

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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LIST OF ABBREVIATIONS

9cRA,	9- <i>cis</i> retinoic acid
ABCA1,	ATP-binding cassette transporter protein, sub-family A member 1
ABCG1,	ATP-binding cassette sub-family G member 1
ADH,	alcohol dehydrogenases
AIF,	apoptosis-inducing factor
ALDH,	aldehyde dehydrogenases
AP-1,	activator protein complex-1
ATRA,	all- <i>trans</i> retinoic acid
BAI1,	brain specific angiogenesis inhibitor 1
BCL-2,	B-cell lymphoma 2
BH3,	BCL-2 homology 3
BID,	BH3-interacting domain death agonist
BMDM,	bone marrow derived macrophage
C1qb,	complement 1qb
C/EBP,	CCAAT/enhancer binding protein
CFDA,	carboxyfluorescein diacetate succinimidyl ester
CHX,	cycloheximide
CMTMR,	5-(and-6)-(((4-chloromethyl)benzoylamino)tetramethylrhodamine
CYP27,	mitochondrial sterol 27-hydroxylase
CRT,	calreticulin
DEAB,	4-diethylamino-benzaldehyde
DEX,	dexamethasone acetate
DN,	double negative
DP,	double positive
DSF,	disulfiram
FN,	fibronectin
FKN,	fractalkine
GAPDH,	glyceraldehyde-3-phosphate dehydrogenase
Gas6,	growth-arrest-specific 6
GCs,	glucocorticoids
GEF,	guanine nucleotide exchange factor
GR,	glucocorticoid receptor
GRE,	glucocorticoid response elements
HSP90,	heat shock protein 90
LDL,	low density lipoprotein
LOX-1,	lectin-type oxidized LDL receptor 1
LRP,	low density lipoprotein receptor-related protein
LXR,	liver X receptor
MBL,	mannose binding lectin
MERTK,	c-Mer proto-oncogene tyrosine kinase
MFG-E8,	milk fat globule epidermal growth-factor 8
MHC,	major histocompatibility complex
MILIBS,	metal ion-dependent ligand binding site

MOMP,	mitochondrial outer membrane permeabilization
NR,	nuclear receptor
PAF,	platelet activating factor
PGE ₂ ,	prostaglandin E ₂
PPAR,	peroxisome proliferator-activated receptor
PS	phosphatidylserine
RA,	retinoic acid
RALDH,	retinaldehyde dehydrogenases
RAR,	retinoic acid receptor
RARE,	retinoic acid response element
RXR,	retinoid X receptor
RXRE,	RXR response element
SP,	single positive
SH2,	SH3 Src homology 2
SDR,	short-chain dehydrogenase/reductase
SP-A, SP-D,	lung surfactant proteins A and D
SR-A ,	scavenger receptor A
SREBP,	sterol response element binding protein
TAM,	Tryo3–AXL–Mer
TCR,	T cell receptor
TIM,	T cell immunoglobulin and mucin-domain-containing molecule
TG2,	transglutaminase 2
TNFR1,	tumour necrosis factor receptor 1
TRAIL,	TNF-related apoptosis-inducing ligand receptor
TSP-1,	trombospondin-1
UCP2,	uncoupling protein 2

List of synthetic compounds (Inhibitors, Agonists and Antagonists)

Name	Target
AGN109	pan RAR antagonist
AM580	RAR α agonist
DSF (disulfiram)	Alcohol dehydrogenase inhibitor
DEAB (4-diethylamino-benzaldehyde)	Retinaldehyde dehydrogenase inhibitor
GSK3787	PPAR δ antagonist
GW1516	PPAR δ agonist
GW3965	LXRs agonist
LG268	RXR agonist
RSG (rosiglitazone)	PPAR γ agonist
CHX (cycloheximide)	inhibitor of new protein synthesis

INTRODUCTION

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 maintains 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* (Koemer et al., 2009; Marino et al., 2014). 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 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 preventing secondary necrosis; while the engulfing cells do not produce inflammatory cytokines (Savill and Fadok, 2000; Kurosaka et al., 2003; Elmore 2007). However, if the apoptotic cells are not promptly cleared, the membrane integrity is lost over time and apoptotic cells can progress to secondary necrosis. The unregulated release of intracellular contents from necrotic cells can cause inflammatory responses, production of autoantibodies particularly toward intracellular antigens and DNA released from the dying cells (Elliot and Ravichandran, 2010).

Apoptosis can be initiated via two different pathways: either at the plasma membrane by death receptor ligation (extrinsic pathway) or at the mitochondria (intrinsic pathway). Extrinsic apoptosis occurs in response to ligation of the so-called death receptors, such as CD95 (also known as FAS), tumor necrosis factor receptor 1 (TNFR1) or TNF-related apoptosis-inducing ligand (TRAIL) receptor. This results in the recruitment of several proteins to the ligated receptors leading to activation of effector caspases - that function as cysteine proteases to cleave other protein substrates within the cell, to trigger apoptosis - or truncate the B-cell lymphoma 2 (BCL-2) homology 3 (BH3)-only protein BID (BH3-interacting domain death agonist), which co-activates the intrinsic pathway of apoptosis by translocating to mitochondria (Marino et al., 2014).

The more complex intrinsic signaling pathway of apoptosis, also known as mitochondrial pathway, involves non-receptor-mediated intracellular signals. This is marked by one central event – the mitochondrial outer membrane permeabilization (MOMP) - which results in the release of cytochrome C from the mitochondria to the cytosol, and triggers the execution of cell death by promoting caspase activation. Apoptosis-inducing factor (AIF) and endonuclease G also released as a result of MOMP can promote caspase-independent cell death (Marino et al., 2014). This pathway is regulated by members of the BCL-2 family consisting of pro- and anti-apoptotic proteins. Cellular stress, such as DNA damage, leads to an increase in pro-apoptotic protein levels which in turn allows pro-apoptotic BCL-2 proteins to induce the release of cytochrome c from mitochondria, causing activation of the caspase cascade (Schlossmacher et al., 2011).

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.

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. The concentration of retinoids in the body is maintained within a very narrow range, generally very low and usually 100 to 1,000 times less than that of retinol. The lipid soluble retinol is required to move in the cells in association with a number of carrier proteins, which are either in intracellular compartments or in the extracellular environment (Huang et al., 2014; O'Byrne et al., 2013)

RETINOIC ACID BIOSYNTHESIS

All-*trans* RA (ATRA) is produced from retinol in a two-step oxidative pathway (Fig. 1). First step is the oxidation of retinol to retinaldehyde which is generally considered to be the rate-limiting step (Napoli et al. 1986) 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 the oxidation of retinol to retinaldehyde: the cytosolic alcohol dehydrogenases (ADH) and the microsomal short-chain dehydrogenase/reductase (SDR) (Parés et al., 2008). The current consensus appears to be that the ADH enzymes are not essential for ATRA biosynthesis, since there is a physiologically relevant supply of vitamin A during embryogenesis or adulthood, but ADHs play a role as backup enzymes under extreme dietary conditions. At least one member of the SDR group is clearly important for retinoid metabolism *in vivo*. This important SDR enzyme is 11-*cis*-retinol dehydrogenase (SDR9C5), which was proven to be essential for the regeneration of 11-*cis*-retinaldehyde during the retinoid visual cycle, because mutations in this gene (RDH5) have been linked to a rare form of stationary night blindness (Simon et al., 1995; Yamamoto et al., 1999)

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). In addition, another member of ALDH family, ALDH8A1 (RALDH4), was shown to recognize 9-*cis*-retinaldehyde, but not all-*trans* -retinaldehyde as a substrate (Lin et al., 2000; Lin et al., 2003). ALDH1A2 is the primary enzyme responsible for ATRA biosynthesis at most sites during embryogenesis (Niederreither et al., 1999). ALDH1A1 is not essential for embryogenesis, but it may have a role in ATRA biosynthesis during adulthood. 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 (Fan et al., 2003; Molotkov et al., 2003). ALDH1A1 and ALDH1A2 have similar catalytic efficiency, but the K_m value of ALDH1A2 is much lower than that of ALDH1A1 (Gagnon et al., 2003; Gagnon et al., 2002). 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 (Kedishvili, 2013).

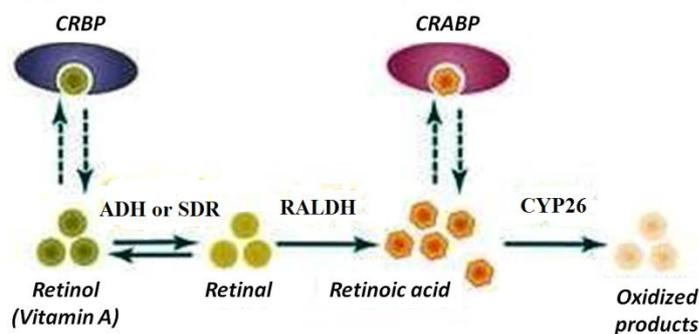


Figure 1. Synthesis and degradation of retinoic acid

In a reversible reaction, retinal is synthesized from retinol (vitamin A) by either alcohol dehydrogenases (ADHs) or short-chain dehydrogenase/reductases (SDRs). Cellular retinol binding proteins (CRBPs) can bind retinol, whereas cellular retinoic acid binding proteins (CRABPs) can bind RA. Finally, endogenous RA is degraded by CYP26 enzymes. (Marlétaz et al., 2006)

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) (Fig. 2). Other nuclear receptors also bind to lipophilic molecules, including diverse steroids, thyroid hormone, a variety of dietary lipids and their metabolic derivatives.

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 by deacetylating histones associated with the target sequences thus increasing chromatin condensation. 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 (Marlétaz et al., 2006). 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 (Mic et al., 2003; Kane, 2012). By contrast, ATRA is easily detectable in many tissues. Our group has found that a non-conventional retinoid is produced in the thymus – a dihydroretinoic acid derivative – capable of activating retinoid receptors, which may serve as an endogenous RXR ligand (Sarang et al., 2013). Possible other candidates as physiological RXR ligands are the unsaturated fatty acid docosahexanoic acid (de Urquiza et al., 2000),

linolenic acids (Wolf, 2006) or phytol metabolites (Kitareewan et al., 1996). All of these compounds can activate RXR, but none of these studies have fully confirmed the physiological relationship of the ligand with the receptor (Huang et al., 2014). There are three separate genes encoding both RAR (RAR α , RAR β and RAR γ) and RXR (RXR α , RXR β and RXR γ) that are conserved throughout vertebrates. In macrophages, RAR α , RAR γ , RXR α and RXR β have been found to be expressed (Barish et al., 2005).

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 the thyroid hormone receptor, vitamin D receptor, 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 (Huang et al., 2014). LXRs similarly to 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 (Zelcer and Tontonoz 2006).

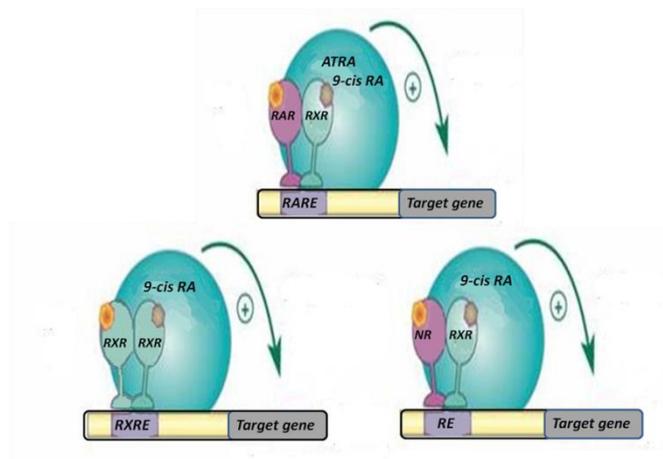


Figure 2. Mode of action of retinoic acid

Retinoic acid signaling is mediated by RAR and RXR receptors. The ligated RAR/RXR heterodimer or RXR/RXR homodimer binds to retinoic acid response element (RARE) and RXR response element (RXRE) respectively in the regulatory regions of target genes. RXR, activated by 9- cis RA, forms heterodimers with other nuclear receptors (NR) including LXRs and PPARs and bind to specific response elements (RE) present in the promoters of their target genes.

LXRs - LXR α and LXR β - play central role in the transcriptional control of lipid and cholesterol metabolism (Kalaany and Mangelsdorf, 2006). LXRs function as oxysterol sensors (Willy et al., 1995) and are essential components of a physiological feedback loop regulating cholesterol metabolism and transport (Edwards et al., 2002). LXR α is highly expressed in the liver and at lower levels in the intestine, adipose tissues and macrophages etc., whereas LXR β is ubiquitously expressed. LXR ligands promote reverse cholesterol transport, in which excess cholesterol is transferred from macrophages via induction of cholesterol transporter proteins (ABCA1 and ABCG1), and also via increased production of cholesterol acceptors (apoE and apoC) and lipoprotein remodelling proteins (Castrillo and Tontonoz, 2004). 27-hydroxycholesterol is one of the several oxysterols identified as potential endogenous ligands of LXR. 27-hydroxycholesterol is produced by a mitochondrial 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 (Szántó et al., 2004).

PPARs (including three different proteins: PPAR α , PPAR β/δ and PPAR γ) regulate genes important in cell differentiation and various metabolic processes, especially lipid and glucose homeostasis (Grygie-Górniak, 2014). PPAR δ (also called PPAR β) is ubiquitously expressed, but its highest expression was found in intestinal epithelium, liver and keratinocytes, and it is abundantly found in heart, spleen, skeletal muscle, lung, brain and thymus as well (Girroi et al., 2008). PPAR δ participates in fatty acid oxidation, in skeletal and cardiac muscles, but it also regulates blood glucose and cholesterol levels (Grygiel-Górniak, 2014). In addition, activation of PPAR δ (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 (Wang et al., 2003). Administration of synthetic PPAR δ ligands was further shown to promote fat burning and to protect against the development of obesity (Castrillo and Tontonoz, 2004).

GLUCOCORTICOIDS

Glucocorticoids (GCs) are essential steroid hormones for human life, and very effective anti-inflammatory and T cell apoptosis inducing agents. Cortisol is a naturally occurring glucocorticoid, and it is one of the corticosteroids (adrenal cortical steroids) produced by

adrenal glands. GCs are important for many physiological processes including the utilization of carbohydrate, fat and protein as well as for normal response to stress.

GCs exert a wide range of anti-inflammatory and immunosuppressive activities after binding to the glucocorticoid receptor (GR). Synthetic derivatives of GCs, such as dexamethasone (DEX) or prednisolone, are widely used in the treatment of inflammatory disorders including rheumatoid arthritis, multiple sclerosis, psoriasis and eczema, respectively they are used in the treatment autoimmunity and cancer (Tuckermann et al., 2005). A well known immunosuppressive activity of GCs is their ability to efficiently enhance phagocytosis of apoptotic cells by human and mouse macrophages (Liu et al., 1999). 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 c-Mer proto-oncogen receptor tyrosine kinase (MERTK) phagocytosis receptor and milk fat globule epidermal growth factor - 8 (MFG-E8) and complement 1q (C1q) serum proteins (Ehrchen et al., 2007).

Because 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 (for example HSP90, immunophilin etc.) which block the nuclear localization sequence of the GR, thus inhibiting GR nuclear translocation, keeping the GR in an inactive state. Upon glucocorticoid binding, the GR undergoes conformational changes and then dissociates from its chaperone proteins. Consequently, nuclear localization sequence and the dimerization domain are exposed and enable the formation of GR homodimer, which translocates to the nucleus, and binds the GC response elements in the promoters of its target genes. Beside the direct transactivating effect of GR, it can also transrepress gene transcription by directly binding negative GC response element, or it can indirectly modulate gene transcription by interacting with other proteins in the nucleus, such as transcription factors like activator protein complex-1 (AP-1).

Besides the nuclear effects, some of the glucocorticoid effects are mediated by non-genomic signaling. Non-genomic effects were defined as any action that does not affect gene expression initially or directly, but that does induce rapid effects, such as inhibition of signal transduction pathways (Mitre-Aguilar et al., 2015). For example, GR and HSP90 play an

essential role in the formation of antigen activated T cell receptor (TCR) -associated protein complexes. The ligand binding of glucocorticoid receptor causes the dissociation of multiprotein complex and results in impaired TCR signaling in T cells (Löwenberg et al., 2006).

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. Positive and negative selection steps in the thymus enable the differentiation and selection of T cells, which bear functional T cell receptors. TCRs (composed of an α and a β chain) recognize antigen only in the context of cell-cell interaction presented by correct MHC. The TCR is closely associated with a group of 5 proteins collectively called the CD3 complex. The CD3 complex is composed of a γ , a δ , two ϵ and two ξ chains. The CD3 complex is necessary for cell surface expression of the TCR during T cell development. In addition, the CD3 complex transduces activation signals to the cell following antigen interaction with the TCR (Mayer and Nyland, 2010).

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) precursor 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” (Szondy et al., 2012). To generate single positive cells, CD4⁺CD8⁺ thymocytes undergo negative selection (death) and positive selection (survival). Thymocytes expressing a TCR that interact too strongly with self-peptides presented by self MHC molecules are eliminated by TCR-mediated apoptosis (negative selection). Thymocytes bearing TCRs with low-to-moderate avidity for self peptide/MHC are rescued (positive selection) (Coutinho et al., 2011).

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 E₂ (PGE₂) (Fadok et al., 1998) and adenosine

(Köröskényi et al., 2011) 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 (Kiss et al., 2006; Mastino et al., 1992; Szondy et al., 2003). 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 signaling, ensures the induction of apoptosis of neglected thymocytes (Fig. 3) (Szondy et al., 2012).

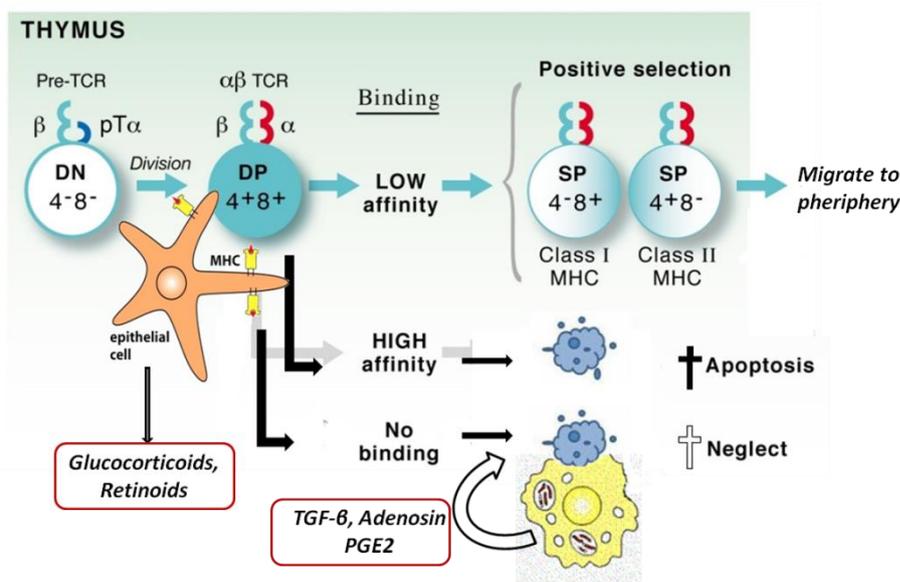


Figure 3. Thymocyte development in the thymus. CD4⁻CD8⁻ (DN) cells expressing pre-TCR undergo divisions and become $\alpha\beta$ TCR⁺CD4⁺CD8⁺ (DP) at which stage they interact with self-peptides presented by class I and II MHC molecules expressed on thymic stromal cells. Cell death of non-functional DP thymocytes (no interaction between TCR-pMHC) are affected by glucocorticoids and retinoids produced by MHC presenting thymic epithelial cells. In addition, macrophages engulfing apoptotic cells produce TGF- β , adenosine and PGE2, which also influence the DP thymocyte's cell death. Those thymocytes whose TCRs interact with high affinity to pMHC undergo apoptosis, while those bound to pMHC with low affinity mature to become MHC class I-restricted CD8⁺ SP or class II-restricted CD4⁺ SP cells. These mature thymocytes then migrate to the periphery. Partly used: (Fridkis-Hareli and Reinherz, 2004)

ROLE OF GLUCOCORTICOIDS IN THE DEVELOPMENT OF THYMOCYTES

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 (Purton et al., 2004) or to influence thymocyte selection.

The mechanism of glucocorticoid-induced apoptosis in thymocytes seems to occur via the mitochondrial pathway. This is confirmed by the observation that GCs can induce apoptosis in T cells by increasing the expression of Bim, the pro-apoptotic member of BCL-2 family (Wang et al., 2003). In addition, thymocytes from double knock-out mice lacking Bak and Bax (two other pro-apoptotic BCL-2 protein) are resistant to GC-induced apoptosis (Rathmell et al., 2002). Moreover, it was reported that GC treatment disrupts the outer membrane of mitochondria, releasing pro-apoptotic molecules such as cytochrome c and AIF. These molecules finally activate caspase-3 to induce apoptosis (Chung et al., 2002).

GCs also modulate thymocyte selection by communicating with TCR-mediated signaling events. They are able to increase the TCR signaling thresholds required to promote positive and negative selection (Ashwell et al., 2000), on the other hand, activation of the TCR/CD3 signaling is necessary and sufficient to protect primary T cells from the GC-induced apoptosis (Jamieson and Yamamoto, 2000).

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 (Csősz et al., 2009; Odii and Coussons, 2014). TG2 is composed of four distinct domains: an NH₂-terminal B-sandwich domain, a catalytic core domain, and two COOH-terminal B-barrel domains with a phospholipase C binding sequence.

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 (Hasegawa et al., 2003; Mishra et al., 2004; Murthi et al., 1999). In the GDP-bound form, the transamidation activity is inhibited by the bound nucleotide, while Ca²⁺ binding favours the transamidation activity. In the apoptotic cells, the normally high intracellular GTP concentration decreases and the increasing Ca²⁺ level leads to activation of transamidase activity of TG2 (Liu et al.,

2002). Furthermore, TG2 has calcium-independent 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 (Odi and Coussons, 2014). One of these surface proteins is fibronectin (FN). Transglutaminase 2 has a very high affinity for FN, and together they mediate many adhesion-dependent phenomena, such as cell migration, matrix assembly, and signaling. Transglutaminase 2 can also bind many integrin receptors, via the extracellular domains of the $\beta 1$ and $\beta 3$ integrin subunits, to maintain cell-extracellular matrix (ECM) interactions independently from fibronectin binding. In cell-matrix interactions, TG2 can also function as a co-receptor for various isoforms of integrin and FN to form ternary complexes, where all the three proteins successfully interact with each other (Akimov et al., 2000).

Transglutaminase 2 has been shown to be induced and activated in cells undergoing apoptosis (Fésüs et al., 1987). Several signaling pathways can lead to the parallel induction of apoptosis and TG2 expression (Fésüs et al., 1996). 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 (Fésüs et al., 1989; Melino et al., 2000). 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 (Fésüs and Szondy 2005). It does so by affecting many pathways, for example in the thymus TG2 is induced during the early phase of apoptosis, initiated by distinct signaling pathways (via glucocorticoid receptor, T-cell receptor or p53 signal), and not only the increased amount but the increased activity of the *in vivo* enzyme can be detected (Szondy et al., 1997). On T cells TG2 promotes apoptosis by increasing the mitochondrial Ca^{2+} levels (Hsieh et al., 2013) Further proapoptotic function of TG2 is the acceleration of the mitochondrial cell death acting as a BH3-only protein. TG2 is able to interact with the pro-apoptotic BCL-2 family members Bax and Bak, even in the absence of any apoptotic stimuli. Following apoptosis induction, the early increase of calcium concentration at the mitochondrial level activates the cross-linking activity of TG2, which by polymerizing Bax stabilizes its pore-forming conformation resulting a more rapid release of cytochrome C thereby promoting apoptosis (Rodolfo et al., 2004).

Besides thymocytes, TG2 also facilitates apoptosis in neurons or red blood cells, but it has a protective role against the induction of massive cell death in hepatocytes and cardiac cells

(Fésüs and Szondy 2005). Moreover, TG2 can support the migration of macrophages to the site of apoptosis by crosslinking the ribosomal S19 protein that functions as a chemoattractant (Nishiura et al., 1998). TG2 can also support the recognition of apoptotic cells by facilitating the exposure of phosphatidylserine (PS) (primary marker of apoptotic cells) to the surface (Sarang et al., 2007).

The majority of the cellular TG2 pool is present in the cytoplasm, some are also found in the mitochondria and in the nucleus, – no TG2 is detected in the ER or Golgi compartments – and a significant fraction of TG2 (1–20%) is localized extracellularly, both on the plasma membrane and in the ECM. TG2 is constitutively externalized from undamaged cells in various cell types including fibroblasts, osteoblasts, endothelial cells, smooth muscle cells, and monocytes/macrophages. While these cell types contain TG2 on their surface and deposit TG2 in the ECM, no secretory signal sequences, hydrophobic or transmembrane domains are found in TG2, and little is known regarding the factors that control its secretion (Belkin, 2011). An unconventional pathway of cytoplasmic TG2 secretion has been described involving phospholipid-dependent delivery into recycling endosomes (Zemskov et al., 2011).

Recognition of apoptotic cells via PS receptors - which is the first step of apoptotic cell phagocytosis - triggers latent TGF- β release from macrophages (Fadok et al., 1992). Simultaneously, phagocytes produce TG2, which activates TGF- β (Upchurch et al., 1991). 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 (Szondy et al., 2003; Ritter et al., 1988). Induction of TG2 by TGF- β in macrophages results in an autoregulatory loop leading to further TGF- β formation and release (Fésüs and Szondy, 2005).

ENGULFMENT OF APOPTOTIC CELLS

Every day billions of cells die in our body to eliminate those that are harmful, useless, or senescent (Nagata, 2010). Examples of unwanted cells include excess cells generated during development, cells infected with intracellular bacteria or viruses, transformed or malignant cells capable of tumorigenesis, and cells irreparably damaged by cytotoxic agents (Hochreiter-Hufford and Ravichandran, 2013). Phagocytic removal of apoptotic cells is also prominent during the resolution phase of inflammation, when large numbers of infiltrating

granulocytes and lymphocytes undergo apoptosis and must be cleared to terminate an inflammatory response (Lemke, 2013).

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 (Hochreiter-Hufford and Ravichandran 2013). Since the phagocytosis of apoptotic cells has distinctive morphologic features and unique downstream consequences, Peter Henson and colleagues have suggested the unique term *efferoctosis* (taken from the Latin *effero*, meaning to take to the grave or to bury) to specifically refer to the engulfment of apoptotic cells (Vandivier et al., 2006).

A phagocyte is any cell that is capable of engulfment, including “professional” phagocytes such as macrophages, immature dendritic cells and neutrophils. The “non-professional” phagocytes are neighbouring cells in a tissue that can also mediate the clearance of apoptotic targets. An important difference between the phagocytic process of professional and non-professional phagocytes is the response of professional phagocytes to phagocytic stimuli. This response includes the generation and release of pro-inflammatory and anti-inflammatory mediators and respiratory burst or release of toxic and microbicidal molecules by degranulation. Additionally, professional phagocytes are capable of recognizing a wide variety of apoptotic targets and ingesting them rapidly and efficiently (Rosales, 2005).

The elimination of apoptotic cells and cell bodies by phagocytes represents an evolutionarily conserved way to prevent exposure of the surrounding tissue to potentially cytotoxic, immunogenic, or inflammatory cellular contents (Chung et al., 2006) Uncleared corpses can undergo secondary necrosis, promoting inflammation and autoimmunity (Hochreiter-Hufford and Ravichandran 2013). What distinguishes the phagocytosis of apoptotic cells from the phagocytosis of most bacteria or necrotic cells is the release of anti-inflammatory cytokines IL-10, TGF- β , platelet activating factor (PAF), and PGE₂ and inhibition of production of pro-inflammatory cytokines TNF- α , GM-CSF, IL-12, IL-1 β , and IL-18 which suppress autoimmune responses (Chung et al., 2006; Hochreiter-Hufford and Ravichandran, 2013). Mice deficient in specific engulfment receptors, such as MERTK, bridging molecules, such as MFG-E8, or C1q fail to clear apoptotic cells leading to the generation of autoantibodies and the development of an autoimmune response. In patients with systemic lupus erythematosus, there is a strong correlation between disease progression and the failed clearance of apoptotic cells (Ravichandran and Lorenz, 2007).

STEPS OF APOPTOTIC CELL CLEARANCE

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 (Fig. 4) (Erwig and Henson, 2008).

“FIND ME” SIGNALS

Apoptotic cells are rarely seen *in situ*, even in tissues with high cellular turnover such as bone marrow or thymus. This is thought to be due to the release of “find me” signals by apoptotic cells that recruit motile phagocytes leading to efficient clearance of dying cells. To date, several proposed “find me” signals released by dying cells have been reported (Hochreiter-Hufford and Ravichandran 2013; Elliott et al., 2009).

Before the tethering/engulfment phases of macrophage-mediated apoptotic-cell clearance, phagocytes are required to navigate effectively to sites of apoptosis. Active release of chemoattractant (“find me”) signals from apoptotic cells occur in an early stage after engagement of the cell-death program (Truman et al., 2008).

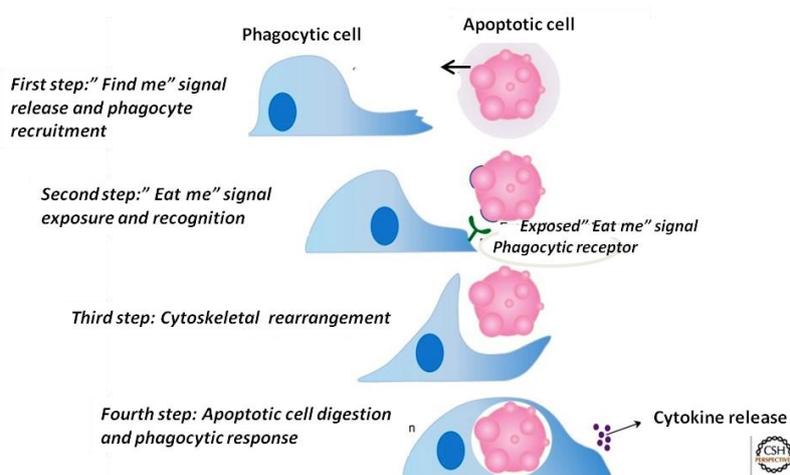


Figure 4. The steps of apoptotic cell clearance

First step is the sensing stage, stimulating phagocyte migration to the apoptotic site. Second, phagocytes recognize exposed “eat me” signals on the surface of apoptotic cells via their phagocytic receptors leading to downstream signaling events culminating in Rac activation and cytoskeletal rearrangement. Finally, further signaling events within the phagocyte regulate the digestion and processing of the apoptotic cell meal and the secretion of anti-inflammatory cytokines.

(Hochreiter-Hufford and Ravichandran, 2013)

Lysophosphatidylcholine (LPC) was the first discovered lipid ‘find me’ signal. LPC is released from apoptotic cells due to the caspase-3 mediated activation of the calcium-independent phospholipase A2 (Lauber et al., 2003). The crucial receptor for binding of LPC is the G-protein-coupled receptor G2A, which promotes the chemotaxis of monocytic cells (Peter et al., 2008).

Sphingosine-1-phosphate (S1P), a sphingolipid metabolite produced by the ubiquitously expressed sphingosine kinase 1 enzyme, is another chemotactic factor secreted by apoptotic cells. Low nanomolar concentrations of S1P were shown to elicit chemotactic movement of macrophages. Moreover, S1P markedly induces macrophage cytoskeletal rearrangements (Gude et al., 2008).

The chemokine and adhesion molecule CX3CL1 also known as fractalkine (FKN), together with its cognate receptor CX3CR1 plays an active role in the chemotaxis of macrophages towards apoptotic cells (Truman et al., 2008). FKN is unique among chemokines because it is synthesized as a transmembrane molecule consisting of an extracellular N-terminal domain, a mucin-like stalk, a transmembrane α helix, and a short cytoplasmic tail. The soluble form consists of the chemokine domain and the extracellular mucin-like stalk, while the membrane-bound form functions as an adhesion molecule and promotes shear-resistant adhesion of CX3CR1 leukocytes. The soluble fractalkine has chemoattractive activity for monocytes, natural killer cells, and T cells and is generated by two sheddases, the disintegrin-like metalloproteinases ADAM 10 and ADAM 17. The membrane-bound fractalkine supports integrin-independent leukocyte adhesion (Jones et al., 2010).

ATP and UTP have been identified as critical and non-redundant “find me” signal released by apoptotic cells (in equimolar quantities) during the early stages of apoptosis in a caspase-dependent manner. Nucleotides bind to P2Y2 receptor and induce monocyte recruitment (Elliott et al., 2009). Plasma membrane channel pannexin 1 opens during apoptosis after the activation by effector caspases and mediates the regulated release of ATP and UTP (Chekeni et al., 2010).

“EAT ME” SIGNAL EXPOSURE AND ENGULFMENT RECEPTORS

Apoptotic cells express specific markers on their surface (“eat me” signals) for recognition via specific receptors on the phagocytes. 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 (Fig. 5). These extracellular bridging molecules (opsonins) enhance the susceptibility of apoptotic cells to phagocytosis and provide additional recognition (binding site – receptor) arrangements (Erwig and Hanson 2008). In contrast to “eat me” signals that are expressed by apoptotic cells, molecules expressed specifically on the surface of living cells, such CD31 and CD47, might actively prevent engulfment (“don’t eat me” signals) (Ravichandrand and Lorenz, 2007).

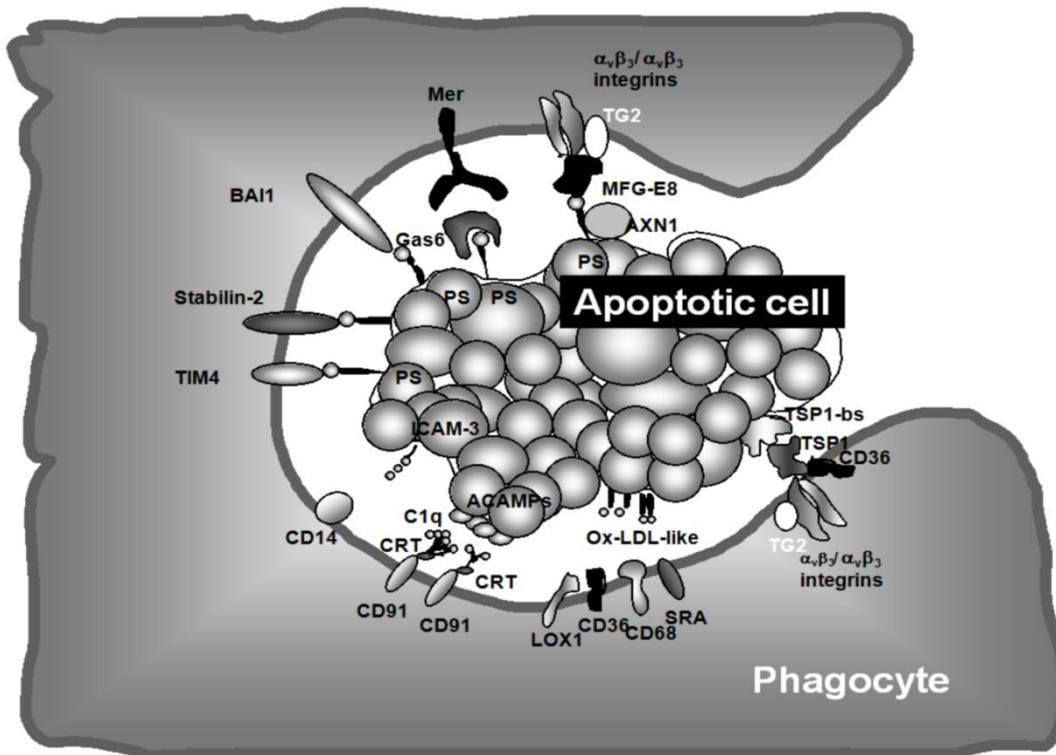


Figure 5. Molecules serving for recognition of apoptotic cells

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

Apoptotic cells display several changes including alteration of membrane lipid molecules and carbohydrates. The best characterised “eat me” signal is the exposure of PS. Living cells display minimal PS because it is actively transported from the outer leaflet to the inner leaflet by the aminophospholipid translocase. Apoptotic cells lose membrane phospholipid asymmetry and expose PS on the outer leaflet of the plasma membrane (Fadok et al., 1992). This process occurs very early during apoptosis (Martin et al., 1995). The appearance of outer leaflet PS results from calcium-dependent phospholipid flip-flop along with the loss of aminophospholipid translocase activity (Bratton et al., 1997). For instance change in PS concentrations on the outer leaflet is increased about 280 fold at apoptotic Jurkat cells compared to living ones. The appearance of PS appears to be a universal phenomenon in cells undergoing apoptosis, or programmed cell death. Since many cell types expose PS during apoptosis and blocking PS recognition attenuates engulfment, it has clearly been established that PS represents an essential “eat me” signal for the clearance of apoptotic cells (Ravichandran 2011). In addition to PS, there appear other “eat me” signals on the surface of the apoptotic cells including other phospholipids, such as lyso-PC (Gardai et al., 2006), or oxidized low-density lipoprotein (oxLDL)-like moiety. Its oxidized sites mimic oxLDL, binding sites for thrombospondin or C1q and sites capable of binding different collectins. Depending on cell type and activation status, changes in surface glycosylation can either directly mediate cellular engulfment or enhance phagocytosis by cooperation with further engulfment signals. During apoptotic cell death, changes in the glycocalyx (decreased exposure of sialic acids) also serve as an “eat me” signal on the surface of apoptotic lymphocytes (Meesmann et al., 2010). Apoptotic cells display altered intercellular adhesion molecule 3 (ICAM-3), a highly glycosylated Ig-superfamily member. This transmembrane glycoprotein is constitutively and abundantly expressed by all leukocytes (Moreira and Barcinski, 2004).

In addition to changes in the cell surface molecules, some intracellular proteins are also exposed during the apoptotic process including calreticulin or annexin I. The endoplasmic reticulum chaperone calreticulin serves as a crucial ‘eat me’ signal on a number of apoptotic cell types. It is a highly conserved protein, with broad localization within the cells (present in the ER/SR membranes and in the nucleus), and on the cell surface, where it serves as a key cellular receptor for C1q. It is also recognised by LDL-receptor-related protein (LRP) on the engulfing cell and it acts also as a co-receptor for LRP ligands such as C1q and alpha-2-

macroglobulin (Gardai et al., 2005; Fricker et al., 2012). Similarly to calreticulin, annexin I is also recruited from the cytosol and exported to the outer plasma membrane leaflet where it colocalizes with PS. Caspase activity and intracellular calcium release were found to be essential for the recruitment and export of cytosolic annexin I to the PS-rich cell surface (Arur et al., 2003).

Depending on the nature of the induction of apoptosis, there might be differences in exposure of “eat me” signals that in turn may dictate the usage of particular combination of engulfment receptors by a given phagocyte. More than a decade ago, Peter Henson suggested a model of “tethering and tickling”, where macrophages express multiple different engulfment receptors binding to multiple ligands on apoptotic cells, some of them have only an adhesion function, whereas the others may mediate signalling (Henson et al., 2001). Similarly, not all engulfment receptors are expressed on a given phagocyte and therefore multiple modes of recognition are necessary during phagocytosis (Ravichandran, 2011).

DIRECT BINDING OF APOPTOTIC CELL BY PHAGOCYTES

Phagocytes recognise and bind PS via two different ways. There are receptors capable of recognizing PS directly and receptors which recognize PS indirectly via soluble bridging molecules. PS can be recognised directly via members of T cell immunoglobulin and mucin-domain-containing molecule (TIM) protein family (including TIM1, TIM3 and TIM4), brain-specific angiogenesis inhibitor 1 (BAI1) or stabilin-2. The members of the TIM family contain a conserved binding pocket, termed as metal ion-dependent ligand binding site (MILIBS). The MILIBS motif recognizes the PS. All of the TIM proteins, except TIM-2 which does not contain MILIBS, can directly bind PS. TIM-4 is highly expressed by professional phagocytes, while TIM-3 is expressed on antigen presenting cells, including DCs, macrophages and human monocytes (DeKruyff et al., 2010). BAI1 also functions as a PS binding engulfment receptor. BAI1 is a seven-transmembrane protein belonging to the adhesion-type G-protein-coupled receptor family. The thrombospondin type 1 repeats within the large extracellular region of BAI1 mediating direct binding to PS. Another PS binding phagocytosis receptor Stabilin-2 has been identified as a multifunctional scavenger receptor that endocytoses modified LDL and evidences display its Gram-negative and Gram-positive bacterial-binding activities as well (Park et al., 2008; Kim et al., 2012).

Several other phagocytic receptors bind directly to lipid or sugar motifs of apoptotic cell membranes, these include a number of scavenger (cleaner) receptors, which are able to bind

modified forms of LDL. Scavenger receptor A (SR-A) is expressed mainly on macrophages and binds oxidized or acetylated LDL molecules (Platt et al., 1998). Another scavenger receptor, the class E scavenger receptor lectin-type oxidized LDL receptor 1 (LOX-1), was also identified as an oxidized low-density lipoprotein receptor which functions as an engulfment receptor for apoptotic cells (Oka et al., 1998). Both SR-A and LOX-1 bind oxLDL-like sites on apoptotic cells. Another scavenger receptor CD68 - its mouse homologue macrosialin – can also recognize the previously mentioned oxidized sites on apoptotic cells (Erdoesova et al., 2002). Besides scavenger receptors, the lipopolysaccharide receptor CD14 - which recognizes the altered intracellular adhesion molecule-3 (ICAM-3) on apoptotic cell – also binds the phagocytes directly to apoptotic cells (Gregory et al., 1998).

APOPTOTIC CELL BINDING VIA BRIDGING MOLECULES TO THE RECEPTORS ON THE PHAGOCYTES

Recognition and tethering of apoptotic cell can also occur through PS binding bridging molecules including MFG-E8, protein S and growth-arrest-specific 6 (Gas6), which physically link phagocytic receptors to PS molecules on apoptotic cells. These opsonins are recognized by their cell surface receptors on phagocytes, such as the integrin $\alpha_v\beta_3$ and members of the Tryp3–AXL–Mer (TAM) receptor tyrosine kinase family. The glycoprotein MFG-E8 was originally discovered as a mammalian milk fat globule membrane component. MFG-E8 is expressed and secreted by professional phagocytes such as macrophages and immature dendritic cells (Nagata et al., 2010). On one hand, C1 and C2 domains in the C-terminal region of MFG-E8 can bind PS on apoptotic cells with high affinity; on the other hand MFG-E8 can simultaneously engage integrin $\alpha_v\beta_3$ or $\alpha_v\beta_5$ on phagocytes via its RGD (arginine-glycine-aspartate) motif on the N-terminal end of the protein. Through this bridging function, MFG-E8 can trigger PS dependent integrin-mediated phagocytosis of apoptotic cells (Hanayama et al., 2002; Akakura et al., 2004). Interestingly, MFG-E8 is not expressed on resting macrophages, but is upregulated in activated macrophages. It is suggested that “find me” signals drive expression of MFG-E8 on macrophages (Ravichandran, 2011).

Integrins are heterodimeric receptors (composed of an α - and a β -subunit) expressed on the surface of phagocytes. To date, 18 α - and 8 β -subunits have been described in human cells. The integrins that are involved in apoptotic cell phagocytosis are $\alpha_v\beta_3$ and $\alpha_v\beta_5$ (Dupuy and Caron, 2008). The integrin β_3 co-receptor TG2 was found to be required for the accumulation of β_3 integrin at the engulfing portals of macrophages generating TG2/integrin β_3 /MGF-E8

complexes during apoptotic cell uptake (Tóth et al., 2009). In the absence of TG2, integrin β_3 cannot properly recognize the apoptotic cells, it is not accumulated in the phagocytic cup, and its signalling is impaired. Moreover, TG2 enhances the activation of RhoG and Rac1, the downstream signalling targets of β_3 integrin supporting this way the efficient phagocytosis (Tóth et al., 2009).

Two other PS binding bridging molecules, Protein S and Gas6 bind and activate TAM receptors – generally MERTK or AXL expressed on the surface of the phagocytes – enhancing the phagocytosis of apoptotic cells. Protein S is a vitamin K–dependent protein, present in the blood at a concentration of around 300 nmol/L and it is best known for its anticoagulant property, serving as a cofactor for protein C. Protein S is responsible for the serum-stimulated phagocytosis of apoptotic cells (Anderson et al., 2003). It has been identified as a ligand for the TAM receptors in addition to Gas6. Protein S binding to Tyro3 and MERTK shows a high degree of species specificity. Peculiar is that human protein S shows only weak or no affinity for the different human TAM receptors, whereas bovine protein S displays good affinity to human Tyro3 (Van der Meer et al., 2014).

Gas6 was initially identified as a gene product and its expression in fibroblasts increases during the growth-arrested state and it potentiates cell proliferation and prevents cell death. It has high structural homology (~42%) with protein S and its modular composition is the similar. The plasma concentration of Gas6 is much lower than that of Protein S (around 0.25 nmol/L). Gas6 binds the TAM receptors with different affinities: $AXL \geq Tyro3 \gg MERTK$ (Ishimoto et al., 2000; Van der Meer et al., 2014). At the N-terminal region the Gla domain (γ -carboxyglutamic domain) of Gas6 and Protein S mediate the Ca^{2+} -dependent binding to PS on the apoptotic cell membrane. The C-terminal region of these bridging molecules bind to TAM receptors causing phosphorylation of the intracellular tyrosine side chains.

Recently, Tubby, Tubby-like protein 1 (Tulp1) and galectin-3 have been identified as new ligands for TAM receptor–mediated efferocytosis (Caberoy et al., 2010 and 2012). Tubby and galectin-3 specifically bind to MERTK, whereas Tulp1 can activate all 3 of the TAM receptors (Van der Meer et al. 2014). Similarly to integrins, the MERTK post-receptor signalling cascade resembles to that of the $\alpha\beta_5$ integrin receptor, as MERTK activation stimulates the formation of a p130Cas-CrkII-Dock180 ternary complex in an $\alpha\beta_5$ -dependent manner (Wu et al., 2005).

An another bridging molecule is trombospondin-1 (TSP-1), an extracellular matrix glycoprotein which binds to TSP-1 binding sites on apoptotic cells and class B scavenger receptor CD36/ α v β 3/ α v β 5 integrin complex on the phagocytes (Savill et al., 1992; Savill et al. 1990).

Collectins (collagen-containing C-type lectins) are normally involved in non-self pattern recognition but they also function as opsonins for apoptotic cells. Collectins are pattern recognition receptors, members of the lectin superfamily, which recognize carbohydrate patterns (Moreira and Barcinski, 2004). Members of the family include mannose binding lectin (MBL) and the lung surfactant proteins A and D (SP-A, SP-D). These opsonins bind the collectin-binding sites of apoptotic cells (Lauber et al., 2004; Ravichandran and Lorenz 2007). Complement component C1q shares structural and functional homology with the collectins, although it does not exhibit lectin activity, and also functions as an opsonin (Ogden et al., 2001). Complement cascade is the major effector system of the innate immune response. C1q is the recognition component of the classical pathway of complement activation. It is a soluble macromolecule, circulating in the blood. C1q is composed of 18 polypeptide chains within six subunits, each subunit contains three similar but distinct polypeptide chains: the A-, B-, and C-chain (Sontheimer et al., 2005). Each heterotrimeric triplehelical fiber is prolonged by a C-terminal globular region, which specifically recognizes PS (Paidassi et al., 2008). C1q is expressed by monocytes/macrophages, microglia and dendritic cells (Galvan et al., 2012). C1q and MBL bind to apoptotic cell and interact with Collagen C1q Receptor on the phagocyte surface - also known as Calreticulin (CRT) or collectin receptor - , which in turn forms complexes with LRP/CD91 (Vandivier et al., 2002) .

ENGULFMENT PATHWAYS

During the engulfment of apoptotic corpses two distinct signalling pathways lead to the rearrangement of cytoskeleton by the activation of Rac1 and in turn the pseudopod extension (Fig. 6). Activation and deactivation of Rac1 is controlled by “engulfment synapses”. That is, when a phagocyte starts to engulf an apoptotic cell, activated Rac1 and integrins are recruited to the portal and induce the formation of phagocytic cups consisting of an actin patch. As soon as the dead cell sinks into the phagocyte through one of these cups, Rac1 is inactivated and the actin is depolymerised (Kitano et al., 2008; Nagata et al., 2010). The first pathway regulates actin polymerization – and is involved not only in apoptotic cell engulfment but also in cell migration, neurite growth, and myoblast fusion – is the CrkII-Dock180-ELMO-Rac

signalling pathway (Nagata et al., 2010). CrkII is a cytoplasmic protein, which functions as an adaptor for signal transduction using its Src homology 2 (SH2) and SH3 domains. Dock180-ELMO are a guanine nucleotide exchange factor (GEF) complexes for the Rac in mammalian cells. Rac is a small G-protein and is a member of Rho family GTPases. Rac activation at the sites of the apoptotic cell recognition subsequently leads to actin polymerization and cytoskeletal rearrangement. Several phagocytic receptors including $\alpha\beta3$ and $\alpha\beta5$ integrins or MERTK use the CrkII–Dock180–ELMO pathway for the clearance of apoptotic cells (Hochreiter-Hufford and Ravichandran, 2013). The BAI1 also cooperates with the ELMO/Dock180/Rac signalling pathway to promote cytoskeletal rearrangement and engulfment of apoptotic cells (Park et al., 2007).

The second signalling pathway for the uptake of apoptotic cells includes LRP1 (CD91), GULP and ABCA1. LRP1 cytoplasmic tail interacts with phosphotyrosine binding domain of the adaptor protein GULP (Su et al., 2002). The 12-transmembrane ATP-binding cassette transporter protein ABCA1, appears to be required on the engulfing as well as on the dying cell but the exact signalling pathway is currently unclear (Hochreiter-Hufford and Ravichandran, 2013). It seems that both ABCA1 and CD91 can activate Rac1 after interaction with GULP (Moreira and Barcinski, 2004). The phagocytic receptor stabilin-2 also interacts with the adaptor protein GULP to activate Rac1 and initiate apoptotic cell uptake following PS binding (Park et al., 2008; Kim et al., 2012).

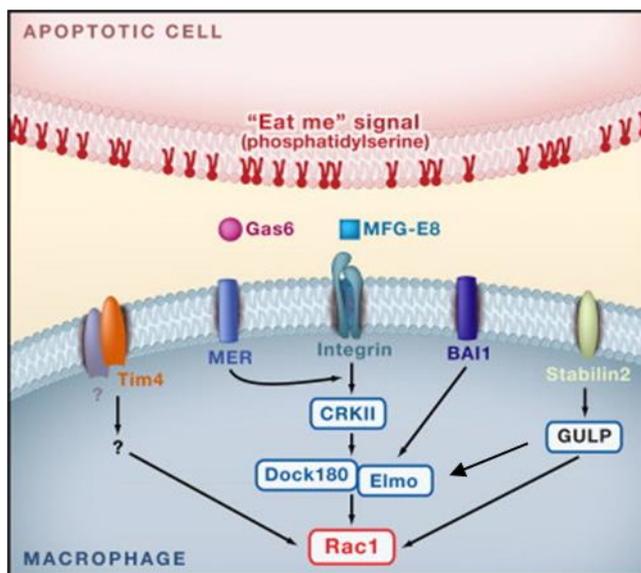


Figure 6. Post-receptor signaling cascades following phagocytes receptor activation Besides the $\alpha\beta3$ and $\alpha\beta5$ integrins, MERTK, BAI1 and Stabilin2 also contribute to PS-dependent phagocytosis of opsonised apoptotic cells. Rac1 activation leads to actin polymerization and engulfment of the opsonized dying cell. (Nagata et al, 2010).

APOPTOTIC CELL DIGESTION AND PHAGOCYTTIC RESPONSE

Following apoptotic cell recognition and uptake, the process of efferocytosis is not complete, because several downstream events (phagosome maturation) from internalization can influence the engulfment capacity of phagocytes. The maturation of phagosome is the end of the phagocytic process. Once a cell is internalized, it “matures” through a series of increasingly acidified membrane-bound structures called phagosomes, ultimately leading to efficient degradation of apoptotic cells. At the end of a series of events the phagosome fusions with the acidic lysosome structures and acidic proteases and nucleases get activated ensuring degradation of apoptotic cell targets (Kinchen and Ravichandran, 2008). Apoptotic cells are digested into their basic cellular building blocks including nucleotides, fats, sterols and peptides.

There are several consequences of apoptotic cell engulfment, such as phagocytes must handle the internalized components of the apoptotic cell. One of these components is the cellular lipid content of the apoptotic cells able to activate lipid sensing receptors in engulfing macrophages. LXRs or PPARs respond to this lipid uptake and through transcriptional regulation they increase the number of phagocytic receptors as well as activate 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. In the absence of LXRs or PPAR γ/δ , mouse macrophages show impaired engulfment of apoptotic cells (A-Gonzalez et al., 2009; R szer et al., 2011; Mukundan et al., 2009).

Accumulation of apoptotic cell membrane derived cholesterol in macrophages activates LXRs and provides positive feedback to promote further clearance of apoptotic corpses via upregulation of MERTK expression (A-Gonzalez et al., 2009). In addition, RAR α is a direct LXR target gene and ligation of LXRs enhances RAR α expression and activity. The combination of LXR and RAR agonists has a synergistic effect on some RAR α target genes. One of these is TG2 and its increased expression enhances the capacity of macrophages to engulf apoptotic bodies (R b  et al., 2009). In addition LXR activation leads to the synthesis of a nonclassical retinoid, which - by ligating retinoic acid receptors - contributes to the upregulation of further phagocytic receptors including TG2, C1q, Tim-4 and stabilin-2. On the other hand agonists of RXRs also enhance the expression of LXRs, MERTK, and ABCA1, very likely by acting via the RXR ligand binding site of the LXR’s heterodimer

(Sarang et al., 2014). Activation of LXRs promotes efflux of the excess apoptotic cell-derived cholesterol via ABCA1 (A-Gonzalez et al., 2009).

Another nuclear receptor, PPAR δ also functions as a sensor of modified fatty acids and sterols derived from the dying cells – like LXRs – to orchestrate the apoptotic cell phagocytosis thereby apoptotic cells enhance their own clearance. Apoptotic cells can induce and activate PPAR δ , which then enhances the expression of opsonins, such as C1q, MFG-E8 and thrombospondin (Mukundan et al., 2009). Cholesterol and fatty acids from apoptotic cells can activate PPAR γ , too. PPAR γ /RXR α heterodimer induces the transcription of genes involved in phagocytosis, such as CD36, MERTK, AXL or TG2 (Röszer et al., 2011).

Metabolites derived from the ingested apoptotic cells influence mitochondrial function in the phagocyte. The relative mitochondrial membrane potential significantly increased within those phagocytes that contained engulfed apoptotic cell compared to those phagocytes without ingested targets. Changing the phagocyte mitochondrial membrane potential significantly affects phagocytosis, with lower potential enhancing engulfment and higher membrane potential by inhibiting the uptake.

It has been published that uncoupling protein 2 (UCP2), a mitochondrial membrane protein, that acts to lower the mitochondrial membrane potential, is upregulated in phagocytes engulfing apoptotic cells. Uncoupling proteins, UCPs (UCP1, UCP2, UCP3) are expressed most abundantly in brown adipose tissue, the major tissue for adaptive thermogenesis that dissipates energy as heat via UCPs. Macrophages from UCP2^{-/-} mice were less efficient to engulf apoptotic cells (Park et al., 2011). The UCPs regulate the mitochondrial membrane potential via dissipation of the proton gradient across the inner mitochondrial membrane, without the generation of ATP. The lack of ATP generation concomitant with a decrease in mitochondrial membrane potential subsequent to engulfing apoptotic cells suggested that phagocytes possess a rapid mechanism for uncoupling nutrient oxidation from ATP generation (Park et al., 2011).

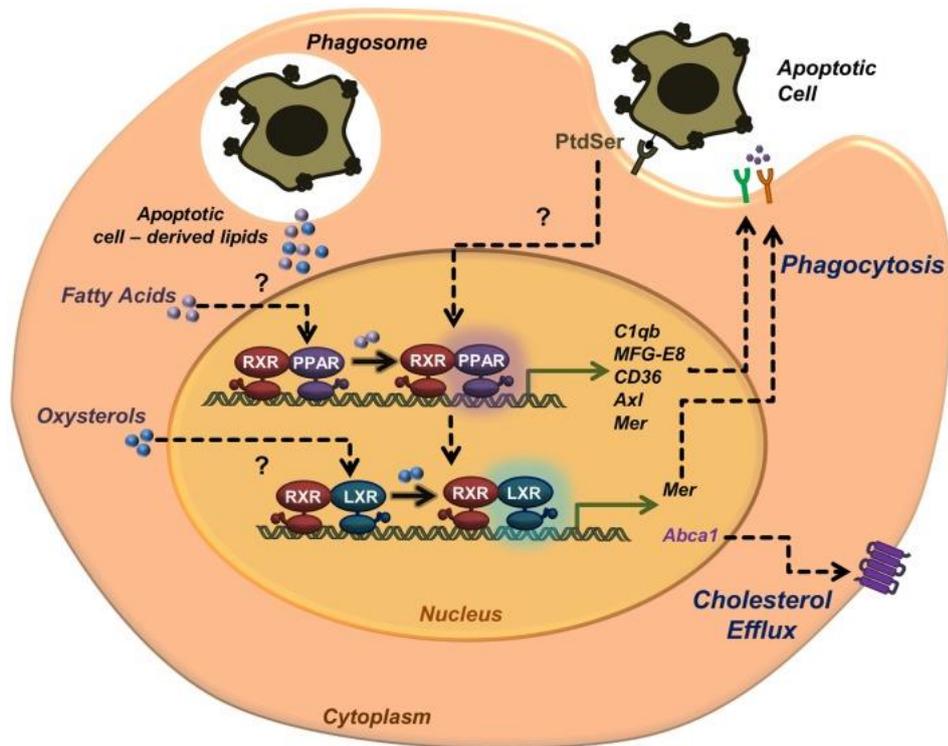


Figure 7. Activation of nuclear receptors in phagocytes during apoptotic cell clearance. Apoptotic cell recognition and engulfment promote the transcriptional activity of LXR and PPAR nuclear receptors. Lipids derived from the engulfed apoptotic cells serve as source of endogenous ligands to activate PPARs (fatty acids) and LXRs (oxysterols). Nuclear receptor activation upon apoptotic cell phagocytosis leads to the upregulation of phagocytic receptors (e.g., MERTK, CD36) and opsonins (e.g., MFG-E8 and C1qb), and phagocyte metabolism. (A-Gonzales and Hidalgo, 2014)

AIMS OF THE STUDIES

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.

MATERIALS AND METHODS

REAGENTS

All reagents were obtained from Sigma-Aldrich except when indicated otherwise.

EXPERIMENTAL ANIMALS

The experiments were carried out on 4-weeks-old or 2- to 4-months-old C57BL/6 mice. In some experiments RAR α knock-out mice (Chapellier et al., 2002) and RARE-hsp68-lacZ reporter transgenic mice (Rossant et al., 1991) were also used. RARE-hsp68-lacZ mice harbor a tetrameric repeat of the RAR β 2 RARE linked to the hsp68 minimal promoter, which has been widely used as a RA-reporter transgene. Due to the hsp68 minimal promoter the lacZ has a background expression. The induced expression of the lacZ gene depends on the simultaneous presence of retinoid receptor ligands and the endogenous RARs. 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-well plates (1.5×10^6 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 μ M 2-mercaptoethanol, 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C in 5% CO₂ for 2 days before use. Macrophages were treated with 1 μ M GW3965 (Glaxo Smith Kline) a synthetic liver X receptor (LXR) agonist, 1 μ M Rosiglitazone (SPI-Bio), a peroxisome proliferator-activated receptor (PPAR)- γ agonist, 1 μ M GW501516 (Alexis Biochemicals), a synthetic PPAR δ agonist for 4 hrs, or 0.1 μ M 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% CO₂. Non-adherent cells were washed away after three days. BMDMs were treated with 1 μM DEX, 25 μM diethylaminobenzaldehyde (DEAB) to block aldehyde dehydrogenase enzyme activities, or vehicle (DMSO) for the indicated time periods. In some experiments 1 μM GSK3787 (Tocris), 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 (CHX) 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 (DSF) (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 (eBioscience) 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 TG2 expression experiments thymocytes (10⁷ 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% CO₂ 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 (AbD Serotec), 0.3 μM all-trans retinoic acid, 0.3 μM 9-*cis* retinoic acid, 0.3 μM AM580 (Tocris Bioscience) or 1nM LG268 (from R. Heyman, Ligand Pharmaceuticals).

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, PPAR δ and C/EBP β . Separated proteins were transferred to an Immobilon-P transfer membrane (Millipore) and were probed with rabbit polyclonal anti-TG2 antibody (SantaCruz), anti-mouse LXR α/β (ABGENT), PPAR δ (Abcam), C/EBP β (Biolegend) and mouse anti- β -actin or GAPDH antibodies were used as loading control. Protein bands were visualized by Immobilon Western Chemiluminescent HRP substrate (Millipore).

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 μ M As₂O₃ –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 for additional 6 hrs or 18 hrs and afterwards RNA was isolated from the samples, RALDH1 and 2 were detected by qRT-PCR as describe later. In some experiments, macrophages were preincubated with 5 μ g / ml actinomycin D for 30 min to block transcription, with 50 μ M cytochalasin D or recombinant Annexin V (10 μ g/ 10^5 cells) for 15 min prior to phagocytosis to block it.

DETECTION OF *IN VITRO* PHAGOCYTOTIC CAPACITY OF BMDMs

BMDMs were stained for 24 hrs with 5 μ M 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 μ M 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). Total RNA (1 μ g/samples) was reverse transcribed into cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instruction. QRT-PCR was carried out in triplicate using pre-designed FAM-MGB-labelled assays for all genes (Applied Biosystems) except for lacZ 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) 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 (Invitrogen) was used for normalization of fluorescent reporter signal. Relative mRNA levels were calculated using comparative C_T method and were normalized to cyclophilin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. ABI Prism SDS2.1 software was used for data analysis (Applied Biosystems).

IMMUNODETECTION OF RALDH1 AND 2 IN MACROPHAGES OF THE THYMUS

Fluorescence light microscopic detection of macrophages (F4/80-immunopositive cells), RALDH1 or RALDH2 was performed in frozen thymus sections from mice exposed to

dexamethasone for 24 hrs. Thymuses were frozen in embedding medium (Shandon Cryomatrix, Thermo Scientific) by liquid nitrogen. Tissue was sectioned at 7 μm (Leica Instruments), mounted on Superfrost Ultra Plus microscope slides (Menzel) 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 (Abcam) or RALDH2 (SantaCruz) antibodies in 1:150 dilution in 2% BSA-PBS 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 (Hycult Biotech) in 1:250 dilution in 2% BSA-PBS in a humid chamber at RT for 1hr. Following washes sections were further incubated with secondary antibodies (anti-goat NI557 conjugated IgG polyclonal antibody, 1:250, R&D) 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 (Nikon). Acquired and presented images are representative of all the samples examined. For documentation, images were processed using Adobe PhotoShop software (Version 5.5).

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°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 LXR α and LXR β or C/EBP β and ON-TARGETPLUS Non-targeting Control Pool (Dharmacon) using the DharmaFECT 1 Transfection Reagent (Dharmacon) 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 μ l/100 μ L well). After 2 hrs, transfection efficiency (fluorescent signal) was detected by FACS analysis. At 24/48 hrs after transfection, cells were treated with 1 μ M DEX or vehicle (DMSO) for 18/24 hrs and harvested for detecting the mRNA/protein levels of LXR or CCAAT/enhancer binding protein (C/EBP) β of transfected BMDMs by quantitative PCR/western blot analysis, respectively. To determine the effect of LXR or C/EBP β gene silencing on the mRNA expression levels of various glucocorticoid-induced genes, at 48 hrs after transfection, cells were treated with DEX (1 μ M) 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 \pm SD or SEM. P values were calculated by using unpaired Student's t-test.

RESULTS

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 (Figure 8A) 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 (Szondy et al., 1998 and 2003). As shown in Figure 8A, addition of TGF- β or ATRA alone slightly increased the mRNA expression of TG2, while 9-*cis* RA was effective to induce the TG2 mRNA expression in thymocytes.

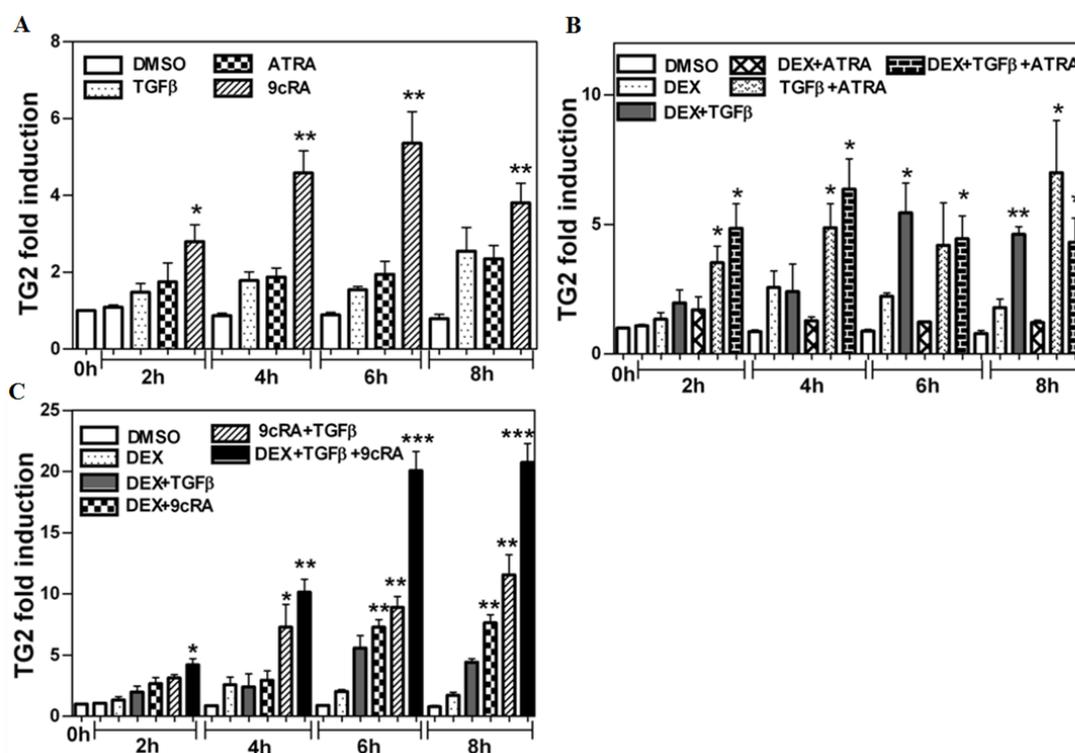


Figure 8. Retinoids and TGF- β significantly enhance the TG2 mRNA expression in isolated mouse thymocytes induced to die by dexamethasone acetate. Thymocytes were exposed to the indicated compounds (DMSO, 0.5%; DEX, 0.1 μ M; rTGF- β , 5 ng/ml; ATRA, 0.3 μ M; 9cRA, 0.3 μ M) for the indicated time period. TG2 gene expression levels were measured by qRT-PCR using cylophilin as reference gene. Results are expressed as mean \pm S.D. of four independent experiments (* p <0.05, ** p <0.01, *** p <0.001).

As we reported previously (Szegezdi et al., 2000), exposure of thymocytes to DEX, a very efficient apoptosis inducer of thymocytes (Wyllie, 1980) 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 (Fig. 8B). ATRA added together with either TGF- β or in combination with DEX and TGF- β could significantly elevate the TG2 mRNA (Fig. 8B). In the case of 9-*cis* RA, 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 (Fig. 8C). The most effective TG2 inducer in DEX-exposed thymocytes was 9-*cis* RA added together with TGF- β (Fig. 8C), 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 9-*cis* RA and TGF- β also contributes to the appearance of TG2.

SYNTHETIC LIGANDS OF RAR α AND RXR RECEPTORS INDUCE TG2 EXPRESSION IN DEX-EXPOSED APOPTOTIC THYMOCYTES

Since 9-*cis* RA (acting as a ligand for both RAR and RXR receptors) appeared more effective in each experimental setting than the RAR agonist ATRA, we used synthetic RXR agonist LG268 and the synthetic RAR α 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 (Fig. 9). 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.

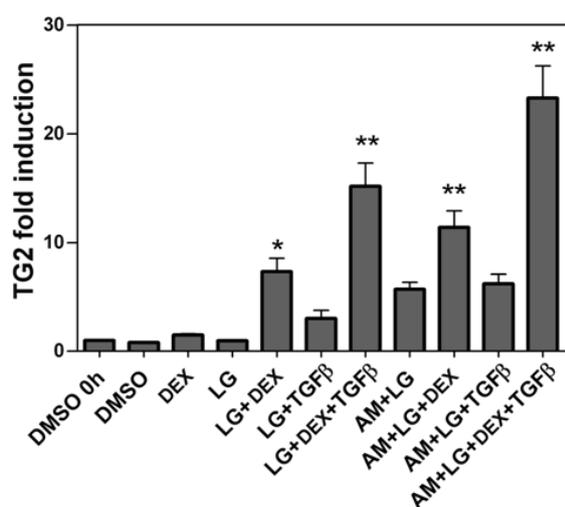


Figure 9. Synthetic agonists of RA receptors effectively increase TG2 mRNA in DEX-exposed thymocytes Thymocytes were exposed to the indicated compounds (DMSO, 0.5%; DEX, 0.1 μ M; rTGF- β , 5 ng/ml; LG268, 1nM; AM580, 0.3 μ M;) for 8 hrs. TG2 gene expression levels were measured by qRT-PCR using cylophillin as reference gene. Results are expressed as mean \pm S.D. of four independent experiments (* p <0.05, ** p <0.01).

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 (DSF), 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. As seen in Figure 10, 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.

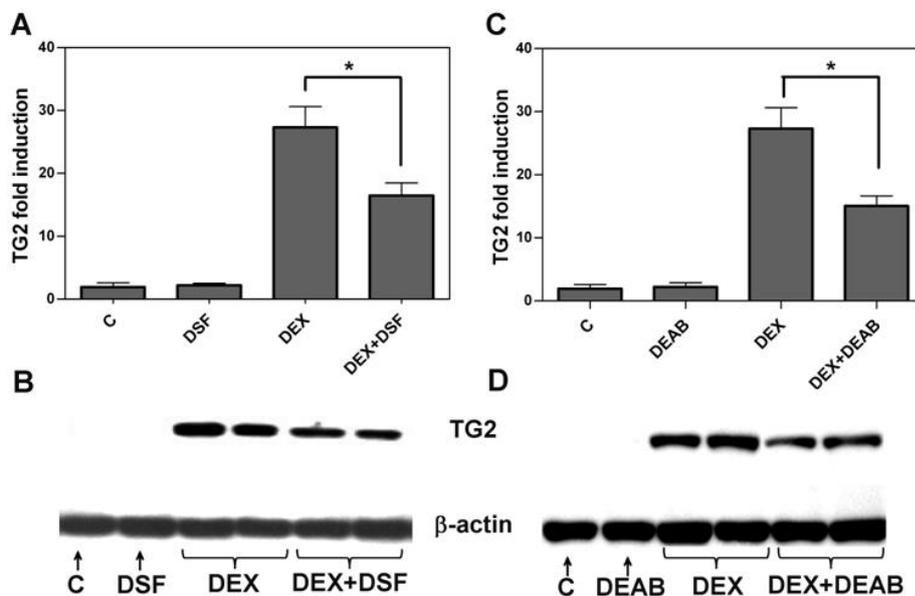


Figure 10. *In vivo* inhibition of either alcohol dehydrogenases or RALDHs significantly attenuates the apoptosis-linked TG2 expression in the mouse thymus. After 2 days pretreatment with disulfiram, an alcohol dehydrogenase inhibitor (A and B), or DEAB, an inhibitor of RALDHs (C and D), *in vivo* thymocyte apoptosis was induced by intraperitoneal injection of 0.3 mg DEX in 4-weeks-old C57BL/6 mice. Thymuses were collected 24 hrs later. TG2 gene expression levels were measured by qRT-PCR using cylophilin as reference gene, while TG2 protein levels were determined by Western blot using β -actin as loading control. Results are expressed as mean \pm S.D. of three independent experiments (* $p < 0.05$).

THE *IN VIVO* APOPTOSIS INDUCTION IS COUPLED TO ENHANCED RETINOIC ACID DEPENDENT TRANSCRIPTION IN THE MOUSE THYMUS

To demonstrate that both 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 (Rossant et al., 1991). Same than in 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 (Fig. 11A and B). Thymic β -galactosidase mRNA expression was nearly fully inhibited by DEAB (Fig. 11B), but only partially by disulfiram (Fig. 11A) 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.

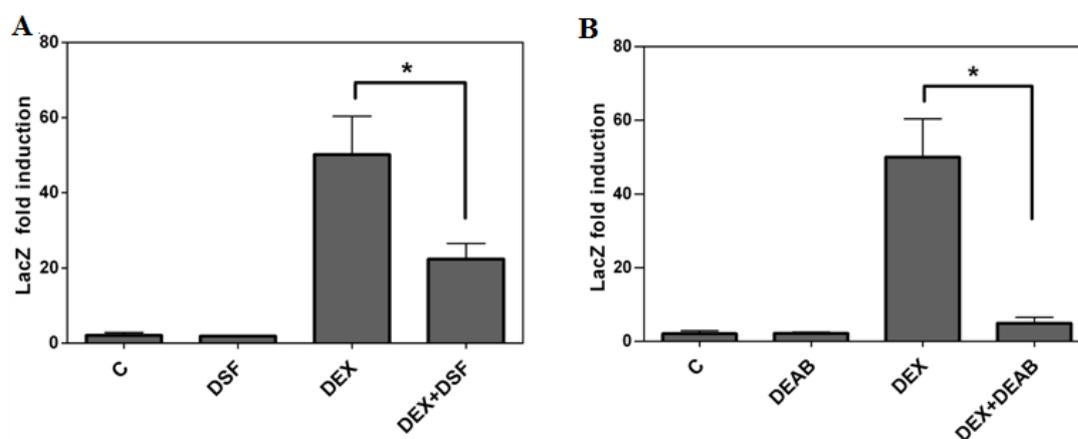


Figure 11. Inhibition of *in vivo* retinoic acid synthesis by DSF or DEAB significantly reduces the DEX-induced LacZ expression in the mouse thymus. After 2 days pretreatment with disulfiram (DSF) an alcohol dehydrogenase inhibitor (A), or DEAB, an inhibitor of RALDHs (B), *in vivo* thymocyte apoptosis was induced by intraperitoneal injection of 0.3 mg DEX in 4-weeks-old RARE-hsp68-lacZ reporter mice. Thymuses were collected 24 hrs later. LacZ gene expression levels were measured by qRT-PCR using GAPDH as reference gene. Results are expressed as mean \pm S.D. of three independent experiments (* p <0.05, ** p <0.01).

IN VIVO THYMOCYTES APOPTOSIS IS ACCOMPANIED BY ENHANCED EXPRESSION OF 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. As seen in Figure 12A, 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 more effective apoptosis inducer DEX.

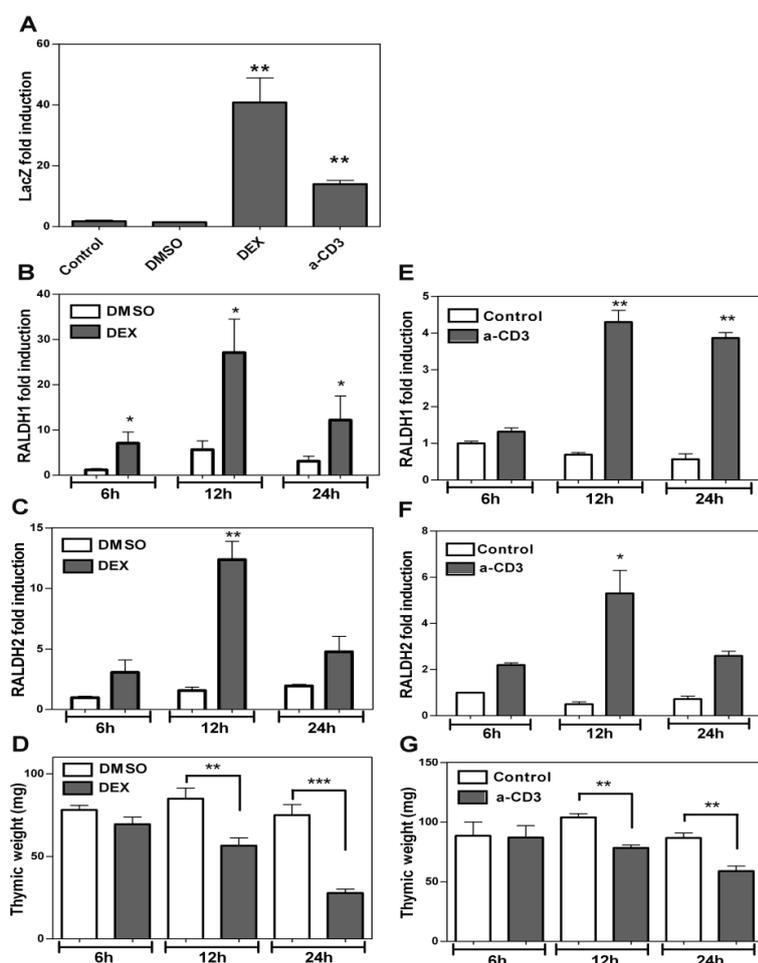


Figure 12. Induction of the apoptosis of thymocytes *in vivo* causes an enhanced expression of RALDH1 and 2, and increased retinoid-dependent transcriptional activity in the mouse thymus. *In vivo* thymocyte apoptosis was induced by intraperitoneal injection of 0.3 mg DEX or by that of 50 μ g anti-CD3 antibodies in RARE-hsp68-lacZ reporter mice. LacZ (A),

*RALDH1 (B and D), RALDH2 (C and E) relative gene expressions were determined at 24 hrs or at the indicated time points by qRT-PCR analysis using cyclophilin as reference gene. Thymic weight (F and G) were measured at 24 hrs or at the indicated time points. Results are expressed as mean \pm S.D. of four independent experiments ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$).*

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 to be expressed by the mouse thymus (Kiss et al., 2008) following *in vivo* apoptosis induction. As shown in Figure 12B and C, 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 (Fig.12E-F). 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 response 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 (Fig. 12D and G).

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 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 able to express RALDHs (Kiss et al., 2008), 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 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 (Mukundan et al., 2009, A-Gonzalez et al., 2009, Röszer et al., 2011). 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. As shown in Figure 13A, phagocytosis of apoptotic thymocytes enhanced the mRNA expression of RALDH1, but did not alter that of RALDH2 in engulfing macrophages (Fig.13B). As it can be seen on Figure 13C and D the expression of

RALDH1 was further increased and RALDH2 mRNA also induced, when same macrophages engulfed the larger apoptotic NB4 cells, so the induction was not thymocyte specific.

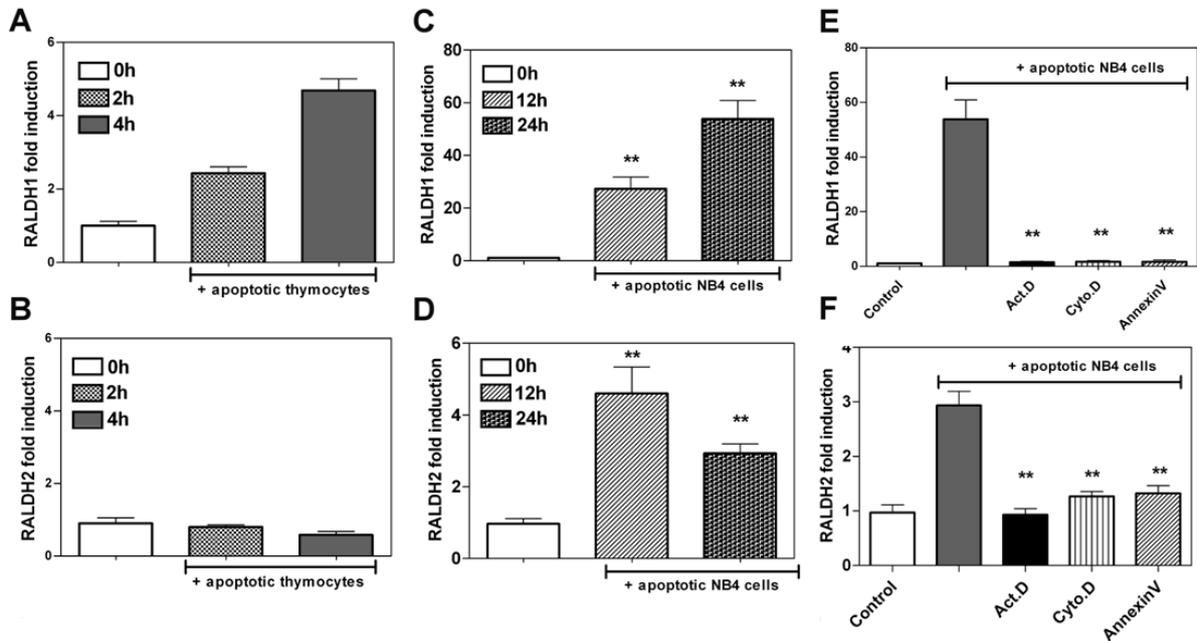


Figure 13. Engulfment of apoptotic cells induces the expression of RALDHs in macrophages. Macrophages were exposed to apoptotic thymocytes (A and B) or to apoptotic NB4 cells (C -F) for the indicated time period or for 6 hrs (E, F). Macrophages were pretreated for 30 min in the presence of actinomycin D (5 $\mu\text{g/ml}$), cytochalasin D (50 μM) or recombinant Annexin V (10 $\mu\text{g}/10^5$ apoptotic cells) (E, F), and the relative expression levels of RALDH1 and 2 were determined by qRT-PCR analysis using cyclophilin as reference gene. Results are expressed as mean \pm S.D. of four independent experiments (* $p < 0.05$, ** $p < 0.01$).

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 (Fig. 13E and F). 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 (Cvatanovic and Ucker, 2004), or PS on the apoptotic cell surface was masked by preincubating the apoptotic cells with recombinant annexin V (Hoffmann et al., 2001). Both cytochalasin D and recombinant annexin V inhibited the induction of RALDH mRNA levels by apoptotic cells (Fig. 13E and F) suggesting that engulfment of apoptotic cells, rather than their recognition *per se*, that triggers RALDH expression.

Since the three lipid-sensing nuclear receptors (LXR α , PPAR γ and δ) have been implicated in the macrophage response to the engulfed apoptotic cells (Mukundan et al., 2009; A-Gonzalez et al., 2009; R szer et al., 2011), 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 PPAR γ agonist and GW1516 a PPAR δ agonist, promoted the mRNA expression of RALDH1 in peritoneal macrophages (Fig. 14A) and only GW1516 was ineffective in inducing RALDH2 expression (Fig. 14B). These observations indicate that all the three lipid sensing receptors might mediate the effect of apoptotic cell engulfment on the expression of RALDHs.

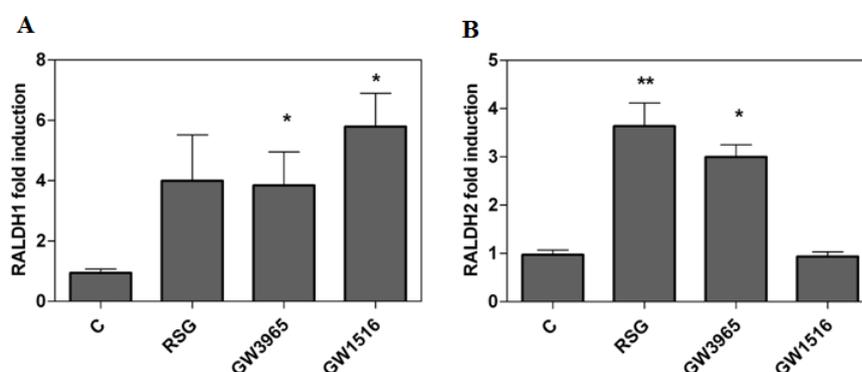


Figure 14. Ligation of three lipid sensing receptors induce the expression of retinoic acid synthesizing enzymes in macrophages. Macrophages were exposed to 1 μ M rosiglitazone (RSG), 1 μ M GW3965 or 1 μ M GW1516 for 4 hrs, and the relative expression levels of RALDH1 and 2 were determined by qRT-PCR analysis using cylophillin as reference gene. Results are expressed as mean \pm S.D. of four independent experiments (* p <0.05, ** p <0.01).

RALDHs ARE EXPRESSED BY MACROPHAGES 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. The green colour shows the macrophage marker F4/80 while the red colour demonstrates the RALDH1 or 2 enzymes in tissue section. As it can be seen on Figure 15, both enzymes were co-stained with macrophages following DEX induced apoptosis, suggesting that macrophages express RALDHs and are capable for retinoid synthesis.

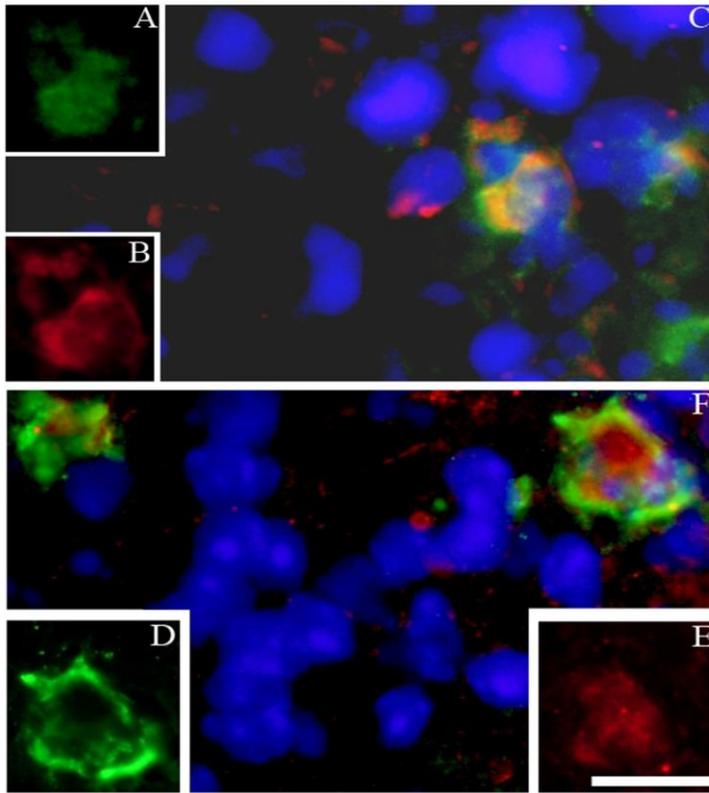


Figure 15. Colocalisation of RALDH1 and 2 with macrophages in tissue sections of mouse thymus exposed to dexamethasone acetate for 24 hrs. (A) F4/80 staining of a macrophage (green) in the thymus at 24 hrs DEX injection. (B) The endogenous red labeling of RALDH1. (C) Double-labeled macrophages (green, F4/80, and RALDH1 red) along with staining for nuclei (DAPI, blue) to confirm both the cellular localization of the label in macrophages and the anatomical location of these cells in the section presented. (D) Macrophages (F4/80), (E) RALDH2 (red labeling), and (F) the dual-labeled correlation (yellow), along with DAPI (blue) staining, respectively. Scale bar = 15 μ m.

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 (Fig. 12B), we investigated whether *in vitro* DEX treatment affects RALDH1 expression in macrophages. As shown in Figure 16, RALDH1 mRNA is significantly increased following DEX treatment of macrophages, while had no effect on RALDH2 expression (data not shown). Our data indicate that triggering of thymocyte apoptosis induces a RALDHs expression in the thymus (Fig. 12, 15) and the apoptosis inducer DEX itself can contribute to the increase of the RALDH1 mRNA expression in macrophages.

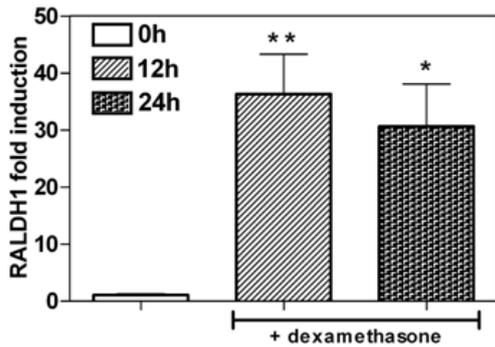


Figure 16. Dexamethasone treatment of macrophages causes RALDH1 mRNA induction. Macrophages were exposed to 0.1 μ M DEX for 24 hrs, and the relative expression levels of RALDH1 was determined by qRT-PCR analysis using cylophilin as reference gene. Results are expressed as mean \pm S.D. of four independent experiments (* p <0.05, ** p <0.01).

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 μ M DEX for 24 hrs either alone or in combination with 25 μ M DEAB. As shown in Figure 17A, 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 (Sarang et al., 2014). That is why we decided to investigate whether DEX-induced increase in the phagocytosis of apoptotic cells (Liu et al., 1999) 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 (Liu et al., 1999), exposure of BMDMs to 1 μ M DEX significantly enhanced their capacity to engulf apoptotic thymocytes by increasing the percentage of macrophages engulfing apoptotic cells (Fig. 17).

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. As seen in Figure 17B-E, 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 continuously 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 (Fig. 17D, E). 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 glucocorticoids on the enhancement of efferocytosis during prolonged phagocytosis of apoptotic cells.

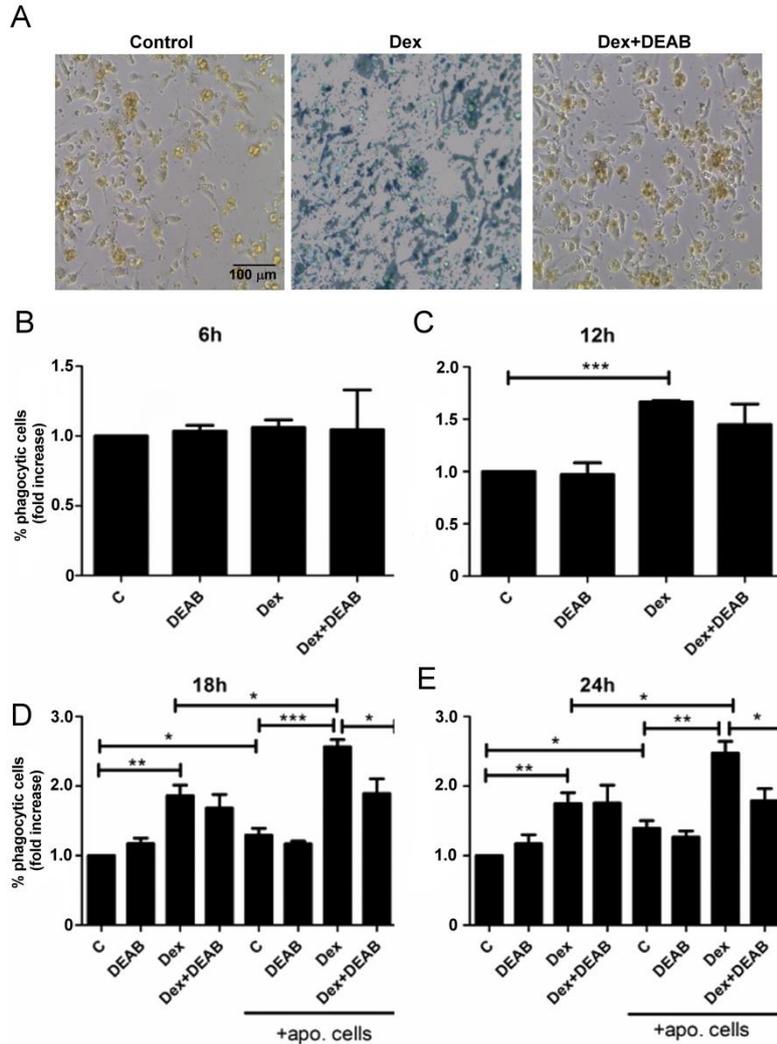


Figure 17. Inhibition of retinoic acid synthesis prevents the glucocorticoid-induced enhancement in long-term efferocytosis. (A) Bone marrow derived macrophages from RARE-hsp68-lacZ reporter-carrying mice produce physiologically relevant concentrations of retinoids when they are exposed to 1 μ M DEX for 24 hrs, and this pathway is completely inhibited by addition of DEAB (25 μ M), an RALDH inhibitor. The appearance of the β -galactosidase protein was demonstrated by X-gal staining. Images were taken using an AMG EVOS inverted microscope at 20 X magnification. Bone marrow derived macrophages were treated with DEAB (25 μ M), DEX (1 μ M) alone or together for (B) 6 hrs, (C) 12 hrs, (D) 18 hrs and (E) 24 hrs. To study continued efferocytosis, in the indicated experiments macrophages were first exposed to non-fluorescent apoptotic thymocytes during the last 3 hrs of DEX treatment before being exposed to CFDA-labelled thymocytes. % of macrophages engulfing apoptotic cells was then determined as described in Materials and Methods. The increase in the percentage of engulfing macrophages is compared to that of the non-treated controls (10.9 \pm 0.8%) and is expressed as fold induction showing the mean \pm SEM of 3 separate experiments. Significantly different from respective control: * p < 0.05, ** p < 0.01, *** p < 0.001.

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 (Fig.14), 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 (Fig.18A). As shown in Figure 18A and B, DEX significantly induced both the mRNA and protein expression of LXRs and PPAR δ , as well their heterodimerization partner RXR α with a peak at around 12 hrs. The expression of PPAR γ , another lipid sensing nuclear receptor, was downregulated (data not shown). 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 (Szántó et al., 2004), we also tested the expression level of CYP27 in DEX exposed macrophages. The enzyme was readily induced following 4 hrs DEX treatment of macrophages (Fig. 18C). Moreover the protein synthesis inhibitor cycloheximide only partially inhibited the induction of CYP27 (Fig. 18D), 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 (Araya et al., 2003). On the other hand, the induction of the investigated lipid sensing nuclear receptors was completely inhibited by pretreatment with 20 μ M cycloheximide (data not shown), suggesting that synthesis of new protein(s) (e.g. a new transcription factor) is/are needed for their increased expression.

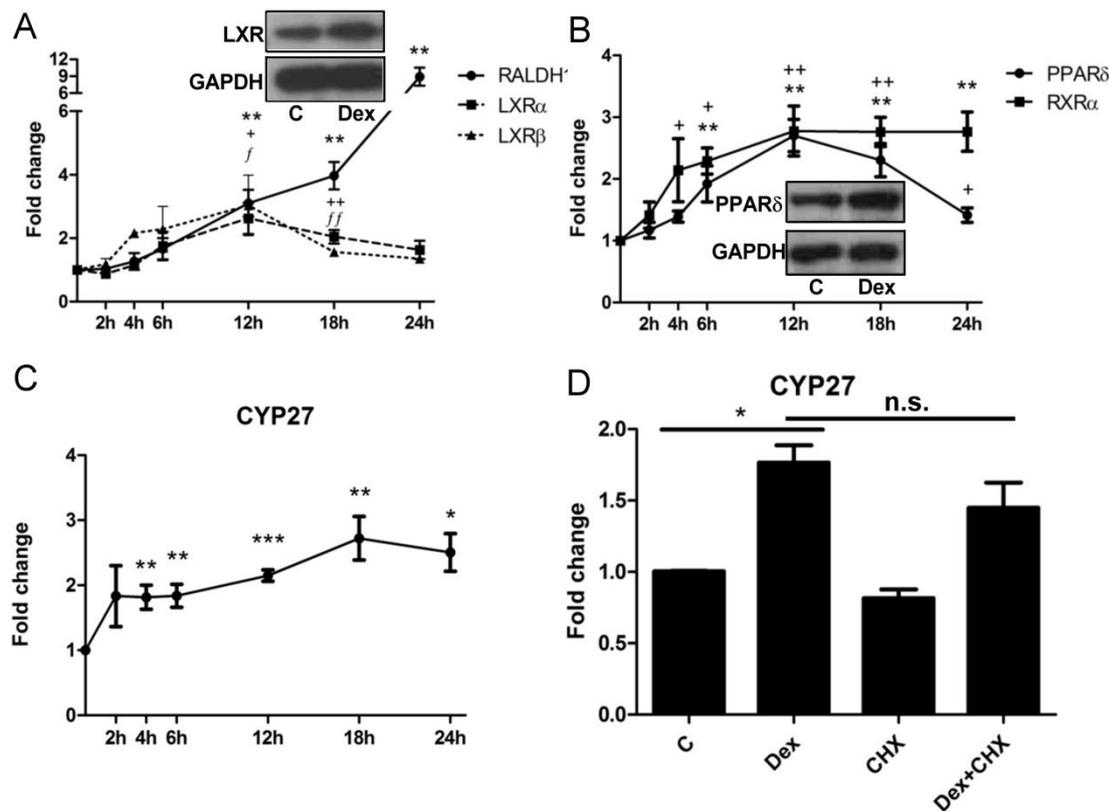


Figure 18. Glucocorticoids induce the expression of CYP27, several lipid sensing nuclear receptors and RALDH1 in macrophages. Macrophages were treated with DEX ($1\mu\text{M}$) for the indicated time periods. mRNA expressions of (A) RALDH1, LXR α and β , (B) PPAR δ and RXR α and (C) CYP27 were determined by qRT-PCR analysis. (D) Macrophages were treated with DEX ($1\mu\text{M}$) together with cycloheximide ($20\mu\text{M}$) for 4 hrs to inhibit new protein synthesis. mRNA levels of CYP27 were determined by qRT-PCR analysis. GAPDH was used as reference gene. Results are expressed as mean \pm SEM of at least 3 separate experiments. Significantly different from respective control: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ for RALDH1, RXR α or CYP27, + $p < 0.05$, ++ $p < 0.01$ for LXR α or PPAR δ , ^f $p < 0.05$, ^{ff} $p < 0.01$ for LXR β . Protein levels detected by Western blot for LXRs and PPAR δ following a 24 hrs DEX treatment are also shown in (A) and (B), respectively.

DEXAMETHASONE INDUCES RAR α AND C/EBP β 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 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 (Yoshikawa et al., 2001) and then SREBP-1c can drive the

RALDH1 expression (Huq et al., 2009). On the other hand RAR α and CCAAT/enhancer binding protein (C/EBP) β simultaneously can act as regulators of RALDH1 expression (Elizondo et al., 2009) where the LXR controls the RAR α gene expression level (Rebé et al., 2009). That is why we investigated whether LXRs are involved in the DEX-induced increase in the expression of RALDH1 and the mRNA level of above mentioned genes were measured in DEX exposed BMDMs. In our experimental system the expression of SREBP-1c was not altered by DEX (data not shown) but, as shown in Figure 19A, the expression of both RAR α and C/EBP β was enhanced in macrophages following DEX exposure. While preincubation with cycloheximide completely inhibited the induction of RAR α by DEX (data not shown), administration of it resulted in increased C/EBP β mRNA levels. This observation suggests the involvement of a labile negative protein factor(s) in the regulation of the C/EBP β mRNA levels (Fig. 19B), and that glucocorticoids might promote the induction of C/EBP β by suppressing the transcription of this labile inhibitory protein(s).

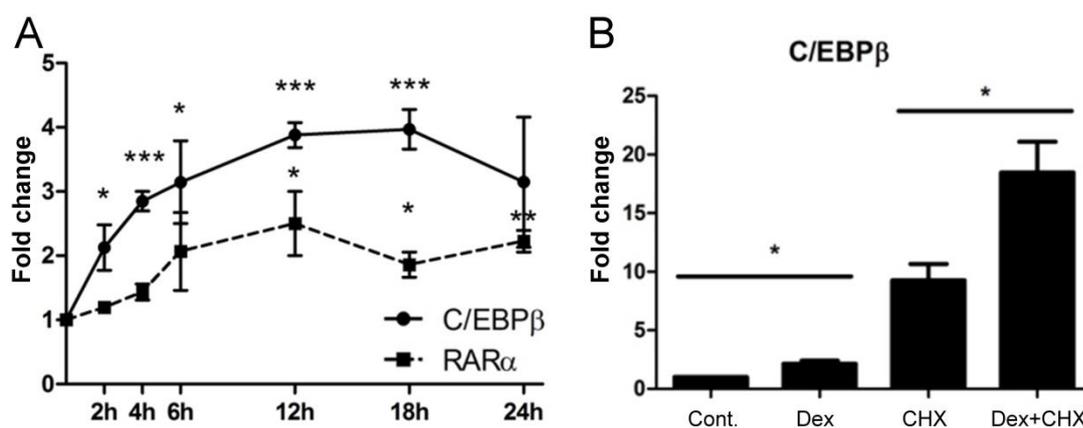


Figure 19. C/EBP β and RAR α gene expression level are enhanced in DEX exposed BMDMs. (A) Macrophages were treated with DEX (1 μ M) for the indicated time periods. mRNA expression levels of C/EBP β or RAR α were determined by qRT-PCR analysis. (B) Macrophages were treated with DEX (1 μ M) together with cycloheximide (20 μ M) for 4 hrs to inhibit new protein synthesis. mRNA levels of C/EBP β were determined by qRT-PCR analysis. GAPDH was used as reference gene. Each bar represents mean \pm SEM of 3 separate experiments. Significantly different from respective control: * p < 0.05, ** p < 0.01, *** p < 0.001.

DELIVERY OF LXRS AND C/EBP β siRNA INTO BMDMs RESULTS IN EFFECTIVE GENE SILENCING

To investigate further the role of C/EBP β , LXRs and RAR α induction in DEX-induced efferocytosis, the effect of silencing of LXRs and C/EBP β expressions was studied in DEX exposed BMDMs. Figure 20A shows the siRNA transfection efficiency in BMDMs achieved with Dharmafect transfection reagent. Figure 20B and C show the silencing efficiency of siRNA against LXRs and C/EBP β . Silencing of both genes was successful as demonstrated on mRNA and protein levels in control and in DEX treated samples, too.

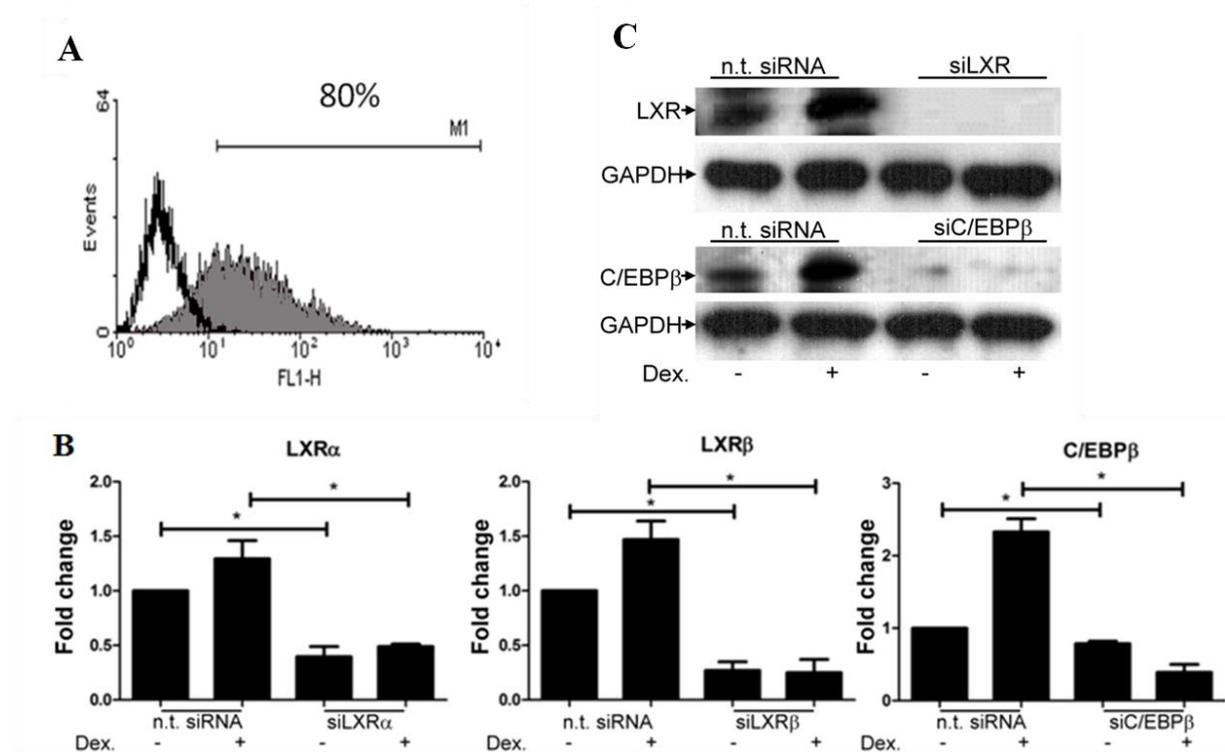


Figure 20. Efficiency of LXRs and C/EBP β siRNA transfection of BMDMs using Dharmafect transfection reagent.

(A) Macrophages were transfected with siGLO Green transfection indicator (Dharmacon) by Dharmafect transfection reagent to determine the transfection efficiency of cells. (B) Macrophages were transfected with non-targeting siRNA, LXR α and LXR β siRNA together or with C/EBP β siRNA as previously. 24 hrs after the transfection, cells were treated with DEX for 24 hrs and mRNA levels of LXR and C/EBP β were determined by qRT-PCR analysis. (C) Macrophages were transfected with non-targeting siRNA, LXR α and LXR β siRNA (Dharmacon) together or with C/EBP β siRNA (Dharmacon). 48 hrs after transfection, cells were treated with DEX for 24 hrs and protein level of LXRs and C/EBP β was determined by Western blot analysis. Each bar represents mean \pm SEM of 3 separate experiments. Significantly different from respective control: * $p < 0.05$.

INDUCTION OF RALDH1 REQUIRES THE EXPRESSION OF LXR-INDUCED RAR α AND DEX-INDUCED C/EBP β

As shown in Figure 21A, silencing of C/EBP β prevented the DEX induced LXR α expression indicating that DEX-induced LXR α mRNA enhancement is mediated by C/EBP β . Figure 21B demonstrates, that DEX-induced increase in RAR α mRNA expression is inhibited by LXRs silencing which is in line with the observation that RAR α is an LXR target gene (Rebé et al., 2009). In addition, silencing of C/EBP β also prevented the DEX-induced RAR α expression (Fig. 21B) since RAR α is induced in LXR dependent manner and LXR induction is inhibited by C/EBP β siRNA (Fig. 21A). On the other hand, silencing of LXRs did not prevent the DEX-induced enhanced RXR α expression indicating that RXR α is not a direct LXR target gene. However, its induction by DEX was also C/EBP β -dependent (Fig. 21C).

Silencing of LXRs or C/EBP β which are required for DEX-induced RAR α production (Fig. 21B), impaired the RALDH1 expression in DEX treated BMDMs (Fig. 21D). Similarly, administration of AGN109, a pan RAR antagonist (Fig. 21E) or genetic ablation of RAR α (Fig. 21F) also prevented the induction of RALDH1 by DEX. These data suggest a determining role of RAR α in the regulation of RALDH1 expression. Altogether these data indicate that glucocorticoids induce the expression of C/EBP β in macrophages which contributes to the induction of both LXRs and RXR α . Glucocorticoids also induce the expression of CYP27, which might provide an endogenous ligand for the LXRs. RAR α is induced in an LXR/RXR-dependent manner and is required for the induction of RALDH1. Though previous studies indicated that C/EBP β can also be directly involved in the induction of RALDH1 (Elizondo et al., 2009), since it is also involved in the induction of RAR α via the DEX regulated LXR pathway, our data cannot provide evidence for its direct involvement in the regulation of RALDH1 expression upon DEX treatment.

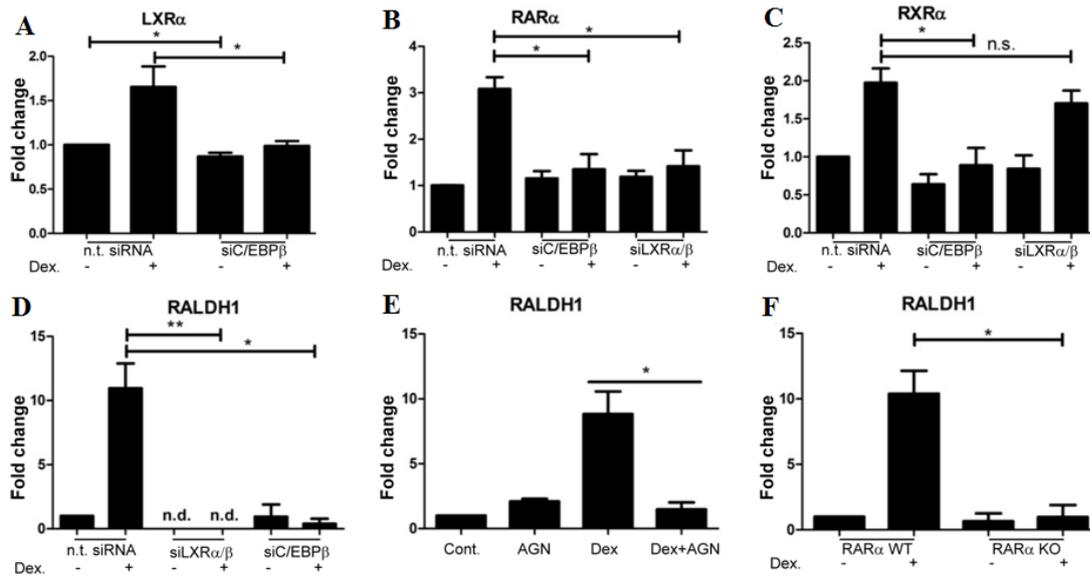


Figure 21. C/EBP β , LXRs and RAR contribute to the glucocorticoid-induced RALDH1 induction in bone marrow derived macrophages. (A-D) Macrophages were transfected with the indicated siRNA. 48 hrs after the transfection, cells were treated with DEX for 18 hrs to determine mRNA expression levels of (A) LXR α (B) RAR α (C) RXR α and (D) RALDH1 was determined by qRT-PCR analysis. (E) Macrophages were treated with DEX (1 μ M) and with the pan RAR antagonist AGN109 (1 μ M) as indicated for 24 hrs. mRNA expression levels of RALDH1 were determined by qRT-PCR analysis. (F) Macrophages derived from wild type or RAR α KO mice were treated with DEX (1 μ M) for 24 hrs. mRNA expression levels of RALDH1 were determined by qRT-PCR analysis. GAPDH was used as reference gene. Each bar represents mean \pm SEM of 3 separate experiments. Significantly different from respective control: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. n.s. = not significant. n.d. = not determined. nt=non-targeted.

GLUCOCORTICOIDS INDUCE THE EXPRESSION OF MERTK, C1Q AND UCP2 IN BMDMs IN A C/EBP β –DEPENDENT MANNER. THE LONG-TERM INDUCTION OF UCP2 INVOLVES PPAR δ , 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 (McColl et al., 2009; Zahuczky et al., 2011) we also found, that MERTK expression was the most prominent among phagocytosis-related genes following DEX treatment in BMDMs (Fig.22A). We found that MERTK was induced very early after DEX exposure (Fig. 22A) and its induction could be decreased by preincubation with cycloheximide (Fig. 22B) and by C/EBP β silencing (Fig. 22D) indicating that C/EBP β contributes to its induction. Though MERTK was found to be a direct LXR target gene (A-Gonzalez et al., 2009), silencing of LXRs did not affect its DEX-induced expression

significantly (Fig. 22D) indicating that C/EBP β contributes to its induction independently of its effect on LXR expression. Since in the case of PPAR δ $-/-$ mice there is a reduced MERTK expression in macrophages (Mukundan et al., 2009) we measured the DEX-induced MERTK expression in the presence of PPAR δ antagonist GSK3787. In our experiments PPAR δ inhibition had no effect on MERTK expression suggesting that PPAR δ does not regulate the MERTK expression upon DEX treatment (Fig. 22C).

In line with a previous publication (Zahuczky et al., 2011), DEX also induced the expression of the bridging molecule C1q (Fig. 22A). Induction of C1q by DEX was not inhibited by cycloheximide indicating that new protein synthesis is not required for its early induction (Fig.22B). But the presence of C/EBP β and LXRs was required for its long term induction detected at 21 hrs (Fig. 22D), while PPAR δ did not seem to be involved in its DEX-induced regulation (Fig. 22C).

Finally, we detected the glucocorticoid-dependent induction of UCP2, which also was very early induced following DEX treatment (Fig. 22A). Though its short term induction, similar to that of C1q, was independent of new protein synthesis (Fig. 22B), its long-term induction detected at 21 hrs required both PPAR δ (Fig. 22C) and C/EBP β (Fig. 22D). The expression of other phagocytosis-related molecules in bone marrow derived macrophages, such as MFG-E8, CD36, CD91, stabilin-2 and CD14 was not altered by DEX treatment, while that of TG2, integrin β 3, and TIM4 was decreased (data not shown). 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/EBP β and lipid sensing nuclear receptors.

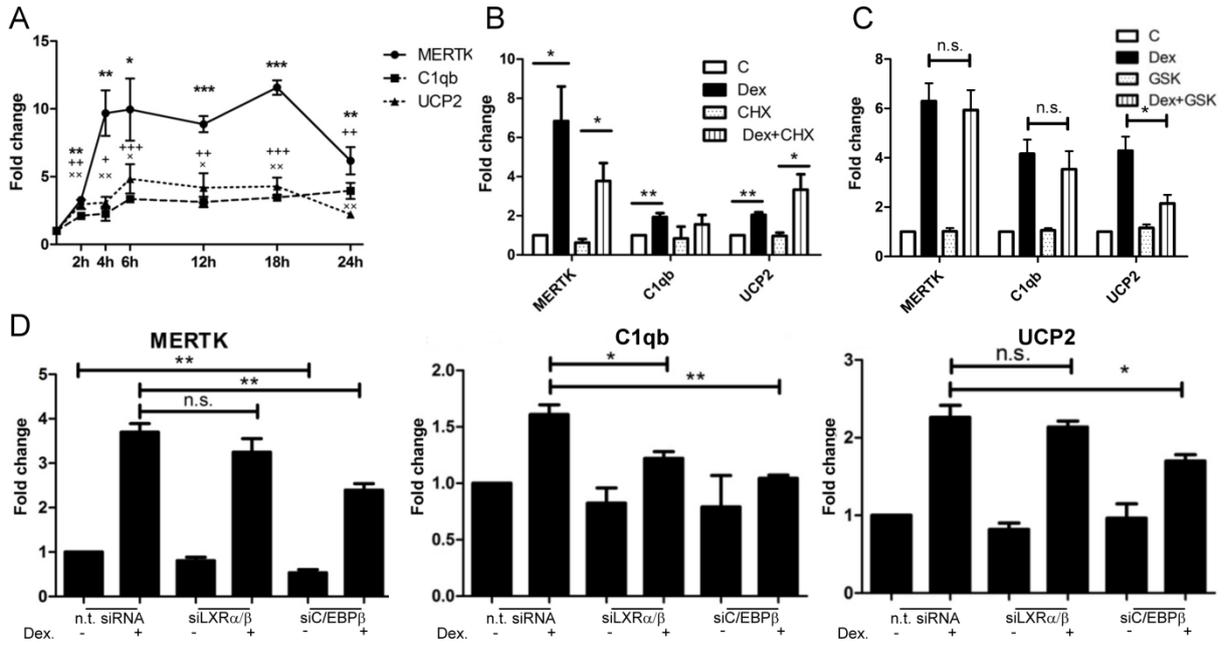


Figure 22. Glucocorticoids induce the expression of MERTK, C1q and UCP2 in BMDMs in a C/EBP β -dependent manner. The long-term induction of UCP2 involves the PPAR δ , while that of C1q the LXR nuclear receptors. (A) Macrophages were treated with DEX (1 μ M) for the indicated time periods. mRNA expressions of MERTK, C1qb, and UCP2 were determined by qRT-PCR analysis using GAPDH as reference gene. Gene expression changes are expressed as mean \pm SEM of at least 3 separate experiments. Significant differences, * p < 0.05, ** p < 0.01, *** p < 0.001, are shown for MERTK, + p < 0.05, ++ p < 0.01, +++ p < 0.001 for C1qb and x p < 0.05, xx p < 0.01 for UCP2. (B) Macrophages were treated with DEX (1 μ M) alone or in combination with CHX (20 μ M) for 4 hrs to inhibit new protein synthesis. mRNA expression levels of the indicated genes were determined by qRT-PCR. (C) Macrophages were treated with DEX (1 μ M) alone or in combination with GSK3787(1 μ M) for 21 hrs, then mRNA expression levels of the indicated genes were determined by qRT-PCR. (D) Macrophages were transfected with the indicated siRNAs, incubated for 48 hrs after transfection, and then they were treated with DEX (1 μ M) for 21 hrs to determine the mRNA expression levels of MERTK, C1qb and UCP2 by qRT-PCR analysis. GAPDH was used as reference gene. Each bar represents mean \pm SEM of at least 3 separate experiments. Significantly different from respective control: * p < 0.05, ** p < 0.01, *** p < 0.001, n.s. = not significant.

LXR_s AND PPAR δ MEDIATE THE GLUCOCORTICOID-INDUCED INCREASE IN THE PHAGOCYTTIC CAPACITY DURING PROLONGED EFFEROCYTOSIS

As described in the introduction part, previous studies have shown that LXRs and PPAR δ are activated in macrophages by the apoptotic cell content when phagocytes engulf apoptotic cells continuously (A-Gonzalez et al., 2009; Mukundan et al., 2009). 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 PPAR δ using PPAR δ antagonist (GSK3787) could interfere with the efferocytosis enhancing effect of glucocorticoids. As shown in Figure 23A, silencing of LXRs or inhibition of the transcriptional activity of PPAR δ 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 (McColl et al., 2009). On the other hand, silencing of C/EBP β 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 PPAR δ interfered with the glucocorticoid effect (Fig. 23B). Silencing of C/EBP β , which we found to be required for the induction of both LXRs and PPAR δ , also prevented the enhancing effect of glucocorticoids on long-term efferocytosis. These data indicate that nuclear receptor LXRs and PPAR δ mediate the enhancing effect of glucocorticoids on the efferocytosis during prolonged phagocytosis.

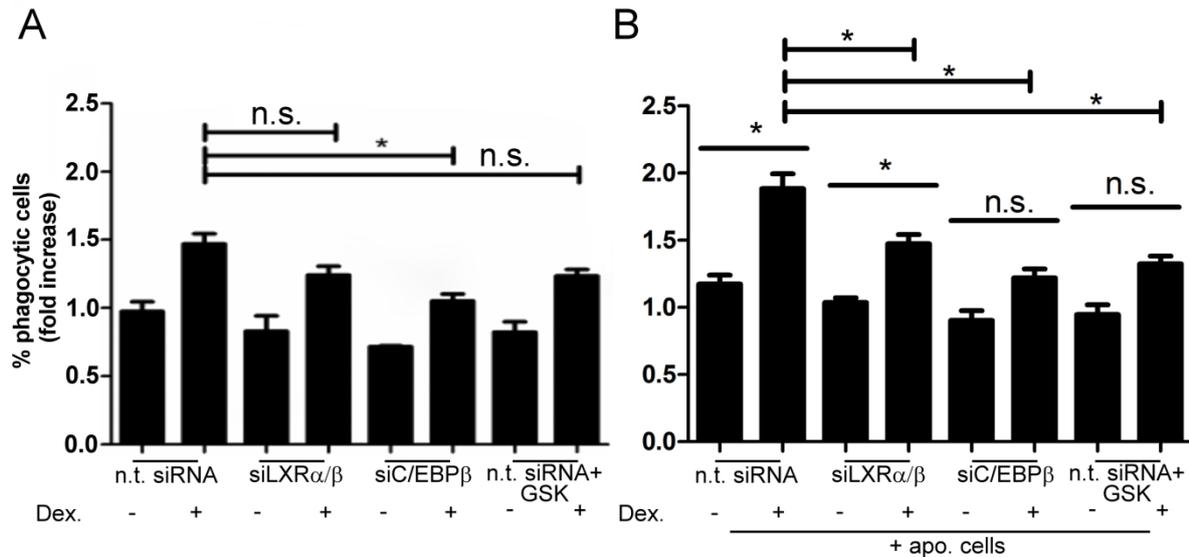


Figure 23. Glucocorticoid-induced long-term efferocytosis is mediated via LXRs and PPAR δ . (A) Macrophages were transfected with non-targeting siRNA, LXR α and β siRNA or C/EBP β siRNA for 48 hrs. Then they were exposed to 1 μ M DEX for 24 hrs. Half of the non-targeting siRNA treated macrophages received DEX treatment in combination with GSK3787(1 μ M), a PPAR δ antagonist. Phagocytosis was determined as it was described in Materials and Methods. (B) To determine prolonged phagocytosis, macrophages were treated as above, but in addition they were also exposed to non-fluorescent apoptotic thymocytes during the last 3 hrs of DEX induction before determining phagocytosis with CFDA-labelled thymocytes. The increase in the percentage of engulfing macrophages is compared to that of the non-treated controls (20.6 \pm 1.9%) and is expressed as fold induction showing the mean \pm SEM of 3 separate experiments. *Significantly different from respective control $p < 0.05$. n.s.= not significant.

PROLONGED PHAGOCYTOSIS INDUCES THE EXPRESSION OF LXR α , RXR α , RAR α AND PPAR δ , 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. As it is shown in Figure 24, prolonged phagocytosis for 3 hrs alone induced the expression of LXR α , PPAR δ , RAR α and RXR α , since the lipid content of the apoptotic cells activated both the LXR and the PPAR δ signaling pathways. The induction of LXR α and RXR α were further increased in DEX-exposed macrophages following the prolonged phagocytosis. The expression of UCP2 was also enhanced after apoptotic cell feeding of macrophages which slightly increased further its DEX-induced expression.

INHIBITION OF RALDH_s 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. As shown in Figure 24, 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 PPAR δ target gene as well. Our data confirm our previous findings that the LXR signaling pathway involves a retinoid-dependent autoregulatory amplification loop (Sarang et al., 2014) and we extended now this observation also for the glucocorticoid-induced lipid sensing signaling pathways.

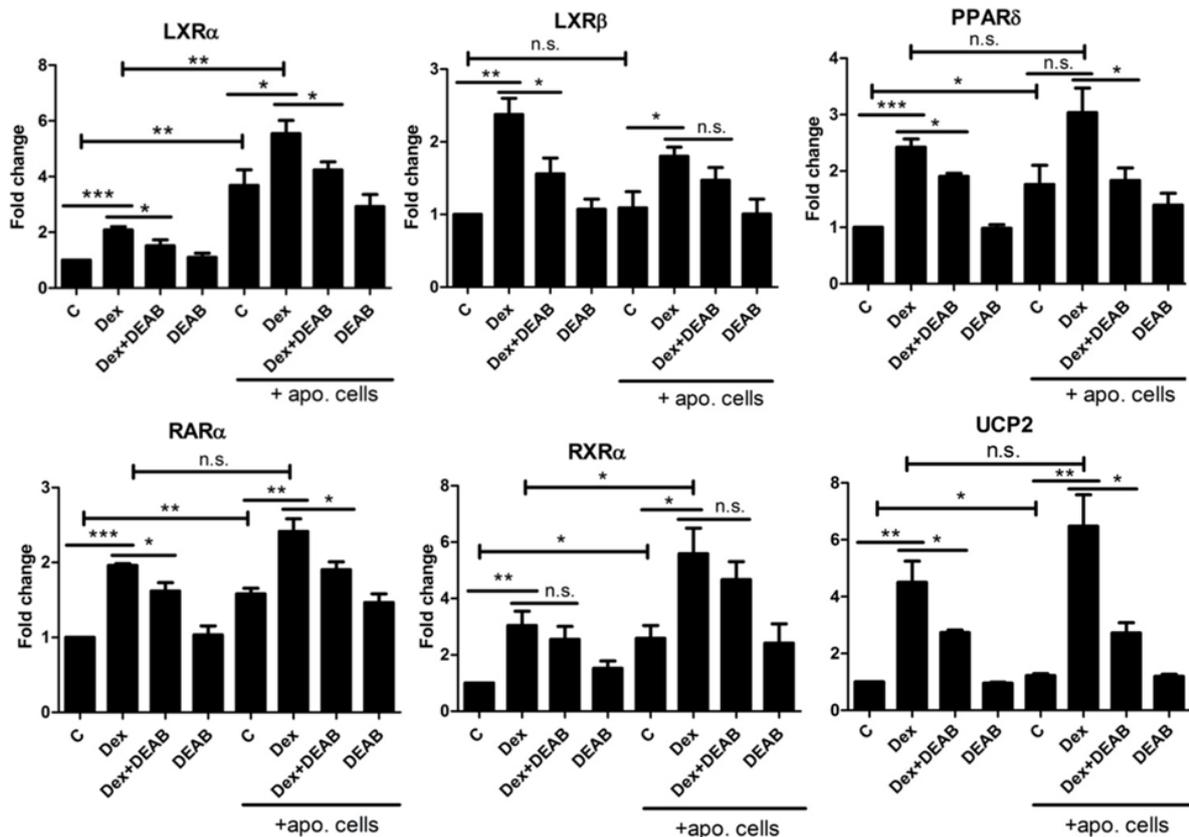


Figure 24. Inhibition of retinoic acid synthesis prevents glucocorticoid-induced expression of lipid sensitive nuclear receptors and UCP2. Macrophages were treated with DEX (1 μ M) and/or DEAB (25 μ M) for 24 hrs. Half of the macrophages were additionally exposed to apoptotic thymocytes during the last 3 hrs of incubation. mRNA levels of the indicated genes were determined by qRT-PCR analysis using GAPDH as reference gene. Each bar represents mean \pm SEM of at least 3 separate experiments. Significantly different from respective control: * p < 0.05, ** p < 0.01, *** p < 0.001. n.s. = not significant.

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 (Xi et al., 2013). 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 (Szondy et al., 1997), indeed, TG2 is able to promote T cell death (Hsieh et al., 2013). In addition, TG2 contributes to the formation of cross-linked protein polymers in apoptotic corpses that prevents the leakage of the harmful cell content (Piredda et al., 1997). 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 (Szegezdi et al., 2000).

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 (Szondy et al., 2003) 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 regulators of TG2 gene transcription (Nagy et al., 1997) and *in vivo* findings from our laboratory have also shown that retinoids are produced in the mouse thymus (Kiss et al., 2008), 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 (Kiss et al., 2008), 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, PPAR δ and PPAR γ). Based on these results, following 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 (Sarang et al., 2014).

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 (Sarang et al., 2014), 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 (Liu et al., 1999; McColl et al., 2009; Zahuczky et al., 2011; Lauber et al., 2013).

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 (Szondy et al., 2014). Most of these diseases respond very well to glucocorticoid treatment. Though many mechanisms have been discovered through which glucocorticoids mediate downregulation of inflammatory responses (Schleimer, 1993), 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 (Zahuczky et al., 2011), the expression level of MFG-E8 (Lauber et al., 2013) and the cell surface expression of the phagocytic receptor CD91 (Nilsson et al., 2012). 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 dexamethasone 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 (Lauber et al., 2013). On the other hand in thioglycollate-elicited macrophages the dexamethasone response was found to be MFG-E8 dependent (Lauber et al., 2013), 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 (Botto et al., 1998). C1q was reported to bind to and activate the CD91 phagocytic receptor (Ogden et al., 2001), and its cell surface expression was shown to be induced by DEX (Nilsson et al., 2012). While CD91 was reported to trigger the GULP-dependent (Su et al., 2002) GULP dependent signalling pathway of Rac1 activation, MERTK contributes to the Dock/Elmo-dependent signalling pathway of Rac1 activation by promoting integrin β_5 signalling (Wu et al., 2005). Thus simultaneous induction of MERTK and C1q by DEX might enhance efferocytosis by simultaneously promoting both signalling pathways that lead to Rac1 activation. In addition to being a CD91 ligand, C1q was reported recently to promote MERTK-dependent efferocytosis also via triggering the adiponectin signalling pathway (Galvan et al., 2014). This signalling pathway involves activation of the AMP activated protein kinase (Thundiyil et al., 2012), which also promotes phagocytosis of apoptotic cells (Bae et al., 2011). Since the induction of both MERTK and C1q required the presence C/EBP β transcription factor induced by DEX (Fig. 22D), silencing of C/EBP β prevented the enhancing effect of DEX on the short term phagocytosis (Fig. 23A).

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, RXR α and PPAR δ , together with CYP27, all of them in a C/EBP β -dependent manner (Fig. 25). The C/EBP β transcription factor has been previously shown to be critical for macrophage differentiation and function (Natsuka et al., 1992) and my data indicate that glucocorticoids also upregulate C/EBP β to initiate or to promote glucocorticoid-dependent transcription.

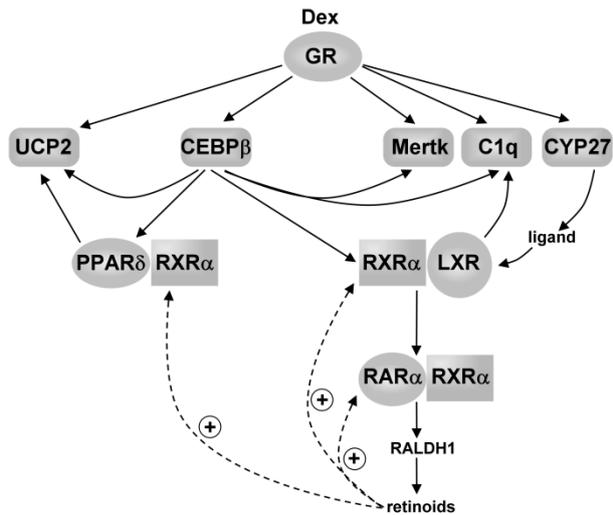


Figure 25. Proposed model for the glucocorticoid-induced enhancement of phagocytosis of apoptotic cells by macrophages. Triggering of glucocorticoid receptors leads to the up-regulation of potential glucocorticoid-target genes such as MERTK, UCP2, CYP27 and C1q, and induces the expression of C/EBP β possibly by repressing the transcription of its labile transcription inhibitory protein. C/EBP β then contributes to the expression of all these genes as well as to the induction of lipid sensing nuclear receptors LXR/RXR α and PPAR δ /RXR α . CYP27 produces an endogenous ligand for triggering LXR leading to the up-regulation of RAR α and then to the consequent up-regulation of RALDH1. The retinoids produced act back via a positive autoregulatory loop to enhance the expression of all the lipid sensing receptors. PPAR δ then contributes to the long-term induction of UCP2, while LXRs to that of C1q.

I have demonstrated that UCP2 mRNA expression was induced in DEX - exposed BMDMs, in a PPAR δ dependent manner (Fig. 22C), while LXRs were required for upregulation of C1q and RAR α , and consequently for that of RALDH1 (Fig. 21). 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 (Fig. 24). The mechanism of this feedback induction was not studied in our experiments, but it is known that both LXRs and PPAR δ are permissive nuclear receptors, thus they can be activated by the RXR retinoid ligand binding site as well (Mangelsdorf et al., 1995). In addition, at least for LXR α 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 (Li et al., 2002). But DEX-induced short-term efferocytosis did not require *de novo* retinoid synthesis (Fig. 17). Neither was the short-term phagocytosis LXR- or PPAR δ -dependent (Fig. 23A). The reason for this is very likely that 1.) the induction of MERTK that drives the enhancement in efferocytosis by DEX (McColl et al., 2009) was LXR- and PPAR δ - 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 PPAR δ -dependent (Fig. 23B) and required the retinoid production induced by LXRs (Fig. 24), 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 exposure to the lipid content of the apoptotic cells (A-Gonzalez et al., 2009; Rószler et al., 2011; Mukundan et al., 2009), nuclear receptors promote prolonged phagocytosis very likely also via inducing alterations in metabolism (Calkin and Tontonoz, 2012; Wolf, 2003), as well as via increasing the expression of UCP2 (Park et al., 2011) 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 PPAR δ , 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.

Retinoids produced by engulfing macrophages might contribute to the thymic homeostasis

T cells differentiate in the thymus. Though the quality of the T cell receptor expressed by thymocytes and the self-antigen presenting cells play a determining role in shaping the T cell repertoire, additional factors might also contribute. Thus vitamin A-deficient animals show marked decreases in cellularity in lymphoid organs and in phagocytosis, resulting in an increase in the frequency and severity of infections (Ross, 1992; West et al., 1989). In addition, retinoids play a role also in the maintenance of the *in vivo* tolerance (Malkovsky et al., 1985; Trentham and Brinckerhoff, 1982). Since most of the symptoms of vitamin A deficiency could be prevented by addition of retinoic acids (RAs), suggestions have been made

that the active metabolites of vitamin A, that mediate its effects on the immune system, are the RAs - all-*trans* RA (ATRA) and 9-*cis* RA (Zhao and Ross, 1995).

Though cells are capable of taking up RAs from the circulation, brain and liver relying almost entirely on the plasma RA pool (Kurlandsky et al., 1995), data coming from our and Agace's laboratory indicate that cells located in the thymus are capable of producing RAs generating an endogenous RA milieu within the tissue (Kiss et al., 2008; Sitnik et al., 2012). Thus we detected an age-dependent mRNA expression of retinaldehyde dehydrogenases (RALDH1 and 2), cellular RA binding protein-II and CYP26A1, proteins responsible for the synthesis, nuclear transport and degradation of RA in the postnatally developing thymus. At the same time, by measuring the induction of an RAR-responsive transgene in two independent transgenic mouse strains, we demonstrated the production of an RAR-activating ligand, which was again age and RALDH dependent. The expression of RALDHs was detected in the thymic epithelial cells and in the mesenchymal cells both in the developing thymus and in a subset of mesenchymal cells in the postnatal thymus (Kiss et al., 2008; Sitnik et al., 2012; Sitnik, 2013). In addition, I have demonstrated that in the postnatal thymus the expression of RALDHs and the production of retinoids were further and significantly induced when apoptosis was initiated *in vivo*. Both the basal and the enhanced expression of RALDHs was concentrated to the cortical layer of the thymus, where most of the apoptosis and phagocytosis takes place, and was localized in the engulfing macrophages (Kiss et al. 2008). Based on the magnitude of the response we propose that apoptosis drives the majority of retinoid production in the postnatal thymus.

However, the amount of ATRA in mouse thymic homogenates is close to our very low detection limit (Kiss et al., 2008) and does not alter with the *in vivo* apoptosis induction (Sarang et al., 2014) suggesting that in this tissue not ATRA is the main RAR-regulating product of retinol metabolism. In fact, a so far unknown retinoid peak is detectable in the mouse thymus, and its size is sensitive to RALDH inhibition (Kiss et al., 2008). Recently we found that RetSat is expressed by macrophages and is induced during *in vivo* apoptosis induction (Sarang et al. 2014). Because dihydroretinol is metabolized via the same RALDHs as retinol, and dihydroretinoic acids are also capable of activating retinoid receptors (Moise et al., 2009), our data might indicate that in this tissue derivatives of the dihydroretinol produced endogenously mediate the effects of vitamin A.

It was shown by three independent laboratories that macrophages engulfing apoptotic cells sense the amount of apoptosis by detecting the lipid content of the engulfed cells via four lipid sensing receptors (LXR α and β , PPAR γ and δ), and respond to it by enhanced phagocytosis (A-Gonzalez et al., 2009; Röszer et al., 2011; Mukundan et al., 2009). Macrophages recognize apoptotic cells via the total pattern of cell surface changes occurring on the apoptotic cells. As a result, their recognition and uptake involves simultaneous action of numerous macrophage phagocytic receptors. Many of these receptors recognize phosphatidylserine or its oxidized form either directly, or indirectly using bridging molecules (Hochreiter-Hufford and Ravichandran, 2013)

Triggering of three lipid sensing receptors enhances phagocytosis via induction of a different set of phagocytic receptors. Thus ligation of LXRs results in the induction of MERTK (A-Gonzalez et al., 2009) and TG2 (Rébé et al., 2009), activation of PPAR δ results in the induction of MFG-E8, Gas6 and thrombospondin (Mukundan et al., 2009), while ligation of PPAR γ leads to the enhanced expression of CD36, MERTK and TG2 (Röszer et al., 2011). Since I found that the expression of RALDHs in engulfing macrophages is also induced by the lipid sensing receptors, it was tested by inhibiting RALDHs with a synthetic inhibitor, whether RAs are involved in the enhancement of phagocytosis. Inhibition of RALDHs strongly attenuated the enhancement of long-term phagocytosis induced by these lipid sensing receptors (Sarang et al., 2014) indicating that RAs are involved in mediating their effect. Since all these lipid sensing receptors are activated by the RXR site as well, production of RAs in macrophages might be part of an autoregulatory feed back loop, which enhances signaling via lipid sensing receptors. In addition, endogenously produced retinoids enhance the expression of a number of phagocytic receptors (Sarang et al., 2014)

Retinoic acids are lipophilic molecules and can be released from the producing cells. While thymocytes do not express RALDHs (Kiss et al. 2008; Sitnik et al, 2012), tight contact of cortical thymocytes with the surrounding macrophages (Rezzani et al., 2008) might allow retinoid signaling induced by macrophage released RAs in these cells as well. Thymocytes indeed express RARs. The expression of RAR α is detectable from gestation day 17, and its level correlate with the proportion of DP cells among the fetal thymocytes (Yagi et al., 1997). Since RAR β and γ are not detectable at this stage, DP cells seem to be the main target of RA action during fetal development. While RAR β expression continues missing in postnatal thymocytes as well, adult DN thymocytes were reported to express RAR α (de Grazia et al.,

1994), while DP thymocytes start to express low levels of RAR γ and the expression increases in both the CD4 $^+$ and the CD8 $^+$ single positive thymocytes, CD8 $^+$ cells expressing 2-3 fold more RAR γ than CD4 $^+$ cells (Dzhagalov et al., 2007).

Thymocytes have been known for a long time to respond to retinoic acid treatment. Thus exposure to RAs induces proliferation of double negative and single positive cells (Dillehay et al., 1989), while in DP thymocytes it induces apoptosis (Fésüs et al., 1995; Xue et al., 1997) and accelerates their glucocorticoid-induced death (Fésüs et al., 1995). On the other hand, simultaneous triggering of TCRs and retinoid receptors results in the survival and further differentiation of DP cells (Iwata et al., 1992; Fésüs et al., 1995; Xue et al., 1997; Szondy et al., 1998; Szegezdi et al., 2003). These observations indicate that retinoids will enhance the rate of cell death by neglect, but cells whose TCR is able to engage peptide-loaded self MHC, are partially protected. Interestingly, glucocorticoids produced by the thymic epithelial cells were shown to modify the outcome of thymocyte selection in a similar way. While signaling through either the TCR or glucocorticoid receptor individually results in apoptosis, simultaneous exposure to both antagonizes the death pathway in a glucocorticoid dose-dependent manner (Vacchio et al., 1994; Zacharchuk et al., 1990). Thus, by modulating the signaling threshold during thymic selection, glucocorticoids were shown to influence the range of selectable avidities for a particular TCR, thereby directly influencing the antigen-specific T cell repertoire (Vacchio et al. 1999). Since retinoids act in a similar dose dependent manner (Szondy et al., 1998), retinoid receptor signaling might be similarly capable of modulating the signaling threshold during thymic selection. In this context it is worth to note that the majority of RALDHs expression is found in the cortical layer of the thymus indicating that the effect of retinoids on the selection processes will occur in this region. In accordance, using a RA sensitive reporter mouse it was shown that RAR transcriptional activity is induced in CD69 $^+$ CD4 $^+$ CD8 $^+$ thymocytes undergoing positive selection and lineage commitment, and continues to be present in both CD4 $^+$ and CD8 $^+$ SP cells (Sitnik, 2013). The presence of retinoids seems to be required for the proper differentiation or survival of these cells, RAR α promoting the CD4 $^+$, while RAR γ the CD8 $^+$ cell differentiation, as in a dnRAR α -CD4Cre mice all the thymocyte subsets had decreased numbers except the CD8 $^+$ thymocytes (Sitnik, 2013), while overexpression of the RAR γ driven by the T-cell specific Ick promoter enhances the CD8 $^+$ thymocyte cell number (Pohl et al., 1993).

As retinoids are known to contribute to the TGF- β -induced pTreg formation (Benoist and Mathis, 2012), it is interesting to speculate whether they could also contribute to the formation of thymus-derived Treg cells. On one hand because RAs are known to enhance the activation of the Foxp3⁺ locus (Benson et al., 2007), on the other hand because by antagonizing the biological activity of Nur77 by RAR α in DP thymocytes (see below), they might promote the survival of those CD4⁺ thymocytes that have TCR specificities with intermediate affinity for self-antigens and express only intermediate levels of Nur77 (Moran et al., 2011).

Experiments carried out in our laboratory during the past years revealed some aspects of the molecular mechanism of retinoid action in thymocytes. Thus initiation of apoptosis is mediated via RAR γ (Szondy et al., 1997) and involves the induction of Nur77, as Nur77 null thymocytes are resistant to retinoid-induced apoptosis (Kiss et al., 2015). Nur77 is known to regulate the expression of various apoptosis-related genes (Rajpal et al., 2003). Retinoids promote glucocorticoid-induced apoptosis because ligated RAR α /RXR enhances the transcriptional activity of the glucocorticoid receptor via directly targeting it (Tóth et al., 2011). Ligation of RAR α , on the other hand, inhibits negative selection of DP thymocytes (and perhaps promotes Treg formation) by preventing the upregulation of Bim, a proapoptotic member of the Bcl-2 family, and by binding to Nur77 and inhibiting its transcriptional activity (Szegezdi et al., 2003).

In summary based on all our results we believe (Fig.26) that in the postnatal thymus the main source of the endogenous retinoid production are the engulfing macrophages, and the amount of retinoid formed is related to the rate of apoptosis. The retinoid produced enhances the phagocytic capacity of macrophages by enhancing the expression of the phagocytic receptors, thus when the rate of thymocyte apoptosis is accelerated (for example after stress), the phagocytic capacity adapts to it. In addition, since the proliferation of pre-TCR expressing DN cells is enhanced by retinoids (Dillehay et al., 1989), RAs produced by macrophages in an engulfment-dependent manner might also promote the replacement of the dying DP cells by promoting the proliferation of their precursor cells. Retinoids produced by the engulfing macrophages contribute to the thymic selection processes as well, by inhibiting the negative selection of those thymocytes that have TCR specificities with intermediate affinity for self-antigens and thus perhaps contributing also to the thymic Treg formation. In addition ligation of RAR α contributes to the CD4⁺, while that of RAR γ to the CD8⁺ thymocyte development.

They also promote the “cell death by neglect” alone or acting together with the glucocorticoid hormone to make sure that the useless cells die fast. In addition, in apoptotic thymocytes macrophage-derived retinoids also contribute to the appearance of TG2, which participates in the efficient induction and completion of the apoptotic program (Fésüs and Szondy, 2005). Our data together with the other laboratories indicate the existence of a complex crosstalk between thymocytes and engulfing macrophages mediated by retinoids to regulate differentiation, removal and replacement of dying DP cells in the thymus.

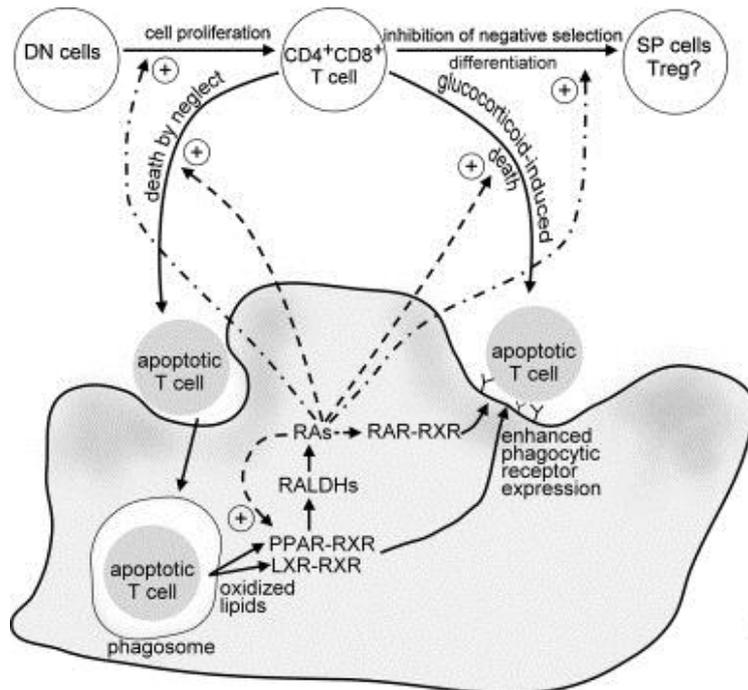


Figure 26. Suggested crosstalk between thymocytes and engulfing macrophages mediated by the macrophage-derived retinoids. Suggested crosstalk between thymocytes and engulfing macrophages mediated by the macrophage-derived retinoids. Macrophages produce RAs in an engulfment-dependent manner regulated by their lipid sensing receptors (PPARs and LXRs). In macrophages RAs enhance engulfment by promoting the transcriptional activity of both the lipid sensing and the retinoid nuclear receptors. The RAs released enhance “death by neglect” alone or by enhancing the glucocorticoid-induced death of DP thymocytes, while they interfere with the negative selection of thymocytes that have TCR specificities with intermediate affinity for self-antigens. This way they might contribute also to the thymic Treg formation. In addition, RAs seem to be required for the survival or differentiation of SP cells. Ligation of RAR α contributes to the CD4⁺, while that of RAR γ contributes to the CD8⁺ thymocyte development. RAs might also stimulate proliferation of the pre-TCR expressing DN thymocytes thus promoting the replacement of the engulfed cells by newly formed DP thymocytes

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 dexamethasone. 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/EBP β . C/EBP β contributes to the further induction of the phagocytosis-related genes, and is required for the induction of lipid sensing receptors LXRs, PPAR δ , RAR α , RXR α and RALDH1, the latter one in an LXR- and RAR α -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 PPAR δ upregulation.

Retinoids, produced by macrophages can be released and affect TG2 expression as well as the selection of developing thymocytes. In the last part of my thesis I summarize what is known about the involvement of retinoids in the regulation of thymic selection processes, and how engulfment-regulated retinoid production might contribute to the maintenance of thymic homeostasis.

ÖSSZEFOGLALÁS

Az apoptotikus sejthalál hatékony megvalósítását követő eredményes eltakarítás a professzionális fagociták és nem professzionális szomszédos fagociták által, egy kulcsfontosságú mechanizmus a szöveti homeosztázis fenntartásához. Minden nap a sejtek billiói halnak el és fagocitálódnak anélkül, hogy gyulladást és immunválaszt idéznének elő. Az apoptotikus és az őket felvevő sejtek által termelt transzglutamináz 2 biztosítja az elhaló sejtek gyors felismerését és eltávolítását, valamint közreműködik a káros sejt tartalom kibocsátás megakadályozásában is. A T sejtek a tímuszban differenciálódnak és szelekciójuk folyamán az újonnan képződő sejtek 95%-a elhal majd eltakarítódik. Ezért az egér tímuszban az apoptózis és a fagocitózis *in vivo* folyamata kitűnően tanulmányozható.

Az értekezésben bemutattam, hogy habár a TG2 indukálódik az *in vivo* elhaló timocitákban, nem fejeződik ki az *in vitro* apoptotikus timocitákban. A TG2 *in vivo* indukációjához a szöveti környezetben jelenlévő faktorokat igényel. Azt találtam, hogy ezeknek a faktoroknak egyike egy A vitamin származék mely a makrofágokban termelődik az aldehid és retinaldehid dehidrogenázok által, az apoptotikus sejtek fagocitózisa közben. Ezen retinoidok termelését a lipid érzékelő magreceptorok szabályozzák, úgy mint a PPAR-ok és LXR-ok, és elsősorban a fagocitáló makrofágok apoptotikus sejt felvevő képességére hatnak.

Azt találtam, hogy a retinoidokat nem csak az apoptotikus sejteket felvevő makrofágok termelik, hanem a dexametazonnal kezelt makrofágok is képesek rá. Bemutattam, hogy a glükokortikoidok elősegítik mind a rövidtávú mind a hosszútávú apoptotikus sejt felvétel folyamatát. Úgy tűnik a glükokortikoidok közvetlenül indukálják fagocitózis-kapcsolt gének kifejeződését, mint a Mer tirozin kináz, a C1q, az UCP2 és a C/EBP β transzkripció faktor. A C/EBP β közreműködik a fagocitózis-kapcsolt gének további növelésében és a lipid érzékelő receptorok, mint az LXRs, PPAR δ , RAR α , RXR α valamint a RALDH1 indukációjában, az utóbbihoz LXR- és RAR α -függő módon hozzájárulva. A hosszútávú efferocitózis glükokortikoid indukálta növekedése a lipid érzékelő receptorok indukációjától függ, melyekről ismert, hogy a bekebelezett sejtek lipid tartalma által indukálódnak, majd fokozzák az apoptotikus sejtek fagocitáló képességét. A retinoidok nem befolyásolják a glükokortikoid-indukálta rövidtávú apoptotikus sejt fagocitózist, de szükségesek a glükokortikoid-indukálta efferocitózis fokozáshoz az apoptotikus sejtek hosszú időn keresztül zajló eltakarításához, azáltal, hogy biztosítják az LXR és PPAR δ receptorok megfelelő szintű kifejeződését.

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PUBLICATIONS, CONFERENCES



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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.
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2. Sarang, Z., **Garabuczi, É.**, Joós, G., Kiss, B., Tóth, K., Rühl, R., Szondy, Z.: Macrophages engulfing apoptotic thymocytes produce retinoids to promote selection, differentiation, removal and replacement of double positive thymocytes.
Immunobiology. 218 (11), 1354-1360, 2013.
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3. **Garabuczi, É.**, Kiss, B., Felszeghy, S., Tsay, G.J., Fésüs, L., Szondy, Z.: Retinoids produced by macrophages engulfing apoptotic cells contribute to the appearance of transglutaminase 2 in apoptotic thymocytes.
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List of other publications

4. Kiss, B., Tóth, K., Sarang, Z., **Garabuczi, É.**, Szondy, Z.: Retinoids induce Nur77-dependent apoptosis in mouse thymocytes.
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5. Szondy, Z., **Garabuczi, É.**, Joós, G., Tsay, G., Sarang, Z.: Impaired clearance of apoptotic cells in chronic inflammatory diseases: Therapeutic implications.
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