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

The effect of dihydroretinol during monocyte-macrophage differentiation and the lack of heme oxygenase-1 on the efferocytosis process

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1. ABSTRACT

Our body is renewed in every single day: harmful, redundant, aged or useless cells are eliminated for maintaining tissue homeostasis. Uncleared apoptotic corpses can promote inflammation, so their rapid and non-immunogenic removal by macrophages (efferocytosis) is a key step in maintaining tissue integrity. Defective efferocytosis has been demonstrated to associate with the pathogenesis of various inflammatory disorders such as asthma, systemic lupus erythematosus or rheumatoid arthritis. Macrophages play a crucial role in the efferocytosis process: they sense, recognize, engulf and digest the dying or dead cells. Normally efferocytosis is an immunologically silent process, however, if dead cells are removed in an inflammatory environment, the efferocytosis process promotes the phenotypic change of macrophages to be converted from pro-inflammatory to regenerative cells that drive the resolution of inflammation and organize tissue regeneration.

My research has focussed on bone marrow-derived macrophages which, *in vivo*, are generated from bone marrow-derived monocytes at the site of infection or tissue injury. In the first part of my thesis I investigated, how dihydroretinol (DHR), a new retinoid, which is produced by the retinol saturase enzyme upregulated during the differentiation of monocytes, affects the efferocytosis of the generated macrophages. My data reveal that retinoids promote a differentiation program that contributes to the formation of a regenerative macrophage phenotype.

Previously we found, that macrophages engulfing apoptotic thymocytes strongly upregulate heme oxygenase-1, which is an inducible microsomal enzyme that degrades heme. In the second part of my project I investigated the mechanism of HO-1 induction and the consequences of the loss of HO-1 during efferocytosis. My data indicate that the induction of HO-1 not only degrades heme, but is part of the anti-inflammatory program induced by the apoptotic cell uptake. Since efferocytosis plays a cental role in the regulation of inflammation every step that brings us closer to its more precise understanding can be useful in treatments of autoimmune and inflammatory diseases.

2. Introduction

2.1. Efferocytosis

Every day billions of cells die in our body to eliminate those that are harmful, useless, infected with bacteria/viruses or senescent. This regulated process called apoptosis, which produces apoptotic bodies, which are then cleared by phagocytes without initiating inflammation or an immune response. The clearance of apoptotic cells by phagocytes (efferocytosis) plays a crucial role in maintaining tissue homeostasis and in initiating the resolution of inflammation and tissue repair in case of infection or tissue injury.

In the pathogenesis of efferocytosis, dead/dying cells were taken up by phagocytes. A phagocyte can be any cell that is capable of engulfment, including „professional" phagocytes like macrophages, immature dendritic cells or neutrophils. In tissues, neighbouring „nonprofessional" cells such as epithelial cells and fibroblasts can also mediate the removal of dead cells. The difference is that the professional phagocytes can much more easily and rapidly sense and remove apoptotic bodies.

2.1.1. Recruitment of the phagocytes to apoptotic cells

In the first step, the dying cell releases 'find-me' signals to establish a chemotactic gradient stimulating the migration of phagocytes to the apoptotic cell. These 'find-me' signals can be proteins (fractalkine), lipids (lysophosphatidylcholine, LPC and sphingosine-1-phosphate, S1P), or nucleotides (ATP and UTP).

Then the phagocytes distinguish the apoptotic cell from healthy living cells via different specific receptors, which leads to migration toward the dying cell. The released fractalkine is sensed via CX3C chemokine receptor 1, while the LPC is recognated via the G-protein-coupled receptor G2A. S1P stimulates chemotaxis by binding to the G protein-coupled receptors S1P-R1 through S1P-R5 and macrophages are known to express all the five S1P receptors. Most evidence suggests that the P2Y2 receptor on monocytes binds the released nucleotides and stimulates phagocyte chemotaxis.

2.1.2. Recognition and sensing of apoptotic cells

Next, when in the proximity of their targets, phagocytes must be able to distinguish live from dead cells. Apoptotic cells display specific 'eat me' signals on their surface, which phagocytes detect via engulfment receptors on their cell surface. Lots of 'eat-me' signals have been identified to date such as changes in glycosylation of surface proteins or changes in surface charge, binding of serum proteins (thrombospondin and complement C1q) to the apoptotic cell, expression of intercellular adhesion molecule 3 (ICAM3) and oxidized low-density lipoprotein (LDL)-like moiety, and the exposure of certain intracellular proteins like calreticulin and annexin I. The best-studied 'eat-me'

signal on apoptotic cells is the exposure of the lipid phosphatidylserine (PS). In living cells, PS kept on to the inner leaflet of the plasma membrane via ATP-dependent translocaces. However, when the cell is dying, PS occur on the outer leaflet of the lipid bilayer in a caspase-dependent manner. The concentration of PS on the surface of apoptotic cells is estimated to increase by more than 280-fold within a few hours after induction of apoptosis.

Multiple apoptotic cell recognition and engulfment receptors are expressed on the surface of phagocytic cells that recognize ’eat me’ signals displayed on dying cells. These include complement receptor 3 and 4, members of the T-cell immunglobulin and mucin domain familiy (TIM), mannose receptors, cluster of differentiation (CD) 36, scavanger receptors and integrin $\alpha_v\beta_3/\alpha_v\beta_5$ receptors. The lectins bind altered sugars on apoptotic cells, CD14 binds ICAM3, while the scavenger receptors bind oxidized LDL. Several receptors recognize PS directly, such as stabilin 1 and 2, TIM4 or brain-specific angiogenesis inhibitor 1 (BAI1). In addition, bridging molecules such as milk fat globule EGF factor 8 (MFG-E8), growth-arrest-specific 6 (Gas6), and protein S have also been shown to bind PS on the surface of apoptotic cells and promote engulfment via their cognate receptors on phagocytes. MFG-E8 associates with the $\alpha_v\beta_3/\alpha_v\beta_5$ integrin receptors, transglutaminase 2 (TG2) binds MFG-E8 as a co-receptor of integrin β3, Gas6 and protein S bridge PS on dead cells with the Tyro-3-Axl-Mer family of receptors (TAM receptors) on phagocytes. CD36, in conjunction with integrins $\alpha_v\beta_3/\alpha_v\beta_{5,}$ binds thrombospondin, while the LRP1/CD91, in conjunction with calreticulin binds complement C1q.

Living cells express ’don’t eat me’ signals (CD47, CD31 and CD24), so phagocytes can distinguish them from the dying or dead cells. One of the most well known ’don’t eat me’ signal is CD47 expressed by living cells. CD47 is recognized by SIRPα, which then inhibits the engulfment by phagocytes. During apoptosis, the expression of CD47 is decreased to help the engulfment.

2.1.3. Engulfment of apoptotic cells

Once the apoptotic target is captured, the phagocyte undergoes cytoskeletal rearrangements that initiates engulfment. The Rho family GTPases play a determinant role in this process. They are members of the Ras superfamily of small signaling proteins (RhoA, Cdc42, and Rac). Regulation of removal by RhoA is executed via the Rho-associated coiled-coil-containing protein kinase (ROCK). Active (GTP-bound) Rho increases the kinase activity of ROCK, which mediates phosphorylation of the myosin light chain and promotes cell contraction.

The amount of GTP-bound Rac is increasing after the recognition of apoptotic cells. Rac activation has an evolutionarily conserved positive effect on engulfment, and several proteins involved in it like a Dock180 (Dedicator of cytokinesis 180 kDa protein) and ELMO (Engulfment and Cell Motility protein). ELMO binds directly to Dock180 to increase the guanine nucleotide exchange

factort activity of Dock180. This results in a direct attachment to the carboxyl terminus of the transmembrane receptor BAI1. ELMO1 alone is not able to induce the uptake of apoptotic cells, however together with the Dock180 protein, they strongly increase the GTP-bound Rac level, thus the cell uptake. Several phagocytic receptors introduced to use the Dock180-ELMO pathway for clearance of apoptotic cells such as integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$, the TAM receptor Mer (MerTK) and BAI1. Currently, the exact role of Cdc42 is not completely clear, but certain proteins that bind to Cdc42-GTP have been associated with the engulfment of apoptotic cells.

2.1.4. What happens following the engulfment of apoptotic cells?

After recognition and engulfment, the process of apoptotic cell clearance is not complete, the engulfed apoptotic material have to be degraded. Following internalization, membrane-bound compartments containing the engulfed target (phagosomes) fuse with the acidic lysosomes, which are full with digestive enzymes. As a result, apoptotic bodies are digested into their basic cellular building blocks, like nucleotides, fats, sterols, peptides and amino acids. Efferocytosis actively suppresses inflammation, because engulfing macrophages secrete anti-inflammatory cytokines such as transforming growth factor (TGF)-β and interleukin (IL)-10. At the same time, the presence of apoptotic cells or their engulfed lipid content inhibit the production of pro-inflammatory cytokines, such as tumor necrosis factor (TNF), IL-1 and IL-12.

2.2. Macrophages

Macrophages are important in regulating both tissue homeostasis and inflammation, in performing essential tissue-specific functions as well as protecting the organism from infection. Most mature tissue macrophages are derived from embryonic precursors that seed the tissues before birth, maintaining their numbers in adults by self-renewal. They develop from three sources, which correspond to three generations of hematopoietic (blood-forming) stem cells. The first generation of hematopoietic stem cells are differentiated into specialized macrophages, the microglias; the second generation are erythro-myeloid progenitors, and the third generation develops from the hemogenic endothelium of the aorta-gonad-mesonephros zone and colonize various organs such as the liver.

Monocytes are short-lived, dynamic cells that complement the classic tissue-resident mononuclear phagocytic cells. They circulate in the blood. When they sense cytokines and growth factors, they migrate to tissues, where they differentiate into macrophages according to the signals they sense. While tissue-resident macrophages play a role in the formation and transformation of tissues and in maintaining their homeostasis, monocyte-derived macrophages primarily help to protect the host and direct the biological response to tissue damage.

It is worth noting, that most published macrophage works use *in vitro* environment. In the case of mice, bone marrow-derived macrophages (BMDM) are the most studied. In the *in vitro*, artificial environment we use different stimuli to induce differentiation, for example macrophage colony stimulating factor (M-CSF), which has substantial polarizing effects. This method is generally accepted, and many researchers use it, however we must not forget that the microenvironment has a great effect on macrophages, and we can not create the same environment like in tissues.

2.2.1. Macrophage phenotypes

Macrophages can exhibit two main phenotypes depending on the stimuli and microenvironment: a pro-inflammatory M1 phenotype, or a pro-resolving M2 (alternatively activated) phenotype. Basically M1 macrophages participate in the removal of pathogens; and the alternatively activated M2 macrophages participate in cell proliferation and tissue regeneration.

When macrophages are activated by pro-inflammatory cytokines such as IL-12, pathogen-associated molecular patterns, TNF-α or interferon (IFN)-γ, the polarization shifts towards the M1 phenotype. M1 macrophages fight against intracellular pathogens. M2 macrophages, on the other hand, play a role in wound healing and reducing inflammation. Different subsets of macrophages produce different growth factors, cytokines and chemokines.

Efferocytosis shifts macrophages towards the M2 phenotype, which is characterized by reduced levels of pro-inflammatory cytokines such as TNF-α, chemokine "C-X-C motif" ligand (CXCL)-8, IL-6, and enhanced levels of anti-inflammatory mediators, such as IL-10 and TGF-β. During efferocytosis, dying or dead cells disappear. Otherwise, dead cells may become secondarily necrotic, releasing harmful autoantigens in normal tissues, thereby increasing the chances of neurodegenerative disorders, kidney problems, various types of cancer, or asthma.

2.3. Retinol saturase enzyme and its connection to efferocytosis

In vivo macrophages are exposed to varying number of apoptotic cells, so they need a mechanism to prepare them for increased levels of apoptotic cell uptake. The lipid sensing receptors like liver X receptor (LXR) and the peroxisome proliferator-activated receptors (PPAR)s sense the amount of engulfed apoptotic cell material, and in response to it, they can enhance the phagocytic capacity of macrophages by inducing the expression of several efferocytosis-related molecules. All these receptors function as retinoid X receptor (RXR) heterodimers.

Previous work in our laboratory has shown that activation of these lipid sensing receptors increases retinoic acid synthesis in macrophages, which partially mediates their effect on the efficacy of apoptotic cell phagocytosis. However, in the apoptosing thymus, our lab was unable to demonstrate

production of the classically known retinoic acids. Surprisingly, they found a compound produced in a retinaldehyde dehydrogenase-dependent manner, which could have been a dihydroretinol derivative based on its molecular weight. At the same time, the induction of the retinol saturase enzyme (RetSat) was also detected.

The RetSat is an oxidoreductase, which performs a stereospecific saturation of the C13–C14 double bond of all-trans-retinol (retinol, ROL) to generate (13R)-all-trans-13,14-dihydroretinol (dehydroretinol, DHR). DHR is oxidized *in vivo* to all-trans-13,14-dihydroretinoic acid, a highly selective agonist of the retinoic acid receptor (RAR), and to 9-cis-13,14-dihydroretinoic acid, a highly selective agonist of the RXR receptor. Some experimental work indicates that 9-cis-13,14-dihydroretinoic acid might be physiological RXR ligand.

When studying RetSat null mice, my colleagues found that after 1 hour uptake of apoptotic cells (short-term phagocytosis) was not affected by the loss of RetSat, however, after 5 hours of continuous efferocytosis, the phagocytic capacity of RetSat-null macrophages was decreased compared to the wild-type control macrophages. In addition, its product, DHR, added during the generation of bone marrow-derived macrophages (BMDMs) upregulated TG2 during the last 2 days of the 5-day differentiation process and promoted efferocytosis by the resulted macrophages. These data indicate that RetSat contributes to efficient efferocytosis of apoptotic cells.

Though DHR is oxidized *in vivo* to all-trans-13,14-dihydroretinoic acid, my collegues could not detect the induction of other retinoid-sensitive efferocytosis-related genes by DHR that they identified previously in mature BMDMs. Since previous reports indicated that the RetSat pathway might function independently of RAR receptors by regulating the activity of peroxisome proliferator-activated receptor (PPAR)γ or that of carbohydrate response element binding protein, my aim of the studies was to identify how DHR promotes efferocytosis administered during monocyte/macrophage differentiation.

2.4. Heme oxygenases

During total RNA sequencing of RetSat$^{+/+}$ and RetSat$^{-/-}$ BMDMs, my colleagues found that macrophages produce lower amount of MFG-E8 molecules in the absence of RetSat. In addition, they noticed that macrophages engulfing apoptotic thymocytes strongly upregulate heme oxygenase 1 (HO-1), an enzyme that degrades heme, although the apoptotic thymocytes contain low amount of heme, and the constantly expressed HO-2 would have been enough to degrade it. We were curious: could this protein have another function in efferocytosis?

Heme is essential for all nucleated cells that sense or use oxygen. However, free heme is toxic, so it must be removed or transformed as soon as possible. Heme oxygenases catalyze heme

degradation. Two HO isoforms are known in humans, heme oxygenase-1 and 2. Both enzymes can neutralize heme by converting it to carbon monoxide, ferrous iron, and biliverdin. The latter is immediately converted into bilirubin by the biliverdin reductase enzyme. The importance of HO-1 is shown by the fact that its absence is almost incompatible with life. Mating of HO-1+/- mice does not result in the expected Mendelian offspring ratio, in most cases the null deletion of the HO-1 gene is lethal.

HO-2 is a constitutively expressed isoform, while HO-1 is readily inducible by multiple mechanisms and by various stimuli, such as UV radiation, oxidative stress, lipopolysaccharides, etc. In macrophages, the two main transcription factors that regulates HO-1 transcription are Nrf2 (Nuclear factor erythroid 2-related factor 2) and the signal transducer and activator of transcription 3 (Stat-3). Conversely, BTB and CNC homolog 1 (BACH1) and JunD can repress HO-1 expression. With no stress stimuli, Nrf2 is accumulated in the cytosol and bound to Kelch-like ECH associated protein-1 (Keap1), which inhibits Nrf2 translocation to the nucleus and directs it to proteosomal degradation. Parallely, in the nucleus BACH1 forms a heterodimer with a small Maf protein and blocks HO-1 transcription by binding to the Maf recognition site (MARE). At high heme concentrations, BACH1 dissociates from DNA, is exported from the nucleus and is inactivated, which enables MAFK to bind to NRF2 and form an activation complex for Hmox1 expression via binding to the antioxidant response element (ARE). Nrf2 can be regulated and activated independently of Keap1 by phosphorylation, which can be triggered by a number of signals, including the association of Nrf2 with other proteins or epigenetic factors such as microRNAs. In addition, tyrosine 486 phosphorylation of BACH1 can also promote the nuclear export of BACH1, thus allowing Nrf2 access to the ARE.

HO-1 is highly expressed in tissues with high numbers of degrading red blood cells, such as the spleen, liver, and bone marrow. Metalloporphyrins, such as tin protoporphyrin-IX (SnPPIX), competitively inhibit HO-1 activity both *in vitro* and *in vivo*.

In our experiments, we investigated how regulated the expression of HO-1 in macrophages engulfing apoptotic cells, and what is the role of HO-1 protein in this process.

3. AIMS OF THE STUDY

I. Previous work in our laboratory has shown that the retinol saturase enzyme is induced during phagocytosis and during the differentiation of monocytes. In the absence of retinol saturase, the efferocytic capacity of macrophages was decreased, while its product, dihydroretinol (DHR), enhanced the phagocytic capacity. Thus, I investigated the mechanism of DHR action during the differentiation of bone marrow monocytes.

II. During our previous work, we found that heme oxygenase-1 (HO-1) is significantly induced in macrophages after the uptake of apoptotic thymocytes, which contain low amount of heme. Therefore, I investigated how and why HO-1 is induced during the recruitment of apoptotic cells in macrophages.

4. MATERIALS AND METHODS

4.1. Animals

Most of the experiments were carried out with bone marrow-derived macrophages differentiated from the bone marrow of 2–5-month-old C57BL/6 mice. In some experiments, BMDMs were differentiated also from BACH1, HO-1, or adenosine A_{2A} receptor or adenosine A_3 receptor knock out mice and from their C57BL/6, C57BL/6xFVB, FVB, and C57BL/6 littermates, respectively. Mice were maintained under specific pathogen-free conditions in the Central Animal Facility, University of Debrecen, except for the HO-1 knock out mice initially provided by Dr. Anupam Agarwal, University of Alabama, Birmingham, USA which were bred and maintained under SPF conditions at the animal facility of the Faculty of Biochemistry, Biophysics and Biotechnology of the Jagiellonian University since 2004. All animal experiments were approved by the Animal Care and Use Committee of the University of Debrecen (DEMÁB) with a permission number 7/2016/DEMÁB .

4.2. Generation of bone marrow-derived macrophages (BMDM)

Mice were sacrificed by isoflurane overdose. Bone marrow progenitors were obtained from the femur of 2 to 5-month-old mice by lavage with sterile physiological saline. In every experiment, cells were differentiated and maintained at 37 ◦C in 5% CO_2.

For investigation of HO-1, cells were differentiated for 5 days in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 20% conditioned medium derived from L929 cells, as a source for macrophage colony-stimulating factor (M-CSF), 2 mM glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin. On the third day, non-adherent cells were washed away, and the same culture medium described above was readded.

In case of HO-$1^{+/+}$ and $^{-/-}$ mice for the eryptotic red blood cells (eRBC) phagocytosis and cytokine release experiments, bone marrow progenitors were differentiated for 5 days in DMEM high-glucose medium supplemented with 10% FBS, 10 ng/mL purified human recombinant M-CSF, 100 U/mL penicillin, and 100 µg/mL streptomycin.

During investigation of the effects of retinoids, cells were differentiated for 5 days in DMEM medium supplemented with 10% conditioned medium derived from L929 cells, 2 mM glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin as I described above. In the experiments, 1 µM ROL, 1 µM DHR, and 30 nM all-trans retinoic acid (ATRA) or 50 ng/mL recombinant bone morphogenetic protein (rBMP)-2 alone or together with 0.5 µM AGN194310, 0.3 µM LDN193189, 2.5 µM PD98059, 10µM SIS3, 10 µM disulfiram or 25 µM N,Ndiethylaminobenzaldehyde (DEAB) were added to the culture at the start of day 4 of the differentiation, and the cells were collected either 2–6 h later for

either total mRNA sequencing or qRT-PCR determinations, or for 48 h for total mRNA sequencing and for efferocytosis assays.

4.3. Generation of apoptotic thymocytes and eryptotic red blood cells (eRBCs) *in vitro*

Thymi were collected from 4-weeks-old C57BL/6 mice, thymocytes were isolated and cultured for 24 h in the absence of serum to generate apoptotic thymocytes. As a result of the 24 hours serum starvation, the degree of cell death was more than 80% based on propidium iodide/annexinV-FITC staining.

Blood was obtained from C57BL/6 mice under isoflurane anesthesia by supra-orbital punctuation or drained from heart and anticoagulated with heparin. After anticoagulation and centrifugattion, the plasma and buffy-coat were discarded. RBCs were washed three times with Hanks' balanced salt solution at 4 ◦C and exposed to ionomycin for 2.5 h. The degree of cell death was around 80% in this cases, too.

4.4. Generation of apoptotic thymocyte supernatant

After 24 h, the cell culture medium, in which apoptotic thymocytes were generated alone or in the presence of 20 μM Z-VAD-FMK, was collected after harvesting thymocytes by centrifugation and was filtrated through a 0.22 μm sterile syringe filter to remove apoptotic bodies. This supernatant was added to macrophages in DMEM supplemented with 20% FBS in 1:1 ratio.

4.5. Efferocytosis assays

Apoptotic thymocytes were stained with DeepRed dye for 24 h, while eRBCs were stained with PKH26 Red Fluorescent Cell Linker Kit after the ionomycin treatment according to the protocol provided by the manufacturer. Apoptotic thymocytes and eRBCs were added to macrophages in 1:5 macrophage:target cell ratio.

During HO-1 investigation, apoptotic thymocytes or eRBCs were added to macrophages in the continuous presence or absence of 20 μM SnPPIX. In some experiments during studying retinoids effect on macrophage differentiation, LDN193189 and SIS3 were added together with the target cells. For short-term phagocytosis assays, after coculture for 1 h, apoptotic cells were washed away. For mid- or long-term phagocytosis assays, macrophages were first exposed to unstained apoptotic cells for 6 h or 24 h, and then to the stained apoptotic cells for an additional hour. Then, macrophages were detached by trypsinization, and their fluorescence was analyzed using Becton Dickinson FACSCalibur. Macrophages were gated according to their forward and side scatter properties.

Engulfing macrophages were identified within the macrophage population based on their high fluorescent emission detected in the FL2 and FL4 channels in the case of PKH26 and DeepRed dye, respectively.

For detecting gene expressions with real-time quantitative PCR, macrophages and target cells without labeling were cultured in a 1:5 target ratio for the indicated times as described above. Then, the cells were collected in TRI reagent. In some experiments, macrophages were exposed to RpcAMP (cAMP analog), forskolin (adenilate cyclase activator), or SB203580 (p38 MAP kinase inhibitor) 30 min prior to the start of efferocytosis.

4.6. Fluorescent microscopy

Apoptotic thymocytes were stained with DeepRed dye for 24 h, while eRBCs were labeled with PKH26 Red Fluorescent Cell Linker Kit according to the protocol provided by the manufacturer. BMDMs were stained with carboxyfluorescein diacetate succinimidyl ester or after phagocytosis, BMDMs were fixed by 1% paraformaldehyde and stained with NucBlue Live Cell Stain Ready Probes reagent according to the manufacturer's instructions. Apoptotic thymocytes and eryptotic RBCs were added to macrophages in a 1:5 macrophage/target cell ratio for 1 h; then, the remaining cells were washed away. BMDMs were fixed with 1% paraformaldehyde, then pictures were taken on fluorescent microscope.

4.7. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis of mRNA expression

Total RNA was isolated from BMDMs cultured alone or exposed to apoptotic thymocytes, eRBCs, apoptotic cell supernatants, and various compounds using the TRI reagent according to the manufacturer's guidelines. Before reverse transcription PCR, the RNA concentration of the samples was adjusted to 100 ng/µl with nuclease-free water. Total RNA was reverse transcribed into cDNA using a High-Capacity cDNA Reverse Transcription Kit according to the manufacturer's instruction. qRT-PCR was carried out in triplicate using pre-designed FAM-labeled MGB assays including LightCycler 480 Multiwell 384 white plates, sealed with adhesive tapes on a Roche LightCycler LC 480 real-time PCR instrument. Relative mRNA levels were calculated using the comparative CT method and were normalized to cyclophilin A or β-actin mRNA.

4.8. SDS-PAGE and Western-blot analysis

To receive the total cellular proteins, cells were harvested and lysed in ice cold lysis buffer including protease inhibitor. The protein content of the samples were determined by Bio-Rad Protein

Assay Dye. Apoptotic thymocyte, eRBC, and BMDM lysates were run on 12% SDS polyacrylamide gels, and the separated proteins were electroblotted onto polyvinylidene difluoride membranes. For blockin 5% low-fat milk powder in TTBS was used. Membranes were probed with monoclonal anti-HO-1 and monoclonal anti-β-actin antibodies. Membranes were washed with TTBS buffer at room temperature and then incubated with HRP-conjugated secondary antibodies. The proteins were detected using Immobilon Western Chemiluminescent HRP substrate, and the pixel density was determined using ImageJ software.

4.9. Determination of the heme oxygenase activity

Eryptotic RBCs were added to C57BL/6 macrophages in a 1:5 macrophage/target cell ratio in the presence and absence of SnPPIX for 6 h, as it was described in the efferocytosis assays. Then, macrophages were collected from 6 wells, and 10^6 macrophages were taken up in ice-cold PBS and frozen immediately to −70 ◦C. Heme oxygenase activity was determined later as described by Balla et al.

4.10. Determination of small G-proteins (Rac1, Cdc42, RhoA) activity in macrophages

As it was described in the efferocytosis assays, apoptotic thymocytes or eryptotic RBCs were added to C57BL/6 macrophages in a 1:5 macrophage/target cell ratio in the presence and absence of SnPPIX for 24 h. Macrophages were collected, and the activity of the three G-proteins were determined by the G-LISA Rac1, Cdc42, and RhoA activation assay kits according to the manufacturer's instructions.

4.11. Determination of cytokine production

Wild-type and HO-1 null bone marrow-derived macrophages were exposed to apoptotic thymocytes (isolated from C57BL/6 mice) or eRBCs (isolated from C57BL/6xFVB mice) for 6 h in a 1:5 macrophage/target cell ratio. Then, dying cells were washed away, and the macrophages were cultured for an additional 18 h in DMEM medium. At the end of culture, cell culture media were collected and analyzed by Mouse Cytokine Array and the pixel density in each spot of the array was determined by Image J software.

4.12. mRNA sequencing

To obtain global transcriptome data from differentiating monocytes in the presence and absence of ROL or DHR, high throughput mRNA sequencing analysis was performed on an Illumina

sequencing platform. The quality of total RNA samples was checked on Agilent BioAnalyzer using a Eukaryotic Total RNA Nano Kit, according to the manufacturer's protocol. Samples with RNA integrity number (RIN) values >7 were accepted for the library preparation process. RNA-Seq libraries were prepared from total RNA using the TruSeq RNA Sample preparation kit, according to the manufacturer's protocol. Briefly, poly-A RNAs were captured by oligo-dT conjugated magnetic beads, then the eluted mRNAs were fragmented at 94 Celsius. The first cDNA strand was generated by random priming reverse transcription, and after the second strand synthesis step double-stranded cDNA was generated. After repairing ends, A-tailing and adapter ligation steps, adapter-ligated fragments were amplified in enrichment PCR, and finally, sequencing libraries were generated. Sequencing runs were executed on an Illumina HiSeq2500 instrument using single-end 50 bp sequencing in the Genomic Medicine and Bioinformatics Core Facility of the University of Debrecen.

4.13. Functional analysis of differentially expressed genes (DEGs)

In order to gain insight into the biological function of the given transcripts, we used the online webtool Search Tool for the Retrieval of Interacting Genes v11.5 to identify over-represented gene ontology biological processes among the DEGs. Statistically significant enrichment of genes within the GO category was determined using the Aggregate Fold Change test with Benjamini and Hochberg FDR multiple test correction ($FDR < 0.05$).

4.14. Statistical analysis

All the data are representative of at least three independent experiments carried out with macrophages isolated from three different mice. Values are expressed as mean ± S.D. For differences between 2 groups, a two-tailed unpaired Student's t-test was used; for comparisons $n > 2$ groups, one-way ANOVA (with Tukey's multiple comparisons test) was used. All statistical analyses were performed using GraphPad Prism 6.01 and p value < 0.05 was considered as significant and is indicated by asterisk.

5. RESULTS

5.1 Retinoids promote mouse bone marrow-derived macrophage differentiation and efferocytosis via upregulating bone morphogenetic protein-2 and Smad3

5.1.1. Dihydroretinol (DHR) administered during monocyte differentiation enhances efferocytosis of macrophages by upregulating the expression of several efferocytosis-related molecules

First, we tested whether addition of DHR during monocyte differentiation indeed affects efferocytosis of the resulting macrophages. We found that DHR significantly enhanced the phagocytic capacity of macrophages tested after 5 days of differentiation. Since our previous studies indicated that DHR upregulates TG2 and MFG-E8 only during the last 2 days of differentiation, we also tested whether DHR is able to enhance efferocytosis if it is only added from the start of day 4 of differentiation. DHR was able to induce the same increase in the efferocytic capacity as if it was present during the whole 5 day-period of differentiation, indicating that DHR acts only during the last two days to enhance efferocytosis. Fluorescent images of engulfing macrophages revealed that exposure to DHR not only enhanced the phagocytic uptake of apoptotic cells by macrophages but also promoted the tethering of apoptotic cells to them.

To identify which efferocytosis-related genes are upregulated by the end of differentiation, DHR was added to differentiating monocytes from the start of day 4, and the differentiated macrophages were collected 2 days later for total mRNA sequencing. From the collected cells we identified 781 DEGs between DHR-treated and non-treated BMDMs (based on at least 1.5-fold change and corrected p value < 0.05). A total of 357 transcripts showed an increase, and 424 transcripts showed decreased gene expression in the DHR-treated cells. The mean FC of decreased and increased transcripts was -2.66 ± 1.89 and 6.55 ± 22.52, respectively. The median FC value of decreased and increased transcripts was -2.08 and 2.36, respectively. Functional analysis revealed that genes related to monocyte differentiation, phagocytosis, and wound healing response are overrepresented among the upregulated DEGs. Among the genes showing enhanced expression in DHR-treated macrophages, we found 10 related to phagocytosis of apoptotic cells. Five of these were efferocytosis receptors or coreceptors that included stabilin-2, TG2, Axl, MARCO, and CD36, one was a bridging molecule (THBS-1), and one was a Rab family member (Rab20) contributing to phagosome maturation. In line with our previous findings, besides TG2 and THBS-1 the expression of the other retinoidsensitive phagocytosis genes that we identified in mature BMDMs (Tim-4, C1q, CD14) were not affected by DHR administered during monocyte differentiation. In addition, we detected a very strong induction of the calmodulin-dependent kinase 1 (Camkk1) enzyme and that of the purinergic receptor P2X

(P2X1). We also found the induction of Smad3, a transcription factor known to enhance efferocytosis by acting directly during efferocytosis, as well as by enhancing the expression of several efferocytosis-related genes. Interestingly, this screen did not pick up the bridging molecule MFG-E8 that we found in our previous study, which was also DHR-sensitive. Upregulation of three tethering receptors (CD14, Tim-4, and P2X1) might explain the observed increase in the tethering of apoptotic cells following DHR exposure of macrophages during their differentiation.

To verify the findings, we determined the mRNA expression of five DHR-induced efferocytosis-related molecules (TG2, Axl, THBS-1, CD36 and stabilin-2) in BMDMs by qRT-PCR and found their increased expressions as compared to the non-treated. The expression of Tim-4, C1q, or CD14, three previously identified retinoic acid-sensitive phagocytosis receptors, however, was not induced, even if the ATRA concentration was raised to 300 nM.

5.1.2. DHR administered during monocyte differentiation promotes efferocytosis via directly affecting RARs

Since previous studies indicated that DHR could mediate its effects via being converted to dihydroretinoic acids that activate RARs, we tested whether ROL that can be converted to ATRA or ATRA that is the primary ligand of RARs could replace DHR in regulating efferocytosis at the end of the 5 day-monocyte differentiation, if added at the start of day 4 of differentiation.

We found, that both ROL and ATRA added at the start of day 4 during macrophage differentiation enhanced phagocytosis of apoptotic thymocytes, ATRA being more effective. ROL and ATRA were also capable of inducing the same 5 efferocytosisrelated genes tested 48 h later, which were induced by DHR. In addition, AGN194310, a highly selective pan-RAR antagonist, attenuated the DHR-induced enhancement both in the efferocytic capacity of macrophages, and in the expressions of several efferocytosis-related genes, similar to its inhibition of those induced by ROL and ATRA. Interestingly, however, not all the retinoid-induced expression of phagocytosis-related genes was prevented by administration of the pan-RAR transcription antagonist compound indicating that only the expression of TG2 and THBS-1 was induced clearly in an RAR transcription dependent manner. Interestingly, these were the two phagocytic molecules that we previously identified to be induced in an RAR-dependent manner in mature BMDMs. AXL, stabilin-2 or CD36, however, seem to be induced by a different mechanism. This mechanism might involve direct interaction of RARs with other transcription factors leading to their transrepression or transactivation with which a transcription antagonist does not necessarily interfere. The RAR involved is very likely RARβ, since it was the only RAR induced by DHR, and we could confirm its induction by all of the three retinoids detected by qRT-PCR as well. In addition, these data also demonstrate that different genes are open

for RAR-mediated regulation at the start of day 4 of differentiation, compared to the differentiated macrophages.

To convert ROL or DHR to the relevant retinoic acid (ATRA/all-trans dihydroretinoic acid), the subsequent action of the same aldehyde dehydrogenases and retinaldehyde dehydrogenases is required. Interestingly, however, neither the aldehyde dehydrogenase inhibitor disulfiram, which inhibits the first step, nor the retinaldehyde dehydrogenase inhibitor DEAB, which inhibits the second step of this conversion, prevented the ROL- or the DHR-induced efferocytosis of differentiating macrophages. These data indicate that ROL or DHR might regulate RAR activity by direct binding to the receptor, rather than being converted to retinoic acid. These compounds had no effect on the ATRA-induced efferocytosis either.

5.1.3. Retinoids added from day 4 of monocyte differentiation induce the expression of bone morphogenetic protein 2 (BMP-2)

To identify which genes might mediate the effect of retinoids on efferocytosis added on day 4 of differentiation, DHR and ROL were added to differentiating macrophages at the start of day 4, and cells were collected 2 h later for total mRNA sequencing.

We identified 106 DEGs between DHR-treated and non-treated differentiating macrophages (based on at least 1.5-fold change and corrected p value <0.05). 74 transcripts showed an increase, and 32 transcripts showed decreased gene expression in the DHR-treated cells. The mean FC of decreased and increased transcripts was -2.28 ± 0.72 and 3.34 ± 4.4, respectively. The median FC value of decreased and increased transcripts was −2.08 and 2.15, respectively. The same genes were induced by ROL as well, indicating that DHR and ROL affect the same transcriptional pathways. From the upregulated genes, we selected the BMP-2 growth factor as a candidate gene because it was found to be strongly upregulated in the 48 h samples as well, and it was also shown previously to be a RAR target gene.

Thee mRNA expression of BMP-2 was induced in a time-dependent manner by all the three retinoids added on day 4 of differentiation. The addition of AGN194310 completely prevented the induction of BMP-2 mRNA by all the three retinoids tested 6 h after retinoid addition, proving the transcriptional involvement of RARs. Inhibition of retinaldehyde dehydrogenases by DEAB had no effect on the retinoid-induced induction of efferocytosis, and DEAB did not affect the BMP-2 mRNA induction either, indicating again that ROL and DHR might regulate RAR activity by direct binding.

5.1.4. BMP-2 contributes to the retinoid-induced efferocytic capacity in differentiating macrophages

To test the potential involvement of BMP-2 in the retinoid-induced efferocytosis, retinoids were added together with the BMP receptor (ALK2/3 serine/threonine kinase) inhibitor LDN193189. Though the LDN193189 added alone during macrophage differentiation increased the basal efferocytosis by the resulted BMDMs, the retinoid-induced enhancement in efferocytosis was significantly inhibited by it. If the BMP-2 receptor inhibitor was added to non-treated BMDMs at the start of efferocytosis, it did not affect the 1 h efferocytosis, indicating that the BMP-2 receptor signaling must act during the macrophage differentiation prior to efferocytosis.

5.1.5. Smad3 is also induced by retinoids

We observed that following DHR administration, the mRNA induction of Smad3, a transcription factor known to mediate the effect of transforming growth factor (TGF)-β and known to be associated in macrophages with efferocytosis. Indeed, the mRNA expression of Smad3 was induced in a timedependent manner by all three retinoids added on day 4 of differentiation. The addition of AGN194310 completely prevented the induction of Smad3 mRNA by all the three retinoids tested 6 h after retinoid addition, proving the transcriptional involvement of RARs. On the other hand, the inhibition of retinaldehyde dehydrogenases by DEAB, as in the case of efferocytosis or that of the mRNA expression of BMP-2, had no effect on its retinoid-induced induction.

Then we tested whether the induction of Smad3 mRNA by retinoids involves BMP2. However, neither was its induction by retinoids affected by the BMP-2 receptor inhibitor LDN193189 nor was it induced by recombinant BMP-2 added alone or together with ATRA.

Previous studies indicated that TGF-β is produced and signals in an autocrine manner by differentiating monocytes, and TGF-β can upregulate Smad3 expression via mitogen-activated protein kinase kinase-1 (MEK1). However, inhibition of MEK1 by PD98059 had no effect on the retinoid-induced Smad3 expression. These data indicate that neither BMP-2 nor TGF-β1 mediates the effect of retinoids on the expression of Smad3.

5.1.6. Smad3 also contributes to the retinoid-induced efferocytosis during monocyte differentiation

To test whether the Smad3 transcription factor is involved in retinoid-induced efferocytosis during macrophage differentiation, at day 4 of differentiation retinoids were added together with SIS3, a TGFβ-1 receptor (ALK5 kinase) inhibitor known to interfere with Smad3 signaling, and the efferocytosis capacity of the cells was determined 2 days later. We found that SIS3 significantly inhibited basal efferocytosis, and prevented the induction of efferocytosis by retinoids implying a

determining role of TGFβ receptor/Smad3 signaling in the upregulation of efferocytosis during macrophage differentiation. In addition, it inhibited efferocytosis of fully maturated BMDMs added at the start of efferocytosis to a similar degree, indicating a determining role of Smad3 during the efferocytosis process itself. However, we have to note that exposure to SIS3 for 2 days significantly reduced (by 45% as compared to controls) the number of viable macrophages in the 5-day cultures, in accordance with the observation that TGF-β1, which signals in an autocrine manner in differentiating monocytes strongly contributes to their survival.

5.1.7. Retinoids also upregulate the expression of vascular endothelial growth factor A and other M2-phenotype associated genes

Among the early upregulated genes, in addition to those that mediate the effect of retinoids on efferocytosis, we also found genes like vitamin D receptor or vascular endothelial growth factor (VEGF) A, which are associated with the M2 polarization of monocytes/macrophages. Vitamin D receptor is known to promote M2 conversion of macrophages, while VEGFA is a growth factor known to be produced by M2 pro-reparative monocytes/macrophages that drives angiogenesis. Thus, we also looked at the regulation of VEGFA mRNA expression.

Similar to BMP-2 and Smad3, retinoids induced the mRNA expression of VEGFA in a time- and RAR transcription-dependent manner, and DEAB had no effect on this induction either. Lack of effect of LDN193189 or PD98059 indicated that ALK2-, ALK3-, or MEK1-mediated signaling are not involved in the regulation of VEGFA mRNA expression. However, previous studies using Smad3 null macrophages demonstrated the involvement of Smad3 in the regulation of VEGFA in macrophages, indicating that retinoids might indirectly increase its expression via elevating Smad3 levels.

Previous studies indicated that BMDMs lie further toward the M1 end of the M1–M2 polarization spectrum. Interestingly, BMP-2 itself was shown to be produced by M2 macrophages. Thus, we searched for M2 marker genes among those upregulated by the last 48 h DHR treatment during macrophage differentiation to determine whether retinoid-treatment might promote M2 polarization. DHR-exposure upregulated the expression of several M2-associated macrophage genes, such as Aldh1a2, Marco or Clec7a.

5.2. Heme oxygenase-1 contributes to both the engulfment and the anti-inflammatory program of macrophages during efferocytosis

5.2.1. Apoptotic thymocytes and eryptotic red blood cells induce the expression of HO-1 in engulfing macrophages

First, we selected two types of dying cells: apoptotic thymocytes the heme content, which is below the detection limit, and eryptotic red blood cells that contain a very high amount of heme, as hemoglobin makes up about 96% of the red blood cells' dry content (by weight). Prior to the experiments, we decided to determine whether these cells express the HO-1 protein. We found, that HO-1 protein is not expressed by apoptotic thymocytes or eRBCs in such an amount that would interfere with the assays, so they are suitable to study the effect of apoptotic cell uptake on the expression of HO-1 specifically in the engulfing macrophages.

Independently of their heme content, both types of dying cells induced the mRNA expression of HO-1 in engulfing macrophages within 6 h, and the level of the protein remained elevated even 24 h later. Surprisingly, we have not found a significant difference in the degree of induction during the first 6 h uptake of the two cell types despite the big difference in their heme content.

5.2.2. HO-1 expression in engulfing macrophages is induced by apoptotic thymocytes via soluble signals, while the induction by dead RBCs is cell uptake-dependent

If the heme content of dead cells plays a role in the induction of HO-1 in engulfing macrophages, the dead cells have to be taken up first. Thus, to assess the involvement of heme in the induction of HO-1 by the apoptotic cell uptake, we decided to block the uptake of apoptotic cells by administering the actin polymerization inhibitor cytochalasin-D. We found, that cytochalasin-D strongly blocked the efferocytosis of both apoptotic thymocytes and RBCs. However, only the eRBC uptake-related HO-1 induction was inhibited in the presence of cytochalasin D.

Since these data indicated that intracellular heme is not the main inducer of HO-1 expression by apoptotic thymocytes, we decided to test whether apoptotic recognition signals are involved. If we assume that the cell surface signals provided by apoptotic thymocytes and eRBCs are similar (we have reported that they both express the universal cell death signal phosphatidylserine), these data indicate that eRBCs upregulate HO-1 entirely internally, while apoptotic thymocytes might use only soluble signals. Indeed, the culture medium collected from dying thymocytes was able to induce alone the expression of HO-1 in BMDMs, while that of eRBCs was not able to do so. Although the induction of HO-1 by the apoptotic supernatant was less than that by the apoptotic thymocytes, which could indicate the involvement of a phagocytic receptor that is not triggered by eRBCs, the degradation or

dilution of the soluble factor, which in the context of efferocytosis is directly released at high concentration around the macrophage, might also explain the observed difference. However, when the supernatant was collected from serum-starved thymocytes incubated in the presence of Z-VAD-FMK, a pan-caspase inhibitor, the supernatant was not able to induce HO-1 mRNA expression. In contrast, Z-VAD-FMK added together with the apoptotic thymocyte supernatant did not affect the apoptotic thymocyte supernatant-induced induction of HO-1 mRNA expression, proving that Z-VAD-FMK does not affect the macrophage.

These observations indicate that the soluble signals responsible for HO-1 induction are generated in a caspase-dependent manner in apoptotic thymocytes. Our finding is in harmony with a previous report which indicated that one of the 'find me' signals released by apoptotic thymocytes in a caspase-dependent manner, sphingosine-1-phosphate, not only regulates migration of macrophages, but is also involved in the upregulation of their HO-1 expression in a p38 MAPK-dependent manner.

5.2.3. BACH1 might be involved in the upregulation of HO-1 by both apoptotic thymocytes and eRBCs

BACH1 has a very strong suppressive effect on the basal expression of HO-1 in BMDMs, since we detected already in the nontreated BACH1 null macrophages a significantly elevated HO-1 expression at both mRNA and protein levels, as compared to their wild-type controls. As we expected, exposure to eRBCs could not induce further this elevated HO-1 expression. Surprisingly, there was a similar finding when the uptake of apoptotic thymocytes was tested. These observations indicate that either the competition between the two transcription factors, Nrf2 and BACH1, controlling the expression of HO-1 is such an important element in its regulation that when the negative regulator is missing, its transcription is already maximally activated, or both signaling pathways induced by either the dying RBCs or by the dying thymocytes target BACH1. Altogether, these data indicate that eRBCs induce HO-1 in engulfing macrophages following their uptake via their heme content, while apoptotic thymocytes use soluble signals, and both signaling pathways might target BACH1.

5.2.4. Adenosine released during efferocytosis is not involved in the induction of HO-1 expression in macrophages engulfing apoptotic cells

During efferocytosis, the ATP released from apoptotic cells in a caspase-dependent manner is converted to adenosine on the surface of macrophages to trigger adenosine receptors. Macrophages have been reported to express adenosin A2A, A2B, and A3 receptors (A2AR, A2BR, A3R). During efferocytosis, A2ARs are up, while A3Rs are downregulated, indicating that the dominant receptors that mediate the effect of adenosine during efferocytosis at the start of phagocytosis are the A3Rs, while later, they are the A2ARs. Accordingly, previous studies from our and other laboratories have

indicated that the adenylate cyclase pathway activated by adenosine A2A receptors contributes to the anti-inflammatory program of apoptotic cell uptake, while the A3Rs are involved in the chemotactic navigation of macrophages. In addition, reports have indicated that the adenylate cyclase pathway might be involved in the regulation of HO-1 expression.

Thus, we decided to investigate whether adenosine also contributes to the soluble signals that regulate HO-1 expression in engulfing macrophages. To answer the question, wild-type and A2AR null macrophages were exposed to either apoptotic thymocytes or dying RBCs, and their HO-1 expressions were compared. We found that the loss of A2ARs did not affect the basal HO-1 expression of macrophages. In addition, the loss of A2ARs did not affect the apoptotic thymocyte or the dead RBC-induced HO-1 expression either, indicating that the soluble adenosine signal does not contribute to the apoptotic cell-induced upregulation of HO-1.

Next, we investigated whether the adenylate cyclase pathway contributes to the induction of the HO-1 expression during efferocytosis at all. Thus, we tested the effect of RpcAMP (a competitive inhibitor of cAMP) on the apoptotic cell-induced HO-1 expression. The administration of RpcAMP did not affect either the basal or the induced expression of HO-1 in BMDMs engulfing dying thymocytes or RBCs independently of their A2AR expression. These data indicate that no other soluble signal is generated during efferocytosis that would contribute to the HO-1 expression via activating the adenylate cyclase pathway.

Finally, we decided to test whether the adenylate cyclase pathway has any effect on the HO-1 expression in BMDMs by exposing them to forskolin, which is a strong adenylate cyclase activator. Forskolin added alone or during the phagocytosis of apoptotic cells could not enhance HO-1 expression, indicating that the adenylate cyclase pathway does not contribute to the regulation of HO-1 expression in dead cell engulfing BMDMs.

In addition to the inhibition of the adenylate cyclase pathway, A3Rs might activate the p38 MAPK signaling pathway as well in a cell type-dependent manner. Since previous studies indicated the involvement of sphingosine-1-phosphate and also the p38 MAPK pathway by the apoptotic thymocyte supernatant, we decided to test whether the apoptotic supernatant-induced HO-1 expression involves the A3Rs. We found that inhibition of the p38 MAPK pathway nearly completely prevented the induction of HO-1 in macrophages induced by both the apoptotic thymocyte uptake and by the exposure to the apoptotic thymocyte supernatant. These data confirm the determining role of the p38 MAPK pathway in the apoptotic thymocyte-induced HO-1 expression, and they underline the involvement of soluble signals that trigger the p38 MAPK pathway. However, loss of A3Rs did not affect the induction HO-1 by the apoptotic thymocytes or by their supernatant. Inhibition of the p38 MAP kinase pathway or loss of A3R had no effect on the HO-1 expression induced by the uptake of eRBCs either. The different results received following inhibition of the p38 MAP kinase pathway

indicate that if both eRBCs and apoptotic thymocytes induce HO-1 mRNA expression in a BACH1-dependent manner, the mechanism of targeting BACH1 is different in the two signaling pathways. Our data altogether exclude the involvement of adenosine in HO-1 induction of engulfing macrophages.

5.2.5. Loss of HO-1 activity results in a decrease of the phagocytic capacity of macrophages after long-term phagocytosis of dying red blood cells

Next, we investigated whether the loss of HO activity affects the phagocytosis of apoptotic cells. For this purpose, we inhibited HO-1 and HO-2 activities by preincubating macrophages with their competitive inhibitor SnPPIX. Since in line with a previous report, SnPPIX induced an increase in the HO-1 mRNA expression in both engulfing and non-engulfing macrophages. We tested first whether the applied concentration of the inhibitor is sufficient to inhibit the superinduced HO activity in eRBC-exposed macrophages, and we found that SnPPIX applied in 20 μM concentration efficiently inhibited HO activities.

The phagocytosis of both apoptotic thymocytes and eryptotic red blood cells was determined immediately after exposure to macrophages (short-term phagocytosis), after continuous phagocytosis for 6 h (mid-term phagocytosis), or after continuous phagocytosis for 24 h (long-term phagocytosis). The phagocytic capacity of macrophages engulfing apoptotic thymocytes was not affected up to 24 h by SnPPIX, even though both HO activities were inhibited, while the phagocytic capacity of macrophages engulfing the high heme containing RBCs under the same conditions was inhibited at the same time point. SnPPIX reduced not only the percentage of eRBC engulfing macrophages but also their mean fluorescence, indicating that the lower number of engulfing macrophages took up less eRBCs. This decrease was not related to a decreased viability of the engulfing macrophages detected by Annexin V-fluorescein isothiocyanate/propidium iodide staining, and it was confirmed by testing the long-term phagocytic capacity of HO-1 null macrophages as well. These data indicate that the observed decrease was not related to a potential side effect caused by the inhibitor.

The phagocytosis of apoptotic cells involves numerous phagocytic receptors. Many signals that affect efferocytosis regulate the expression of the phagocytic receptors in macrophages. Thus, we tested whether the engulfment of RBCs in the presence of HO-1 activity inhibitor affects the expression of any of these genes. However, none of the tested key phagocytic receptors or bridging molecules (Tim4, TG2, MerTK, integrin β3/β5, stabilin2, CD14, MFG-E8, CD36, thrombospondin) were expressed differently after 24 h of RBC efferocytosis. Thus, the loss of HO activity must inhibit efferocytosis via a different mechanism.

Signaling pathways initiated by the phagocytic receptors were reported to regulate the GTP loading of several small G proteins to achieve efficient efferocytosis. These small G proteins work in a temporally regulated fashion in which Rac1 and Cdc42 are activated early to work together in facilitating phagocytic cup formation through actin polymerization followed by RhoA activation, which drives mechanical retraction and phagosome internalization. Thus, we decided to determine the general activity of these three G proteins following the 24 h phagocytosis of RBCs in wild-type macrophages exposed or not to SnPPIX. We found an increase in the amount of activated Rac1 and no alterations in the amount of activated RhoA and Cdc42 after 24 h of RBC efferocytosis. The inhibitor alone did not affect the activity of these G proteins. However, in the presence of the inhibitor, the phagocytosis-induced activation of Rac1 was prevented.

5.2.6. While apoptotic cell uptake inhibits basal pro-inflammatory cytokine production of macrophages, pro-inflammatory cytokine release of engulfing macrophages is not altered or enhanced if HO-1 protein is not expressed

Apoptotic cells do not simply fail to provide pro-inflammatory signals; they actively interfere with the inflammatory program. Since it has been reported that HO-1 has anti-inflammatory properties, we decided to investigate whether the upregulation of HO-1 contributes to the anti-inflammatory program induced by apoptotic cells by detecting the pro-inflammatory cytokine formation of both dying thymocyte- or RBC-engulfing wildtype and HO-1 null macrophages. Unfortunately, due to the induction of HO-1, we could not repeat the experiments in the presence of SnPPIX to investigate separately the effect of the HO-1 activity loss, because we could not have separated the effect of the activity loss and that of the HO-1 protein induction.

Evaluation of the cytokine secretion profile of unstimulated macrophages was performed using a highly sensitive cytokine antibody array method, enabling the simultaneous detection of low concentrations of multiple cytokines in one assay. The cytokines released were first evaluated by experiments using untreated wild-type and HO-1 null macrophages *in vitro*. Non-engulfing macrophages show that most of the available 40 cytokines on the filters were detectable, even though some were at very low levels. The loss of HO-1 did not affect significantly the composition of most of the cytokines released, but we found great individual differences in the amount of pro-inflammatory cytokines produced both within the HO-1$^{+/+}$ and HO-1$^{-/-}$ macrophages derived from different mice. However, when macrophages were exposed to either apoptotic thymocytes or eryptotic RBCs, we found several cytokines, the production of which was suppressed in wild-type engulfing macrophages, but increased or remained unaltered in their HO-1 null counterparts. Nine of these cytokines (IL-1α and β, IL-17, MIG, RANTES, MCSF, IL-13, IL-23 and KC) were identified during both thymocyte

and RBC phagocytosis as pro-inflammatory cytokines, the downregulation of which was affected by the loss of HO-1 in three independent experiments. Interestingly, we found the IL-1R antagonist to respond in a similar manner, not only in this study, but also in a previous study, which is in line with the observation that IL-1 production and activity are regulated separately.

To confirm our results, we selected three of these cytokines to determine their mRNA levels in the same macrophages from which the supernatants were collected. Since the individual differences in cytokine productions were reflected in individual differences in the mRNA levels as well, we demonstrated their change as fold expression following eRBC uptake. The mRNA expressions of MCSF and KC were also decreased in eRBC-exposed wild type but not in HO-1 null macrophages. We did not find the same for IL-1β. However, IL-1β protein levels are also affected by the degree of caspase-1 activation, and HO-1 was shown to attenuate NOD-like receptor containing a pyrin domain 3 inflammasome activity.

Altogether, our data indicate that the induction of HO-1 contributes to the antiinflammatory effects induced by dying cells in engulfing macrophages.

6. DISCUSSION

Macrophages perform two main roles during efferocytosis of healthy dying cells: (1) engulfment and degradation of the dying cell and (2) suppression of inflammation. Previous studies from our laboratory have indicated that DHR acting during the last 2 days of mouse bone marrow-derived macrophage differentiation enhances the phagocytic capacity of the resulting BMDMs, but it affects a different set of efferocytosis genes as compared to retinoic acid-exposed, mature BMDMs. That is why we concluded that DHR might act via a different signaling pathway in differentiating macrophages than retinoic acid does in mature BMDMs in the promotion of efferocytosis. However, we found that DHR acts on the same signaling pathway as ROL and ATRA mediated by RARs, but a different set of genes is sensitive to retinoid treatment in differentiating as compared to the differentiated macrophages. Interestingly, inhibitors of the conversion of ROL or DHR to retinoic acids did not prevent their effect on gene expression or on efferocytosis, indicating that ROL and DHR might directly activate RARs in differentiating macrophages without being converted to the relevant retinoic acids. Our data also reveal that not all effects of retinoids on the phagocytosis receptor gene expressions were mediated by the direct transcriptional activity of RARs. It is known that in addition to promoting transcription, ATRA-bound RARs can mediate AP1 transrepression, and we also have shown that RARs are also capable of promoting the transcriptional activity of the glucocorticoid receptor acting via direct receptor/receptor interaction. Certain synthetic retinoids can differentiate between these biological activities because different parts of the ATRA-bound receptor participate in these activities, and synthetic retinoids do not necessarily compete with all of them. In addition, retinoid-bound RARs might also have non-genomic effects, which indirectly affect transcription.

We identified two mediators of retinoid signaling in monocytes that contribute to the observed increase in efferocytosis capacity: BMP-2 and Smad3. Both BMP-2 and Smad3 have already been shown to be direct RAR-regulated target genes, and according to our data neither the retinoid-induced BMP-2 nor the differentiation-related TGF-β1 signaling contribute the upregulation of Smad3 by retinoids. BMP-2 generally signals via the Smad 1/5/8 and possibly 9 transcription factors. In addition, however, in differentiating cells BMP-2 was found to signal also via a noncanonical pathway that involves BMP-2/TGF-β1 receptor heterodimers and Smad3. Though we have not investigated this possibility in detail, based on our results we cannot exclude that retinoids might promote efferocytosis not only via BMP-2 and TGF-β but also in a BMP-2/ BMP-2 and TGF-β1 heterodimer receptor/Smad3-dependent manner in differentiating macrophages.

Monocytes are bone marrow-derived innate immune cells that circulate for approximately 2 days before being cleared from circulation. Circulating monocytes scan for pathogens and respond to inflammatory signals needed to perform their phagocytic functions, or monocytes can tissue migrate

to become macrophages. The resulting macrophages are not a uniform cell population; and there is significant macrophage heterogeneity determined by the immune environment. In mice, monocyte derived macrophages are typically classified as either pro-inflammatory M1 or M2 pro-reparative cells generated following efferocytosis. The M2 macrophages can be divided into further groups participating in the resolution of inflammation, antigen presentation, and tissue repair, and the later are known to release growth factors and pro-angiogenic and tissue repair factors, such as VEGFA, growth differentiationfactor 3, or insulin-like growth factor-1.

Monocyte differentiation to macrophages *in vivo* is an adherence-mediated maturation, as inhibition of this adherence results in monocyte formation. Thus, BMDM differentiation partially mimics the *in vivo* monocyte-derived macrophage differentiation process. Bone marrow-derived monocytes very often appear and differentiate into macrophages in tissues where an increased rate of cell death occurs, such as during skeletal muscle injury or in the case of high fat diet-induced death of adipocytes. Thus, a retinoid-induced increase in their efferocytosis capacity will contribute to the efficient clearance of dead cells in these tissues to prevent chronic inflammation and the development of autoimmunity. In addition, Smad3 is also a determinant mediator of the anti-inflammatory effects of TGF-β, known to be released by engulfing macrophages during efferocytosis.

Upregulation of the expression of vitamin D receptor or that of VEGFA by retinoids during monocyte differentiation also indicates that retinoids promote the generation of such M2-type macrophages that participate in the regulation of tissue repair. Upregulation of TG2, or ALDH1a2 detected 48 h after retinoid addition also supports this view. Interestingly, the involvement of Smad3 in mediating tissue repair responses of macrophages was just reported in an infarcted myocardium model.

In line with our observations, M2 polarization of differentiating monocytes by ATRA released by a mouse tumor itself was observed in the environment of the tumor, where the resulting tumor-associated M2-like macrophages mediated immune-suppression and promoted tumor growth. ATRA was also found to promote M2 polarization of differentiating monocytes during *L. major* infection in mice. Altogether our data provide further proof that retinoids acting during macrophage differentiation promote the generation of M2-like, immune-suppressive, pro-reparative macrophages, and we demonstrate that the effects of retinoids are partially mediated via BMP-2 and Smad3.

Previously in our laboratory showed that long-term phagocytosis of RetSat null bone-marrow derived macrophages was impaired. To reveal the cause of the defect, total RNA sequencing was performed on $RetSat^{+/+}$ and $RetSat^{-/-}$ BMDMs. During the analysis of the data, it was found that the HO-1 enzyme, which is responsible for the degradation of heme, was significantly increased in the low heme-containing apoptotic thymocyte engulfing macrophages. Thus, in the second half of my project I investigated why HO-1 was so enhanced and could HO-1 have another role in efferocytosis.

Two different types of dying cells were used: the low amount of heme-containing apoptotic thymocytes and the high amount of heme-containing eryptotic red blood cells.

Altogether, the data presented here indicate that dying cells upregulate HO-1 in macrophages: apoptotic thymocytes via soluble signals that do not include adenosine or other cAMP elevating ones but require the p38 MAPK signaling pathway, while eRBCs following their uptake. Both regulatory pathways might involve the regulation of BACH1. In case of the inhibited HO activity, the uptake of those cells that have low heme content did not affect the phagocytic capacity of macrophages even after 24 h continuous engulfment, while the same resulted in an inhibition of phagocytosis in case of high heme-containing cells' uptake. In the latter case, the loss of HO activity affected the engulfment-induced Rac1 activation. While apoptotic cell uptake suppressed the basal pro-inflammatory cytokine production of wild-type macrophages, in the absence of HO-1, we detected an enhanced or unaltered pro-inflammatory cytokine release during the engulfment of both cell types. The anti-inflammatory effect of HO-1 might explain why even low heme-containing dying cells upregulate HO-1 in macrophages and why they use alternative signals than intracellular heme accumulation to act so. Our data altogether demonstrate that HO-1 contributes to both the engulfment and the anti-inflammatory program of macrophages during efferocytosis.

Our finding is in harmony with a report that has shown that the thymic structure and function of HO-1 null mice is not altered, where thymic cells continually die and are cleared, indicating proper heme metabolism, while these mice are characterized by splenomegaly and by a strongly damaged fibrotic structure in the spleen, where the majority of aging RBCs are cleared. In these spleens, eRBC-engulfing tissue resident macrophages die due to heme accumulation. The different sensitivity of macrophages to heme accumulation in the two tissues *in vivo* might be explained by the constant and not altered expression of HO-2 in HO-1$^{-/-}$ macrophages, the activity of which might be sufficient to handle the low amount of heme generated during efferocytosis of apoptotic thymocytes in the thymus, while not during efferocytosis of the high heme-containing eRBCs in the spleen. Accordingly, when both HOs were inhibited by SnPPIX, we observed a more significant inhibition in the long-term eryptotic red blood cell uptake than in that when HO-1 was lost alone, underlying that the degree of efferocytosis inhibition is dependent on the residual HO activity.

Our findings could not confirm the results of a report that indicated that the accumulation of heme in macrophages (in their case during hemolysis) can overactivate Cdc42 by binding to its guanine nucleotide exchange factor dedicated of cytokinesis 8 (DOCK8). Our data indicate rather that in the absence of HO-1 activity, Rac1 activation is influenced during phagocytosis. Since previous studies demonstrated that carbon monoxide, the product of HO activity, promotes efferocytosis, our data might indicate that at physiological levels of heme uptake, the accumulation of heme and loss of

production of carbon monoxide together might affect long-term efferocytosis in the absence of HO activity influencing dominantly Rac1 activity.

7. SUMMARY

The clearance of apoptotic cells by macrophages (efferocytosis) plays a key role in the maintenance of tissue homeostasis and in eliminating the inflammation. Previous work in our laboratory has shown that (13R)-all-trans-13,14- dihydroretinol (DHR) that is produced by retinol saturase enzyme, administered during bone marrow-derived macrophage (BMDM) differentiation increased the efferocytic capacity of the generated mature macrophages. In this study, I aimed to determine the mechanism of DHR action. Total gene expression analysis of BMDMs, which were differentiated in the presence or absence of retinol (ROL) or DHR was carried out by total mRNA sequencing. Gene set enrichment analysis identified several efferocytosis-related genes that were upregulated by both compounds. However, none of them were the retinoid-regulated phagocytic receptors, except transglutaminase 2 (TG2), that my collegues identified previously in mature macrophages. That is why we checked the early target genes of DHR during macrophage differentiation, and found 74 upregulated genes. To mediate the effect of retinoids, the most promising candidates were BMP-2 and Smad3, whose expressions were increased significantly. During monocyte differentiation DHR, retinol, and retinoic acid all induced the expression of these two genes in a RAR-dependent manner, DHR being the less, retinoic acid being the most effective inductor. We also found, that retinoids upregulate the expression of the vitamin D receptor and the vascular endothelial growth factor A. Altogether our data demonstrate that retinoids during monocyte differentiation promote the generation of pro-reparative M2 macrophages and increase their efferocytotic capacity by inducing a BMP-2 and smad signaling via activating RARs.

Heme oxygenase-1 (HO-1) plays an important role in the catabolism of heme and yields equimolar amounts of biliverdin, carbon monoxide, and free iron. In a previous study in our research group has found that the low amount heme-containing apoptotic thymocytes strongly increased the expression of HO-1 in engulfing macrophages. Therefore, in the second half of my research work, I aimed to investigate the role of HO-1 in the efferocytosis program.

In this project, I used two types of dead cells, apoptotic thymocytes, which barely contains heme, and the high amount of heme-containing eryptotic red blood cells (eRBCs). I found that both of them strongly upregulate HO-1 in macrophages. The induction by apoptotic thymocytes is fully dependent on soluble signals, while in the case of eRBCs, it is cell uptake dependent. Both pathways involve the regulation of BACH1 transcription factor, the repressor of the HO-1 gene. The loss of HO-1 has no effect on the long-term continuous efferocytosis of apoptotic thymocytes, while that of eRBCs is inhibited. This latter is connected to an internal signaling pathway induced by the accumulated heme that prevents the increase in Rac1 activity triggered by the efferocytosis receptors and required for proper efferocytosis. Normally, the clearance of apoptotic cells suppresses the basal pro-inflammatory

cytokine production in wild-type macrophages. However, in the absence of HO-1, we found enhanced amounts of pro-inflammatory cytokines produced by engulfing macrophages. Altogether our data indicate that HO-1 is necessary for both the proper degradation of heme and engulfment of high heme-containing apoptotic cells, and generally for the anti-inflammatory effects mediated by the apoptotic cells.

8. LIST OF PUBLICATIONS

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Registry number: DEENK/347/2023.PL
Subject: PhD Publication List

Candidate: Éva Vincze-Fige
Doctoral School: Doctoral School of Dental Sciences

List of publications related to the dissertation

1. **Vincze-Fige, É.**, Sarang, Z., Sós, L., Szondy, Z.: Retinoids Promote Mouse Bone Marrow-Derived Macrophage Differentiation and Efferocytosis via Upregulating Bone Morphogenetic Protein-2 and Smad3.
   Cells. 11 (18), 1-22, 2022.
   DOI: http://dx.doi.org/10.3390/cells11182928
   IF: 6

2. **Vincze-Fige, É.**, Szendrei, J., Sós, L., Kraszewska, I., Potor, L., Balla, J., Szondy, Z.: Heme Oxygenase-1 Contributes to Both the Engulfment and the Anti-Inflammatory Program of Macrophages during Efferocytosis.
   Cells. 10 (3), 652-669, 2021.
   DOI: http://dx.doi.org/10.3390/cells10030652
   IF: 7.666

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List of other publications

3. Garabuczi, É., Tarban, N., **Vincze-Fige, É.**, Patsalos, A., Halász, L., Szendi-Szatmári, T., Sarang, Z., Király, R., Szondy, Z.: Nur77 and PPARγ regulate transcription and polarization in distinct subsets of M2-like reparative macrophages during regenerative inflammation.
Front. Immunol. 14, 1-14, 2023.
DOI: http://dx.doi.org/10.3389/fimmu.2023.1139204
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4. Szondy, Z., Al Zaeed, N., Tarban, N., **Vincze-Fige, É.**, Garabuczi, É., Sarang, Z.: Involvement of phosphatidylserine receptors in the skeletal muscle regeneration: therapeutic implications.
J. Cachexia Sarcopenia Muscle. 13 (4), 1961-1973, 2022.
DOI: http://dx.doi.org/10.1002/jcsm.13024
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