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

INVOLVEMENT OF MACROPHAGE-DERIVED RETINOIDS IN THE REGULATION OF TRANSGLUTAMINASE 2 EXPRESSION AND THE PHAGOCYTOSIS ENHANCING EFFECT OF DEXAMETHASONE

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The Examination takes place at Department of Restorative Dentistry,
Faculty of Dentistry, University of Debrecen, at 9:00 a.m. on 4th of December, 2015.

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The PhD Defense takes place at the Lecture Hall of In Vitro Diagnostics Building,
Faculty of Medicine, University of Debrecen, at 1:00 p.m. on 4th of December, 2015.

1. INTRODUCTION

1.1. Apoptosis

'Apoptosis' is the original term introduced by Kerr et al. in 1972 to define a type of cell death with specific morphological features. Apoptosis is one of the several types of programmed cell death and it is characterized by a series of morphological changes, including rounding-up of the cell, reduction of cellular and nuclear volume (pyknosis) and nuclear fragmentation (karyorrhexis), as well as plasma membrane blebbing (but it maintenances its integrity until the final stages of the process), which lead to the formation of apoptotic bodies and finally engulfment by resident phagocytes *in vivo*. Apoptotic bodies consist of cytoplasm with tightly packed organelles with or without a nuclear fragment. The organelle integrity is still maintained and all of this is enclosed within an intact plasma membrane. These bodies are engulfed by phagocytes and degraded within phagolysosomes. There is no inflammatory reaction associated neither with the process of apoptosis or with the removal of apoptotic cells since apoptotic cells do not release their cellular constituents into the surrounding tissue; they are quickly phagocytosed by the surrounding cells thus likely preventing secondary necrosis; while the engulfing cells do not produce inflammatory cytokines.

In the following parts I would like to describe two compounds the retinoids and glucocorticoids, which were used to promote apoptosis in our work. In addition, I also would like to give some information about thymocytes what we used to investigate the regulation of tissue transglutaminase (TG2) expression in dying cells.

1.2. Retinoids

Retinoid literally means "like retinol." Retinoids are the vitamin A (retinol) and its natural and synthetic derivatives. Retinol must be obtained directly through dietary intake, but may also be derived in its provitamin A forms. Retinoic acid (RA) the active metabolite of vitamin A, is very important for embryogenesis and is involved in many cellular activities such as proliferation, differentiation and apoptosis.

All-*trans* RA (ATRA) is produced from retinol in a two-step oxidative pathway. First step is the oxidation of retinol to retinaldehyde which is generally considered to be the rate-limiting step followed by the oxidation of retinaldehyde to ATRA. Retinaldehyde can be converted back to retinol, but the oxidation of retinaldehyde to ATRA is irreversible. Two types of enzymes are

responsible for oxidation of retinol to retinaldehyde: the cytosolic alcohol dehydrogenases (ADH) and the microsomal short-chain dehydrogenase/reductase (SDR).

For the second step of the reaction, which is the oxidation of all-*trans*-retinaldehyde to ATRA three members of the aldehyde dehydrogenases (ALDH) 1A family of proteins are physiologically important: ALDH1A1 (RALDH1), ALDH1A2 (RALDH2), and ALDH1A3 (RALDH3). The *in vivo* role of ALDH1A1 in ATRA biosynthesis is evidenced by the fact that whereas ALDH1A1 -/- mice are viable and have normal morphology of the retina, their liver shows reduced ATRA biosynthesis and increased serum retinaldehyde levels after treatment with retinol. ALDH1A1 and ALDH1A2 have similar catalytic efficiency, but the *K* m value of ALDH1A2 is much lower than that of ALDH1A1. Thus, ALDH1A1 is the least potent retinaldehyde dehydrogenase of the three enzymes. ATRA induces its own catabolism by upregulating CYP26 (cytochrome P450, family26) enzyme – a member of the cytochrome P450 superfamily of enzymes – responsible for the degradation of excessive ATRA.

1.3. Nuclear receptors activated by retinoids

In the nucleus retinoids bind to nuclear receptors in mammals, which function as ligand-activated transcription factors. Ligand binding results in a conformational change in the nuclear receptor, switching on their transcriptional activities. Retinoic acids activate the retinoic acid receptors (RARs) and retinoid X receptors (RXRs). RARs form heterodimers with retinoid X receptors. In the absence of ligand, the RAR/RXR heterodimer is constitutively bound to DNA on the retinoic acid response elements (RARE) and associated with co-repressor complexes that induce transcriptional silencing. Binding of RA to the receptor induces a conformational change allowing the association of co-activators and the release of co-repressors, resulting in the decondensation of chromatin and activation of target gene expression. It is believed that a high affinity ligand for RXRs is 9-*cis*-retinoic acid (9-*cis* RA), which is generated by a simple isomerisation from ATRA. The RARs are able to bind both ATRA and 9-*cis* RA. However the physiological role of 9-*cis* RA as a natural RXR ligand is controversial because endogenous detection of this molecule is very difficult in embryos or in most adult tissues. By contrast, ATRA is easily detectable in many tissues. There are three separate genes encoding both RAR (RARα, RARβ and RARγ) and RXR (RXRα, RXRβ and RXRγ) that are conserved throughout vertebrates. In macrophages, RARα, RARγ, RXRα and RXRβ have been found to be expressed.

RXR has a special role in the nuclear receptor family, because it acts as a common heterodimerization partner of RAR and many other nuclear receptors, such as liver X receptors (LXRs) and peroxisome-proliferator-activated receptors (PPARs) etc. RXR is a silent transcriptional partner in RAR-RXR heterodimers. This heterodimer is transcriptionally not active in the absence of an RAR ligand, even if an agonist binds to the RXR. Other heterodimeric partners of RXR can be activated by RXR ligands alone and these complexes are referred to as "permissive" nuclear receptors. LXRs similarly PPARs form permissive heterodimers with the RXRs, so the complex can be activated by ligands of either partner and have additive or synergistic downstream effects.

LXRs - LXRα and LXRβ - play central role in the transcriptional control of lipid and cholesterol metabolism. LXRs function as oxysterol sensors and are essential components of a physiological feedback loop regulating cholesterol metabolism and transport. LXRα is highly expressed in the liver and at lower levels in the intestine, adipose tissues and macrophages etc., whereas LXRβ is ubiquitously expressed. LXR ligands promote reverse cholesterol transport, in which excess cholesterol is transferred from the macrophages via induction of cholesterol transporter proteins (ABCA1 and ABCG1), and also via increased production of cholesterol acceptors (apoE and apoCs) and lipoprotein remodelling proteins. 27-hydroxycholesterol is one of the several oxysterols identified as potential endogenous ligands of LXR. 27-hydroxycholesterol is produced by a mithochondrial p450 enzyme, CYP27. Induction of the enzyme's expression results in an increased level of 27-hydroxycholesterol and upregulation of LXR-mediated processes. CYP27 gene is under coupled regulation by retinoids and ligands of PPARs via a PPAR-retinoic acid receptor response element in its promoter.

PPARs (including three different proteins: PPARα, PPARβ/δ and PPARγ) regulate genes important in cell differentiation and various metabolic processes, especially lipid and glucose homeostasis. PPAR*δ* (also called PPAR*β*) is ubiquitously expressed. PPARδ participates in fatty acid oxidation, in skeletal and cardiac muscles, but it also regulates blood glucose and cholesterol levels. In addition, activation of PPARδ (in white and brown adipose tissue) specifically induces expression of genes required for fatty acid oxidation and activates uncoupling proteins (UCPs) in brown fat for energy dissipation as heat, which in turn leads to reduced adiposity.

1.4. Glucocorticoids

Glucocorticoids (GCs) are essential steroid hormones for human life, and very effective anti-inflammatory and T cell apoptosis inducing agents. GCs exert a wide range of anti-inflammatory and immunosuppressive activities after binding to the glucocorticoid receptor (GR). GCs are

lipophilic substances, they can passively diffuse across the plasma membrane and bind to the GR in cytoplasm. In the absence of glucocorticoid ligand, cytoplasmic GR forms a heterocomplex with chaperons, keeping the GR in an inactive state. Upon glucocorticoid binding, the GR dissociates from its chaperone proteins and consequently translocates to the nucleus, where it binds the GC response element in the promoters of its target genes.

Synthetic derivatives of GCs, such as dexamethasone (DEX) or prednisolone, are widely used in the treatment of inflammatory disorders. A well known immunosuppressive activity of GCs is their ability to efficiently enhance phagocytosis of apoptotic cells by human and mouse macrophages. GCs achieve the augmented phagocytosis, at least partially, acting on gene expression of several target genes. Short time (16 hrs) glucocorticoid treatment of human macrophages have been shown to regulate the expression of over 100 genes, including those known to be associated with apoptotic cell phagocytosis, such as and Mer tyrosine kinase (MERTK) phagocytosis receptor and MFG-E8 and C1q serum proteins.

1.5. Thymocytes development and selection

The primal role of the thymus is the generation of mature T cells due to the elimination of non-functional or harmful developing thymocytes. The thymocytes that ultimately develop into mature T cells are derived from pluripotent hematopoietic stem cells that migrate into the thymus from either the foetal liver or bone marrow. During T cell development, thymocytes differentiate from immature CD4-CD8-double negative (DN) precusor to functional CD4+ or CD8+ single positive (SP) thymocytes through the CD4+CD8+ double positive (DP) stage. During their differentiation, 90% of the CD4+ CD8+ thymocytes produced are incapable of recognizing self-peptide loaded self-MHC complexes present in the thymus and undergo a default death pathway named “death by neglect”. To generate single positive cells, $CD4^{+}CD8^{+}$ thymocytes undergo negative selection (death) and positive selection (survival) steps in the thymus enable the differentiation and selection of T cells, which bear functional T cell receptors.

Dying thymocytes are phagocytosed by thymic macrophages. Following engulfment, macrophages release several molecules which influence the apoptosis of double positive thymocytes and prevent pro-inflammatory cytokine production. On one hand, transforming growth factor beta (TGF-β), prostaglandin E_2 (PGE_2) and adenosine are produced as anti-inflammatory molecules and act in an autocrine manner on macrophages. On the other hand, both adenosine and PGE2 can induce apoptosis of the thymocytes, while TGF-β was shown to support both glucocorticoid- and TCR-driven cell death. Since apoptosis and the consequent engulfment of apoptotic cells are continuously

ongoing processes in the thymus, molecules released continuously by engulfing macrophages might contribute to the formation of a thymic milieu that, in the absence of TCR signalling, ensures the induction of apoptosis of neglected thymocytes.

In addition, GCs produced in the thymus by thymic epithelial cells or transferred in an endocrine manner from the adrenal cortex have been known for a long time to affect thymocyte development by inducing apoptosis of the neglected thymocytes or to influence thymocyte selection.

1.6. Transglutaminase 2

Transglutaminase 2 (TG2), also known as tissue transglutaminase (tTG), is the most abundant and most studied enzyme among the nine members of the transglutaminase enzyme family. Transglutaminases catalyse crosslinking of ε-amino group of a lysine residue and a γ-carboxamide group of glutamine residue or formation of intramolecular bond incorporating di- and polyamines into proteins. TG2 is a multifunctional protein with over 130 substrates at various locations inside and outside the cell. Besides its crosslinking activity, TG2 possesses protein disulphide isomerase and protein kinase activities and it can act as a G-protein in various signaling pathways. Furthermore, TG2 has non-enzymatic activities, especially extracellularly, where it interacts with a number of cell surface proteins, taking part in cell adhesion processes and stabilization of the extracellular matrix.

Transglutaminase 2 has been shown to be induced and activated in cells undergoing apoptosis. Generally, the main role of TG2 *in vivo* is to ensure that once the apoptotic process is initiated, it is finished without causing inflammation or tissue injury. The main function of TG2 in apoptosis is that, by using its crosslinking activity, it produces highly cross-linked protein polymers in apoptotic corpses, where the irreversible formation of scaffolds stabilizes the dying cells and prevents the leakage of harmful intracellular components. In the thymus, TG2 is induced during the early phase of apoptosis, and not only the increased amount but the increased *in vivo* activity of the enzyme can be detected.

Recognition of apoptotic cells via phosphatidylserine receptors - which is the first step of apoptotic cell phagocytosis - triggers latent TGF-β release from macrophages. Simultaneously, phagocytes produce TG2, which activates TGF-β. Both macrophages and apoptotic cells possess TGF-β receptors. In dying thymocytes TGF-β promotes apoptosis induced by specific signals and induces TG2 expression, while in macrophages TGF-β promotes phagocytosis and downregulates pro-inflammatory cytokine formation. Induction of TG2 by TGF-β in macrophages results in an autoregulatory loop leading to further TGF-β formation and release.

1.7. Engulfment of apoptotic cells

Every day billions of cells die in our body to eliminate those that are harmful, useless, or senescent. The term "phagocytosis" refers to an internalization process by which larger particles, such as bacteria and dead/dying cells, are engulfed and processed within a membrane-bound vesicle called the phagosome. The rapid and effective clearance of apoptotic cells by phagocytes prevent exposure of surrounding tissue to potentially cytotoxic, immunogenic, or inflammatory cellular contents. Uncleared corpses can undergo secondary necrosis, promoting inflammation and autoimmunity.

Phagocytic clearance of apoptotic cells can be divided into four distinct steps: accumulation of phagocytes at the site where apoptotic cells are located; recognition of dying cells through a number of bridging molecules and receptors; engulfment by a unique uptake process; and processing of engulfed cells within phagocytes.

Apoptotic cells express specific markers on their surface ("eat me" signals) for recognition via specific receptors on the phagocytes. The best characterised and essential "eat me" signal for clearance of apoptotic cells is the exposure of phosphatidylserine (PS) on the outer leaflet of the plasma membrane. These "eat me" signals on the cell surface can then either interact directly with receptors on the macrophage surface or bind bridging molecules which serve as a bridge to link apoptotic cell to phagocytes. During the engulfment of apoptotic corpses two distinct signalling pathways lead to rearrangement of cytoskeleton by activation of Rac and in turn the pseudopod extension. Following apoptotic cell recognition and uptake, the process of phagocytosis is not complete, because several downstream events, such as phagosome maturation, degradation of dead cells can influence the engulfment capacity of phagocytes. Apoptotic cells are digested into their basic cellular building blocks including nucleotides, fats, sterols, and peptides.

One of these components is the cellular lipid content of the apoptotic cells able to activate lipid sensing receptors in engulfing macrophages. Thus LXRs or PPARs, as lipid sensing nuclear receptors, can respond to this lipid uptake and through transcriptional regulation they increase the number of phagocytic receptors and opsonins as well as the metabolism of engulfing macrophages. This way the engulfing macrophages sense the amount of apoptosis and can promote the rapid and early removal of dying cells.

2. AIMS OF THE STUDY

Transglutaminase 2 has been known for a long time to be associated with the *in vivo* apoptosis program of various cell types including T cells. *In vivo* induction of apoptosis results in the appearance of TG2 in dying thymocytes. Though thymocyte apoptosis can also be induced *in vitro*, upregulation of TG2 is not detectable. This indicates that tissue factors present only in the tissue environment are required for the *in vivo* induction of TG2 in apoptotic thymocytes. Previous studies have shown that one of these factors is transforming growth factor-β which is released by macrophages engulfing apoptotic cells. Besides TGF-β, the TG2 promoter consists response elements for retinoic acid receptor, as well. Retinoids were shown to be produced in the thymus.

- Since *in vitro* TGF-β alone was unable to promote significant upregulation of TG2 in apoptotic thymocytes, we decided to test the hypothesis that during *in vivo* apoptosis retinoids present in the thymus together with TGF-β produced by macrophages might contribute to the *in vivo* induction of TG2.

We found during these studies that following DEX-induced *in vivo* thymocyte apoptosis, thymic engulfing macrophages produce retinoids to regulate TG2 expression of apoptotic cells and to promote phagocytosis of apoptotic cells by macrophages. Moreover DEX treatment itself induces RALDH1 expression in macrophages.

- Since previous studies have shown, that GCs exert their immunosuppressive activity partly via enhancing the phagocytosis of apoptotic cells, and a work in our laboratory has shown that retinoids promote phagocytosis of apoptotic cells, we decided to investigate whether DEX-induced retinoid production of macrophages contributes to the enhanced phagocytosis of apoptotic cells induced by DEX.

3. MATERIALS AND METHODS

Experimental animals

The experiments were carried out on 4-weeks-old or 2- to 4-months-old C57BL/6 mice. In some experiments RARα knock-out mice and RARE-hsp68-lacZ reporter transgenic mice were also used. RARE-hsp68-lacZ mice harbor a tetrameric repeat of the RARβ2 RARE linked to the hsp68 minimal promoter, which has been widely used as a RA-reporter transgene. Mice were maintained in specific pathogen-free condition in the Central Animal Facility and all animal experiments were approved by the Animal Care and Use Committee of University of Debrecen (DEMÁB).

Macrophage cell culture and treatment

Peritoneal macrophages were harvested from 2- to 4-months-old C57BL/6 mice by peritoneal lavage with sterile physiological saline. Cells were plated onto 12-wells plate ($1.5x10^6$ cells/well) and after 3-4 hrs incubation the non-adherent cells were washed away, while adherent macrophages were cultured in RPMI 1640 medium supplemented with 10% FBS, 2 mM glutamine, 1 mM Na-pyruvate, 50 μM 2-mercaptoethanol, 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C in 5% CO_2 for 2 days before use. Macrophages were treated with 1 μM GW3965 a synthetic liver X receptor (LXR) agonist, 1 μM Rosiglitazone a peroxisome proliferator-activated receptor (PPAR)-γ agonist, 1 μM GW501516 a synthetic PPARδ agonist for 4 hrs, or 0.1 μM dexamethasone-acetate (DEX) for 12 hrs or 24 hrs.

To generate bone marrow derived macrophage (BMDM), bone marrow progenitors were obtained from the femurs of 2- to 4-month-old mice by lavage with sterile physiological saline. Cells were allowed to differentiate for 5 days in DMEM medium supplemented with 10% FBS, 20% conditioned medium derived from L929 cells, 2 mM glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C in 5% CO_2. Non-adherent cells were washed away after three days. BMDMs were treated with 1 μM DEX, 25 μM 4-diethylamino-benzaldehyde (DEAB) to block aldehyde dehydrogenase enzyme activities, or vehicle (DMSO) for the indicated time periods. In some experiments 1 μM GSK3787 a PPARδ antagonist, 1 μM AGN193109 a pan RAR antagonist was used. For prolonged phagocytosis experiments apoptotic thymocytes were added to BMDMs in 5:1 (apoptotic cells: macrophage) ratio for 3 hrs, then apoptotic cells were washed away extensively. For the inhibition of new protein synthesis 20 μM cycloheximide was used.

Inhibition of *in vivo* retinoid synthesis

To inhibit the endogenous retinoid synthesis in the thymus, 4-weeks-old mice were injected intraperitoneally with disulfiram (1.33 mg/g body weight on the first day, 0.33 mg/g on the 2nd and 3rd day) or with DEAB (0.2 mg/g body weight on the first day and 0.1mg/g on the 2nd and 3rd day). Thymic apoptosis was induced with the apoptosis inducer added together with the third dose of the inhibitor.

Induction of thymic apoptosis *in vivo*

4-weeks-old mice were injected intraperitoneally with 0.3 mg DEX - dissolved in DMSO/physiological saline - or with 50μg of anti-CD3 antibodies to induce thymic apoptosis. Controls received the same dose of DMSO/ physiological saline. Thymuses were removed at the indicated time points. Thymic apoptosis was evaluated by measuring the change in the amount of thymic weight.

Induction of thymocyte apoptosis *in vitro*

For experiments of TG2 expression, thymocytes (10^7 cells/ml) were cultured in RPMI 1640 medium supplemented with 10% charcoal stripped FBS, 2 mM glutamine, 1 mM Na-pyruvate, 50 μM 2-mercaptoethanol, 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C in 5% CO_2 for indicated time periods. Apoptosis was induced by addition of 0.1 μM DEX. In some experiments thymocytes were also exposed to 5ng/ml recombinant human TGF-β1, 0.3 μM all-*trans* retinoic acid, 0.3 μM 9-*cis* retinoic acid, 0.3 μM AM580 or 1nM LG268.

Western blot analysis

Collected thymuses or BMDMs were homogenized in ice-cold lysis buffer containing 0.5% Triton X-100. Protein concentration of samples was diluted to 2 mg/ml, then the samples were boiled with an equal volume of Laemmli buffer. Electrophoresis was performed in 10% SDS-polyacrylamide gel for TG2 or in 15% SDS-polyacrylamide gel for LXR, PPARδ and C/EBPβ. Separated proteins were transferred to an Immobilion-P transfer membrane and were probed with rabbit polyclonal anti-TG2 antibody, anti-mouse LXRα/β , PPARδ , C/EBPβ and mouse anti-β-actin or GAPDH antibodies were used as loading control. Protein bands were visualized by Immobilon Western Chemiluminescent HRP substrate.

Detection of RALDHs expression of peritoneal macrophages followed by *in vitro* phagocytosis of apoptotic cell

Thymocytes or NB4 cells (acute promyelocytic cell line) were used as apoptotic cells**.** To generate apoptotic thymocytes, thymus was collected from 4-weeks-old C57BL/6 mice, cells were isolated and cultured for 24 hrs (10^7 cells/ml) in RPMI 1640 medium supplemented with penicillin/streptomycin in the absence of serum. In case of NB4 cells the apoptosis was induced by 10 μM As_2O_3 –treatment for 12 hrs. This method results in >80% apoptotic cells (as assessed by propidium iodide/AnnexinV-FITC staining). Apoptotic thymocytes were added to the peritoneal macrophages in 10:1 (apoptotic cells: macrophage) ratio and they were coincubated for 2 or 4 hrs. In case of apoptotic NB4 cells in 5:1 ratio was used and following 6 hrs of coculture, apoptotic cells were washed away and macrophages were incubated additional 6 hrs or 18 hrs and afterwards RNA was isolated from the samples. In some experiments, macrophages were preincubated with 5 μg / ml actinomycin D for 30 min to block transcription, with 50 μM cytochalasin D or recombinant Annexin V (10 μg/10^5 cells) for 15 min prior to phagocytosis to block it.

Detection *of in vitro* phagocytic capacity of BMDMs

BMDMs were stained for 24 hrs with 5 μM CellTracker Orange (5-(and-6)-[([4-chloromethyl]benzoylamino)tetramethylrhodamine]; Invitrogen). To generate apoptotic thymocytes, thymi were collected from 4-weeks-old C57BL/6 mice, thymocytes were isolated and cultured for 24 hrs (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 5:1 (apoptotic cells/macrophage) ratio for 30 min (or for 45 min in case of siRNA transfected macrophages). After coculture, apoptotic cells were washed away and macrophages were detached by trypsinization. Percentage of macrophages engulfing apoptotic cells were analyzed on a Becton Dickinson FACSCalibur. For determining prolonged phagocytosis, macrophages were exposed to non-labelled apoptotic thymocytes for 3 hrs and phagocytic activity was measured in the following 30 min by replacing the non-labelled apoptotic cells with CFDA labelled ones.

Analysis of mRNA expression

Total RNA was isolated from control and treated thymuses, isolated thymocytes or macrophages by TRI reagent according to the manufacturer's guidelines. The concentration and purity of RNA were obtained by means of NanoDrop spectrophotometer (Thermo Scientific, Schwerte, Germany). Total RNA (1µg/samples) was reverse transcribed into cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Budapest, Hungary) according to the manufacturer's instruction. QRT-PCR was carried out in triplicate using pre-designed FAM-MGB-labelled assays for TG2 and RALDHs (Applied Biosystems, Budapest, Hungary) on an ABI Prism 7900 or 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-CAT-ATC-CTG-ATC-TTC-C-3', probe FAM-ACT-CCA-ACG-CAG-CAC-CAT-CAC-CGC-TAMRA. ROX reference dye was used for the normalization of fluorescent reporter signal. Relative mRNA levels were calculated using comparative C_T method and were normalized to cyclophilin or glycerinaldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. ABI Prism SDS2.1 software was used for data analysis.

Immunodetection of RALDH1 and 2 in macrophages of the thymus

Flourescence light microscopic detection of macrophages (F4/80-immunopositive cells), RALDH1 or RALDH2 was performed in frozen thymus sections from mice exposed to DEX for 24 hrs. Thymuses were frozen in embedding medium in liquid nitrogen. Tissue was sectioned at 7 µm, mounted on Superfrost Ultra Plus microscope slides and stored at -20°C until use. Before IF staining, slides were kept at room temperature (RT) for 1 hour, fixed in ice-cold acetone for 10 min and washed with PBS containing 0.1% Triton X-100. To minimize the nonspecific staining by antibodies, sections were incubated with 2% BSA in PBS for 30 min at 37°C in a humid chamber. Sections were then incubated with goat polyclonal RALDH1 or RALDH2 antibodies in a humid chamber for overnight at 4°C. After washings the samples were incubated with rat anti-mouse FITC-conjugated monoclonal F4/80 antibody in a humid chamber at RT for 1hr. Following washes sections were further incubated with secondary antibodies and mounted in DAPI aquamount. Appropriate primary delete control immunohistochemical reactions were performed. The region of fluorescent images were captured with a digital camera (Olympus DP50) connected to Nikon Eclipse 800. Acquired and presented images are representative of all the samples examined. For documentation, images were processed using Adobe PhotoShop software.

X-gal staining of dexamethasone-treated BMDMs

BMDMs derived from RARE-hsp68-lacZ reporter transgenic mice were treated with 1 μM DEX, 25 μM DEAB to block aldehyde dehydrogenase enzyme activities, or vehicle (DMSO) for 24 hrs. Cells were then fixed with 0.25 mM glutaraldehyde for 5 minutes on ice. Fixed cells were incubated at 37 ^{o}C for 24 hrs in X-gal staining solution (35 mM potassium ferrocyanide, 35 mM potassium ferricyanide, 2 mM $MgCl_2$, 0.02% Nonidet P-40, 1 mg/ml X-gal). Images were taken using an AMG EVOS inverted microscope at 20 X magnification.

siRNA transfection of BMDMs

Five-day-matured BMDMs were transfected with ON-TARGETplus SMARTpool siRNA specific for mouse LXRα and LXRβ or C/EBPβ and ON-TARGETplus Non-targeting Control Pool using the DharmaFECT 1 Transfection Reagent according to the DharmaFECT's Transfection Protocol. siGLO Green (6-FAM) Transfection Indicators are specially designed for nuclear localization for a clear signal of successful transfection. siGLO Green Transfection Indicator (50 nM) was transfected into BMDMs (0.3 μl/100 μL well). After 2 hrs, transfection efficiency (fluorescent signal) was detected by FACS analysis. At 24/48 hrs after transfection, cells were treated with 1 μM DEX or vehicle (DMSO) for 18/24 hrs and harvested for detecting the mRNA/protein levels of LXR or C/EBPβ of transfected BMDMs by quantitative PCR/western blot analysis, respectively. To determine the effect of LXR or C/EBPβ gene silencing on the mRNA expression levels of various glucocorticoid-induced genes, at 48 hrs after transfection, cells were treated with DEX (1μM) or vehicle (DMSO) for the indicated time periods.

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 or SEM. P values were calculated by using unpaired Student's t-test.

4. RESULTS

4.1. MACROPHAGES ENGULFING APOPTOTIC CELLS PRODUCE RETINOIDS WHICH CONTRIBUTE TO THE INDUCTION OF TG2 EXPRESSION IN THYMOCYTES

Retinoic acids and TGF–β together effectively enhance the mRNA expression of TG2 in dexamethasone exposed thymocytes dying *in vitro*

First we tested the possible effect of retinoic acids and TGF-β on TG2 mRNA expression added alone, or in different combinations with DEX, which was used as apoptosis inducer at thymocytes. Both retinoids and TGF-β were applied in a concentration that was shown to be effective in our previous studies to initiate apoptosis of thymocytes. Addition of TGF-β or ATRA alone slightly increased the mRNA expression of TG2, while 9cRA was effective to induce the TG2 mRNA expression in thymocytes. As we reported previously, exposure of thymocytes to DEX, a very efficient apoptosis inducer of thymocytes only slightly affected the endogenous TG2 expression. Further addition of ATRA was insufficient to promote DEX-induced TG2 expression, while the TGF-β together with DEX efficiently increased the TG2 mRNA in thymocytes. ATRA added together with either TGF-β or in combination with DEX and TGF-β could significantly elevate the TG2 mRNA. In the case of 9cRA, the TG2 induction detected at combination with either DEX or TGF-β, was more pronounced than the one observed with ATRA, indicating that the RXR site takes part in the induction of TG2 expression in dying thymocytes. The most effective TG2 inducer in DEX-exposed thymocytes was 9cRA added together with TGF-β, suggesting that the apoptotic signal is provided by the glucocorticoid hormone, which alone is insufficient to induce significant TG2 expression, but when applied together with 9cRA and TGF-β it also contributes to the appearance of TG2.

Synthetic ligands of RARα and RXR receptors induce TG2 expression in DEX-exposed apoptotic thymocytes

Since 9cRA (acting as a ligand for both RAR and RXR receptors) appeared more effective in each experimental setting than the RAR agonist ATRA, we used the synthetic RXR agonist LG268 and the synthetic RARα agonist AM580 to study the role of RA receptors in TG2 induction. LG268 significantly induced the expression of TG2 in each experimental setting. However, it was more effective, when it was added together with AM580. Altogether these data indicate that *in vitro*

retinoids and TGF-β together can significantly enhance TG2 expression in dying thymocytes, and RAR/RXR heterodimers ligated at both sites might mediate most effectively the effect of retinoids.

Inhibition of the *in vivo* retinoic acid synthesis attenuates apoptosis-induced TG2 expression in the mouse thymus

To prove our hypothesis, that retinoids produced in the thymus can contribute to the TG2 expression of dying thymocytes, two different retinoic acid synthesis inhibitors were used. Inhibition of RA synthesis prior to apoptosis induction should prevent or attenuate TG2 induction. Mice were injected either with disulfiram, an alcohol dehydrogenase, or 4-diethyl amino-benzaldehyde (DEAB), an inhibitor of RALDHs for 2 days, before the apoptosis induction of thymocytes. As described previously, mice were injected intraperitoneally with DEX to induce thymic apoptosis and resulting in massive expression of TG2 in apoptotic thymocytes. Pretreatment with either disulfiram or DEAB, significantly reduced the DEX-induced TG2 expression indicating that endogenously produced retinoids might contribute to its apoptosis-related *in vivo* appearance. However, the DEX-induced expression of TG2 was never fully inhibited indicating that other signals are also contributing to its *in vivo* appearance.

The *in vivo* apoptosis induction is coupled to enhanced retinoid dependent transcription in the mouse thymus

To demonstrate that both disulfiram (DSF) and DEAB are able to efficiently inhibit the *in vivo* retinoid synthesis at the applied concentration, RARE–lacZ transgenic mice were used. These transgenic mice express beta-galactosidase (lacZ) gene under the control of the retinoic acid responsive element (RARE). In these mice lacZ expression demonstrates the *in vivo* retinoid-dependent transcriptional activity. Similar to the previous experimental settings - the inhibition of the *in vivo* retinoid synthesis followed by thymus apoptosis induction - retinoid-dependent transcriptional activity was determined. To our surprise DEX treatment alone induced a significant increase in the lacZ reporter gene expression. Thymic β-galactosidase mRNA expression was nearly fully inhibited by DEAB, but only partially by disulfiram indicating a dramatic inhibition of retinoid-induced transcription by DEAB, but only partial inhibition by disulfiram, confirming the efficiency of the retinoic acid synthesis inhibitors. However, we could not further increase the dose of disulfiram, as its higher doses were lethal for the mice.

***In vivo* thymocytes apoptosis is accompanied by enhanced expression RALDH1 and 2, and by increased retinoid-dependent transcriptional activity**

Our previous finding, that retinoid-dependent transcription is increased after DEX-induced apoptosis in the thymus, raises the question whether this response is related only to DEX treatment, or it is apoptosis specific. To study this phenomenon, beside DEX-induced apoptosis we induced cell death of thymocytes also by injecting the RARE-lacZ mice with anti-CD3 antibodies, which trigger the T cell receptor-mediated cell death pathway. Both apoptotic stimuli caused an enhanced retinoid dependent transcription, but the two different apoptotic stimuli induced different levels of retinoid production in the thymus. Since the effect of DEX was much more pronounced, probably it is proportional to the higher amount of apoptotic cells caused by the more effective apoptosis inducer DEX. Since this LacZ transcription indicates a massive retinoid production in the thymus, we decided to check the expression of RALDH1 and 2, two RA synthesis specific enzymes, which were found previously to be expressed by the mouse thymus following *in vivo* apoptosis induction. We found, that RALDH1 and 2 mRNA expression of the thymus significantly increased following DEX injection in a time dependent manner. The anti-CD3 antibodies also caused an enhanced expression of RALDHs. Moreover, in line with the lacZ results, the expression of RALDH1 increased much more significantly following DEX injection as compared to anti-CD3 antibody injection, while less difference was found in the RALDH2 response. The different responses might be partially related to the fact that DEX induced a more significant cell death in the thymus at each time point demonstrated by the remaining thymic weight.

Macrophages engulfing apoptotic cells express increased levels of RALDHs possibly via activation of three lipid sensing receptors

As the previous results demonstrated, the induction of the two retinoic acid synthesizing enzymes in the thymus following apoptosis induction, suggests an apoptosis-related retinoid production in thymus. During previous studies, it has been found that thymic epithelial cells are able to express RALDHs, but it seems unlikely that they are responsible for an apoptosis-related phenomenon. Our hypothesis was that maybe the thymic macrophages sense the amount of thymocyte apoptosis and respond to it with an increased RALDHs expression. Since, it was recently published that macrophages engulfing apoptotic cells sense the amount of dying cells via three lipid sensing receptors and then promote an enhanced phagocytosis of apoptotic corpses. We decided to test whether the expression of RALDHs can be altered during phagocytosis of apoptotic cells in macrophages. For this purpose peritoneal macrophages were isolated, and exposed to apoptotic

thymocytes or NB4 cells, which are larger and contain more lipids than thymocytes. Phagocytosis of apoptotic thymocytes enhanced the mRNA expression of RALDH1, but did not alter that of RALDH2 in engulfing macrophages. The expression of RALDH1 was further increased and RALDH2 mRNA also induced, when the same macrophages engulfed the larger apoptotic NB4 cells, so the induction was not thymocyte specific.

Incubation of macrophages with actinomycin D, a transcription inhibitor, prevented the apoptotic cell-associated induction of RALDHs mRNA expression, indicating that regulation occurs at the transcriptional level. Next we wanted to know, whether the recognition of PS on the apoptotic cell surface or the ingestion of the apoptotic cells trigger the RALDHs induction. For this purpose phagocytosis was inhibited by cytochalasin D, an inhibitor of actin polymerization, which does not influence the recognition of apoptotic cell, or PS on the apoptotic cell surface was masked by preincubating the apoptotic cells with recombinant annexin V. Both cytochalasin D and recombinant annexin V inhibited the induction of RALDH mRNA levels by apoptotic cells suggesting that engulfment of apoptotic cells, rather than their recognition *per se*, triggers RALDH expression.

Since the three lipid-sensing nuclear receptors (LXRα, PPARγ and δ) have been implicated in the macrophage response to the engulfed apoptotic cells, we decided to test whether triggering of these receptors affects the expression of RALDH1 or 2. Tested at 4 hrs, all the three agonists, GW3965, an LXR agonist, rosiglitazone, a PPARγ agonist and GW1516, a PPARδ agonist, promoted the mRNA expression of RALDH1 in peritoneal macrophages and only GW1516 was ineffective in inducing RALDH2 expression. These observations indicate that all the three lipid sensing receptors might mediate the effect of apoptotic cell engulfment on the expression of RALDHs.

RALDHs are expressed by macrophages engulfing apoptotic cells in the thymus following *in vivo* apoptosis induction

To prove that RALDHs are indeed expressed by macrophages in the thymus following *in vivo* apoptosis induction, frozen thymic sections were co-stained for macrophages and two RALDHs, 24 hrs following apoptosis induction by DEX. We found that both enzymes were co-stained with macrophages following DEX induced apoptosis, suggesting that macrophages express RALDHs and are capable for retinoid synthesis.

Dexamethasone induces the expression of RALDH1 mRNA in macrophages

Since *in vivo* DEX treatment caused much more RALDH1 induction than anti-CD3 antibodies in the thymus, we investigated whether *in vitro* DEX treatment affects RALDH1 expression in macrophages. RALDH1 mRNA is significantly increased following DEX treatment of macrophages, while it had no effect on RALDH2 expression. Our data indicate that triggering of thymocyte apoptosis induces an expression of RALDHs in the thymus and the apoptosis inducer DEX itself can contribute to the increase of the RALDH1 mRNA expression in macrophages.

4.2. DEXAMETHASONE TREATMENT ALSO INDUCES RETINOID PRODUCTION IN MACROPHAGES WHICH ENHANCES PROLONGED PHAGOCYTOSIS OF APOPTOTIC CELLS

Retinoid production is required for the glucocorticoid-induced enhancement of efferocytosis during prolonged clearance of apoptotic cells

In the following experiments we wanted to prove that *in vitro* DEX treatment indeed induces retinoid synthesis in macrophages. For this purpose BMDMs from RARE-hsp68-lacZ reporter transgenic mice were used. In these mice, positive X-gal staining shows the endogenous retinoid-dependent lacZ expression of macrophages. Macrophages were exposed to 1 μM DEX for 24 hrs either alone or in combination with 25 μM DEAB. 24 hrs DEX treatment induced lacZ expression in macrophages, while neither the vehicle control, nor the DEAB co-treated cells did not show positive reaction. These data indicate that DEX induces physiologically relevant retinoid synthesis in BMDMs, and DEAB in the applied concentration can completely block it.

Previous studies from our laboratory have shown that retinoids can increase the efferocytosis capacity of macrophages. That is why we decided to investigate whether DEX-induced increase in the phagocytosis of apoptotic cells is dependent on the DEX-induced retinoid synthesis. For this purpose DEX treated macrophages were exposed to the retinaldehyde dehydrogenase inhibitor DEAB and their phagocytic capacity were determined. As it was published previously, exposure of BMDMs to 1 μM DEX significantly enhanced their capacity to engulf apoptotic thymocytes by increasing the percentage of macrophages engulfing apoptotic cells.

The effect of DEX was time-dependent, it was effective in BMDMs after 12 hrs treatment and the maximal enhancement was seen after 18 hrs treatment. Administration of DEAB did not affect the

glucocorticoid-induced enhancement of efferocytosis, if efferocytosis was detected in the first 30 min after the indicated times of DEX treatment. That is why we tested the effect of DEX not just on short-term phagocytosis (only 30 min engulfment) but also on long-term efferocytosis, when the DEX treated BMDMs were exposed continuosly to non-labelled apoptotic cells for 3 hrs , then fluorescently labeled apoptotic thymocytes were used for 30 min to test the engulfment capacity of macrophages. Prolonged phagocytosis for 3 hrs alone only slightly increased the percentage of macrophages engulfing apoptotic cells. However, when macrophages were pretreated with DEX, the percentage of engulfing cells was increased, and this enhancement was inhibited by DEAB. These data indicate that retinoids mediate the effect of GCs on the enhancement of efferocytosis during prolonged phagocytosis of apoptotic cells.

Glucocorticoids induce the expression of various lipid sensing nuclear receptors prior to that of RALDH1

Since previously we have shown that triggering various lipid sensing nuclear receptors induce the expression of RALDHs, we were wondering, whether DEX affects the RALDHs expression via regulating the expression and activity of lipid sensing nuclear receptors. In line with previous studies, RALDH1 was induced by DEX in macrophages and the expression level was increased until 24 hrs followed by glucocorticoid treatment. DEX significantly induced both the mRNA and protein expression of LXRs and PPARδ, as well as their heterodimerization partner RXRα with a peak at around 12 hrs. The expression of PPARγ however was downregulated. Since, the ligation of LXRs is also required for their signalization, and it is known that mitochondrial sterol 27-hydroxylase (CYP27) produces a ligand (27-hydroxycholesterol) for LXRs, we also tested the expression level of CYP27 in DEX exposed macrophages. The enzyme was readily induced following 4 hrs DEX treatment of macrophages. Moreover the protein synthesis inhibitor cycloheximide only partially inhibited the induction of CYP27, indicating that it might be a direct glucocorticoid target gene. Indeed, in the promoter of the human CYP27 gene the presence of a putative glucocorticoid response element was reported. On the other hand, the induction of the investigated lipid sensing nuclear receptors was completely inhibited by pretreatment with 20 μM cycloheximide, suggesting that synthesis of new protein(s) (e.g. a new transcription factor) is/are needed for their increased expression.

Dexamethasone induces RARα and C/EBPβ gene expression in macrophages

In the next step, we wanted to investigate how RALDH1 expression is regulated by DEX in macrophages. Previously we found that ligation of LXRs increases the RALDH1 mRNA expression in macrophages and data from literature also implicate the involvement of LXRs in RALDH1 induction by two different ways. On one hand LXR can directly promote transcription of sterol response element binding protein (SREBP-1c) through two LXR response elements in its promoter and then SREBP-1c can drive the RALDH1 expression. On the other hand RARα and CCAAT/enhancer binding protein (C/EBP)β simultaneously can act as regulators of RALDH1 expression where the LXR controls the RARα gene expression level. That is why we investigated whether LXRs are involved in the DEX-induced increase in the expression of RALDH1 and the mRNA level of the above mentioned genes were measured in DEX exposed BMDMs. In our experimental system the expression of SREBP-1c was not altered by DEX but the expression of both RARα and C/EBPβ was enhanced in macrophages following DEX exposure. While preincubation with cycloheximide completely inhibited the induction of RARα by DEX, administration of it resulted in increased C/EBPβ mRNA levels. This observation suggests the involvement of a labile negative protein factor(s) in the regulation of the C/EBPβ mRNA levels, and that glucocorticoids might promote the induction of C/EBPβ by suppressing the transcription of this labile inhibitory protein(s).

Induction of RALDH1 requires the expression of LXR-induced RARα and DEX-induced C/EBPβ

To investigate further the role of C/EBPβ, LXRs and RARα induction in DEX-induced efferocytosis, the effect of silencing of LXRs and C/EBPβ expressions was studied in DEX exposed BMDMs. We found that silencing of C/EBPβ prevented the DEX induced of LXRα expression indicating that DEX-induced LXRα mRNA enhancement is mediated by C/EBPβ. We also observed that DEX-induced increase in RARα mRNA expression is inhibited by LXRs silencing which is in line with the observation that RARα is an LXR target gene. In addition, silencing of C/EBPβ also prevented the DEX-induced RARα expression, since RARα is induced in LXR dependent manner and LXR induction is inhibited by C/EBPβ siRNA. On the other hand, silencing of LXRs did not prevent the DEX-induced enhanced RXRα expression indicating that RXRα is not a direct LXR target gene. However, its induction by DEX was also C/EBPβ-dependent.

Silencing of LXRs or C/EBPβ which are required for DEX-induced RARα production, impaired the RALDH1 expression in DEX treated BMDMs. Similarly, administration of AGN109, a pan RAR antagonist or genetic ablation of RARα also prevented the induction of RALDH1 by DEX. These data suggest a determining role of RARα in the regulation of RALDH1 expression. Altogether these data indicate that glucocorticoids induce the expression of C/EBPβ in macrophages which contributes to the induction of both LXRs and RXRα. Glucocorticoids also induce the expression of CYP27, which might provide an endogenous ligand for the LXRs. RARα is induced in an LXR/RXR-dependent manner and is required for the induction of RALDH1. Though previous studies indicated that C/EBPβ can also be directly involved in the induction of RALDH1, since it is also involved in the induction of RARα via the DEX regulated LXR pathway, our data cannot provide evidence for its direct involvement in the regulation of RALDH1 expression upon DEX treatment.

Glucocorticoids induce the expression of MERTK, C1q and UCP2 in BMDMs in a C/EBPβ–dependent manner. The long-term induction of UCP2 involves PPARδ, while that of C1q requires LXR nuclear receptors.

Next we wanted to figure out which efferocytosis-related molecules are affected by DEX treatment of macrophages. We measured the expression of several genes and in line with previous publications we also found, that MERTK expression was the most prominent among phagocytosis-related genes following DEX treatment in BMDMs. We found that MERTK was induced very early after DEX exposure and its induction could be decreased by preincubation with cycloheximide and by C/EBPβ silencing indicating that C/EBPβ contributes to its induction. Though MERTK was found to be a direct LXR target gene, silencing of LXRs did not affect its DEX-induced expression significantly indicating that C/EBPβ contributes to its induction independently of its effect on LXR expression. Since in the case of PPARδ -/- mice there is a reduced MERTK expression in macrophages we measured the DEX-induced MERTK expression in the presence of PPARδ antagonist GSK3787. In our experiments PPARδ inhibition had no effect on MERTK expression suggesting that PPARδ does not regulate the MERTK expression upon DEX treatment.

In line with a previous publication , DEX also induced the expression of the bridging molecule C1q. Induction of C1q by DEX was not inhibited by cycloheximide indicating that new protein synthesis is not required for its early induction. But the presence of C/EBPβ and LXRs was required

for its long term induction detected at 21 hrs, while PPARδ did not seem to be involved in its DEX-induced regulation.

Finally, we detected the glucocorticoid-dependent induction of UCP2, which also was very early induced following DEX treatment. Though its short term induction, similar to that of C1q, was independent of new protein synthesis, its long-term induction detected at 21 hrs required both PPARδ and C/EBPβ. The expression of other phagocytosis-related molecules in bone marrow derived macrophages, such as MFG-E8, CD36, CD91, stablin-2 and CD14 was not altered by DEX treatment, while that of transglutaminase 2, integrin β3, and TIM4 was decreased. Our data confirm previous findings that glucocorticoids enhance a selective set of phagocytosis related molecules and demonstrate that their long-term induction by glucocorticoids is related to the induction of C/EBPβ and lipid sensing nuclear receptors.

LXRs and PPARδ mediate the glucocorticoid-induced increase in the phagocytic capacity during prolonged efferocytosis

As described in the introduction part, previous studies have shown that LXRs and PPARδ are activated in macrophages by the apoptotic cell content when phagocytes engulf apoptotic cells continuously. Since DEX significantly induced the expression of these nuclear receptors, we decided to investigate whether silencing of LXRs or inhibition of the transcriptional activity of PPARδ using PPARδ antagonist (GSK3787) could interfere with the efferocytosis enhancing effect of glucocorticoids. Our results demonstrate that silencing of LXRs or inhibition of the transcriptional activity of PPARδ by GSK3787 had no effect on efferocytosis if it was detected directly after 24 hrs DEX treatment of macrophages. It is in line with our finding that these nuclear receptors do not affect the glucocorticoid-induced expression of MERTK, which was shown previously to drive the glucocorticoid-induced enhancement of short-term efferocytosis. On the other hand, silencing of C/EBPβ which reduced the glucocorticoid-augmented induction of MERTK, also reduced the effect of glucocorticoids on the short-term efferocytosis. If, however, efferocytosis was detected during prolonged phagocytosis, both silencing of LXRs and inhibition of the transcriptional activity of PPARδ interfered with the glucocorticoid effect. Silencing of C/EBPβ, which we found to be required for the induction of both LXRs and PPARδ, also prevented the enhancing effect of glucocorticoids on long-term efferocytosis. These data indicate that nuclear receptors, such as LXRs and PPARδ mediate the enhancing effect of glucocorticoids on the efferocytosis during prolonged phagocytosis.

Prolonged phagocytosis induces the expression of LXRα, RXRα, RARα and PPARδ, which is further enhanced in the presence of dexamethasone

Since our results indicate that lipid sensing receptors are required for the glucocorticoid-induced enhancement of efferocytosis during prolonged clearance of apoptotic cells, we decided to investigate, how the expression of the nuclear receptors does alter during prolonged clearance. We observed that prolonged phagocytosis for 3 hrs alone induced the expression of LXRα, PPARδ, RARα and RXRα, since the lipid content of the apoptotic cells activated both the LXR and the PPARδ signaling pathways. The induction of LXRα and RXRα was further increased in DEX-exposed macrophages following prolonged pagocytosis. The expression of UCP2 also enhanced after apoptotic cell feeding of macrophages which slightly increased further its DEX-induced expression.

Inhibition of RALDHs by DEAB decreases both the glucocorticoid-induced and the prolonged phagocytosis-induced expression of nuclear receptors

Next we tested, how the inhibition of RALDHs by DEAB affect the expression of retinoid regulated nuclear receptors and UCP2 during DEX-augmented long-term phagocytosis. Inhibition of RALDHs interfered with both the glucocorticoid- and the prolonged phagocytosis-induced increase in lipid sensing nuclear receptor expressions indicating that retinoids play a positive autoregulatory role in both pathways. Similar expression pattern was observed for UCP2, a PPARδ target gene as well. Our data confirm our previous findings that the LXR signaling pathway involves a retinoid-dependent autoregulatory amplification loop and we extended now this observation also for the glucocorticoid-induced lipid sensing signaling pathways.

5. DISCUSSION

Macrophages engulfing apoptotic cells produce retinoids which contribute to the induction of TG2 expression in thymocytes

Cell death is crucially required for the survival and homeostasis of multicellular organism, particularly in organs with high turnover as the bone marrow and intestine. Apoptosis is also needed to eliminate non-functional or autoreactive immune cells e.g. during maturation in the central lymphoid organs (bone marrow and thymus). In healthy individuals between 50 and 70 billions cells die each day by apoptosis. Phagocytosis of these apoptotic cells is fundamentally important throughout life, because non-cleared apoptotic cells become secondary necrotic and the consequent release of proinflammatory cell contents damages the tissue environment and provokes autoimmunity.

The thymus provides a unique microenvironment for the development and maturation of T cells. After a highly regulated developmental process in the thymus, only about 1% of the thymocytes are able to emigrate and join the peripheral lymphocyte pool. Since more than 95% of the differentiating thymocytes die by apoptosis and then they must be phagocytosed rapidly and effectively by thymic macrophages, the efficient induction and completion of the apoptotic program - with participation of TG2 - is essential in the thymus. This complex crosstalk between apoptotic and phagocytic cells can be well studied *in vivo* in the thymus.

TG2 is both induced and activated in cells undergoing apoptotic cell death. Following *in vivo* apoptosis induction in the thymus the enzyme accumulates to high levels in apoptotic thymocytes as an early event of the apoptosis program, before DNA breakdown which indicates that it has a role in the initiation phase of apoptosis, indeed, TG2 is able to promote T cell death. In addition, TG2 contributes to the formation of cross-linked protein polymers in apoptotic corpses that prevents the leakage of the harmful cell content. However, thymocytes fail to upregulate TG2 during their apoptosis *in vitro*, and they die efficiently in the absence of TG2. This observation indicates that in thymocytes TG2 might contribute to, but is not necessary for cell death induction. In addition, factors available only in the tissue environment must regulate its apoptosis-related expression.

In my experiments, I investigated the regulation of TG2 expression in apoptotic thymocytes. Since phagocytosis of dying thymocytes by thymic macrophages is continuously ongoing processes in the thymus, several molecules which are released by the engulfing macrophages as a consequence of

efferocytosis are constantly produced and provide a thymic milieu for the developing thymocytes. As such, they can be potential regulators of the expression of TG2. Previous studies indicated that TGF-β, a cytokine specifically released by macrophages ingesting apoptotic cells, is one of those factors, which can regulate the *in vivo* TG2 expression of dying cells via the TGF-β response element in its promoter. However, previous studies from our laboratory also demonstrated that TGF-β is not a strong inducer of TG2 expression that is why I decided to look for another candidate which might be responsible for the massive TG2 induction of apoptotic thymocytes. Since, it was known, that retinoids also act as a direct regulator of TG2 gene transcription and *in vivo* findings from our laboratory have also shown that retinoids are produced in the mouse thymus, in my experiments, I investigated the possible contribution of retinoids to the induction of TG2 expression in dying thymocytes.

I have demonstrated that *in vitro* RAs, or TGF-β and RAs together can significantly enhance the TG2 mRNA expression in dying thymocytes, and the apoptotic signal contributes to the TG2 induction. I have also demonstrated that inhibition of RA synthesis either by alcohol or by retinaldehyde dehydrogenases significantly attenuates the *in vivo* induction of TG2 following apoptosis induction indicating that retinoids indeed contribute *in vivo* to the enhanced TG2 expression. In fact, I found that retinoids not only contribute to TG2 expression by being constantly present in the thymic environment, but the *in vivo* apoptosis induction of thymocytes is accompanied by an enhanced retinoid-dependent transcriptional activity in the thymus. In line with these observations, *in vivo* apoptosis induction in the thymus was also accompanied by enhanced RALDH1 and 2 expression of thymic engulfing macrophages. Though thymic cortical and medullary epithelial cells were previously shown to express RALDHs, based on our data we propose that similarly to TGF-β, retinoids are produced after birth by macrophages engulfing apoptotic cells in a phagocytosis dependent manner. I have shown that the apoptotic cell-induced retinoid production might be mediated via the three lipid sensing receptors (LXRs, PPARδ and PPARγ). Based on these results, experiments in our laboratory have demonstrated that the LXR signaling pathway promotes phagocytosis of apoptotic cells via retinoid production, which induces the expression of numerous phagocytic receptors.

Dexamethasone treatment also induces retinoid production in macrophages which enhances prolonged phagocytosis of apoptotic cells

I have demonstrated, that *in vivo* DEX treatment was more efficient than that of anti-CD3 in promoting retinoid-dependent transcriptional activity (lacZ mRNA expression), which might be related partially to the fact that DEX is a more potent apoptosis inducer leading possibly to more phagocytosis and more engulfment-dependent retinoid production. However, I also found that DEX alone can induce the expression of RALDH1 in macrophages, independently of the engulfment, thus DEX alone can also contribute to the DEX treatment-induced *in vivo* lacZ expression in the thymus. Since the *in vitro* DEX treatment of macrophages induced RALDH1 expression, and previous studies from our laboratory have shown that retinoids enhance the phagocytic capacity of macrophages, we decided to investigate whether this RALDH1 expression induces a physiologically relevant retinoid synthesis in macrophages which could contribute to the known efferocytosis enhancing effect of DEX.

Apoptotic cells are rarely seen *in situ* in the thymus. This is thought to be the consequence of the highly effective dead cell removal by efferocytosis which prevent secondary necrosis and the consequent release of proinflammatory cell contents that damages the tissue environment and provokes autoimmunity. In addition, sensing and removal of apoptotic cells generally induce an anti-inflammatory response. As a consequence, improper clearance of apoptotic cells being the result of either genetic anomalies and /or a persistent disease state, contributing to the establishment and progression of autoimmune diseases. Most of these diseases respond very well to glucocorticoid treatment. Though many mechanisms have been discovered through which glucocorticoids mediate downregulation of inflammatory responses, increasing evidence suggests that enhancing clearance of apoptotic cells might be one of them, so investigation of the efferocytosis enhancing effect of glucocorticoids might help to understand how they exert their immunosuppressive activity.

Previous studies have shown that glucocorticoids are capable of enhancing short-term phagocytosis of apoptotic cells by increasing the expression of several efferocytosis-related genes, such as MERTK, thrombospondin-2, the expression level of MFG-E8 and the cell surface expression of the phagocytic receptor CD91. In our experimental system, we could confirm the enhancing effect of glucocorticoids on the short-term efferocytosis and the induction of MERTK, but not that of the induction of MFG-E8 in BMDMs. In line with our observation, if MFG-E8 knock-out mice were treated for a week with DEX which affects both macrophages and induces thymocyte apoptosis in the thymus, no alterations were detected in the rate of *in vivo* efferocytosis or in the residual thymic

size. On the other hand in thioglycollate-elicited macrophages the DEX response was found to be MFG-E8 dependent, and we also found an increase in MFG-E8 expression in peritoneal macrophages in response to DEX treatment indicating that the DEX response on MFG-E8 might be macrophage-type specific.

However, we could detect the induction of another bridging molecule C1q. C1q was reported to bind to and activate the CD91 phagocytic receptor, and its cell surface expression was shown to be induced by DEX. While CD91 was reported to trigger the GULP-dependent signaling pathway of Rac activation, MERTK contributes to the Dock/Elmo-dependent signaling pathway of Rac activation by promoting integrin β_5 signaling. Thus simultaneous induction of MERTK and C1q by DEX might enhance efferocytosis by simultaneously promoting both signaling pathways that lead to Rac activation. In addition to being a CD91 ligand, C1q was reported recently to promote MERTK-dependent efferocytosis also via triggering the adiponectin signaling pathway. This signaling pathway involves activation of the AMP activated protein kinase, which also promotes phagocytosis of apoptotic cells. Since the induction of both MERTK and C1q required the presence C/EBPβ transcription factor induced by DEX, silencing of C/EBPβ prevented the enhancing effect of DEX on the short term phagocytosis.

In addition to the phagocytosis receptors and bridging molecules, my novel finding is that DEX also upregulates the expression of several nuclear receptors, such as LXRs, RXRα and PPARδ, together with CYP27, all of them in a C/EBPβ-dependent manner. The C/EBPβ transcription factor has been previously shown to be critical for macrophage differentiation and function and my data indicate that glucocorticoids also upregulate C/EBPβ to initiate or to promote glucocorticoid-dependent transcription.

I have demonstrated that UCP2 mRNA expression was induced in DEX - exposed BMDMs, in a PPARδ dependent manner, while LXRs were required for up-regulation of C1q and RARα, and consequently for that of RALDH1. The regulation of other RALDHs was not investigated in our study, but the retinoids produced by RALDHs promoted the induction of the expression of all these nuclear receptors via a positive autoregulatory loop. The mechanism of this feedback induction was not studied in our experiments, but it is known that both LXRs and PPARδ are permissive nuclear receptors, thus they can be activated by the RXR retinoid ligand binding site as well. In addition, at least for LXRα it was demonstrated that LXR binding sites also exist in its promoter. Thus following ligation, LXRs promote their own transcription via an autoregulatory loop mechanism.

But DEX-induced short-term efferocytosis did not require *de novo* retinoid synthesis. Neither was the short-term phagocytosis LXR- or PPARδ-dependent. The reason for this is very likely that 1.) the induction of MERTK that drives the enhancement in efferocytosis by DEX was LXR- and PPARδ- independent 2.) the metabolic changes including upregulation of UCP2 induced by these nuclear receptors are required only during prolonged phagocytosis, when macrophages not only take up apoptotic cells, but also have to handle the digested material 3.) though the induction of C1q was LXR-dependent, in the presence of serum which contains complement factors, the lack of C1q production by macrophages following LXR silencing could not result in a detectable effect on the DEX-induced short-term efferocytosis. However, glucocorticoid-induced prolonged phagocytosis tested after exposure of macrophages to apoptotic cells for 3 hrs was LXR - and PPARδ-dependent and required the retinoid production induced by LXRs, in line with the observation that the full upregulation and activity of these nuclear receptors was dependent on the endogenously produced retinoids. Besides enhancing the expression of phagocytic receptors known to be triggered by nuclear receptors following the exposure to the lipid content of the apoptotic cells, nuclear receptors promote prolonged phagocytosis very likely also via inducing alterations in metabolism, as well as via increasing the expression of UCP2 needed to handle the digested material during continued clearance of apoptotic cells.

Altogether our data indicate that glucocorticoids promote not only the short-term, but also the long-term phagocytosis of apoptotic cells, and they act so by upregulating the lipid sensing receptors LXRs and PPARδ, and retinoids act in a positive autoregulatory loop to enhance their transcriptional activity. Since retinoids mediate the glucocorticoid effect on long-term efferocytosis, retinoids could be considered as potential promoters of the efficiency of glucocorticoid treatment in inflammatory diseases.

6. SUMMARY

Efficient execution of apoptotic cell death followed by efficient clearance mediated by professional and by nonprofessional neighboring phagocytes, is a key mechanism in maintaining tissue homeostasis. Every day billions of our cells die and get cleared without initiating inflammation and an immune response. Transglutaminase 2 expressed both in apoptotic and engulfing cells ensures fast recognition and removal of apoptotic cells, as well as it contributes to the prevention of the release of the harmful cell content. T cells differentiate in the thymus, and during their selection processes 95% of the newly produced cells die and clear. Thus mouse thymus provides us an excellent *in vivo* system to study apoptosis and phagocytosis *in vivo*.

In my thesis I demonstrate that though TG2 is induced in dying thymocytes *in vivo*, TG2 is not expressed in thymocytes dying *in vitro*. For the *in vivo* induction of TG2 factors found in the thymic environment are required. I found that some of such factors are vitamin A derivatives, which are produced via aldehyde and retinaldehyde dehydrogenases in macrophages, when they engulf apoptotic cells. The production of these retinoids is under the control of lipid sensing nuclear receptors, such as PPARs and LXRs, and they regulate primarily the phagocytic capacity of engulfing macrophages.

I found that retinoids are produced not only in macrophage engulfing apoptotic cells, but also in macrophages exposed to DEX. I show that glucocorticoids promote both short-term and long-term clearance of apoptotic cells. Glucocorticoids seem to directly induce the expression of the phagocytosis-related genes Mer tyrosine kinase, complement 1q, the uncoupling protein-2, and the transcription factor C/EBPβ. C/EBPβ contributes to the further induction of the phagocytosis-related genes, and is required for the induction of lipid sensing receptors LXRs, PPARδ, RARα, RXRα and RALDH1, the latter one in an LXR- and RARα-dependent manner. Glucocorticoid-induced enhancement in long-term efferocytosis was dependent on the induction of lipid sensing receptors known to be triggered by the lipid content of the engulfed cells to enhance phagocytic capacity. Retinoids did not affect the glucocorticoid-induced short term phagocytosis of apoptotic cells, but were required for the glucocorticoid-induced enhancement of efferocytosis during prolonged clearance of apoptotic cells by promoting efficient LXR and PPARδ upregulation.

7. PUBLICATIONS

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Registry number: DEENK/216/2015.PL
Subject: Ph.D. List of Publications

Candidate: Éva Garabuczi
Neptun ID: HD9ZUD
Doctoral School: Doctoral School of Dental Sciences

List of publications related to the dissertation

1. **Garabuczi, É.**, Sarang, Z., Szondy, Z.: Glucocorticoids enhance prolonged clearance of apoptotic cells by upregulating liver X receptor, peroxisome proliferator-activated receptor-[delta] and UCP2.
Biochim. Biophys. Acta, Mol. Cell Res. 1853 (3), 573-582, 2014.
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Oral Presentations:

Garabuczi É. **What regulates the tissue transglutaminase expression in the dying thymocytes in vivo?** 2nd Molecular Cell and Immune Biology (MCBI) Winter School, Krompachy, Slovakia, January 6-9, 2009

Garabuczi É. **Regulation of transglutaminase 2 expression in thymocytes undergoing apoptosis.** 3rd Molecular Cell and Immune Biology Winter School, Mariazell, Austria, January 7-10, 2010

Garabuczi É. **Retinoids might contribute to the upregulation of transglutaminase 2 in apoptotic thymocytes.** 4th Molecular Cell and Immune Biology Winter School, Galyatető, Hungary, January 11-14, 2011

Garabuczi É. **Dexamethasone might promote phagocytosis of apoptotic cells by macrophages via activating nuclear receptor pathways.** 5th Molecular Cell and Immune Biology Winter School, Galyatető, Hungary, January 4-7, 2012

Garabuczi É. **Glucocorticoids enhance phagocytosis of apoptotic cells by upregulating the expression of both phagocytic receptors and lipid sensing receptors.** 6th Molecular Cell and Immune Biology Winter School, Galyatető, Hungary, January 8-11, 2013

Garabuczi É. **Involvement of the LXR receptor in the glucocorticoid-induced enhancement of apoptotic cell phagocytosis.** 7th Molecular Cell and Immune Biology Winter School, Galyatető, Hungary, January 7-10, 2014

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Éva Garabuczi, Adrienn Galuska, Zsolt Sarang, László Fésüs, Zsuzsa Szondy**. Dexamethasone induced enhanced apoptotic cells phagocytosis in macrophages might involve lipid sensing nuclear receptor.** Apoptotic Cell Recognition & Clearance, Gordon Research Conferences and Gordon Research Seminars, University of New England, Biddeford, ME, June 22-28, 2013

8. KEYWORDS

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