O, Mevorach D. 2002. Opsonization of apoptotic cells by autologous iC3b facilitates clearance by immature dendritic cells, down-regulates DR and CD86, and up-regulates CC chemokine receptor 7. *J. Exp. Med.* 196(12):1553-61.
- Voll RE, Herrmann M, Roth EA, Stach C, Kalden JR, Girkontaite I. 1997. Immunosuppressive effects of apoptotic cells. *Nature* 390, 350-1
- Walsh SJ, Algert C, Gregorio DI, Reisine ST, Rothfield NF. 1995. Divergent racial trends in mortality from systemic lupus erythematosus. *J. Rheumatol.* 22(9):1663-8.
- Wan E, Yeap XY, Dehn S, Terry R, Novak M, Zhang S, Iwata S, Han X, Homma S, Drosatos K, Lomasney J, Engman DM, Miller SD, Vaughan DE, Morrow JP, Kishore R, Thorp EB. 2013. Enhanced efferocytosis of apoptotic cardiomyocytes through myeloid-epithelial-reproductive tyrosine kinase links acute inflammation resolution to cardiac repair after infarction. *Circ. Res.* 113(8):1004-12.
- Yamaguchi H, Maruyama T, Urade Y, Nagata S. 2014. Immunosuppression via adenosine receptor activation by adenosine monophosphate released from apoptotic cells. *Elife*. 3:e02172.
- Yamamoto T, Oishi K, Yoshimine H, Tsuchihashi Y, Matsushima K, Nagatake T. 2003. Fourteen-member macrolides promote the phosphatidylserine receptor-dependent phagocytosis of apoptotic neutrophils by alveolar macrophages. *Antimicrob. Agents Chemother.* 47(1):48-53.
- Yang LV, Radu CG, Wang L, Riedinger M, Witte ON. 2005. Gi-independent macrophage chemotaxis to lysophosphatidylcholine via the immunoregulatory GPCR G2A. *Blood*. 105(3):1127-34.

- Yegutkin GG. 2008.
Nucleotide- and nucleoside-converting ectoenzymes: Important modulators of purinergic signalling cascade.
Biochim Biophys Acta. 1783(5):673-94.
- Yoshikawa T, Shimano H, Amemiya-Kudo M, Yahagi N, Hasty AH, Matsuzaka T, Okazaki H, Tamura Y, Iizuka Y, Ohashi K, Osuga J, Harada K, Gotoda T, Kimura S, Ishibashi S, Yamada N. 2001.
Identification of liver X receptor-retinoid X receptor as an activator of the sterol regulatory element-binding protein 1c gene promoter.
Mol. Cell. Biol. 21(9):2991-3000.
- Zahuczky G, Kristóf E, Majai G, Fésüs L. 2011.
Differentiation and glucocorticoid regulated apopto-phagocytic gene expression patterns in human macrophages. Role of MERTK in enhanced phagocytosis.
PLoS One.;6(6):e21349.

10. PUBLICATIONS



UNIVERSITY OF DEBRECEN
UNIVERSITY AND NATIONAL LIBRARY



Registry number:
Subject:

DEENK/144/2017.PL
PhD Publikációs Lista

Candidate: Gergely Joós
Neptun ID: VMCJC9
Doctoral School: Doctoral School of Dental Sciences

List of publications related to the dissertation

1. **Joós, G.**, Jakim, J., Kiss, B., Szamosi, R., Papp, T., Felszeghy, S. B., Sággy, T., Szemán-Nagy, G., Szondy, Z.: Involvement of adenosine A3 receptors in the chemotactic navigation of macrophages towards apoptotic cells.
Immunol. Lett. 183, 62-72, 2017.
DOI: <http://dx.doi.org/10.1016/j.imlet.2017.02.002>
IF: 2.483 (2015)
2. Szondy, Z., Garabuczi, É., **Joós, G.**, Tsay, G. J., Sarang, Z.: Impaired clearance of apoptotic cells in chronic inflammatory diseases: therapeutic implications.
Front. Immunol. 5, 1-8, 2014.
DOI: <http://dx.doi.org/10.3389/fimmu.2014.00354>
3. Sarang, Z., **Joós, G.**, Garabuczi, É., Rühl, R., Gregory, C. D., Szondy, Z.: Macrophages engulfing apoptotic cells produce nonclassical retinoids to enhance their phagocytic capacity.
J. Immunol. 192 (12), 5730-5738, 2014.
DOI: <http://dx.doi.org/10.4049/jimmunol.1400284>
IF: 4.922





List of other publications

4. Sarang, Z., Garabuczi, É., **Joós, G.**, Kiss, B., Tóth, K. Á., Rühl, R., Szondy, Z.: Macrophages engulfing apoptotic thymocytes produce retinoids to promote selection, differentiation, removal and replacement of double positive thymocytes.
Immunobiology. 218 (11), 1354-1360, 2013.
DOI: <http://dx.doi.org/10.1016/j.imbio.2013.06.009>
IF: 3.18

Total IF of journals (all publications): 10,585

Total IF of journals (publications related to the dissertation): 7,405

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.

22 May, 2017

