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

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

by

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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 and its unique, specific molecular features 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.

1.2.The role of efferocytosis in a healthy organism

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. Efferocytosis is closely related to the regulation of inflammation to the maintenance of tissue homeostasis. It has determining role in tissue regeneration (Biancheri et al., 2014) and differentiation (Sarang et al., 2013). 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

Most of the cell types are capable of engulfing dead cells. However, 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 evolutionally but also between different cell types (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). Moreover, most of the experimental data related to efferocytosis research is derived from studies, in which murine or human periferial and/or bone marrow derived macrophages are used. In the further sections of the introduction chapter (and also in the Results part, where I present our own experimental data) efferocytosis is going to refer on to macrophage related efferocytosis or the given cell type will be specifically indicated.

1.4.Steps of efferocytosis

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-infammatory cytokine production in the engulfing phagocyte **(**Savill et al., 2002).

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 like lysophosphatidylcholine **(**LPC), sphingosine-1-phosphate

(S1P), fractalkine (FKN) or adenosine triphosphate (ATP). Whether these "find-me" signals are always formed, or whether they are released in a cell type specific manner is not known yet. 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. 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 receptors are designated adenosine 1 receptor (A1R), adenosine 2A receptor (A2AR), adenosine 2B receptor (A2BR) and adenosine 3 receptor (A3R). Stimulation of the different adenosine receptors is determined by the concentration of adenosine and the cell surface receptor density of the given receptor. Peritoneal macrophages have been reported to express adenosine A2AR, A2BR and A3R receptors (Kronlage et al., 2010).

After phagocytes are recruited to the close proximity of dying cells, dead cells are identified by the phagocytes (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" (Szondy et al., 2011).

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 cells (Fadok et al., 2003). Many of the macrophage receptors recognize PS or its oxidized form binding to it directly or indirectly via bridging molecules. Macrophages are capable of both expressing the cell surface receptors and releasing the bridging molecules for the recognition and engulfment of apoptotic cells. Previous work in our laboratory has shown that transglutaminase 2 (TG2) acting as a coreceptor for integrin β3 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).

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 enhance 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). Triggering of these receptors leads to enhanced

expression of phagocytic receptors and bridging molecules. 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 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. 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). 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 RARα (Rébé et al., 2009). In addition, ATP-binding cassette transporter (ABCA1) and Mer tyrosine kinase (Mertk) – two efferocytosis related genes- were also found to be a direct LXR target genes (Costet et al., 2000; 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).

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 retinoic acid production, are the engulfing macrophages. Indeed, apoptotic cell uptake *in vitro* or LXR ligation by synthetic agonist also triggered the expression of RALDHs in macrophages indicating that the uptake of apoptotic cells is coupled to retinoid synthesis (Garabuczi et al., 2013).

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. 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 efferocitotic 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 hallmark feature of apoptotic cell clearance, (on the) contrary to necrotic cell clearance, is the non-inflammatory, tolerogenic nature of the process. 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 immunosupressive effects that involves inhibition of pro-inflammatory cytokine release and secretion of anti-inflammatory compounds (Fadok et al., 1998).
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 immunosupressive 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).

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. 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). Several other pathological disorders including lung and airway defects (COPD, asthma and cystic fibrosis) (Henson et al., 2008), atherosclerosis (Seimon et al., 2009), neurodegenerative diseases (Parkinson's, Alzheimer's, and Huntington's disease (Mattson et al., 2000)), type 2 diabetes and obesity (O'brien et al., 2002; Li et al., 2009) and periodontitis (Joós G., 2015) have been associated with defective efferocytosis.

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.

Different therapeutic approaches have been already tested with promising results. These include strategies to affect the recognition and binding of apoptotic cells to the phagocytes or to target lipid-sensing nuclear receptors with the aim of increasing the expression of phagocytic receptors or their bridging molecules. Affecting the Rac-1/RhoA balance and to alter the membrane lipid composition of macrophages both bear exciting possibilities that can contribute to future therapies of the above mentioned diseases (Szondy et al., 2014).

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 β3 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 retionoid, which's producion 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 bread 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. $2x10^5$ 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% CO_2 on 20-mm glass bottom dishes (In Vitro Scientfic, Sunnyvale, California, USA). After 4 h incubation, the non-adherent cells were washed away. The cells were than 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 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 administrating 1 μM 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 $6x10^6$ apoptotic thymocytes. Through the Whatman GF/C paper filter the apoptotic thymocyte-derived find-me signals smaller than 0.7 μm, 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 μg/ml streptomycin at 37°C in 5% CO2. Non-adherent cells were washed away every second day. BMDMs were treated with 1 μM GW3965 (Glaxo Smith Kline, Budapest, Hungary), a synthetic LXR agonist, 1 μM LG268 (kind gift of John Schwabe, University of Leicester), a synthetic RXR agonist, 1 μM 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 25μM 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 μg/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% CO_2 /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) CO_2 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 (×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 system[36] 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 substraction 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 paralell 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, than 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% H_2O_2 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 ^{o}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 μM 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 μM 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 glycerinaldehyde-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 Immobilion-P transfer membrane (Millipore, Budapest, Hungary) and were probed with rabbit polyclonal anti-TG2 (SantaCruz, Heidelberg, Germany), anti-mouse LXRα/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 β3 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 β3 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, 1.5x106 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 µl OptiMem, 6 µl TransITsiQUEST reagent and 37.5 µl (1µM) scrambled or LXRα 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 oC. 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 µl of methanol, vortexed, shaked, diluted with 40 µl 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 ± 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

Proper i*n vitro* chemotactic migration of macrophages toward apoptotic cells is affected by adenosine receptor signaling

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 with an average velocity of 0.3±0.19 μm/min. 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.

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. While the loss of individual adenosine receptor signaling did not affect the migration of macrophages in RPMI alone, 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 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. 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. 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.

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 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, NECA slightly increased the chemotactic index of A3R null macrophages migrating toward the apoptotic cells. 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.

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 5'-N-

ethylcarboxamidoadenosine (NECA), a non metabolisable analogue of adenosine. NECA was administered at 10mM concentration to activate all the adenosine receptors. 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).

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. Similar to wild type cells, removal of purine nucleotides and nuleosides resulted in lower migration velocity (0.1 ± 0.04 μm/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.

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. 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.

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. The difference was more pronounced at the lowest injected apoptotic cell number ($2x10^6$), where two times more wild types macrophages were engulfing than A3R null cells. When the injected apoptotic cells number was increased to $30x10^6$, the percentage of wild type engulfing macrophages also increased (from 21±4% to 93.5±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.

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, 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 and the accumulation of apoptotic Annexin V+ thymocytes 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. 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 3h, 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

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, only ATRA was capable of significantly inducing the engulfment of apoptotic cells. 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.

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. From the additional phagocytosis-related molecules tested, retinoids did not have significant effect on the expression of integrin β1, MFG-E8, thrombospondin, CD91, or CD36, whereas the expression levels of scavenger receptor A, integrin α_v, β_3, and β_5 were decreased.

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 and the percentage phagocytosis. 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. 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. This indicates that very likely upregulation of all the ATRA-sensitive phagocytic receptors together results in enhanced engulfment also in the abscence of CD14.

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, PPARγ, and PPARδ) 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. 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. 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.

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. 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.

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, and that of LXRα, 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). 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 2 h. However, the genes (TG2, C1q, stabilin-2, Tim4, and CD14) found to be induced by the pan RAR agonist AM580 were not induced by GW3965 at 2 h. 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.

If, however, we determined the expression of the same genes 24 h later, we found that all the phagocytic receptors, which were induced by ATRA or 9cRA, 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. 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 synthetized 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 RARα, in line with a previous publication, which has shown that RARα is an LXR target gene (Rébé et al., 2009).

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. 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. DEAB tested at 24 h 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 synthetized 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 LXRα/β silencing as well. Silencing of LXRs efficiently reduced the protein levels of LXRα and LXRβ. Silencing of LXRs prevented the induction of SREBP-1c, Mertk, Tim4, and TG2 by GW3965.

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

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.

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 (∼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. 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. Retinol saturase was expressed by the mouse thymus, and its expression was significantly increased after dexamethasone injection.

5. DISCUSSION

Cell-turnover is coupled phisiologically in most of the tissues to programmed cell death. When the clearence 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 clearence.

Chronic inflammation of periodontal tissues, called periodontitis is a morbiditiy with such characteristics. It is a classical multifactorial disease, that definitely can not proceed in the abscence 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 basicly 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 patways 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 synthetized (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 p38α 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 p38α 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 prooved 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 clearence 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 clearence 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 b3 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 crutial 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 PPARδ 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 collague 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.

We wanted to exactly identify the retinoid, which is produced during effrocytosis 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. 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 ont he 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.

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, Gaipl 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 engulment 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

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8. PUBLICATIONS

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2. Szondy, Z., Garabuczi, É., **Joós, G.**, Tsay, G. J., Sarang, Z.: Impaired clearance of apoptotic cells in chronic inflammatory diseases: therapeutic implications.
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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.
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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.
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