Nuclear hormone receptors and lipid antigen presentation in dendritic cells

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UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF MOLECULAR CELL AND IMMUNE BIOLOGY
DEBRECEN, 2018
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The examination took place at the Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Debrecen, Debrecen; at 11:30; 12th of January 2015.

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen at 1:00 p.m. on 18th of February, 2019.
1. INTRODUCTION

1.1. Dendritic cells (DCs)

1.1.1. General feature of DCs

The immune system has evolved to exert acute and systemic inflammatory responses and to protect the host with layered defenses of increasing specificity. Humans and mice have two types of immune defense: innate- and adaptive immunity. The innate phase of immunity is rapidly followed by an antigen–specific adaptive immune response, initiated by antigen presenting cells (APCs). As APCs, dendritic cells (DC) are unique innate immune cells that function as an indispensable link between the innate- and adaptive immunity, and are important sentinels of the host immune system. Immature DCs (iDCs) constantly sample the peripheral tissues for invaders and host cell-associated self-antigens. Some DCs migrate in immature state to draining lymph nodes (LNs), present self-antigens and are involved in the maintenance of the peripheral tolerance. Mature DCs (mDCs) induce adaptive immune responses in the host by a highly specific antigen presentation process, mediated by their cell surface major histocompatibility complex class (MHC) I molecules that present self-antigens/viruses or by MHCII molecules, required for exogenous antigen presentation. mDCs migrate to LNs from the periphery, present antigens to naïve T cells and activate them. They also present glycolipids of infectious invaders or self-lipid antigens through the cluster of differentiation 1 (CD1) molecules. Despite of enormous scientific effort, lipid antigen presentation is less characterized at molecular level in DCs. To solve a part of these uncovered mechanisms, we dedicated this work to analyze the importance of nuclear hormone receptors in affecting the differentiation and immunogenicity of human and murine DCs.

1.1.2. Ontogeny of DCs

DCs are classified as plasmacytoid DCs (pDCs) or conventional DCs (cDCs). They develop from common myeloid progenitors (CMPs) in the bone marrow (BM), during hematopoiesis. Further studies have indicated that monocytes and DCs may share a common intermedier progenitor, known as the macrophage and dendritic cell progenitor (MDP). MDPs become commitment to produce monocytes or common DC progenitors (CDPs). CDPs give rise only conventional pre-dendritic cells (pre-cDCs) or plasmacytoid pre-dendritic cells (pre-pDCs). It has been suggested that pDCs complete their development before leaving the BM, whereas pre-cDCs constantly released from bone, circulate through the blood to localize into non-lymphoid or lymphoid tissues and fill the DC compartment.

The development and expansion of DC subtypes are determined by a combination of cytokines and transcription factors (TFs). Fms-related tyrosine kinase 3 ligand (Flt3L) was found to be a key cytokine for the myeloid DCs development. Besides the common progenitor (MDP), the common origin of macrophages and DCs has been further supported by the requirement for the macrophage colony-stimulating factor/Colony stimulating factor 1 receptor (M-CSF/CSF-1R) during their development.
Monocytes under the influence of this cytokine give rise to CD103+ DCs in the lamina propria (LP). Although human circulating monocytes are excellent source of DCs in vitro, their contribution to DC homeostasis is still not fully characterized in vivo. CD14+ monocytes can be differentiated to monocyte-derived DCs (mo-DCs) in the presence of interleukin-4 (IL-) IL-4/IL-13 and granulocyte-macrophage colony-stimulating factor (GM-CSF/CSF-2), which is the most frequently utilized human DC differentiation model. Previously, this differentiation process was found to be restricted to inflamed or infected environments, suggesting that the in vitro generation process models mainly the tissue inflammatory and not the steady state DC development. Subsequent experiments have identified the development of monocyte-derived intestinal CD103+/CD11b+/-, splenic CD11b+/ endothelial cell-specific adhesion molecule (ESAM)low- and muscular FC gamma Receptor 1 (FcγRI) bearing DCs under steady state conditions.

Adoptive transfer experiments supported, that DC subsets in the intestinal LP are originated from different precursors (monocytes or pre-cDCs). Monocytes give rise exclusively to CD103/C-X3-C Motif Chemokine Receptor 1 (CX3CR1)+ DCs under the control of M-CSF and Flt3L, while GM-CSF and Flt3L are critical factors for the CD103+/CX3CR1+ DC differentiation form Pre-DCs.

Genome wide gene transcriptional- and cell surface receptor profiling of small intestinal LP, blood and splenic (Sp-) Sp-DC subpopulations identified a co-ordinately regulated TF profile that directs subtype specification and development of these cells in various non-lymphoid and lymphoid tissues. This analysis also revealed that human intestinal CD103/signal regulatory protein alpha (Sirpa)+ cDCs have a gene expression profile consistent with mo-DCs.

1.2 Lipid antigen presentation by DCs

1.2.1. Group 1 and Group 2 CD1 molecules

Beside their peptide antigen presentation capacity, DCs acquire the ability to stimulate lipid-mediated T cell responses. Antigenic lipids are presented by the family of CD1 molecules, evolutionary conserved lipid antigen-presenting molecules. Five CD1 proteins are expressed in humans, classified into two groups based on their nucleotide and amino acid sequence homology. Group 1 contains CD1a, b, c and e; and the only Group 2 member is CD1d. Mice express only CD1d. CD1 genes encode integrated membrane proteins that are structurally similar to MHC1 molecules. CD1 isoforms are consisting of a heavy chain with α1, α2, and α3 extracellular domains, associated non-covalently with β2-microglobulin (β2m). Crystallographic analysis of human and mouse CD1 molecules revealed that these antigen presenting molecules have narrow, deep hydrophobic ligand binding pockets, optimized for lipid presentation. CD1 molecules can adopt different conformations, facilitating the binding of structurally related lipids, hence allowing the presentation of multiple CD1d-bound lipids and increasing the antigen repertoire to CD1-reactive T cells.
1.2.2. CD1d molecules

High level of CD1d can be detected on human blood monocytes; this expression is rapidly down-regulated during their differentiation to DCs. Despite of the low cell surface expression level, CD1d stimulates the expansion and cytokine secretion of CD1d-restricted, invariant natural killer T cells (iNKTs) when mo-DCs are loaded with α-galactosylceramide (αGC) (a lipid activator ligand for iNKT cells). In vivo, immunohistochemistry (IHC) data has demonstrated the CD1d expression on dermal DCs in the skin. CD1d are also present on murine APCs such as Sp-DCs, macrophages, B cells and thymocytes. In contrast to human mo-DCs, GM-CSF and IL-4 induce moderately the surface expression of CD1d on BM-DCs. Furthermore, Flt3L-stimulated BM-DCs, differentiated in the presence of LPS and INFα, have enhanced surface expression of the protein and under inflammatory condition colonic LP-DCs have increased level of CD1d. CD1d also associates with β2m before exiting the ER, although functional CD1d can also be detected on the cell surface in β2m-independent manner. CD1d molecules reach the plasma membrane following two different secretory routes. In the intrinsic pathway, CD1d molecules travel directly to cell surface and present self-antigens, while during the extrinsic pathway, a portion of CD1d molecules in association with the invariant chain (Ii), traffic first to the endosomal compartments. This second pathway is critical for CD1d molecules to be loaded by antigenic self- or exogenous-lipids that are presented to autoreactive CD1d-restricted NKT cells and is required for the positive selection of NKTs.

Murine cell surface CD1d is reinternalized in activatin protein 2 (AP-2)/AP-3-dependent manner. Studies using murine CD1d mutants, revealed that this motif is critical for the lysosomal targeting and iNKT autoreactivity. Contrast to mouse CD1d; the cytoplasmic tail of human CD1d molecule is not associated with AP-3. Moreover human and murine CD1d molecules showed distinct intracellular trafficking properties. The components of the alternative internalization pathway are remained to be characterized. A fraction of human CD1d can be sorted to MHC class II compartments (MIICs) by MHCII and Ii molecules. The association with MHCII does not affect the intracellular localization of CD1d, but facilitates its internalization rate from the cell surface. Ii deficiency resulted in reduced cell surface level of CD1d in MHCII+ cell lines, suggesting that CD1d can be complexed both with Ii and MHCII at the cell surface. MHCII/Ii recruits CD1d into membrane lipid rafts, enriched for costimulatory molecules and this makes CD1d a more potently stimulators to iNKTs.

DCs have the ability to present lipid antigens and efficiently activate CD1-restricted T cells regardless of their maturation state, while the MHC-dependent peptide antigen presentation requires maturation of the cells and fast mobilization of MHC molecules to plasma membrane. CD1d-mediated exogenous antigen presentation is more efficient in iDCs, which actively recycle MHCII/CD1d complexes through the endocytic system, compared to mature DCs that have stabilized MHCII cell surface expression. Both iDCs and mDCs present αGC, which does not require intracellular processing, although iDCs are more active in this presentation. iDCs also have the capacity to present galactosyl(1-2) galactosylceramide (αGGC) (requires lysosomal activation to generate αGC).
1.2.3. Lipid antigen processing and loading

For presentation, lipids have to be extracted from their intra cellular milieu, processed and loaded into the antigen binding pocket of CD1 molecules. These processes are assisted by hydrolases and lipid transporter proteins (LTPs). Some lipid antigens require partial degradation to become antigenic. The synthetic glycolipid αGGC processing requires the removal of the terminal galactose of the precursor lipid to become αGC by α-galactosidase hydrolase enzyme, located in late endosomes. Lipid extraction to the lumen is mediated by LTPs that facilitate loading or replacement of lipid antigens to CD1 molecules in the endosomal compartments.

CD1d lipid antigens are edited by saposins (Saps), membrane-perturbing sphingolipid activator proteins. Active saposins (SapA-D) are generated from the precursor prosaposin in the late endosomes. This process is catalyzed by cathepsins (Cats). Saposins directly bind lipid antigens, extract them from endosomal membranes, and transfer them to CD1 proteins, while the loading of the lipids onto CD1 molecules is an indirect process. The in vivo relevance of Sap-mediated lipid presentation was determined in Sap⁻/⁻ mice. Sap deficiency led to defective iNKT development. In contrast to the impaired iNKT development, the number of the type II NKT cells was normal. Importantly, CD1d expression, cellular distribution and iNKT cell autoreactivity were not altered by Saps but the presentation of αGC was affected to CD1d-restricted iNKT cells. αGC can bind to cell surface CD1d molecules without endosomal recycling, but Saps facilitated the intracellular CD1d/αGC complex formation in lysosomes. Sap deficiency leads to dysregulated lysosomal lipid accumulation, lipid traffic and exchange between membranes. The impaired lipid metabolism results in lysosomal storage diseases which affect CD1d loading.

1.2.4. Lysosomal Cathepsins in DCs

Immunogenic lipid presentation is depended on lysosomal proteolytic mechanism in DCs. Most lysosomal proteases are known as Cats, which are essential for both peptide and lipid antigen presentation. Cysteine proteinases-mediated proteolysis is critical for the antigen-presentation in DCs, wherein controls the lipid editing by cleaving of Ii and pro-saposins in late endosomes. In response to maturation signals, DCs acquire higher capacity for lipid editing by enhanced lysosomal activity (elevated antigen processing and lipid/CD1 complex formation).

CatL, S, and B have shown to be expressed in DCs. Amongst them only CatL and S have been connected to lipid presentation to date. In CatS⁻/⁻ mice, MHCII complexes were accumulated in endosomal vesicles that might affect the intracellular CD1d transport to the membrane. CatS deficiency led to reduced level of cell surface CD1d in thymic DCs. Moreover the intracellular trafficking of CD1d was also affected in CatS⁻/⁻DCs. iNKTs were not reactive to αGC. The number of iNKT cells was also reduced in CatL⁻/⁻ mice but in contrast to CatS⁻/⁻ animals, CatL deficiency failed to alter the CD1d level on the cell surface. CatL in thymocytes was essential for the positive selection and the enzyme was involved in APC-mediated negative selection of iNKTs. In the periphery, CatL in APCs was critical for
the terminal differentiation of iNKTs. In both cases, the deficiency in Cat proteins appeared to impede endosomal events required for potent CD1d-mediated antigen presentation.

DCs have been reported to express lysosomal aspartic proteases such as CatD. Similar to cysteine proteases, CatD is synthetized in inactive form and are activated by autocatalysis in ceramide-dependent manner. Activated CatDs are abundant in lysosomes where cleave LTPs as prosaposin. The significance of this protease has not previously tested in the context of lipid presentation.

1.2.5. CD1d-restricted NKT cells

Unlike conventional T cells, T cell receptors (TCRs) on NKTs are reactive to lipid antigens in the context of the CD1d. These unique self-reactive T cells express both natural killer cell (NK) markers and TCRs on their surface; hence they are termed as NKTs. There are two types of CD1d-restricted T cell populations: invariant NKT (iNKT) cells and type II NKTs.

iNKTs represent a unique population of evolutionarily conserved subset of innate lymphocytes which express highly restricted set of TCR, composed of a semi invariant α chain (Vα14-Jα18 in mice and Vα24-Jα18 in humans) paired with a restricted repertoire of β chains (Vβ2, Vβ7, and Vβ8.2 in mice, or Vβ11 in humans).

In the periphery their subsequent activation results in a rapid cytokine burst within hours by which transactivate other lymphocytes. Indeed, iNKTs are involved in a wide range of immune relevant processes such as maturing DCs, activating NK or B cell or biasing T cell responses, hence iNKTs regulate both innate and adaptive immunity, modulate the ongoing immune responses that can influence the outcome of various disease from autoimmune responses, bacterial or viral infection and cancer.

The potent role of iNKTs in providing tumor immune surveillance was demonstrated by αGC injection, and in several studies without administration of the antigenic lipid ligand, supporting the notion that iNKTs can recognize endogenous antigenic lipids produced by tumor cells. The essential function of iNKTs was demonstrated in Jα18−/− mice, in which adoptively transferred iNKT cells elicited protection against tumors. Depending on the tumor model, resident iNKT reactions can lead to effective anti-tumor immunity through down-stream activation other immune cells by initiating Th1 cytokine cascade in the tumor-associated stroma (TAS), thus orchestrating local activation of effector cells, such as NK and CD8+ T cells, which kill tumor cells. iNKT activation also contributes to DC activation through the CD1d-TCR and CD40-CD40L interactions, which induce DC maturation and IL-12 expression. Secreted IL-12 stimulates NK and iNKT cells to produce even more INFγ, and the two cytokine together trigger NK and CD8+ T cells. Co-administration of peptide antigens with iNKT agonist has adjuvant effect.

Conversely, type II NKTs have polyclonal TCR repertoire. The limited reagents to monitor type II NKTs and the absence of specific surface markers have limited the functional characterization of this NKT population. The immune functions of a fraction of type II NKTs can be analyzed by sulfatide/CD1d tetramers. The in vivo relevance of NKT activation can be characterized in CD1d−/− mice
lacking both iNKTs and type II NKTs. These animal models have been very useful in defining the unique role of type II NKTs in several pathological conditions. Once activated, type II NKTs have the capacity to override iNKT-mediated immune responses. In contrast to the protective role of iNKTs in most murine tumor models, type II NKTs have been shown to be sufficient to suppress tumor immune surveillance and had tumor promoting activity. When sulfatide and αGC were administered together, sulfatide-reactive murine type II NKTs can antagonize the potent αGC-dependent protective iNKT responses. This immune regulatory axis between the two NKT populations and that type II NKTs favor tumor growth by releasing IL-4 and IL-13 were demonstrated in several mouse models.

1.3. Nuclear hormone receptors

1.3.1. Nuclear hormone receptors in human mo-DCs

A global gene expression analysis revealed that some nuclear hormone receptors including peroxisome proliferator-activated receptor γ (PPARγ) and liver X receptor α (LXRα) are expressed and induced in differentiating DCs. Later our microarray profiling demonstrated that 20 out of the 48 nuclear hormone receptors are expressed in human mo-DCs. Retinoid X receptors (RXRs) are unique nuclear hormone receptors with the ability to form heterodimers with one third of the nuclear hormone receptor family. In most cases, RXRs act as obligate partner for high affinity binding transactivation. RXR heterodimers are classified into functionally distinct non-permissive and permissive subgroups.

Among RXR partners, the role of PPARγ/RXR and RARα/RXR heterodimers in developing mo-DCs will be introduced in the next chapter.

1.3.2. The role of retinoid acid receptors in DC biology

Vitamin A/retinol and its derivatives are collectively known as retinoids. Deviation from optimal retinoid level is associated with a variety of human diseases, All-trans-retinoic acid (ATRA), the highly potent biologically active metabolite of retinol, prevents and rescues the main defects caused by Vitamin A deficiency in adult animals. Retinoids exerts their effects in target cells via nuclear retinoid receptors. RARs consist of three subtypes involving RARα, β and γ, form heterodimers with RXRs, which also have three subtypes, RXRα, β and γ. Although both ATRA and 9-cis retinoic acid activate RARs, RXR binds 9-cis retinoic acid only.

1.3.2.1. Retinoids in target cells (cellular up-take and transport)

Vertebrates do not have the ability for de novo retinol synthesis but can obtain this vitamin from the diet as retinyl ester or carotenoids, absorbed by enterocytes and transported predominantly to the liver. Retinyl esters are continually hydrolyzed into retinol from the liver store, binds to Retinol binding protein 4 (RBP4), which delivers it to target tissues. Retinol target cells express a highly conserved, stimulated by retinoic acid-6 (STRA6) transporters, for retinol uptake. Cells may take up retinol through
the recently identified RBP4 receptor-2 (RBPR2) transporters. The structure of RBPR2 is related to human and murine STRA6 and therefore the receptor may be an alternative retinol transporter for STRA6 negative cells.

Within the cell, retinoids are bound by cellular retinol binding proteins (CRBPs). CRBP1 targets retinol for storage or toward metabolic enzymes such medium-chain alcohol dehydrogenases (ADHs) or short-chain dehydrogenase/reductases (RDHs) that convert retinol to retinal. Subsequently, retinal dehydrogenase (RALDH) enzymes synthetize ATRA form retinal. ATRA associates with cellular retinoic acid-binding protein 1 (CRABP1), CRABP2 or fatty acid-binding protein 5 (FABP5) transporters. Cytosolic CRABP2 delivers ATRA directly to RARs and enhances the transcriptional activity of the receptors. CRABP1 moderates the cellular response to ATRA by transporting it to enzymes involved in the ATRA catabolism. Intracellular ATRA level is tightly regulated by the members of cytochrome P450 gene family: CYP26A1, B1 and C1. Polar metabolites generated by these catabolic enzymes are more easily excreted from cells. Importantly, the proximal upstream promoter region of the Cyp26a1 gene contains a functional retinoic acid response element (RARE), therefore Cyp26a1 gene expression is directly regulated by RARs. FABP5 transfers ATRA to PPARβ/δ and activates PPARβ/δ-mediated transcription.

1.3.2.2. Retinol metabolism

For exerting physiological function, retinol has to be converted during two consecutive oxidative reactions for metabolic activation. In the first rate-limiting oxidative step retinol is converted to retinal, catalyzed either by alcohol dehydrogenases (ADH1,3, and 4), or by retinol dehydrogenases (RDH1 and RDH10). 80-94% of cellular retinal-generating capacity resided in the microsomes (intracellular localization of RDHs), rather than the cytosol (site of ADHs). Currently at least three RDHs are seem to be physiologically participating in converting retinol to ATRA: RDH1, RDH10 and DHRS9. RDH10 was identified as all-trans retinol dehydrogenase in the RPE BX and was purified from the microsomal fraction of rMC-1 cells. The role of RDH10 for embryonic ATRA synthesis was identified in RDH10−/− mice, which had severe organ abnormalities and an embryonic lethal phenotype at embryonic day 13.5. The fundamental role of RH10 in embryonic development has been confirmed utilizing RDH10−/− mice, carrying the RARE-lacZ-reporter transgene, which monitor endogenous sites of retinoid signaling.

The second enzymatic step is the irreversible oxidation of retinal to ATRA, mediated by RALDH1,2 or 3, members of the aldehyde dehydrogenase family. Genetic deletion experiments in mice have established the physiological contribution of RALDH isoforms to ATRA production and vital functions of RALDH2 during the embryonic development. RALDH2−/− mice showed early lethality suggesting that this enzyme was essential for embryonal ATRA production. Moreover, the sites of RDH10 expression overlapped with RALDH2 sites, suggesting that co-expression of the two metabolic enzymes was required for active ATRA generation in developing embryos.
1.3.2.3. ATRA synthesis in DCs

Numerous data have demonstrated that multiple factors including ATRA, retinol, GM-CSF, IL-4, TLR ligands can promote or in case of prostaglandin E2 (PGE2) can inhibit the ATRA synthesis in the cells. These imprinting factors promote the expression of Raldh genes, indispensable markers for active ATRA synthesis in DCs. First, Iwata demonstrated that PP- and MLN-DCs synthetized ATRA from retinol and released ATRA enhanced the expression of CCR9 and integrin α4β7 gut homing markers on responding CD4^+ T cells. According to their results, Adh5 was expressed ubiquitously and PP-DCs expressed Adh1 and 4, suggesting that these enzymes might be responsible for retinol to retinal conversion in adult mice. For the retinal to ATRA oxidation step, PP-DCs expressed Raldh1 and to a lower extent Raldh2, while MLN-DCs expressed Raldh2. Subsequent studies demonstrated, that Raldh2 expression in GALT-associated DCs was enriched in CD103^+ DC subsets. It soon became evident that ATRA synthesis is not a universal DC property and only certain DC subsets acquire the ability to produce this retinoid.

The relevance of examining intestinal retinoid metabolism in DCs was further intensified by the detection of CD103^+ DCs in human MLNs. These cells displayed a more mature phenotype by the cell surface expression of CD83 as compared with their CD103^- counterparts and induced α4β7 or CCR9 expression on responding CD8^+ T cells. The DCs-mediated gut-homing expression could be inhibited by LE540 (RARα antagonist), suggesting that human DCs similarly to mouse cells acquire active ATRA metabolic capacity in the gut. Furthermore, unique APCs within the LP, with macrophage-like morphology and co-expressed macrophage (CD14) and DC (CD209) markers, elicited potent antigen-specific immune responses through ATRA-mediated signals. These cells express RDH10 and RALDH2, suggesting that at least some in vivo APCs may produce ATRA by utilizing these oxidizing enzymes.

Finally, a comprehensive analysis demonstrated that ATRA metabolism could be detected also in extra intestinal tissue-derived DC such as lung and skin DCs. Unexpectedly, the RALDH activity was detected in CD103^- skin migratory DCs, while ATRA producing cells in the lung contained both CD103^+ and CD103^- subsets. These data collectively demonstrated that ATRA synthesis capacity is not restricted to intestinal DCs and at least some migratory DC subsets in the peripheral tissue produce ATRA.

1.3.3. PPARs in DC biology

DCs express PPARs. The PPAR family consists of three isotypes: PPARα, δ/β and γ. These receptors show distinct tissue-specific distribution with different physiological functions. In the nucleus for direct transcriptional activation, PPARs heterodimerize with RXRs and bind to their receptor-specific response elements (PPREs) in the promoter or enhancer regions of their target genes. Beside this fundamental role in metabolism, the receptor is involved in the regulation of immune responses. The idea that PPARγ might transcriptionally integrate lipid metabolism and inflammatory responses in DCs was supported by increasing amount of evidence. In vitro experiments presented that the receptor
was functional in DCs, contributing the subtype- and functional specification and immune phenotype of the cells. However, the receptor was not expressed in all DCs but at least in a subset of these immune cells in vivo.

First, an extensive microarray analysis revealed that PPARγ gene was up-regulated during human monocyte-to-DC development. Subsequently, the expression of PPARγ was confirmed by other investigators. In vitro and partly in vivo experimental data suggested that PPARγ acts as a suppressive TF in DCs that inhibits murine and human DCs immunophenotype at multiple levels.

For further characterization of the role of PPARγ on human DC functions, a suitable in vitro model system is required in which DCs express the receptor. Human DCs can be differentiated either for CD34+ hematopoietic stem cells, from CD14+ peripheral monocytes or from myeloid CD1c+ blood DCs. According to previous results of our laboratory, PPARγ expression was immediately induced in a narrow developmental period during the mo-DC differentiation. Freshly isolated monocytes failed to express the receptor, while PPARγ protein was detectable after 4 hours in cultured cells. The receptor was active in this model, because its agonists induced the expression of its bone fide target gene FABP4. To assess the global PPARγ-dependent gene expression profile during DC differentiation we utilized microarray experiments. These results revealed that PPARγ was not a simple inhibitory TF of the DC development because more than 1000 transcripts were regulated by the receptor and the half of these transcripts was up-regulated in the RSG-treated samples. PPARγ-activated genes in the first 6 hours of the differentiation program were involved primarily to the lipid metabolism and transport and were most likely directly/transcriptionally regulated by the receptor. Conversely, immune function-related genes were regulated in the later developmental period, suggesting that PPARγ might alter the DC immune phenotype indirectly through activation of intracellular lipid metabolism and signaling pathways.

1.4. Co-ordinated regulation of retinoid signaling by RARα and PPARγ in DCs

PPARγ and RARα regulate the expression of genes participating in lipid antigen presentation (CD1a and d) in mo-DCs. We confirmed that both receptors regulated the lipid antigen presentation through up-regulated CD1d expression in cultured DCs.

Based on literature data, endogenous serum derived lipids skewed mo-DC development to the generation of CD1a+ cells. Lysophosphatidic acid and cardiolipin were identified human serum lipids that could potently modulate the expression profile of Group 1 CD1 molecules but not that of CD1d. CD1 mRNA expression was regulated by PPARγ. DCs differentiated in serum-supplemented medium expressed CD1d in PPARγ-, and RAR-dependent manner. Administration of lipoproteins during DC development also affected the CD1 profile of the cells, suggesting that the uptake of lipids resulted in intracellular endogenous PPARγ agonists that induced transcriptional events, co-ordinating lipid metabolism, expression of CD1 molecules and DC immune functions.
PPARγ triggered indirectly CD1d expression by turning on endogenous lipid ligand synthesis in developing mo-DCs. Utilizing global gene expression analysis, we compared the expression pattern of genes in control samples and in PPARγ-ligand treated samples, which might be involved in retinol and retinal metabolism and endogenous ATRA production from retinol. Members of SDRs (RDH10 and DHRS9) were up-regulated by PPARγ during mo-DC differentiation. Furthermore, RSG-treatment induced RALDH2 expression. We determined the intracellular ATRA concentration in RSG-treated differentiated DCs by LC-MS analysis. The co-treatment of DCs with RSG and DEAB (RALDH inhibitor) confirmed, that PPARγ-triggered ATRA synthesis was mediated by RALDH activity. The accumulation of ATRA resulted in the induction of retinoid response by the RARα/RXR heterodimer. We also found that approximately the 30% of all PPARγ-responsive genes are regulated via the induction of retinoid signaling.

Both PPARγ and RARα activation leaded to increased iNKT expansion by αGC-loaded DCs. These DCs triggered selective induction of iNKT proliferation and INFγ secretion in autologous MLR cultures. We concluded that PPARγ-induced CD1d expression could be translated to efficient lipid presentation by DCs and to enhanced iNKT activation under these in vitro conditions. These results linked PPARγ and RARα to iNKT-mediated immune responses.
2. AIMS

During the process of DC differentiation many genes in these cells become up- or down-regulated. The regulation of gene expression requires a complex network of growth factors, signaling pathways and TFs. Environmental factors such as lipids induce signal transduction pathways that act on TFs, which have a fundamental role in controlling and co-ordinating of the expression of multiple genes that regulate physiological functions of DCs. We demonstrated that PPARγ signaling axis altered the lipid metabolism through the activated endogenous ATRA synthesis in mo-DCs, leading to subsequent cell type specification, characterized by enhanced lipid antigen presentation capacity of the cells. Despite of enormous scientific effort, the identity of permissive cell types, the required components of the biological active ATRA synthesis and the steps of lipid antigen processing for CD1d-mediated lipid presentation in DCs are still not characterized to date.

**Therefore the objectives of our studies:**

1. Identification of permissive murine DC subtypes by characterization of the expression of genes required for retinol uptake, ATRA production and signaling.
2. Comprehensive survey of human DC subtypes for ATRA biosynthesis and signaling.
3. Functional validation of our human mo-DCs as a suitable model to characterize the required steps for PPARγ-regulated ATRA synthesis, retinoid signaling and lipid antigen presentation.
4. Determination whether PPARγ also stimulates retinoid signaling through the cellular ATRA transport.
6. Provide functional evidence by gene specific silencing and lipid antigen presentation assay that beside RALDH2, PPARγ-activated RDH10 and CRABP2 are also mechanistically indispensable for the retinoid signaling axis-mediated gene expression.
7. Determination whether PPARγ stimulates the iNKT expansion capacity of DC through a novel signaling axis.
3. MATERIALS AND METHODS

3.1. Ligands

Cells were treated with the following ligands: RSG and GW9662 (Alexis Biochemicals, San Diego, CA, USA), ATRA (Sigma-Aldrich, St. Louis, MO, USA), AGN193109 a gift from Roshantha A. S. Chandraratna, (Allergan Inc. Irvine, CA, USA), AM580 (Biomol, Hamburg, Germany), DEAB from Fluka (Honeywell, Morris Plains, NJ, USA) pepstatin A and OVA 257-264 peptide (Innovagen, Lund, Sweden), bafilomycin (Sigma-Aldrich). αGC was obtained from Kirin Brewery Ltd. (Gunma, Japan), αGGC from P.A. Illarionov (School of Bioscience, University of Birmingham, Edgbaston, UK). The vehicle control (1:1 of dimethyl sulfoxide/Ethanol).

3.2. Generation of bone marrow-derived dendritic cells (BM-DCs)

BM cells were isolated from the femur of C57BL/6 mice. Animals were housed under specific pathogen free conditions and the experiments were carried out under institutional ethical guidelines and licenses (license number: 21/2011/DEMÁB). BM cells were differentiated to BM-DCs in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% Fetal bovine serum (FBS), 500 U/ml penicillin/streptomycin, 2 nM L-glutamine (Reagents obtained from Thermo Fisher scientific, Waltham, MA, USA), 20 ng/ml GM-CSF (Peprotech EC, London, UK) and 20 ng/ml IL-4 (Peprotech) or 20 ng/ml GM-CSF alone for 9 days. Cytokine treatment was repeated at day 3 and 6. After 9 day of culturing period, cells were harvested in Trizol reagent (Thermo Fisher scientific) for RNA isolation.

3.3. Splenic (Sp-DC) and Mesenteric lymph node-dendritic cell (MLN-DC) separation

CD11c+ MLN-DCs were obtained from B16-Flt3L tumor cell-injected C57BL/6 mice. Pooled spleens and MLNs of male C57BL/6 mice were cut into small fragments and digested with Collagenase D (Roche, Basel, Switzerland) for 40 minutes at 37°C. Solutions were passed through a nylon mesh and washed. Cell suspension was pre-incubated for 10 minutes at 4°C with anti-mouse CD16/CD32 Mouse BD FC Block antibody (BD Biosciences Pharmingen, San Diego, CA, USA). CD11c+ cells were obtained followed by anti-CD11c MACS bead (Miltenyi Biotec, Bergisch Gladbach, Germany) separation. CD103+ and CD103- DCs were separated by labeling the cells with anti-CD11c-APC and anti-CD103-PE (BD Biosciences Pharmingen) antibodies and subsequent sorting on FACSVantage (BD Biosciences, San Jose, CA, USA).

3.4. DC/Splenocyte co-culture experiment

Pooled MLN CD103+ DCs were obtained as described above (3.3). Splenocytes were purified from pooled spleens of BALB/c mice. Spleens were placed in Petri dish containing RPMI 1640 medium supplemented with 10% FBS, were squeezed out with glass plunger. After washing, lysing Buffer (BD Pharm Lyse, BD Biosciences) was applied against red blood cells. The DC/Splenocyte ratio was 1:20, corresponding to 1:10 DC: T cell ratio in 2 ml culturing medium/well. After 72 hour incubation at 37°C, MLN CD103+ DCs were separated as described above.
3.5. DC/CD8a⁺ T cell co-culture experiment

CD8a⁺ T cells were obtained from OTI-I mice (as a gift of Dr. Zoltan Pos). Single-cell suspension from spleens of OT-I mice were prepared using gentleMACS Dissocator (Miltenyi Biotech). After washing, CD8a⁺ cells were obtained followed by the CD8a⁺ T Cell Isolation Kit II (Miltenyi Biotech), by autoMACS Separator (Miltenyi Biotech). Pooled CD103⁺ MLN-DCs were isolated as described above (3.3). CD103⁺ DCs were loaded with 20 pM OVA 257-264. DC/T cell ratio was 1:2. After 72 hour incubation, CD103⁺ MLN-DCs were separated by anti-CD11c-APC and anti-CD103-PE antibodies and subsequent sorting on FACSVantage. Cells were harvested in Trizol.

3.6. Human mo-DC culture

Monocytes (98% CD14⁺) were isolated from Buffy coats of healthy volunteers, obtained with the Regional Ethical Board permit from the Regional Blood Bank, by Ficoll gradient centrifugation (Amersham Biosciences, Uppsala, Sweden), followed by separation using anti-CD14-conjugated microbeads (Miltenyi Biotech). Monocytes were differentiated to DCs at the density of 1.5 x 10⁶ cell/ml in RPMI 1640 medium supplemented with 10% FBS, 500 U/ml penicillin/streptomycin, 2 nM L-glutamine, 800 U/ml GM-CSF (Gentaur Ltd. London, UK) and 500 U/ml IL-4. Cells were cultured for 5 days. Ligands or vehicle control were added to the cell culture at day 0 and 3.

3.7. Flow cytometry

Cells were harvested and washed in 1X buffered (phosphate buffered saline) PBS and stained in 1X PBS/0.5% bovine serum albumin (BSA) (Sigma-Aldrich) for 40 minutes at 4°C. Cell staining was performed using PE- or FITC- conjugated antibodies: anti-CD14-FITC, anti-F4/80-FITC, anti-CD11c-PE, anti-CD1d-PE, anti-CD1a-FITC, anti-CD11c-FITC, anti-CD209-PE (BD Biosciences Pharmingen) and anti-Vα24-FITC, anti-Vβ11-PE (Immunotech, Marseille, France) and appropriate isotype-matched controls. Analysis of cell surface expression of proteins was performed using a FACSCalibur and analyzed by CellQuest software (BD Biosciences).

3.8. Microarray analysis

The generation of the microarray data used for Figure 18 and 20 (DC subtypes) was described by Szeles et al. We assessed the genes are expressed in mo-DCs and other DC types using Affymetrix microarray data of DCs. The generation of the microarray data of human mo-DC used for Figure 18 and 20 was described by Szatmari et al. Hybridization of the RNA samples was carried out at the Microarray Core Facility of the European Molecular Biology Laboratory (Heidelberg, Germany). Analysis was carried out using GeneSpring GX7.3.1 software (Agilent Technologies, Santa Clara, CA, USA). Raw data (cell files) were analyzed by the GeneChip robust multiarray analysis algorithm (GC-RMA) and raw signal intensities were normalized per chip (to 50th percentile). All microarray data are available in the public Gene Expression Omnibus database (GEO) under accession no. GSE23618 (DC subtypes and in ArrayExpress database, accession no E-TABM-34), accession no. GSE8658 (mo-DC differentiation).
3.9. Real time quantitative PCR (RT-qPCR)

1µg of total RNAs were reverse transcribed with SuperScript II reverse transcriptase and random primers (Thermo Fisher scientific). This was performed at 42°C for 2 hours, and 72°C for 5 min. Quantitative PCR was performed on LC480 platform (Roche), 40 cycles of 95°C for 10 sec and 60°C for 30 sec for Taqman assays (Applied Biosystems, Thermo Fisher scientific) or 95°C for 10 min, 40 cycles of 95°C for 10 sec and 60°C for 30 sec using Sybr green. Gene expression was quantified by the comparative threshold cycle method and normalized to human or mouse housekeeping gene Cyclophilin A (PPIA and Ppia). All PCR reactions were performed in triplicates. Values are expressed as means ± SD. In addition, TaqMan low-density arrays (TLDAs) (Applied Biosystems, Thermo Fisher scientific) were used according to manufacturer’s instructions. For TLDA analyses a high capacity cDNA archive Kit (Life Technologies, Thermo Fisher scientific) was used. RT-qPCR was performed using real-time PCR (ABI Prism 7900, Applied Biosystems, Thermo Fisher scientific).

3.10. RNA interference

Small interfering RNA (siRNA) delivery was performed using electroporation of monocytes as described earlier. Monocytes were counted and resuspended in Opti-Mem (Invitrogen, Thermo Fisher scientific) without phenol/red at the density of 4 x 10^7 cell/ml. For silencing of gene expression, the following siRNA oligonucleotides were used: On-Targetplus SMART pool siRNA against human RDH10, RALDH2, CRABP2, FABP4 or On-Targetplus non-targeting control siRNA pool (NS) (Dharmacon, Lafayette, CO, USA). Non-silencing siRNA and siFABP4 were used, that did not altered the normalized mRNA level of the examined genes. Oligonucleotides were transferred to a 4-mm cuvette (Bio-Rad Laboratories, Hercules, CA, USA) at 3 µM final concentration. 100 µl cell suspension was added, gently mixed and incubated for 3 minutes at room temperature. Electroporation was performed using a Gene Pulser Xcell (Bio-Rad Laboratories). Pulsing conditions were square-wave pulse, 500 V, 0.5 ms. After electroporation, cells were transferred into RPMI 1640 medium supplemented with 10 % FBS, 500 U/ml penicillin/streptomycin, 2 nM L-glutamine, 800 U/ml GM-CSF and 500 U/ml IL-4. Silencing efficiency was assessed on day 1 and day 2 post electroporation. The average siRDH10 efficiency was 48.58± 8.44%, in the case of siRALDH2 the efficiency was 39.22± 10.81% and the average siCRABP2 efficiency was 44.22± 9.25%.

dDCs were harvested at day 3, washed once with unsupplemented RPMI 1640 and PBS. Cells were resuspended in Opti-Mem without phenol/red at the density of 4 x 10^7 cell/ml. The expression of PPARG was silenced with Qiagen siRNA against PPARG, On-Targetplus SMART pool siRNA against human CATD or On-Targetplus non-targeting control siRNA pool (NS) (Dharmacon). Electroporation was performed using a Gene Pulser Xcell and same pulsing condition that was used for monocyte samples. PPARG and CATD were silenced at an efficiency approximately 60%.
3.11. Aldefluor assay

RALDH activity of mo-DC was determined by ALDEFLUOR Kit (StemCell Technologies Germany, Cologne, Germany). Activity measurement was carried out according to manufacturer’s instructions. Briefly, cells were incubated at the density of 1 x 10^6 cell/ml in assay buffer containing activated substrate with or without DEAB for 40 minutes at 37°C. ALDEFLUOR positive cells were determined in FL1-channel of FACSCalibur compared to DEAB-treated control samples.

3.12. Expansion of iNKT cells

mo-DCs were differentiated for 5 days. Cells were treated with 100 ng/ml αGC or αGGC for 48 hours to obtain αGC- or αGGC-pulsed DCs. Lipid-loaded DCs (1 x 10^5) were co-cultured with monocyte-depleted autologous PBMCs (1 x 10^6) for 5 days (1:10 DC/PBMC cell ratio). In CatD inhibition experiments, DCs were treated with 1 or 10 µM peptstatin A (Sigma-Aldrich) at day 3. Prior to co-culture, DCs were washed extensively and resuspended in fresh RPMI 1640 medium supplemented with 10 % FBS, 500 U/ml penicillin/streptomycin, 2 mM L-glutamine, 800 U/ml GM-CSF and 500 U/ml IL-4. PBMCs were stained with anti-TCR Vα24-FITC and anti-TCR Vβ11-PE monoclonal antibodies and double-positive iNKT population was monitored by FACSCalibur. Additionally, the invariant Vα24-Jα18 (iNKT) TCRα chain was quantified by RT-qPCR. In lysosomal acidification inhibition experiments, DCs were differentiated in the presence of RSG. Cells were treated with 50 nM bafilomycin at day 4.

3.13. Western blot analysis

20 µg protein from whole cell lysate was separated by 12.5% polyacrylamide gel and transferred to PVDF membrane (Millipore, Merck, Darmstadt, Germany). Membranes were probed with anti-CRABP2 (208) antibody, kindly provided by Cecile R.-Egly (IGBMC, INSERM, Illkirch-Graffenstaden, France), and then the membranes were stripped and re-probed with anti-GAPDH antibody (ab8245-100; Abcam, Cambridge, MA, USA) according to the manufacturer’s recommendations. In addition, 50 µg protein whole cell lysate was separated by 12.5% PAGE before being transferred onto PVDF membrane (Bio-Rad Laboratories). Membranes were blocked using 5% nonfat dry milk in tris-buffered saline (TBS)+ Tween 20 (TBST) at 4°C overnight before being probed with anti-CatD antibody (R20, sc-6487; Santa Cruz Biotechnology, Paso Robles, CA, USA), and then membranes were stripped and reprobed with anti-GAPDH.


For IHC, monocytes, vehicle-, or RSG-treated DCs (6 x 10^6 cells/group) were pelleted and fixed in 4% paraformaldehyde for 24 hours at 4°C. Cell blocks were embedded into paraffin. Sections (4 µm from each group) were mounted on glass slides. Sections were treated with 3% H2O2 in methanol for 15 minutes at room temperature to block the endogenous peroxidase. For antigen unmasking, sections were heated in antigen retrieving citrate buffer (pH 6.0, Dako, Thermo Fisher Technologies ) for 2 minutes at
120°C using a pressure cooker. Immunostaining of the cells for CRABP2 were carried out using the standard ABC technique utilizing the primary antibody-specific biotinylated secondary antibodies (Vectastain kits, Vector Laboratories, Burlingame, CA, USA). After blocking the non-specific binding sites, sections were incubated with the primary anti-CRABP2 (208) antibody at dilutions of 1 x 1/50 for 1 hour at room temperature prior to use the biotinylated secondary antibodies. The peroxidase-mediated color development was set up for 5 minutes using the VIP substrate (Vector Laboratories). Finally, the sections were counterstained with methylgreen.

3.15. Double immunofluorescence (DI)

DI was performed on formalin-fixed, paraffin embedded intestinal tissue sections obtained from the archives of surgical specimens of the Department of Pathology, University of Debrecen as described earlier. Briefly, following antigen-retrieving and peroxidase block, the first primary antibody was visualized with antibody-matched peroxidase-conjugated IgG followed by tetramethyl-rhodamine (TMR) tagged tyramide (PerkinElmer, Waltham, MA, USA) treatments (red fluorescence). After washing and blocking the non-specific binding sites, sections were incubated with the second primary antibody which was then developed with the use of matched biotinylated secondary antibody (IgG[Fab]2) and streptavidin-FITC (Vector Laboratories) (green fluorescence). After thorough washings, nuclear counterstaining was made with 4’,6-diamidino-2-phenylindole (DAPI) containing the mounting medium (Vector Laboratories). To check the staining specificities, positive and negative controls were included for each IF reaction as described earlier and as indicated in the result section.

In Figure 35, monocytes, DC, or RSG-treated DCs (6 x 10^6 cells/group) were pelleted and fixed in 4% paraformaldehyde for 24 hours at 4°C. Cell blocks were then embedded in paraffin followed by serial sectioning (4 μm thick). Sections from each group were mounted on the same glass slides and subjected to sequential DI staining for detection of PPARγ and CatD protein expressions, respectively. The following primary antibodies were used: anti-PPARγ (clone E8; Santa Cruz Biotechnology) at 1:75 dilution and polyclonal goat anti-CatD (clone C20; Santa Cruz Biotechnology) at 1:100 dilution. In brief, PPARγ was detected by incubating sections 1 hour at room temperature with primary antibody followed by HRP-labeled anti-mouse secondary (IgG[Fab]2) and FITC-conjugated tyramide (PerkinElmer Life Science) treatment. Following extensive washing and blocking, CatD protein expression was detected by 1 h incubation with primary antibody followed by biotinylated rabbit anti-goat (IgG[Fab]2) and streptavidin-Texas Red (Vector Laboratories). DAPI was used for nuclear counterstaining (Vector Laboratories). For negative controls, isotype-specific control IgG Abs (Dako, Thermo Fisher Technologies) or a mixture of monoclonal antibody to PPARγ and a specific blocking peptide were applied on separate slides in replacement of primary antibodies. Normal human adipose tissue was included as positive control. Fluorescence microphotographs were captured using an Olympus BX51 microscope (Tokyo, Japan) equipped with a tricolor excitation filter and an Olympus DP50 digital camera. For transferring and editing images for documentation, Viewfinder and Studio.
Lite software version 1.0.136 of 2001 Pixera (Pixera UK Digital Imaging Systems, Bourne End, UK) and Adobe Photoshop version 8.0 were used.

3.16. Mixed leucocyte reaction (MLR)

mo-DCs were harvested on day 5 and used as stimulator cells. To obtain mDC, iDCs were treated with a mix of cytokines: 10 ng/ml TNFα, 10 ng/ml IL-1β, 1000 U/ml IL-6 (Peprotec), 1 µg/ml PGE2 (Sigma-Aldrich), and 800 U GM-CSF for 24 hour. Allogeneic PBMCs were labeled in PBS supplemented with 10 µM Carboxyfluorescein succinimidyl ester (CFSE) (Thermo Fisher Technologies) at 37°C for 15 minutes. CFSE-labeled PBMCs (2 × 10^5 cell/ml) and immature or mature DCs (1 × 10^4 cell/ml) were co-cultured in 96-well flat-bottom tissue culture plates (1:20 DC/PBMC cell ratio). Cell proliferation was quantitated on day 5 by FACSCalibur.

3.17. Statistical analyses

Samples for each experiment were performed in triplicate (n=3). Biological repeats for each experiment were performed at least three times. Statistical significance was determined using the GraphPad Prism (GraphPad Software, La Jolla, CA, USA) program. Probability of significance was determined using the two-sample Student t test. The results were considered significant at the level of $p < 0.05$. Standard error bars are shown.
4. RESULTS

4.1. PPARγ-DIRECTED ATRA SYNTHESIS AND SIGNALING IN DENDRITIC CELLS

4.1.1. ATRA biosynthesis in mouse intestinal DCs

We hypothesized that RDH10 might be the primary enzyme that initiates retinol oxidation to retinal and the co-expression of RDH10 and RALDH2 determines ATRA production in mucosal DCs. We test this hypothesis in different in vivo- and in vitro generated DC subtypes. We quantified the expression levels of genes involved in ATRA synthesis by RT-qPCR in MLN-DCs. Raldh2 could be detected only in CD103+ DCs. Rdh10 was expressed in both populations. RAR/RXR target gene Cyp26a1 had a similar transcription pattern to the Raldh2 gene, suggesting a negative feedback mechanism to control active retinoid signaling in cells. CD103+ and CD103- MLN-DC populations expressed Tgm2 and Cd1d1, two well-established ATRA target genes. Unexpectedly, the normalized mRNA levels of the genes did not correlate with the ATRA production capacity of the cells.

We characterized ex vivo differentiated DCs in additional gene expression analyses. We differentiated GM-CSF-DCs or GM-CSF+IL-4-DCs from BM and we used isolated Sp-DCs as negative control (no capacity for ATRA generation). GM-CSF triggered Raldh2 expression, the synergistic effect of the two cytokines was confirmed, while Raldh2 expression in Sp-DCs was barely detectable as it was earlier demonstrated. Next we focused on Rdh10 in in vivo- and ex vivo generated cells and found that all DCs expressed this gene.

All DC subsets expressed Cd1d1, but with no correlation with retinoid signaling, while Cyp26a1 and Tgm2 could be reliable markers of active retinoid signaling.

Next we assessed the expression of genes involved in retinol uptake and transport. We could not quantificated Stra6 in DC subsets, we detected the expression of Rbpr2 gene in all DC subsets, indicating the possibility of retinol uptake through this transporter. We assessed the role of cellular interactions using allogenic splenocytes/CD8+ T cells on Crabp2 and Rbpr2 gene expression co-culturing with ex vivo DCs. In the co-culture experiments, the expression of both Crabp2 and Rbpr2 was induced in CD103+ DCs, as a result of cellular, most likely T cell interactions. We confirmed the role of cellular interactions on Crabp2 expression in CD103+ MLN-DC/CD8a+ T cell co-culture experiment, suggesting a triggered ATRA delivery as a result of cellular interactions.

We concluded that ATRA biosynthesis is not a universal feature of DCs, and that, in line with our hypothesis, Rdh10 expression overlaps with Raldh2 expression, suggesting that DCs expressing both enzymes are likely to have active ATRA synthesis and signaling.

4.1.2. Characterization of retinoid signaling in human DCs

CD103+ DCs are also present in the human small intestinal MLNs with similar functional properties compared to murine CD103+ MLN-DCs. Despite previous data, the human DC phenotypes are not identical and not easy to match up with the ATRA generating DCs in mice. Therefore we considered using human mo-DCs for our mechanistic characterization of the components of retinoid signaling by
functional assays. We proved that these ex vivo cells faithfully replicate the behavior of human in vivo DCs by microarray data set analysis that compared the gene expression pattern of mo-DCs and iv vivo DC subsets. mo-DCs were CD14+/CD11c+/CD209+, thus phenotypically resembled in vivo iDCs.

Next we examined the gene expression profile of a group of select genes involved specifically in ATRA biosynthesis and signaling. RDH10, RDH11, and DHR59 were expressed in mo-DCs. Both RALDH1 and RALDH2 were expressed at high levels in mo-DCs, while a moderate level of the transcription of these genes was observed only in dermal DCs. Among the genes encoding ATRA-transporting proteins, CRABP2 was expressed ubiquitously. We also examined the retinoid signaling by analyzing the expression pattern of target genes: tonsillar CD1c+, blood CD1c+ and mo-DCs expressed CD1D and TGM2. PPARG is expressed in mo-DCs. The detectable level of FABP4 is likely to indicate either the presence of extracellular PPARγ ligand in the serum or the presence of possible endogenous activators inside the cells. Other in vivo DC types failed to express PPARG or FABP4. This systematic analysis suggested that retinoid signaling is only active in mo-DCs that co-express RDH10 and RALDH2, and in these cells PPARγ signaling is connected to the retinoid signaling pathway.

To validate our microarray data, we quantified the transcriptional changes of the genes contributed to ATRA synthesis (RALDHs) during the full differentiation period by RT-qPCR. As expected, ligand treatment induced the expression of RDH10 after 6 h, indicating that PPARγ activates this gene probably via direct molecular interaction. The expression of RDH10 in PPARγ-ligand instructed samples continuously increased during the differentiation period. Both CRABP2 and TGM2 genes were up-regulated in RSG-treated DCs after 24 h in accordance with earlier results and increased CD1D transcription was observed at later time points in RSG-treated samples. PPARG was immediately induced in differentiating cells, the highest expression level was detected at 6 h, and the gene was detectable at a somewhat lower level in DCs. We detected a similar expression pattern of FABP4 compared to RDH10.

In summary, ATRA production and signaling is not a universal feature of human DCs and is tightly regulated. We found evidence that mo-DCs expressed all components required for retinol to ATRA conversion and transport. This ATRA producing ability can be induced by the co-ordinate up-regulation of RDH10, RALDH2, and CRABP2.

4.1.3. Transport of ATRA via CRABP2 to the nucleus is PPARγ-regulated

We investigated whether intracellular ATRA delivery could also be regulated by PPARγ. PPARγ activation induced the transcript levels of CRABP2. We found that monocytes did not express CRABP2, while control-treated mo-DCs expressed a detectable level of the protein, which was highly induced by RSG. We further confirmed the elevated CRABP2 expression at the expression site of the delivery protein by IHC. We postulated that the elevated CRABP2 expression in PPARγ-instructed DCs might contribute to the enhanced ATRA response. Further investigations are required for providing direct evidence for CRABP2-mediated ATRA delivery to the nucleus in mo-DCs.
4.1.4. PPARγ, RDH10, RALDH2, CRABP2, and the ATRA-regulated TGM2 co-localize in DCs of the human GALT

In order to obtain evidence for the physiological relevance to our findings, we systematically surveyed the expression of the components of ATRA biosynthesis and signaling in resting human GALT using DI. We chose GALT, as this is the most likely place where lipid signaling could contribute to DC differentiation and subtype specification in the gut. DI of resting GALT for PPARγ demonstrated that PPARγ was in part co-expressed with Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) in mucosal lymphoid tissue cells with DC phenotype. Interestingly, nuclear PPARγ and the cytoplasmic TGM2 proteins showed co-expression in similar cells of GALT exhibiting cytoplasmic green projection characteristic of DC elements, comparable with the staining pattern as seen for PPARγ-DC-SIGN. Therefore, these cells co-expressing PPARγ/TGM2 should represent the DC population of GALT, similarly to PPARγ/DC-SIGN positive cells. These results indicate that in resting lymphoid tissues some of the PPARγ-positive DCs express TGM2 simultaneously, suggesting that PPARγ might regulate ATRA-dependent transcription in vivo as well.

On the other hand, in the GALT we showed that some PPARγ positive cells co-expressed RDH10. Similarly, we observed few PPARγ-expressing DCs with RALDH2 and CRABP2 co-expression, respectively. RDH10, RALDH2, and CRABP2 also co-localized with DC-SIGN in some mucosal DCs. The number of PPARγ+ DCs was increased in cases of inflammatory bowel diseases (IBDs).

These data collectively strongly suggest that the key components of ATRA synthesis and the PPARγ are expressed together in some of the antigen-presenting cells (APCs) of the mucosal lymphoid tissues, consistent with a previous report which demonstrated that murine intestinal DCs expressed RALDH2.

4.1.5. Increased RALDH activity in PPARγ-activated mo-DCs

We wanted to provide functional evidence that retinoic acid biosynthesis take place in mo-DCs. Utilizing LC-MS method we previously demonstrated that mo-DCs could produce ATRA in a PPARγ-dependent manner. We aimed to further investigate this result using ALDEFLUOR staining assay that is suitable to detect intracellular enzymatic activity of RALDHs. mo-DCs were differentiated and at 120 h RALDH activity was measured by flow cytometry. There were 8% RALDH active cells in control-treated sample. In the presence of RSG, the number of RALDH active cells was increased to approximately 40%. We noted that a much higher enzyme activity was displayed in these treated DCs than even in the positive ones in control DCs. In the RSG and GW9662 co-treated sample, the RALDH activity was similar to vehicle-treated control DCs.

Next, we examined RALDH activity in mo-DCs electroporated at monocyte stage using specific siRNAs against RDH10, RALDH2, CRABP2 and NS control siRNA. At day five ALDEFLUOR staining was quantified. These results suggest that RALDHs are active in mo-DCs, and the enhanced ATRA production capacity of mo-DCs is PPARγ dependent.
4.1.6. PPARγ activation induces RAR signaling/gene expression via RDH10, RALDH2 and CRABP2

Based on these data, we hypothesized that RDH10, RALDH2, and CRABP2 might be required for PPARγ-regulated ATRA production and gene expression. Pharmacological analysis revealed the importance of RALDH2 in PPARγ-enhanced retinoid signaling. We have extended our studies by testing to determine whether the oxidizing enzymes and CRABP2 are indeed mechanistically indispensable for retinoid-regulated gene expression induced by PPARγ. To test this hypothesis, we used siRNA-based approach. Monocytes were electroporated with siRNA against RDH10, RALDH2, or CRABP2 and FABP4 (as a control). After 24 or 48 h of RSG treatment, we quantified the transcript level of CD1D and TGM2 by RT-qPCR. PPARγ-induced CD1D expression was down-modulated by all except FABP4–specific siRNA at both time points (24 and 48 h). TGM2 expression changes were similar at 24 h, but only RDH10-specific siRNA reduced it significantly at 48 h as compared with control electroporated (NS) DCs.

In the next set of experiments, we electroporated monocytes with RDH10-specific siRNA then we measured CD1d cell surface protein expression by flow cytometry. Transient transfection of siRDH10 reduced RSG-up-regulated CD1d levels on DCs and it was still down-regulated at day 5 post-electroporation.

These results strongly suggested that PPARγ-mediated signaling induced retinol conversion by RDH10 in mo-DCs. The produced retinal was oxidized to ATRA by RALDH2. The enhanced retinoid signaling was more effective in the presence of the CRABP2 ATRA transporter. In the nucleus, ATRA activates regulated target genes via RAR/RXR heterodimers due to integrated PPARγ-RAR signaling.

4.1.7. PPARγ-induced iNKT expansion is attenuated by RDH10, RALDH2, or CRABP2 knock down

To investigate whether RDH10 can influence the PPARγ-mediated iNKT expansion capacity of the APCs, we silenced the RDH10 gene in monocytes with siRDH10. Cells were differentiated to mo-DCs in the presence of DMSO/ethanol for the control-treated sample or RSG for PPARγ activation; pulsed with or without αGC for 48 h and then co-cultured with autologous PBMCs. The iNKT proliferation capacity was monitored by Vα24/Vβ11 double staining. As expected, enhanced iNKT expansion was detected in RSG-treated and NS siRNA-transfected samples, while reduced iNKT cell numbers were detected in RDH10 siRNA-treated cells.

mRNA expression of the invariant Vα24-Jα18 (iNKT) TCRα marker gene correlated with the cell surface expression of TCR Vα24 and TCR Vβ11 on iNKT cells. siRNA against RDH10, RALDH2, and CRABP2 reduced the normalized TCR Vα24 mRNA levels in RSG-treated samples as compared with non-silencing control (NS)-treated cells.
In the second part of the Result section, we present our recent published data about PPARγ-regulated CatD expression in human mo-DCS. This project was co-ordinated by Dr. Britt Nakken and Dr. Tamás Varga.

4.2. PPARγ-regulated Cathepsin D (CatD) is required for lipid antigen presentation by DCs

4.2.1. PPARγ-regulated CatD expression in human mo-DCs

Our goal was to uncover how PPARγ modulates lipid antigen presentation events in mo-DCs in addition to regulating the expression of CD1d molecules. If PPARγ enhances lipid presentation by regulating a yet unidentified mechanism, then this must be reflected in the gene expression changes upon PPARγ ligand treatment. Therefore, we analyzed our previous microarray data set of differentiating human mo-DCs. We compared the gene expression profiles of differentiating DCs at 6 h, 24 h, and 120 h in control- or RSG-treated samples. This analysis revealed PPARγ-mediated regulation of genes, participated in lipid antigen presentation.

CatD, L, and S lysosomal proteinases, were up-regulated in RSG-treated samples. Based on this microarray result CATD was up-regulated by PPARγ in mo-DCs. RT-qPCR indicated a robust up-regulation of the CATD in RSG-treated samples.

Western blot analysis confirmed PPARγ-mediated up-regulation of CatD at protein level. Furthermore, DI result demonstrated the nuclear localization PPARγ and cytoplasmic localization of CatD.

To further confirm the PPARγ dependence of CATD expression obtained by pharmacological means, we knocked-down PPARγ in differentiating mo-DCs by electroporation. The PPARγ-dependent up-regulation of CATD was robustly reduced by siPPARγ compared to NS control siRNA transfected samples. Based on these results, we concluded that CatD is regulated by PPARγ.

To identify the exact molecular components of the signaling events from PPARγ activation to CATD up-regulation, we examined the involvement of retinoid signaling in the regulation of CATD. We mapped the signaling pathways required for CATD induction by analyzing the expression levels of FABP4 and TGM2 that are under control of PPARγ or RARα, respectively. We found that both receptor specific ligands could induce CATD.

Our data suggest that CATD is under the control of multiple nuclear receptors in developing DCs and that it is under dual control by both PPARγ and RAR.

We hypothesized also, that the induction of CD1d expression might be not the only mechanism, by which PPARγ signaling can enhance iNKT expansion. The result that silencing of PPARγ in day 3 DCs by siRNA did not alter the CD1d transcript level in DCs, whereas regulation of iNKT activation could still be found under these conditions, suggested that PPARγ signaling pathway in mo-DCs enhanced iNKT expansion by regulating other molecules as well, including CatD. This finding is in line with our previous results that demonstrated that CD1d is an indirect and late target of PPARγ.
4.2.2. Inhibition of CatD leads to decreased iNKT proliferation in response to lipid antigen through reduced lysosomal events important for lipid antigen presentation in the context of CD1d

To investigate the possible function of CatD in lipid antigen presentation and iNKT cell stimulation, we used pepstatin A, a inhibitor of CatD. Upon αGGC administration, RGS-treated mo-DCs acquired a substantial increase in their ability to induce iNKT expansion.

Pepstatin A reverted αGGC-induced iNKT cell expansion in a dose-dependent manner in RSG treated mo-DCs.

Endosomal processes involved in lipid antigen presentation to iNKT cells in DCs remain mostly undefined. We found that inhibiting endosomal acidification by bafilomycin in RSG-treated mo-DCs absolutely reduced iNKT expansion in the presence of the precursor lipid αGGC but not in the presence of αGC. This result indicated that PPARγ could induce an endosomal process included to the effective lipid presentation and enhanced iNKT expansion. Furthermore, we detected that only the αGGC-induced iNKT expansion was sensitive to pepstatin A, suggesting the function of CatD in the lysosomal lipid antigen processing.

5. DISCUSSION

5.1. ATRA synthesis in murine DCs

Mucosal DCs orchestrates intestinal homeostasis. Several of these DC functions are tightly regulated by ATRA. After migration to the intestine, specific DC subsets acquired the ability to sense and respond to ATRA which promoted an anti-inflammatory phenotype.

Our goal was to identify and validate additional key regulatory components of ATRA synthesis in mouse DCs. The enzyme required for the retinal production from retinol has not been evaluated in ATRA-producing DCs. We focused on RDH10 which might be the key enzyme that could initiate retinol metabolism in intestinal DCs. While transcription of Rdh10 gene was detectable in all DC subtypes, irrespective of ATRA-production capacity, overlapping expression of Rdh10 with Raldh2 suggested that RDH10 could be responsible for the initial retinol oxidation to retinal in ATRA-generating DCs. The role of RDH10 enzymatic activity in these DC subtypes is remained to be investigated.

ATRA synthesis itself can be regulated by nuclear receptor-mediated signals. In contrast to human DCs, PPARγ agonist did not significantly induce Raldh2 expression in Flt3L-generated BM-DCs and purified Sp-DCs in the absence of IL-4 or GM-CSF. This apparent disparity between mouse and human data may reflect a species difference but remains to be clarified. These results suggest that caution should be exercised when extrapolating mouse data on the manipulation of ATRA signaling to generate efficient mucosal immune responses in the human intestine.

5.2 ATRA production in human DCs

ATRA synthesis in the human intestinal DC subtypes is less investigated. In contrast to murine gut DCs, ATRA synthesis is not restricted to CD103+ DC subsets in human DCs. Both CD103+ /SIRPa+
and CD103⁺/SIRPα⁺ DCs had high levels of RALDH activity, whereas CD103⁺/SIRPα⁻ DCs had significantly lower RALDH activity. ATRA synthesis was also detectable in DCs isolated from the distal part of the human small intestine or the colonic tissues. RDH10, DHRS9 and RALDH2 was detected in tissue-derived DCs, indicating that human intestinal myeloid DCs acquired the complete enzymatic machinery to generate ATRA from retinol. Mindegyik DC CCR= All DCs expressed CCR7, suggesting that these populations might migrate to organized lymphoid tissue in the gut to activate naïve T cells.

Our laboratory previously identified that PPARγ-regulated lipid metabolic pathways could be associated with the altered immune response of DCs. The activation of the nuclear receptor leaded to a transcriptional program with altered lipid metabolism in mo-DCs via induced expression of genes that are required for endogenous ATRA synthesis. RSG-treated DCs express higher level of RDH10 and RALDH1/2 mRNA level, supporting that key enzymes readily could be regulated in these cells in PPARγ-dependent manner. Thereafter de novo produced ATRA is transported to the nucleus by PPARγ-up-regulated CRABP2 carrier molecules to activate RARα receptors.

Our results suggested that transcriptional events in human mo-DCs that up-regulate the CD1D gene that were co-ordinately mediated by PPARγ and RARα receptors. The consequences of the PPARγ-regulated ATRA signaling was the expression of the lipid antigen presenting CD1d molecules on the surface of the mo-DCs and enhanced lipid antigen presentation capacity of the cells. To assess direct evidence that the primary enzyme in this de novo ATRA synthesis was readily RDH10 and the PPARγ-induced ATRA signaling pathway required RALDH2 and CRAPB2, we used siRNA-based gene silencing technic. These results supported our previous data and also provided an even more detailed analysis of the components of this PPARγ-initiated de novo ATRA synthesis, transport and lipid antigen presentation by evidences at molecular level.

These data indicated that ATRA production is regulated differently in mice and human and the expression of RDH10 might providing an important control point to ATRA synthesis in humans DCs.

5.3. PPARγ-dependent nuclear ATRA transport in DCs

CRABP2 expression was barely or not detectable in the murine DC subtypes by TLDA analysis. Utilizing DC/T cell co-culture experiments, we found that the T cell/DC interactions could be an intrinsic factor in the mucosal environment for CRABP2 expression in the APCs.

We found in our human mo-DC model that PPARγ activation resulted in CRABP2 expression in cultured DCs. These results indicated that PPARγ receptor activation is also involved in the nuclear transport of the active metabolite of retinol. The direct evidence for CRABP2-mediated ATRA transport to the nucleus requires further experimentation.

5.4. PPARγ and retinoid signaling in intestinal DCs

ATRA synthetizing DCs was previously identified in the human gut. DCs endow RALDH activity after entry into the intestinal mucosa. As intestine is a privileged area for intensive lipid absorption and
for PPARγ ligand generation we decided to assess whether PPARγ contributes in the regulation of DC retinoid signaling in the intestinal GALT. The functional relevance of PPARγ was assessed in DSS-induced colitis. In mouse colitis models ATRA production was decreased in CD103+ DCs. Contrary, DCs had enhanced capacity to synthetized ATRA in human colonic DCs, isolated from inflamed colonic tissues of CD patients. This elevated generation of ATRA might contribute to disease pathology. Both CD103+ and CD103- DC subsets expressed enhanced level of RALDH2. RDH10 expression was also measured in the sorted DC populations, indicating that human intestinal DCs have capacity for intracellular ATRA production.

Utilizing DI method we characterized the expression a PPARγ and the components of ATRA signaling in human intestinal tissues. We found that GALT-associated immune cells readily express the key components of ATRA production (RDH10, RALDH2) and transport (CRABP2). PPARγ-positive DC-like cells co-expressed the RAR target gene, TGM2, suggesting that these cells possess the complete enzymatic machinery to generate ATRA from retinol, have an active retinoid signaling system and represent a relevant ATRA-producing APCs. The proportion of PPARγ positive DC-like APCs was increased in IBD samples. Our results suggested that the RALDH activity of human intestinal myeloid DCs was indicative of the generation of ATRA that signals via RARα to modulate T cell function in the gut. These data also suggested that PPARγ and ATRA signaling might be connected in intestinal DCs and our ex vivo mo-DCs may correspond to these in vivo DC-like APCs.

5.5 CatD and lipid antigen presentation in human mo-DCs

It is widely accepted that the primary biological function of CatD is the lysosomal protein degradation. CatB and L, have been implicated in lipid antigen presentation.

Multiple aspects of lipid presentation were linked to these lysosomal proteases such as the cell surface expression of CD1d and processing of lipid antigens. However, the function of nuclear hormone receptors in the regulation of the lipid presentation process was poorly investigated in DCs and might be transcriptionally controlled. We tested what other steps of lipid presentation can be under the control of PPARγ in mo-DCs than CD1d. The expression of CatD in differentiating DCs was regulated by the receptor and its expression was closely matched with that of genes already functionally connected to lipid presentation. We also investigated that CatD was up-regulated by both PPARγ and RARα, regulate the protease dually and likely indirectly. The inhibition of the CatD activity resulted in the blocking of PPARγ-dependent iNKT activation, lower capacity to present αGHC. These experiments provided us with mechanistic links between PPARγ, CatD and lipid antigen presentation.

5.6 Lipid antigen presentation, PPARγ in cancer

PPARγ is one of the most controversial TF in term of tumor progression in DCs. In DC-based vaccination therapy the relevant question is how PPARγ affects DC-controlled immune responses. Transcriptional activation of this receptor is crucial during DC development, resulting in a specific DC
characteristic, suggesting that PPARγ active DCs have a regulatory DC phenotype that would be detrimental during the development or the optimization of in vivo DC-based vaccination therapies.

DCs were demonstrated to elicit effective anti-tumor immune responses by presentation of lipid antigens to iNKTs that have primarily protective function in various experimental tumor models in mice. The success of preclinical results supported the idea to design clinical trials, which either harness the function of resting iNKTs or increase the frequency of the cells by adoptive transfer of ex vivo expanded autologous iNKTs as a vaccine. To solve the unpredicted limited success of these trials and to improve the efficacy of the iNKT vaccine therapy, our ex vivo DC-model offer many advances for optimization. Although most of the key qualities of DCs, which are critical during DC-vaccination design, are negatively affected by PPARγ. Upon RSG treatment DCs express all molecules which required for potent lipid antigen presentation, therefore these model DCs let us to understand the molecular mechanisms essential for clinical harnessing of this iNKT population. Optimal manipulation of these DCs in anti-tumor trials is critically dependent on our knowledge of iNKT– and DC biology and of the factors that activate and regulate these cells. PPARγ+ DCs promote iNKT cell functions through enhanced CD1d thus the receptor could be a potential target for CD1d-restricted iNKT-based cancer therapy.

6. SUMMARY

DC network represent a complex APC family of a number of specific subpopulations. From the first stage of their differentiation, all developmental and differentiation status of DCs are TF-regulated. DCs have to adapt to various environmental cues during homing peripheral tissues or in the course of their shuttle to LNs. As professional APCs, the main function of DCs is the continuous grading of all potent molecules into the state of harmful or self-antigens to sustain effective immune protection (2). In this regard, DCs acquire the capacity to process a huge amount of surrounding information, which triggers specific signaling pathways in the cells. The functional flexibility of DCs is frequently accompanied by TF-mediated transcription. DCs express nuclear hormone receptors that translate intra/extracellular signals to the level of gene expression, required for appropriate immune phenotype of the cells (169).The precise transcription network, which regulates DC immune specificities has to be characterized. Therefore we analyzed the functions of PPARγ and RARα in DCs. PPARγ activation turns on the endogenous ATRA production in DC by up-regulated retinol/retinal oxidizing enzyme genes, namely RDH10 and RALDH2. ATRA is transported to nucleus by the PPARγ-induced CRABP2 transporters, activates RARα, leading to co-ordinated transcriptional regulation of genes, required for lipid antigen presentation, such as CD1d antigen presenting molecules. Lipid antigen-loaded DCs activate CD1d-restricted iNKT cells.
We provided evidence that other step of the lipid presentation could be under the control of the two receptors. We identified CatD as a novel target of the PPARγ and linked this lysosomal protease at molecular- and functional level to DC-based lipid presentation to harness iNKT functions.

Based on preclinical results, DC-activated iNKTs triggered regression/stabilization of advanced tumors. A set of anti-cancer strategies focused on inducing extended iNKT number and activity in patients. The main challenge of these trials was triggering clinically relevant responses in patients without side-effects. These therapies were generally well tolerated and in some patients, forced prolonged survival. For optimization of these iNKT-based trials we have to understand all step of lipid antigen presentation in DCs and its functional consequences on iNKT immunity. Our ex vivo DC/iNKT model allows us to monitor these regulator steps at molecular levels. Collectively our results points out the potential benefit to consider PPARγ as a potential target for CD1d-restricted iNKT-based cancer therapy.
List of publications related to the dissertation

   *J. Lipid Res.* 54 (9), 2458-2474, 2013.
   DOI: http://dx.doi.org/10.1194/jlr.M038994
   IF: 4.73

   DOI: http://dx.doi.org/10.4049/jimmunol.1002421
   IF: 5.786

   DOI: http://dx.doi.org/10.1155/2008/473804
List of other publications

DOI: http://dx.doi.org/10.1007/978-1-4939-1062-5_10

DOI: http://dx.doi.org/10.1038/lab.invest.2011.168
IF: 3.981

Total IF of journals (all publications): 14,479
Total IF of journals (publications related to the dissertation): 10,518

The Candidate's publication data submitted to the IDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

12 September, 2018
8. LIST OF KEYWORDS
Dendritic cell, peroxisome proliferator-activated receptorγ, retinoic acid receptor, all-trans retinoic acid, retinol dehydrogenase, CD1d, lipid antigen presentation, α-galactosylceramide, invariant natural killer T cell, Cathespin D

9. ACKNOWLEDGEMENTS

I would like to express my heartfelt gratitude to my Ph.D. supervisor, Prof. Dr. László Nagy for his continuous support, patience, encouragement of my study and research. His guidance helped me in all the time of research and writing of this thesis.

I would like to thank Prof. Dr. László Fésüs and Prof. Dr. József Tőzsér, the former and recent heads of the Department of Biochemistry and Molecular Biology for the opportunity to work in the department.

I am also indebted to Dr. Britt Nakken, Dr. István Szatmári and Dr. Tamás Varga my collaborators for their contribution to my scientific publications.

I would like to thank our collaborators Dr. Balázs Dezső for performing the IHC analysis and Dr. Zoltán Pós for the mouse co-culture experiment.

I also thank to Ibolya Fürtös and Edit Hathy for outstanding technical help, other members of the NLAB for their support and other members of the Department for any technical helps.

Lastly, I would like to thank my family for all their love, encouragement and unwavering support throughout the years.

Adrienn Gyöngyösi

This research was supported by Grants from the Hungarian Scientific Research Fund (OTKA K100196, OTKA NK72730) and TÁMOP-4.2.2.-2012-0023, TÁMOP-4.4.2/08/01 and TÁMOP-4.2.1/B-09/1/KONYV-2010-0007, co-financed by the European Social Fund and the European Regional Development Fund.

The work was also supported by the Higher Education Institutional Excellence Program of the Ministry of Human Capacities in Hungary (Biotechnology thematic programe, no. 20428-3/2018/FEKUTSTRAT).